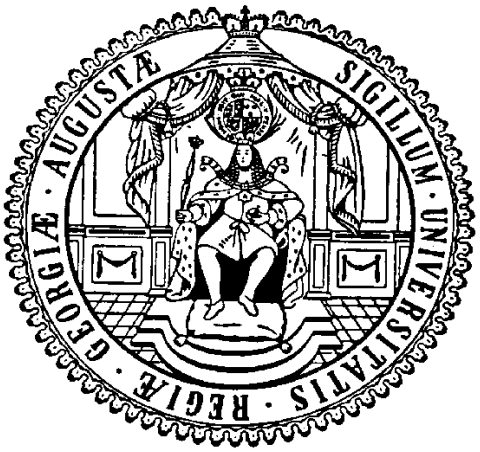
Vitamin B6 metabolism and underground metabolic routes in the Gram-positive bacterium *Bacillus subtilis*

Dissertation

For the award of the degree

“doctor rerum naturalium” (Dr. rer. nat)

of the Georg-August-Universität Göttingen within the doctoral program “Microbiology and Biochemistry” of the Göttingen Graduate Center for Neurosciences, Biophysics, and Molecular Biosciences (GGNB)



submitted by

Björn Richts

From Werl

Göttingen, 2021

Examination board

Thesis advisory committee

Prof. Dr. Fabian Commichau

Institute for Biotechnology, FG Synthetic Microbiology, BTU Cottbus-Senftenberg

Prof. Dr. Jörg Stülke

Institute for Microbiology and Genetics, Department of General Microbiology, Georg-August University Göttingen

Prof. Dr. Rolf Daniel

Institute for Microbiology and Genetics, Department of Genomic and Applied Microbiology, Georg-August University Göttingen

Members of the examination board

Dr. Till Ischebeck

Albrecht-von-Haller-Institute for Plant Sciences, Department of Plant Biochemistry Georg-August University Göttingen

Prof. Dr. Stefanie Pöggeler

Institute for Microbiology and Genetics, Department of Genetics of Eukaryotic Microorganisms, Georg-August University Göttingen

Prof. Dr. Kai Heimel

Institute for Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August University Göttingen

Date of oral examination: 01.07.2021

Statement of authorship

I hereby declare that the doctoral thesis entitled “Vitamin B6 metabolism and underground metabolic routes in the Gram-positive bacterium *Bacillus subtilis*” has been written independently and with no other sources and aids than quoted.

Björn Richts Göttingen, the 16th of May 2021

_____

Danksagung

Zunächst möchte ich mich bei dir bedanken Fabian, dass du mir die Möglichkeit gegeben hast, meine Doktorarbeit unter deiner Aufsicht zu schreiben. Auch nach dem zähen, ersten halben Jahr, warst du immer für Ideen und Diskussion offen und hast so geholfen diese Arbeit weiter zu gestalten. Ich wünsche dir viel Erfolg auf deinem weiteren Weg! Vielen Dank auch an dich Jörg, dass du mir Obdach gewährt hast in Göttingen, nachdem Fabian umgezogen ist. Obwohl du nicht für mich verantwortlich warst, hast du mich mit Rat und Tat unterstützt und auch dazu beigetragen, dass diese Arbeit zu dem geworden ist. Auch möchte ich mich bei Rolf Daniel an der Teilnahme meiner TAC Meetings bedanken, in denen mich die konstruktiven Ideen stets einen Schritt weiterbrachten. Ohne deine Hilfe Sabine wäre ich bei dieser Arbeit nicht so weit gekommen. Auch wenn meine Anweisungszettel manchmal chaotisch waren, hast du mir sehr viel Arbeit abnehmen können und unsere Projekte weit nach vorne gebracht!

Danke auch an meine Studenten Barbora, Georg, Dennis, Joshi und Melvin für die Mitarbeit an den Projekten und an alle Kollaborationspartner: Anja, Elke, Till, Tim, Auke, Alex und Stephan. Natürlich gilt mein Dank auch der gesamten Arbeitsgruppe (HIF+AGS+AGR) für die tolle Zeit. Leider hat Corona das letzte Jahr ein wenig geschmälert, aber dennoch hatten wir viel Spaß und den wissenschaftlichen Austausch untereinander habe ich sehr zu schätzen gelernt.

Vielen Dank auch an dich Patrick. Du hast mich vom ersten Bachelortag bis zum letzten Doktortag in meinen Studien begleitet und gegenseitig konnten wir uns motivieren weiterzumachen, als die Motivation mal wieder einen Tiefpunkt erreicht hatte. Ohne dich wäre das Studium ganz anders gelaufen und ich bin mir sicher, dass wir auch am letzten Lebenstag immer noch mit einem Lächeln zusammen auf die gute Zeit zurückschauen werden.

Danke auch an meine Eltern und an Laura, die mich in jedem Moment meines Studiums unterstützt und mir auch in schlechten Zeiten gut zugesprochen haben. Ohne eure Hilfe wäre das Studium nicht möglich gewesen!

Table of contents

List of abbreviations

General abbreviations

% (v/v)	%(volume/volume)
% (w/v)	% (weight/volume)
2-OG	2-oxoglutarate
4HTP	4-phosphohydroxy-L-threonine
ADP	Adenosine diphosphate
Amp	Ampicillin
AOPB	2-amino-3-oxo-4-(phosphohydroxy)butyric acid
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BGSC	Bacillus Genetic Stock Center
BSA	Bovine serum albumin
BSH	Bacillithiol
CAA	Casamino acid
CAF	Ammonium iron citrate
c-di-AMP	Cyclic di-AMP
CE	Crude extract
cfu	Colony forming unit
DE3	Lysogen that encodes T7 RNA polymerase
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DTT	Dithiothreitol
DXP	Deoxyxylulose-5‘-phosphate
Dxs	1-Deoxyxylulose-5-phosphate-Synthase
E4P	Erythrose-4‘-phosphate
E.C.	Enzyme Commission
Edp	Erythrose-4-phosphate-dehydrogenase
EDTA	Ethylendiaminetetra acetic acid
EL	Erythrocin + Lincomycin
et al.	*Et alii*
Fig.	Figure
G3P	Glyceraldehyde-3-phosphate
$H_2O_{deion.}$	Deionized water
IPTG	Isopropyl-*β*-D-thiogalactopyranoside
LB	Lysogeny broth (medium)
LML	Low molecular weight
Lys	Lysin
NADH/NAD^+	Nicotinamide adenine dinucleotide
NADPH/$NADP^+$	Nicotinamide adenine dinucleotide phosphate
Ni^{2+}-NTA	Nickel-nitrilotri acid
OD	Optical density
OHPB	2-oxo-3-hydroxy-4-phosphobutanoate
ONPG	o-Nitrophenol-*β*-D-galactopyranosid
PAGE	Polyacrylamide gel electrophoresis
PAP	6x SDS loading dye
PCR	Polymerase chain reaction
PdxA	Phosphohydroxy-L-threonine dehydrogenase
PdxB	4-Phosphoerythronate dehydrogenase
PdxH	PNP Oxidase
PdxJ	PNP Synthase
PE	4’-phosphoerythronate
PEP	Phosphoenolpyruvic acid
PFA	Para-formaldehyde
pH	Potential of hydrogen
PHA	3-Phosphohydroxy-1-aminoacetone
PK	Pyruvate kinase
PL	Pyridoxal
PLP	Page Buffer
PLP	Pyridoxal-5’phosphate
PM	pyridoxamine
PMP	Pyridoxamine-5’phosphate
PN	Pyridoxine
PNP	Pyridoxine-5’phosphate
(p)ppGpp	Guanosin-3’,5’-bispyrophosphat
Psi	Pound per square inch
R5P	Ribose-5-phosphate

RT	Room temperature
Ru5P	Ribulose-5-phosphate
SDS	Sodium dodecyl sulfate
SerC	3-Phosphoserine aminotransferase
SNP	Single nucleotide polymorphism
SSB	single strand DNA binding proteins
SP	Sporulation medium
TEMED	N,N,N´,N´-tetramethylethylendiamine
Tm	Melting temperature
TRIS	Tris-(hydroxymethyl)-aminomethane
WT	Wild type
Δ*gene*	Deletion of the gene

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

units

°C	Degree centigrade
Bar	Bar
bp	Base pair
Da	Dalton
g	gram
h	hour
l	liter
M	molar
min	minute
OD	Optical density
rpm	Rounds per minute
sec	seconds

prefix:

m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-11})

Amino acids

A	Alanine (Ala)
C	Cysteine (Cys)
D	Aspartic acid (Asp)
E	Glutamic acid (Glu)
F	Phenylalanine (Phe)
G	Glycine (Gly)
H	Histidine (His)
I	Isoleucine (Ile)
K	Lysine (Lys)
L	Leucine (Leu)
M	Methionine (Met)
N	Asparagine (Asn)
P	Proline (Pro)
Q	Glutamine (Gln)
R	Arginine (Arg)
S	Serine (Ser)
T	Threonine (Thr)
V	Valine (Val)
W	Tryptophan (Trp)
Y	Tyrosine (Tyr)

List of publications

Dormeyer M, Lentes S, **Richts B,** Heermann R, Ischebeck T, Commichau FM (2019) Variants of the *Bacillus subtilis* LysR-type regulator GltC with altered activator and repressor function. *Front Microbiol* 10: 2321. doi: 10.3389/fmicb.2019.02321

Richts B, Rosenberg J, Commichau FM (2019) A survey of pyridoxal 5'-phosphate-dependent proteins in the Gram-positive model bacterium *Bacillus subtilis*. *Front Mol Biosci* 6: 32. doi:10.3389/fmolb.2019.00032

Rosenberg J, **Richts B,** Commichau FM (2020) Fermentative production of vitamin B6 in *v*. Edited by Peter Grunwald. Copyright © 2020 Jenny Stanford Publishing Pte. Ltd.

Richts B, Hertel R, Potot S, Poehlein A, Daniel R, Schyns G, Prágai Z, Commichau FM (2020) Complete genome sequence of the prototrophic *Bacillus subtilis* subsp*. subtilis* strain SP1. *Microbiol Resourc Announc*. 9(32): e00825-20.

Richts B, Lentes S, Poehlein A, Daniel R, Commichau FM (2021) A *Bacillus subtilis pdxT* mutant suppresses vitamin B6 limitation by acquiring mutations enhancing *pdxS* gene dosage and ammonium assimilation. *Environ Microbiol Rep.* 13: 218-233.

Richts B and Commichau FM (2021) Underground metabolism facilitates the evolution of novel pathways for vitamin B6 biosynthesis. *Appl Microbiol Biotechnol* 105(6):2297-2305.

Summary

Genome-reduced bacteria give the opportunity to study essential gene functions but also give the starting point for biotechnological production pathways. The *MiniBacillus* project aims to produce a genome reduced *Bacillus subtilis* strain with only a defined set of necessary genes. To identify new deletable genes, we studied the vitamin B6 metabolic pathway since the active form of vitamin B6 pyridoxal-5'-phosphate (PLP) is involved in about 4% of all known enzymatic reactions. In the Gram-positive soil bacterium *B. subtilis* at least 65 vitamin B6-dependent proteins were identified. In this study, mainly three aspects of vitamin B6 metabolism were studied: The PdxST vitamin B6 synthesis complex, transport and detoxication of vitamin B6 and, the role of the low molecular weight (LMW) thiol bacillithiol (BSH) together with the *ytoQ* gene in a heterologous vitamin B6 synthesis pathway. In *B. subtilis* vitamin B6 is produced by a complex of PdxS and PdxT. The glutaminase domain PdxT cleaves off ammonia from glutamine and transfers it to the synthase domain PdxS. Vitamin B6 even can be produced by PdxS in absence of PdxT, when high concentrations of ammonium are present. A suppressor screen was performed with the *pdxT* mutant on medium containing low amounts of ammonium and an amplification of a 15 kb region including the *pdxS* gene was identified as the main suppression mechanism. Moreover, in some suppressors the ammonium channel *nrgA* was upregulated, leading to a growth advantage especially when *pdxS* was not overexpressed. Besides that, the *pdxS* gene and its promotor region did not acquire beneficial mutations even if the amplification relevant recombinase gene *recA* was deleted.

Furthermore, the nucleobase:cation symporter family proteins and the ECF transporter were excluded as vitamin B6 transporters and an upregulation of the Ars operon was identified as a detoxification mechanism for toxic PL levels.

A PL-auxotroph *B. subtilis* mutant harboring only the last two genes of the DXP-dependent vitamin B6 synthesis pathway from *E. coli*, formed suppressor mutants, which deleted the bacillithiol synthesis gene *bshC* and upregulated the *ytoQ* gene. It was shown that cultivation on medium containing cysteine repealed the beneficial effect. Cysteine can also act as a LMW thiol indicating a role of YtoQ and BSH in oxidative stress response. Furthermore, a proposed underground metabolism pathway for the synthesis of PLP could be excluded, involving the genes *cpgA, serA, serS* and *thrB.*

To increase general fitness and get insights about deleterious effects of the genome reduction in the *MiniBacillus* strains, the two genome-reduced strains PG10 (36% reduction) and PG39 (40% reduction) were evolved in complex medium. For PG39 a deregulation of genes mainly involved in oxidative stress response was identified as suppression mechanism. In PG10 over 25 genes harbored mutations. Interestingly, a downregulation of the *mhqNOP* operon was found, which was identified as highly upregulated in the strain.

1. Introduction

Carbohydrates, proteins and fatty acids play an important role in modern diet as an unvaried diet often leads to severe diseases (*Cena & Calder*, 2020). In addition to this, also micronutrients like vitamins have to be taken up as they cannot be produced by humans or only in inadequate amounts (*Domke et al.*, 2005). Vitamins are organic compounds, which mostly act as co-factors for enzymatic reactions and are normally not catabolized to deliver energy; they promote energy metabolism, though (*Institute of Medicine*, 1998, 2006; *Kanter*, 1994; *Mukherjee et al.*, 2011; *Trumbo et al.*, 2001). 13 characterized vitamins are classified as water- and fat soluble. The group of B-vitamins, B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folic acid), B12 (cyanocobalamin) and vitamin C belong to the water soluble group; vitamin A (Retinol), vitamin D (calciferol), vitamin E (tocopherol) and vitamin K (phylloquinone, menaquinone) dissolve in fat and oils and thereby make up the second group of vitamins (*Lukaski*, 2004). With increasing world population, also the demand on nutritious food grows and as most vitamins are sensitive to environmental influences, the industry has a special interest for the production of vitamins as food additives (*Vandamme & Revuelta*, 2016). Up to today, most vitamins are produced chemically and only for a few vitamins biotechnological synthesis pathways exist, which can compete with the chemical production (*Acevedo-Rocha et al.*, 2019). Nevertheless, the demand on sustainable synthesis of vitamins is rising also because the process can reduce the use of fossil compounds and prevent formation of toxic pathway intermediates (*Revuelta et al.*, 2016; *Schwechheimer et al.*, 2016). Fungi, plants or bacteria possess synthesis pathways for vitamins and do not rely on uptake. Therefore, their metabolic pathways are intensively studied to find possibilities to increase the yield of vitamin production (*Acevedo-Rocha et al.*, 2019; *Commichau et al.*, 2014, 2015; *J. Rosenberg et al.*, 2020).

1.1 The production host *Bacillus subtilis*

The Gram-positive, rod-shaped soil bacterium *Bacillus subtilis* is a common host for various biotechnological applications (*Acevedo-Rocha et al.*, 2019; *Guan et al.*, 2015; *Hao et al.*, 2013; *Rosales-Mendoza & Angulo*, 2015). It belongs to the phylum of the Firmicutes and is thereby closely related to the human pathogens *Listeria monocytogenes* and *Staphylococcus aureus* (*Wolf et al.*, 2004). As a soil bacterium, *B. subtilis* is exposed to environmental changes and adapts to these stress factors by using different lifestyles as endospores, biofilms or motile cells (*González-Pastor et al.*, 2003; *Kearns et al.*, 2005) Moreover, it is able to secrete different antibiotics and proteases to compete against other organisms and to assess nutrients from its surrounding. The 4.2 Mbp big *B. subtilis* genome, containing about 4100 genes, has a low GC content and its sequence was determined in 1997 (*Kobayashi et al.*,

2003; *Kunst et al.*, 1997). *B. subtilis* is well studied and a huge amount of transcriptomic and proteomic data is collected in databases as SubtiWiki (*Eymann et al.*, 2004; *Nicolas et al.*, 2012; *Ravikumar et al.*, 2018; *Zhu & Stülke*, 2018). Besides, also a knockout deletion mutant library exists (*Bacillus Genetic Stock Center*), making it an ideal model organism to study genes of unknown functions (*Koo et al.*, 2017; *Zeigler et al.*, 2008). Due to its natural competence, genetic modifications can easily be made by cultivation of the bacterial cells with foreign DNA (*Hamoen et al.*, 2003).

In contrast to *E. coli*, *B. subtilis* was ranked as a safe bacterium for production in food industries and was certified with the GRAS status (Generally Recognized As Safe), as it is nonpathogenic and does not excrete exo- or endotoxins (*Sewalt et al.*, 2016) Another advantage is that the bacterium can directly secrete proteins into the medium because the cell envelope lacks an outer membrane (*Zweers et al.*, 2008). This makes *B. subtilis* not only a good model organism for studying gene regulation, cellular differentiation processes and metabolism. It is also a valuable production host for proteases in washing detergents, vitamins, recombinant enzymes or other industrial relevant metabolites (*Acevedo-Rocha et al.*, 2019; *Bretzel et al.*, 1999; *Chu*, 2007; *Degering et al.*, 2010; *Hao et al.*, 2013; *Harwood*, 1992; *Simonen & Palva*, 1993; *van Dijl & Hecker*, 2013; *van Tilburg et al.*, 2019; *Van Tilburg et al.*, 2020).

1.2 Vitamin B6

Vitamin B6 is a collective noun for the three vitamers pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM) and their respective phosphate esters pyridoxal-5'-phosphate (PLP), pyridoxine-5'-phosphate (PNP) and pyridoxamine-5'-phosphate (PMP) (*György*, 1956; *I. H. Rosenberg*, 2012). All share the same pyridine-based structure and only differ in their head group at the 4'-C-atom. Pyridoxal carries an aldehyde group, pyridoxine a hydroxy group and pyridoxamine an amino group (see Figure 1) (*Kresge et al.*, 2005; *I. H. Rosenberg*, 2012). PLP is the active compound of vitamin B6 for most of the reactions as its aldehyde group can form a Schiff-base with lysine residues of enzymes and thereby facilitates the reaction with the substrates. (*Christen & Mehta*, 2001; *Eliot & Kirsch*, 2004; *Jansonius*, 1998). More than 160 enzymes require B6 as a co-factor, which is ~4% of all known catalytic activities and 1.5% of free-living prokaryote genes code for PLP-dependent proteins (*Percudani & Peracchi*, 2003, 2009). Most of the PLP-dependent proteins are cofactors in amino acid metabolism, catalyzing *α*,- *β*- and *γ*-elimination and replacements, cleavage of C_a- C_b bonds, racemization or decarboxylation reactions (*Eliot & Kirsch*, 2004; *John*, 1995). Also PM and PMP can take part in enzymatic reactions, either as co-factors or even as substrate (*Mehta & Christen*, 2000; *Romo & Liu*, 2011). The pyridoxamine-pyruvate aminotransferase for example transfers the amino group of pyridoxamine to pyruvate giving pyridoxal

and alanine and *vice versa* (*Yoshikane et al.*, 2006). PMP can be used as a co-factor by CDP-6-deoxy-L-threo-D-glycero- 4-hexulose-3-dehydrase, which catalyzes a step in the biosynthesis of the deoxysugar ascarylose (*K. D. Burns et al.*, 1996).

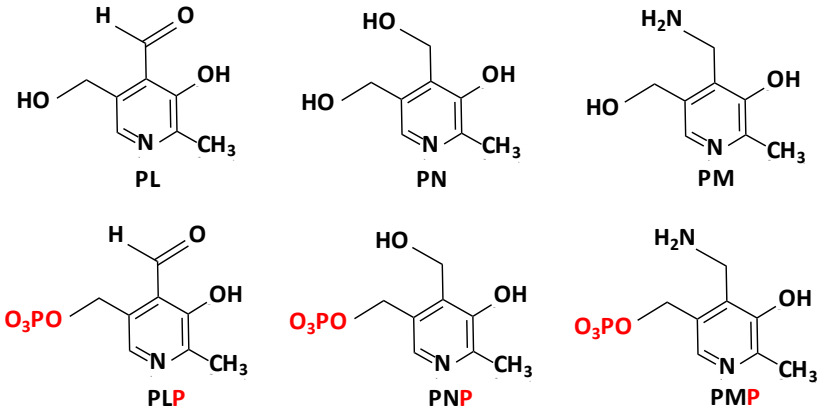


Figure 1 Structure of the B6 vitamers.
The B6 vitamers: pyridoxal (PL), pyridoxal 5'-phosphate (PLP), pyridoxine (PN), pyridoxine 5'-phosphate (PNP), pyridoxamine (PM), and pyridoxamine 5'-phosphate (PMP).

We correlated the Enzyme Commission (E.C.) numbers of the PLP-dependent proteins from the B6 database (http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/bib.pl) with the *B. subtilis* 168 protein E.C. numbers, listed in the SubtiWiki Database (http://subtiwiki.uni-goettingen.de/v4/) (*Zhu & Stülke*, 2018) to describe the PLP-interactome (PLPome (*Hoegl et al.*, 2018)) of *B. subtilis*. In addition to the B6 Database, we took *B. subtilis* specific proteins into consideration whose activities in PLP metabolism were described. In total we were able to identify 61 PLP-dependent proteins (Richts et al., 2019). The colleges from the group of Hoegl *et al*. performed a mass spectrometry experiment with modified B6 analogs and were able to describe 4 more PLP-binding proteins of unknown function in the closely related organism *S. aureus* (*Hoegl et al.*, 2018). In total we came up with 65 PLP-dependent proteins existing in *B. subtilis* of, which 61 are *bona fide* PLP interaction partners and for the others, interactions remain to be experimentally confirmed. Additional PLP-dependent proteins will surely be discovered in the near future as the amount of genome sequences increases continually (https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/).

We categorized the *B. subtilis* PLPome and assigned the proteins to different cellular functions. As seen in Figure 2, the PLP-dependent proteins of *B. subtilis* are mainly active in amino acid biosynthesis and catabolism. PLP is often needed as a catalysator for transaminases, which transfer *α*-amino groups of amino acids to *α*-ketoglutaric acid to form glutamate and the donor amino acid becomes an *α*-keto acid. The reaction can also be reversed and therefore either used for amino acid anabolism or catabolism (*Babitzke et al.*, 1992; *Barb et al.*, 2013; *Kriel et al.*, 2014; *Molle, Nakaura, et al.*, 2003).

PLP-dependent proteins are also involved in other metabolic routes as in the production of antibacterial compounds, iron-and carbon metabolism and nucleotide utilization. The production of the cofactors biotin, folate, heme and NAD^+ are also catalyzed by PLP-dependent enzymes. The PLPome also includes proteins involved in cell wall metabolism, the execution of different cellular lifestyles and in information processing on DNA level. However, about one third of the PLP-dependent proteins in *B. subtilis* are only poorly characterized and it will be interesting for the future to investigate the gene functions. A detailed summary of the PLP-dependent proteins in *B. subtilis* can be found in (*Richts et al.*, 2019).

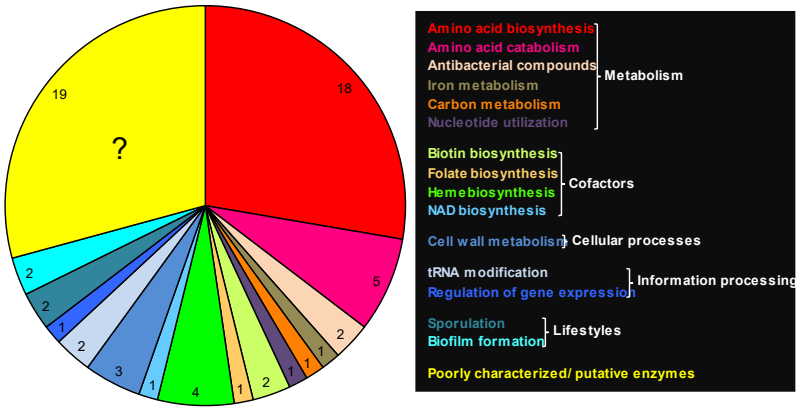


Figure 2 Functional distribution of PLP-dependent proteins in *B. subtilis*.
See Richts et al., 2019c for further information.

PLP cannot only act as a cofactor for enzymatic reactions. The presence of PLP can also alter gene expression by binding to transcription regulators and thereby tweak their binding properties (*Belitsky*, 2004a, 2014; *Martino Luigi Di Salvo et al.*, 2015; *Huq et al.*, 2007; *Oka et al.*, 2001; *Qaidi et al.*, 2013; *Suvorova & Rodionov*, 2016; *Tramonti et al.*, 2017).

As described above, vitamin B6 is an important player in a variety of cellular processes (*Belitsky & Sonenshein*, 2002; *Bramucci et al.*, 2011; *Percudani & Peracchi*, 2009). Moreover, it has been shown that vitamin B6 takes part in oxidative stress response (*Bilski et al.*, 2007; *Moccand et al.*, 2014; *Mooney et al.*, 2009; *Mooney & Hellmann*, 2010; *Vanderschuren et al.*, 2013). Therefore, it is essential to maintain a certain cellular concentration of the B6 vitamer PLP.

For animals and humans vitamin B6 is an essential micronutrient and shortage of B6 intake can lead to severe disease symptoms as impaired glucose tolerance or neuropathy because lack of vitamin B6 produces errors in the so-called “salvage pathway” (*Martino L. Di Salvo et al.*, 2012; *Institute of Medicine*, 1998; *Kraemer et al.*, 2012; *Mills et al.*, 2005). Therefore, vitamin B6 is of special interest for food- and pharmaceutical industry, mostly in forms of pyridoxine hydrochloride, which is supplemented with other vitamins in e.g. bakery products, cereals, baby nutrition or juices (*Acevedo-*

Rocha et al., 2019; *Domke et al.*, 2005; *Eggersdorfer et al.*, 2012; *Fitzpatrick et al.*, 2007, 2010; *Kraemer et al.*, 2012; *J. Rosenberg, Ischebeck, et al.*, 2016). Furthermore, it is often used in farms to foster quick growth of the animals and promote health (*Eggersdorfer et al.*, 2012; *Johnson et al.*, 1950). Moreover, regular vitamin B6 intake in high doses can cause the so called "Megavitamin B6 syndrome", which describes an accumulation of vitamin B6 in the body and thereby causing severe neurological problems (*Callizot & Poindron*, 2008; *Institute of Medicine*, 2006; *Lheureux et al.*, 2005). Most of the symptoms are reversible and disappear after the vitamin B6 intake is lowered, but also irreversible damage can happen (*Barrows et al.*, 2008; *Cupa et al.*, 2015).

Until now biotechnological vitamin B6 production cannot compete with the chemical synthesis as titers of 10 g/l PL would be needed and only ~1 g/l could be obtained. The chemical production follows different synthesis routes, though and partially requires the usage of expensive/toxic compounds so that a shift towards sustainable production is desired (*Acevedo-Rocha et al.*, 2019; *Agranat*, 2009; *Commichau et al.*, 2014, 2015; *Eggersdorfer et al.*, 2000, 2012; *Hoshino et al.*, 2006; *J. Rosenberg et al.*, 2018; *J. Rosenberg, Ischebeck, et al.*, 2016; *Tatsuo et al.*, 2006).

1.3 *De novo* synthesis of PLP

So far, two metabolic pathways involved in biosynthesis of PLP have been described: the deoxyxylulose-5-phosphate (DXP)-dependent pathway, which is evolutionary younger but known for longer time and the DXP-independent pathway, which was later discovered. The DXP-dependent pathway exists in *α*-/*γ*-proteobacteria and the DXP independent pathway in plants, archaea, fungi, bacteria, plasmodium and some sponges species (*Fitzpatrick et al.*, 2007, 2010; *Guédez et al.*, 2012; *Mittenhuber*, 2001; *Mukherjee et al.*, 2011; *J. Rosenberg, Ischebeck, et al.*, 2016; *Seack et al.*, 2001; *Tanaka et al.*, 2005). Nevertheless, *in silico* analyses revealed that the DXP-independent pathway was also present in some in *α*-/*γ*-proteobacteria species, indicating that it is phylogenetically older (*Mittenhuber*, 2001; *Tanaka et al.*, 2005). The acquisition of the DXP-dependent pathway was driven by the emergence of the gene *pdxB* in *γ*-proteobacteria. For the *α*-proteobacteria the *pdxR* (not to confuse with the regulator PdxR) gene was acquired, which catalyzes the same reaction as *pdxB* but is not a homologue (*Mittenhuber*, 2001; *J. Rosenberg, Ischebeck, et al.*, 2016; *Tanaka et al.*, 2005; *Tazoe et al.*, 2006).

The DXP-dependent pathway was identified in *E. coli* and uses seven enzymatic steps to form PLP. Four enzymes catalyze the reaction from erythrose-4-phosphate to 4-hydroxy-threonine phosphate (4HTP) and form the first branch of the pathway (see Figure 3) (*Boschi-Muller et al.*, 1997; *Drewke et al.*, 1996; *Rudolph et al.*, 2010; *Tazoe et al.*, 2006; *Y. Yang et al.*, 1998; *Zhao et al.*, 1995). In the first step, erythrose 4-phosphate (E4P), deriving from the pentose phosphate pathway, is oxidized

by the E4P dehydrogenase Epd to 4'-phosphoerythronate (PE), which is then further oxidized to 2-oxo-3-hydroxy-4-phosphobutanoate (OHPB) by the PE dehydrogenase PdxB (*Boschi-Muller et al.*, 1997; *Rudolph et al.*, 2010; *Zhao & Winkler*, 1995). The released electrons are transferred to NAD^+ in the first step and FAD^+ in the second reaction. Next, the amino group of free glutamine is transferred to OHPB by the 3-phosphoserine aminotransferase SerC, resulting in 4HTP and 2-oxoglutarate (*Drewke et al.*, 1996). Again, electrons are transferred to NAD^+ in an oxidation step catalyzed by the 4HTP dehydrogenase PdxA. The product, 2-amino-3-oxo-4-(phosphohydroxyl)-butyrate is not stable and spontaneously decarboxylates to PHA (*Drewke et al.*, 1996; *Laber et al.*, 1999; *Rudolph et al.*, 2010; *Tazoe et al.*, 2005).

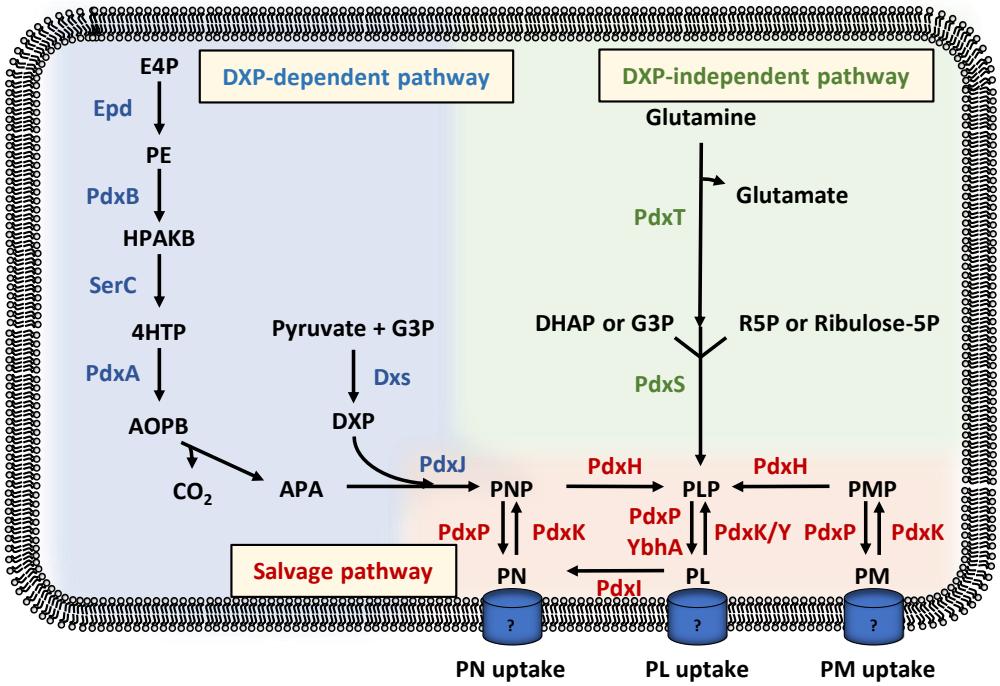


Figure 3 The deoxyxylulose 5-phosphate (DXP)-dependent and DXP-independent vitamin B6 biosynthetic routes and the salvage pathway for the interconversion of the B6 vitamers.
Epd, erythrose 4-phosphate dehydrogenase; PdxB, 4-phosphoerythronate dehydrogenase; SerC, 3-phosphoserine aminotransferase; PdxA, 4-phosphohydroxy-L-threonine dehydrogenase; PdxJ, PNP synthase; Dxs,; PdxH, PNP oxidase; PdxS (PLP synthase subunit) and PdxT (glutaminase subunit) form the PLP synthase complex; PdxK, PL kinase present in *B. subtilis* and E. coli; PdxY, PL kinase present in E. coli. PdxK from *B. subtilis* has PN, PL, and PM kinase activity (see text). E4P, erythrose 4-phosphate; 4PE, 4-phosphoerythronate; OHPB, 2-oxo-3-hydroxy-4-phosphobutanoate; 4HTP, 4-hydroxy-threonine phosphate; AOPB, 2-amino-3-oxo-4-(phosphohydroxyl)-butyrate; PHA, 3-phosphohydroxy-1-aminoacetone; DXP, deoxyxylulose-5-phosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; R5P, Ribose-5-phosphate. Red arrows indicate the steps where promiscuous enzymes may feed into the DXP-dependent and DXP-independent vitamin B6 biosynthetic pathways (*Kim et al.*, 2010; *Oberhardt et al.*, 2016; *J. Rosenberg et al.*, 2018; *Thiaville et al.*, 2016). Figure adapted from (*Richts et al.*, 2019)

In the second branch, the name giving metabolite DXP is formed by the DXP synthase (Dxs) from pyruvate and glyceraldehyde-3-phosphate (G3P). DXP is an essential metabolite because it is also important for thiamine and isoprenoid synthesis (*Cane et al.*, 2001; *Kuzuyama et al.*, 2000). DXP and

PHA are fused to PNP by the PNP synthase (PdxJ) (*Cane et al.*, 1999). Lastly, the PNP oxidase PdxH forms PLP and releases hydrogen peroxide as a byproduct (Zhao & Winkler 1995).

In contrast to the DXP-dependent pathway, the DXP-independent pathway catalyzes the synthesis of PLP in one reaction by the PdxST PLP synthase complex (*Belitsky*, 2004b; *K. E. Burns et al.*, 2005; *Ehrenshaft & Daub*, 2001; *Raschle et al.*, 2005; *Marco Strohmeier et al.*, 2006). The complex is a 24 heteromer consisting of 12 subunits of the PdxS synthase domain and 12 subunits of the PdxT glutminase domain, synthesizing PLP from glutamine, ribose-5-phosphate (R5P) and G3P (*Belitsky*, 2004b; *Smith et al.*, 2015; *Marco Strohmeier et al.*, 2006). They are assembled similar to two gears, which alternate in the patterns of their teeth (see Figure 4). PdxS forms the center with two hexameric rings and six PdxT units attach to the outside of each PdxS ring and by that forming the teeth of the gear (*Marco Strohmeier et al.*, 2006).

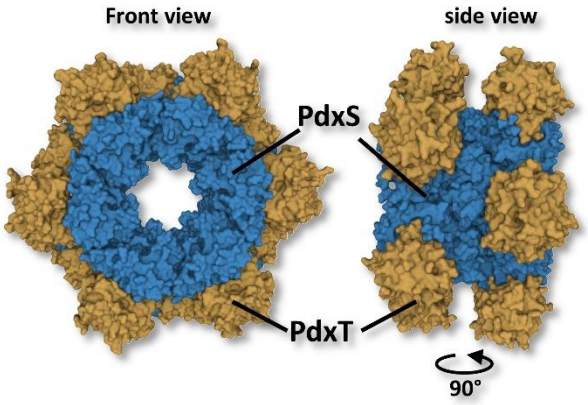


Figure 4 Crystal structure of the PdxST complex.
The PdxST complex shown in front view and rotated by 90°. Subunits of PdxS are depicted in blue and of PdxT in brown. (PDB accession number 2NV2) (*Marco Strohmeier et al.*, 2006)

PdxT is a glutamine amidotransferase (GAT), which can use its cysteine-histidine-glutamate (Cys-His-Glu) motif to hydrolyze ammonium from glutamine (*Belitsky*, 2004b; *K. E. Burns et al.*, 2005; *Raschle et al.*, 2005) (see Figure 5A). PdxT binds with its Cys78 residue to the C_α of glutamine, which leads to the cleavage of ammonia and the formation of a thioester intermediate. With the addition of water, the intermediate reacts to glutamate and ammonia is released (*Smith et al.*, 2015). The function of PdxT depends on the presence of PdxS, which is typical for GATs. In contrast to other GATs, it does not require the substrates of the synthase domain to retain its activity, making it a special member of this group. PdxT attaches only transiently to PdxS in the presence of glutamine. When ammonium has been channeled to PdxS and no more glutamine is present, it leaves the complex (*Guédez et al.*, 2012; *Marco Strohmeier et al.*, 2006).

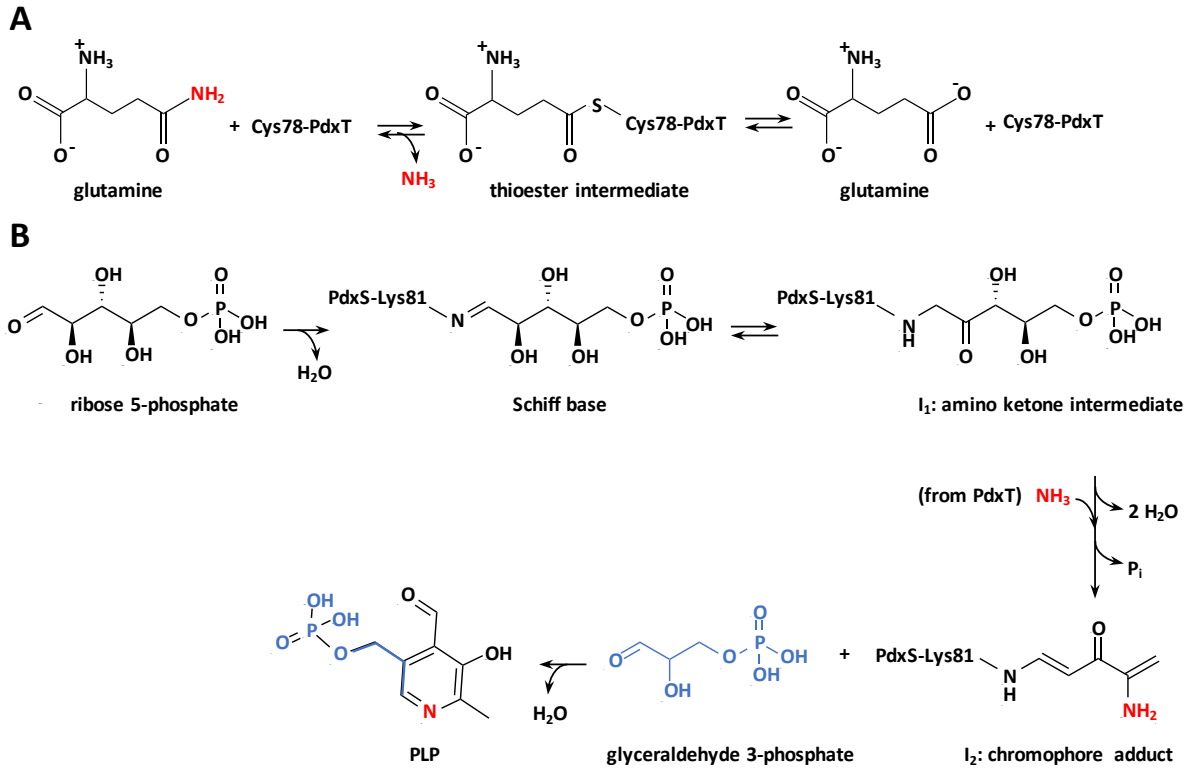


Figure 5 Synthesis of PLP by the PdxST synthase complex.
A: Reaction of the glutaminase domain PdxT. The Cys78 residue can react with glutamine and by that cleaves of its amino group. **B**: Ribose 5-phosphate binds to the Lys81 residue of PdxT and forms a Schiff base, which isomerizes to an amino ketone intermediate I_1. The ammonium derived from glutamine, or free ammonium bind to I_1 and form the stable chromophore adduct I_2. Upon glyceraldehyde 3-phosphate addition PLP is formed (adapted from *Smith et al.*, 2015).

The Lys81 residue of PdxS can form a Schiff-base imine adduct with R5P by replacing the C_1 keto group under dehydration as seen in Figure 5B. This binding occurs independently of PdxT, G3P or glutamine (*K. E. Burns et al.*, 2005). The Schiff base is isomerized without further help of enzymes and forms a stable amino ketone intermediate (I_1) (*Hanes et al.*, 2008; *Moccand et al.*, 2011). Hence, the ammonia deriving from PdxT reacts with I_1 by attaching to the C_2 atom and repelling the phosphate group. Another stable intermediate (I_2) is formed, which is a chromophore and can be detected via its absorption maximum at 320 nm (*Hanes et al.*, 2008; *Raschle et al.*, 2005). In the last step G3P bind at a different site to PdxS and reacts with I2 to PLP in a dehydration reaction. Upon binding of a PLP-dependent enzyme, PLP is released from that site (*Moccand et al.*, 2011; *Smith et al.*, 2015). PdxS can also produce PLP with ammonium directly, independent of PdxT albeit the use of glutamine is more efficient (*Hanes et al.*, 2008; *Raschle et al.*, 2005; *Zalkin & Smith*, 1998). The structure of PdxST promotes the formation of an oxyanion hole consisting of a methionine-rich, hydrophobic region through which the ammonia is shuttled from PdxT to the active center of PdxS. This hole is part of the isomerase barrel fold (TIM), which also confers the ability of PdxS to convert dihydroxyacetone phosphate (DHAP) or ribulose-5-phosphate (Ri5P) to G3P and R5P, respectively (*Marco Strohmeier et al.*, 2006). A similar mechanism could also be shown for other proteins as the imidazole glycerol

phosphate synthase, signalling that the shutteling of ammonia can increase reaction efficiency (*Chaudhuri et al.*, 2001). All in all, the PdxST complex can fulfill several different reactions – triose/pentose isomerization, imine formation, amine addition and ring formation (*K. E. Burns et al.*, 2005; *Raschle et al.*, 2005).

Although The DXP-dependent and the DXP-independent pathways are different, a comparison of PdxS and PdxJ showed similarities in their structure and their mode of action. In addition, also several substrates are identical or derive from the same metabolic pathways (*Fitzpatrick et al.*, 2007).

Animals are not able to produce vitamin B6 as they lost the *de novo* pathway during evolution and they have to take it up with their diet (*Domke et al.*, 2005). To guarantee compatibility among the different vitamers, a salvage pathway exists for the interconversion (see Figure 3). This salvage pathway is also present in most other organisms but can be different in its constellation (*Martino Luigi Di Salvo et al.*, 2011; *Fitzpatrick et al.*, 2007). Only the unphosphorylated vitamers PL, PN and PM can be taken up but little is known about the transport mechanisms of vitamin B6 and only few transporters could be identified so far (*Stolz et al.*, 2005; *Stolz & Vielreicher*, 2003; *Szydlowski et al.*, 2013; *T. Wang et al.*, 2015; *Yamada et al.*, 1977; *Yamada & Furukawa*, 1981). For *Salmonella enterica* a periplasmic vitamin B6 phosphatases PhoN was identified, allowing uptake of extracellular PLP (*Vu & Downs*, 2020). The imported vitamers are phosphorylated by vitamin B6 kinases as PdxK or PdxY of which the latter phosphorylates only PL. Most of the kinases also show activity for PM and PN (*Di Salvo et al.*, 2011; *Nagahashi et al.*, 2008; *J.-H. H. Park et al.*, 2004; *Sugimoto et al.*, 2017; *Tazoe et al.*, 2005; *White & Dempsey*, 1970; *Y. Yang et al.*, 1996, 1998). The PL reductase from *E. coli* PdxI can reduce PL directly to PN (*Ito & Downs*, 2020). Also in humans indications for PL reductase activity were found but no concrete candidates could be identified (*Ramos et al.*, 2019). This reaction step was just recently found and it was originally thought that conversion of the vitamers can only happen on the level of the phosphate esters by oxidases as PdxH. Besides that, PLP and PMP are interconverted in by transaminase reactions, which involved PLP as co-factor (*Eliot & Kirsch*, 2004). The B6 vitamers can also be exported. But same as for the import, the unphosphorylated forms are preferably transported and only few cases have been described for the export of the phosphorylated esters (*Vu et al.*, 2020). Therefore, PLP and PNP are dephosphorylated by PNP phosphatases as PdxP from *Sinorhizobium meliloti*, which has PNP/PLP activity but no PMP activity (*Tazoe et al.*, 2005).

1.4 Vitamin B6 as a regulator

In *B. subtilis* seven PLP-sensitive regulators exist YcxD, YdeF, YdeL, YdfD, YhdI, and YisV and GabR of which only the latter is well characterized. They mainly regulate genes involved in *γ*-aminobutyrate (GABA), taurine or ecotone metabolism. PLP-dependent transcription factors belong to the group of

MocR-subfamily, deriving from the rizophin regulator MocR in *Rhizobium melioti* and bind DNA with their N-terminal GntR-family binding domain. An aminotransferase-like sensor domain is located at the C-terminus of the proteins (*Bramucci et al.*, 2011; *Milano et al.*, 2015; *Suvorova & Rodionov*, 2016; *Tramonti et al.*, 2018). GABA can be converted by *B. subtilis* to nitrogen by the GABA aminotransferase GabT and the succinic semi-aldehyde dehydrogenase GabD. Both enzymes are encoded in a bicistronic operon and the genes are transcribed in the presence of the MocR-family type transcription regulator GabR, GABA and PLP (*Belitsky*, 2004b; *Belitsky & Sonenshein*, 2002). In absence of GABA, GabR binds to the promoter of the *gabR* gene, thereby preventing transcription (*Belitsky & Sonenshein*, 2002; *Edayathumangalam et al.*, 2013). For its regulatory activity the C-terminal part including the aminotransferase domain is not important but a conformational change has been described to be crucial for the formation of external aldimine formation between GABA and PLP.

When GABA and PLP are present, they form an external aldimine, which is sensed by GabR and leads to a conformational change in the aminotransferase domain leading to a dimerization by which the N-terminus can bind to the *gabT-gabD* DNA at two binding sites. An enzymatic reaction of the aminotransferase-like domain is not necessary for the regulatory effect (*Al-Zyoud et al.*, 2016; *Amidani et al.*, 2017; *Edayathumangalam et al.*, 2013; *Milano et al.*, 2017; *Okuda, et al.*, 2015; *Okuda, et al.*, 2015; *S. A. Park et al.*, 2017; *Wu et al.*, 2017).

PLP-sensitive transcription regulators also exist in other organisms, which even regulate the synthesis of genes involved in PLP metabolism. For instance, PdxR regulates the expression of the *pdxST* genes in bacteria like *L. monocytogenes* and PtsJ controls the expression of the *pdxK* gene in *Salmonella typhimurium* (*Belitsky*, 2014; *Tramonti et al.*, 2017). Moreover, the *Arabidopsis thaliana thi* riboswitch responds to thiamine and PLP (*Han et al.*, 2020).

1.5 Regulation of vitamin B6 synthesis

The necessary amount of PLP is very low and in high doses the B6 vitamer is highly toxic for the cells as its 4'-aldehyde group forms covalent adducts with the thiol or amino groups of PLP-independent proteins (*Commichau et al.*, 2015; *Drewke et al.*, 1993; *Farrington et al.*, 1993; *Hartl et al.*, 2017; *Mizushina et al.*, 2003; *Shames et al.*, 1984; *Vermeersh et al.*, 2004). PLP can inhibit for example enzymes, which are involved in carbon or DNA metabolism, as the acetyl-CoA carboxylase, DNA topoisomerase IB or *α*- and *ε*-DNA polymerase (*W. M. Lee et al.*, 2005; *Mizushina et al.*, 2003; *Vermeersh et al.*, 2004). Besides, PLP can also bind to the adenylsuccinate synthase, the initiation factor 3 of *E. coli* or the PL kinase and thereby inhibit the enzymes (*Dong & Fromm*, 1990; *Ghatge et al.*, 2012; *Ohsawa & Gualerzi*, 1981). Thus, vitamin B6 overdosing leads to severe neurological diseases,

rendering out the importance of proper maintenance of the PLP levels (*Callizot & Poindron*, 2008; *Institute of Medicine*, 2006; *Lheureux et al.*, 2005).

PLP and PMP belong to the 30 most damage-prone metabolites as they can spontaneously lead to condensation, oxidation, transamination of reduction reactions or they can promote spontaneous chemical reactions (*Lerma-Ortiz et al.*, 2016; *Linster et al.*, 2013). It is important to control mainly the amount of PLP in the cells, as it was shown that the other vitamers are less toxic (*J. Rosenberg, et al.*, 2016). This could be achieved by for example regulation of the PLP formation, direct transport of PLP to the targets, export of the toxic vitamer, or by degrading PLP (*Mukherjee et al.*, 2008; *J. Rosenberg, Ischebeck, et al.*, 2016)

PdxR is a regulator of vitamin B6 synthesis, which exists in several bacteria harboring the DXP-independent pathway but not in *B. subtilis* and represses the expression of PLP-synthesis genes in presence of PLP (*Belitsky*, 2014; *Jochmann et al.*, 2011; *Liao et al.*, 2015; *Qaidi et al.*, 2013; *Tramonti et al.*, 2015). At low PLP concentrations the expression of the *pdxST* genes is promoted. When the PLP levels increase, it binds to PdxR and thereby prevents it from binding to the promoter and prevents its own synthesis (*Belitsky*, 2014). But also other regulators for vitamin B6 synthesis have been identified as thiamine, which inhibits vitamin B6 production in yeast (*Minami et al.*, 1982).

The overexpression of *pdxA* and *pdxB* from *E. coli* leads to enhanced growth, suggesting that these enzymes produce B6 in a limited fashion (*Pease et al.*, 2002). The exact reason why the amount of B6 is elevated remains to be elucidated but a regulatory mechanism seems to be feasible. Furthermore, the PNP oxidase PdxH is inhibited by PLP. It can further be acetylated to alter its activity (*Suvorova & Rodionov*, 2016; *Zhao & Winkler*, 1995). PdxH has two PLP binding sites, one in the active center and another one at a non-catalytic site. It was shown that the produced PLP in the active site can be moved through a tunnel to the non-catalytic site, where it can be transferred to other proteins as the apo-serine hydroxymethyltransferase (*Safo et al.*, 2001, 2005; *E. S. Yang & Schirch*, 2000). PdxK in *E. coli* is also inhibited by covalent binding of formed PLP to the active center. By being bound, it is also in a state of resting, until it is be channeled to PLP-dependent proteins (*Martino Luigi Di Salvo et al.*, 2015; *Ghatge et al.*, 2012; *Moccand et al.*, 2011).In *B. subtilis,* the release of PLP from the PdxST complex is triggered by PLP-dependent proteins, such as the aspartate aminotransferase AspB (*Moccand et al.*, 2011). These carrier proteins could be the reason for general low amounts of PLP present in the cells, as the reactive co-factor is directly transported to the targets (*Fu et al.*, 2001). It has been shown in humans that even high intakes of vitamin B6 lead to constant levels of PLP because free PLP is bound to serum albumin and is thereby transported through the body (*Huang et al.*, 2012; *Lumeng et al.*, 1974; *Schaeffer et al.*, 1989). Moreover, the possibility exists that PLP could be bound to amino acids for shuttling the B6 vitamer to the targets (*Martino Luigi Di Salvo et al.*, 2011).

PLP can be salvaged to less toxic PL by phosphatases as PdxP. Recently, the phosphatase YbhA was identified in *E. coli,* which has PLP phosphatase activity but also minor phosphotransferase and phosphatase activity for different metabolites as erythrose-4-phosphate, fructose-1,6-bisphosphate, flavin mononucleotide, thiamine-pyrophosphate, glucose- 6-phosphate and ribose-5-phosphate (*Kuznetsova et al.*, 2006; *Saito et al.*, 2006; *Sugimoto et al.*, 2017). It shares 31% sequence identity with YitU from *B. subtilis, which* belongs to the HAD phosphatases and is able to dephosphorylate a precursor of riboflavin (*Sarge et al.*, 2015).

Besides the B6 specific phosphatase PdxP, also alkaline and acidic phosphatases can dephosphorylate PLP and PMP and thereby lower the levels of PLP in the human plasma (*Bull et al.*, 2002; *M. L. Fonda*, 1992; *Margaret L. Fonda & Zhang*, 1995; *Harris*, 1990; *Jang et al.*, 2003). Following this, humans can also degrade PN to 4-pyridoxate from PN, which is secreted in the urine (*Schuster et al.*, 1984). Organisms as *Mesorhizobium loti*, *Pseudomonas sp. MA-1*, or *Ochrobactrum* can further catabolize vitamin B6 to succinate , ammonia, acetate and CO_2 involving seven enzymes (*Burg et al.*, 1960; *Burg & Snell*, 1969; *Yoshikane et al.*, 2006).

Moreover, the PLP-binding protein Yggs (COG0325) of *E. coli* was found, which is highly conserved among all domains of life (*Ito et al.*, 2013). In *Streptomyces coelicolor* for example it is important for sporulation specific cell division (*J. Zhang et al.*, 2019). Deletion of the *yggS* gene in *E. coli* leads to a PN-sensitive phenotype, PNP accumulation and a disturbance in branched-chain amino acid formation (*Ito et al.*, 2019; *Prunetti et al.*, 2016; *Vu et al.*, 2020). Furthermore, cells became synthetically lethal in a *yggS glyA* double mutant. It could be shown, that glycine metabolism is repressed by PMP, as it competes with PLP for binding in GvcP, an enzyme taking part in the glycine cleavage system. Why PNP accumulates is still unclear (*Ito et al.*, 2020). YggS has 33% sequence identity with YlmE of *B. subtilis*. As *E. coli* synthesizes vitamin B6 *via* the DXP-dependent and *B. subtilis via* the DXP-independent pathway, it is likely that YggS regulates the salvage of vitamin B6 and not the production. Indeed, when the yeast PLP synthase gene was integrated into a *S. enterica yggS pdxH* mutant, the same phenotype was observed as in *E. coli*, indicating that the phenotype is independent on the B6 synthesis. It was hypothesized that PLP binds to Yggs and thereby changes the PLP-PMP recycling in the salvage pathway (*Vu et al.*, 2020). The PtsJ protein (*ycxD* 27.09% in *B. subtilis*) is also present in *S. typhimurium* and modulates the salvage of vitamin B6. It acts as a repressor of the *pdxK* gene, coding for a vitamin B6 kinase (*Martino Luigi Di Salvo et al.*, 2015).

1.6 Fermentative production of vitamin B6

Vitamin B6 belongs to the majority of vitamins, which are still chemically produced (*Acevedo-Rocha et al.*, 2019). This process involves expensive and toxic substrates and does not match with a sustainable

production (*Eggersdorfer et al.*, 2012). Therefore, in former studies it was tried to overproduce vitamin B6 in microorganisms to reach titers of at least 10 g/l, which can compete with the chemical synthesis (*Acevedo-Rocha et al.*, 2019; *Agranat*, 2009; *Commichau et al.*, 2014, 2015; *Eggersdorfer et al.*, 2000, 2012; *Hoshino et al.*, 2006; *J. Rosenberg et al.*, 2018; *J. Rosenberg, Ischebeck, et al.*, 2016; *Tatsuo et al.*, 2006). First attempts were made with different wild type isolates of bacteria and fungi and a yield of 25 mg/ml could be obtained with the yeast *Pichia guilliermondii* NK-2 (*Yocum et al.*, 2005). When genetical engineering established to construct overproducing microbes, the B6 synthesis genes *pdxST* of *B. subtilis* were overexpressed in *E. coli* but the amount of produced vitamin B6 could only be enhanced slightly. The DXP-independent pathway is slower than the DXP-dependent pathway, although only one enzyme participates in the reaction. It is furthermore not regulated and PLP is produced without any precursors. Therefore, toxic levels of PLP are produced quickly and the cells die (*Yocum et al.*, 2005). Thus, further studies focused more on the optimization of the DXP-independent vitamin B6 synthesis pathway, for example the pathway genes of *E. coli* were overexpressed and could thereby enhance B6 production to up to 78 mg/l in 31 h of cultivation (*Hoshino et al.*, 2006). The natural overproducer *S. melioti* can even produce up to 100 mg/l. This organism was further modified by overexpressing its native *dxs* gene and introducing the *E. coli epd* gene. The produced amount of vitamin B6 could be even increased to 1.3 g/l (*Hoshino et al.*, 2006). As *B. subtilis* is an excellent production host, it was used to overexpress vitamin B6. Therefore, a codon optimized version of the *epd* gene from *E. coli* and *pdxR*, serC, *pdxA* and *pdxJ* from *S. meliloti* were introduced into the genome of *B. subtilis*, resulting in a vitamin B6 titer of 41 mg/l PN in 72h (*Commichau et al.*, 2014). As the introduced genes have different activities, it probably comes to a metabolic stuck of toxic PN precursors as 4HTP and the cells suffer from this stress (*Commichau et al.*, 2014). Hence, a *B. subtilis* strain was evolved in presence of 4HT interfering with the threonine and isoleucine biosynthesis (*Commichau et al.*, 2014; *Drewke et al.*, 1993; *Katz et al.*, 1974; *J. Rosenberg, Müller, et al.*, 2016; *Rudolph et al.*, 2010). Indeed, the evolved strain harbored mutations in a transporter of the branched amino acids and thereby preventing 4HT uptake. Furthermore, mutations were found in a promoter of a gene de-regulating threonine biosynthesis. By overexpressing the *thrB* gene in the heterologous pathway mutant, the produced B6 amount could be doubled to 120 mg/l. For a detailed review of bacterial vitamin B6 production see (*J. Rosenberg et al.*, 2020).

1.7 Vitamin B6 underground metabolism

Enzymes are not always substrate specific and often catalyze more than one reaction. In the evolutionary perspective, all organisms derived from a last universal common ancestor, which approximately carried 500 genes (*Kannan et al.*, 2013). *B. subtilis* possesses about 4100 and humans

about 30,000 genes (*Kobayashi et al.*, 2003; *Pertea et al.*, 2018; *Zhu & Stülke*, 2018). Although it is possible that new genes derive from noncoding DNA, most novel gene functions emerged by gene duplication and slight modifications of one of the genes, which can establish new gene functionality. During this step, promiscuous enzyme activities evolved and increases fitness for functions, which are not related to their primary tasks. Next, these promote a duplication of the region to cope selective pressure. When the pressure level normalizes due to the beneficial enzyme version, the original copy is lost (*Bergthorsson et al.*, 2007; *Khersonsky & Tawfik*, 2010). This beneficial effect is ofthen not only on the basis of enzymatic activity but could also confer regulation on gene level. These so-called "trigger enzymes" do have both, an enzymatic function in metabolism but also act as regulators of transcription (*Commichau et al.*, 2007; *Commichau & Stülke*, 2008; *Lewis V. Wray et al.*, 2001). Thus, it is reasonable that multiple metabolic pathways can exist in an organism to fulfill the synthesis of one specific compound, as promiscuous enzymes could carry out tasks apart from their known reactions. This so-called underground metabolism describes the fact that promiscuous enzymes can either carry out the functions they evolved for but also use structural related substrates (*Ari & Casadesu*, 1998; *Notebaart et al.*, 2018). It is for example known that in *E. coli* 65% of the known reactions utilize only 37% of known enzymes (*Nam et al.*, 2012).

This underground metabolism is also interesting for biotechnological approaches because novel gene functions also offer new possibilities to overcome the problems of the actual vitamin B6 production (*Copley*, 2012; *Khersonsky & Tawfik*, 2010). Many different serendipitous pathways could be identified until now, which feed directly into the vitamin B6 metabolism pathway or link different metabolic routes (*Cooper*, 2010; *Kim et al.*, 2010, 2019; *Kim & Copley*, 2012; *Man et al.*, 1996; *Oberhardt et al.*, 2016; *Sachla & Helmann*, 2019.; *Smirnov et al.*, 2012; *Thiaville et al.*, 2016).

The first pathway was found in the mid 90ies by *Man et al.* who identified a mutation in *pdxJ*, which was able to overcome the loss of the PNP oxidase PdxH. Moreover, the overproduction of the native genes *serA*, *yeaB*, *ltaE* and *thrB* in *E. coli* can cope the loss of the *pdxB* gene without further integration of foreign genes and thereby relieve vitamin B6 auxotrophy (see Figure 6) (*Kim et al.*, 2010; *Kim & Copley*, 2012). The genes code for enzymes linking serine to vitamin B6 metabolism by converting 3-phosphoglycerate a starting point for serine synthesis to 4HT involving SerA, YeaB and LtaE. 4HT is then converted by ThrB to 4HTP, which is a substrate in the DXP-dependent pathway. Two other pathways were supposed feeding in at the level of SerC and PdxJ, respectively (*Kim et al.*, 2010). These pathways at least consist of AroA but could also include HisB, Php or YjbQ (see Figure 6). The substrates of these pathways still remain to be elucidated, although It is known that YjbQ has a thiamine phosphatase activity and can take part in both vitamin B1 and B6 synthesis (*Morett et al.*, 2008). In an *in silico* approach ThiG, which is also involved in production of the thiazole moiety in thiamine biosynthesis,

was identified as an promiscuous enzyme being able to replace PdxB when overexpressed (*Oberhardt et al.*, 2016). It is speculated that it could replace the entire pathway and directly produce PL. This hypothesis still has to be verified experimentally but structural analysis revealed that ThiG is similar to the PLP synthase PdxS (*Oberhardt et al.*, 2016)

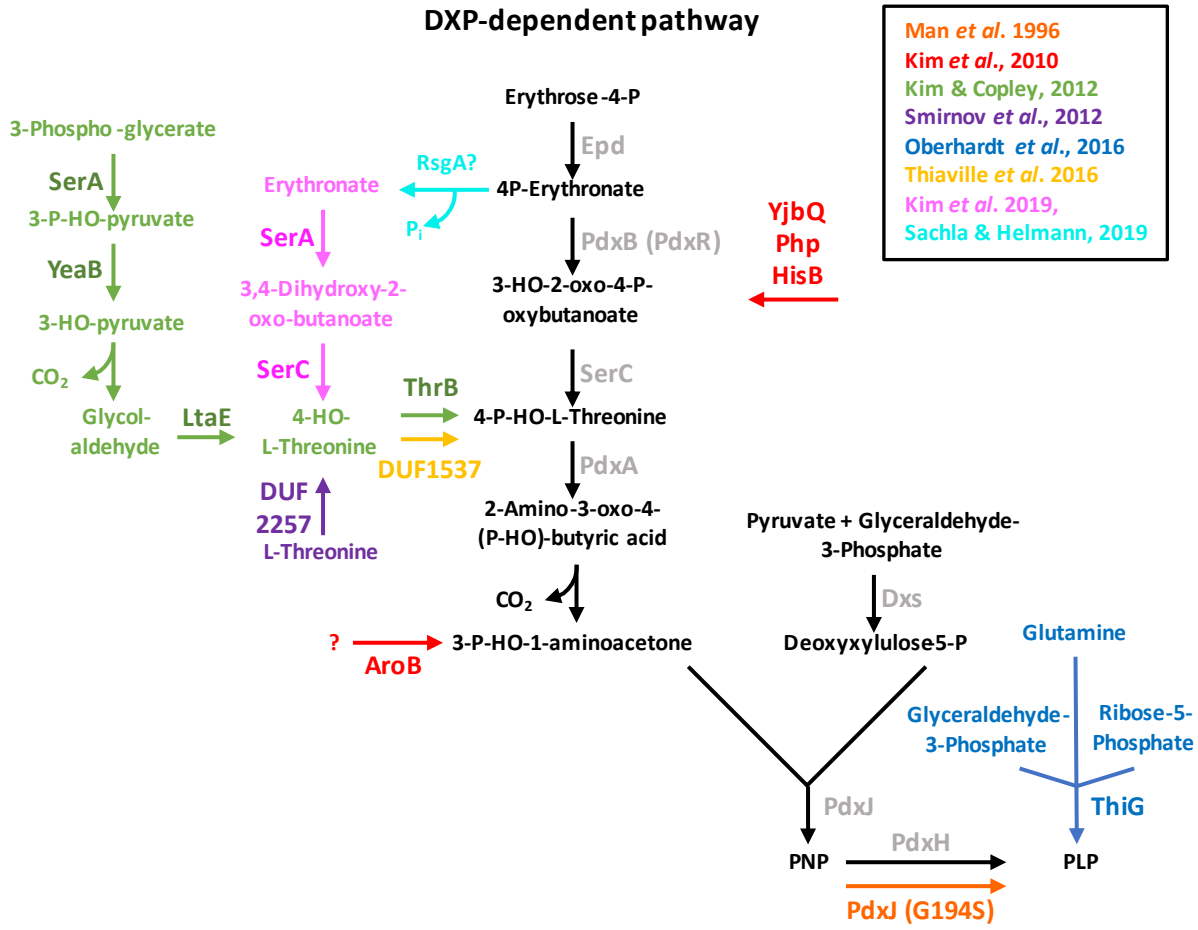


Figure 6 Serendipitous pathways for vitamin B6 synthesis and enzymes that feed into the DXP-dependent pathway in *E. coli*.
Epd, erythrose 4-phosphate dehydrogenase; PdxB, 4-phosphoerythronatedehydrogenase; SerC, 3-phosphoserine aminotransferase; PdxA, 4-phosphohydroxy-L-threonine dehydrogenase; PdxJ, pyridoxine 5'-phosphate synthase; Dxs, 1-deoxyxylulose 5-phosphate synthase; PdxH, pyridoxine 5'-phosphateoxidase; SerA, phosphoglycerate dehydrogenase; NudL, putative NUDIX hydrolase; LtaE, L-allo-threonine aldolase; ThrB (and DUF1537), homoserine kinase; DUF2257, L-threonine dioxygenase; AroB, 3-dehydroquinate synthase; HisB, imidazoleglycerolphosphate dehydratase and histidinol phosphatase; Php, unknown function; YjbQ, unknown function; ThiG, thiazole synthase. RsgA is a GTPase involved in ribosome maturation in E. coli (*Campbell & Brown*, 2008). RsgA Q3 shares 38% overall sequence identify with the *B. subtilis* CpgA protein, which was shown to dephosphorylate 4-phosphoerythronate (*Sachla & Helmann*, 2019). It is tempting to speculate that RsgA is also capable of dephosphorylating 4-phosphoerythronate (adapted from *Richts & Commichau*, 2021)

In an evolutionary approach *E. coli* linages were cultivated for 150 generations, leading to the description of another novel pathway feeding into the DXP-dependent pathway (*Kim et al.*, 2019). The pathway uses 4PE, which is dephosphorylated by an unknown phosphatase to erythronate. Furthermore, SerA and SerC originally are involved in serine metabolism and transform erythronate to

4HT, which is again, utilized by ThrB to 4HTP (*Kim et al.*, 2019). Instead of ThrB also proteins of the DUF1537 kinase family are able to convert 4HT to 4HTP (*Smirnov et al.*, 2012; *Thiaville et al.*, 2016; *X. Zhang et al.*, 2016). These proteins are known to be active in catabolism of carbon acid sugars as L-threonate or D-erythronate (*X. Zhang et al.*, 2016). It was recently shown that in *B. subtilis* the protein CpgA, which is necessary for ribosomal maturation also moonlights as a phosphatase, detoxifying 4PE (*Sachla & Helmann*, 2019).

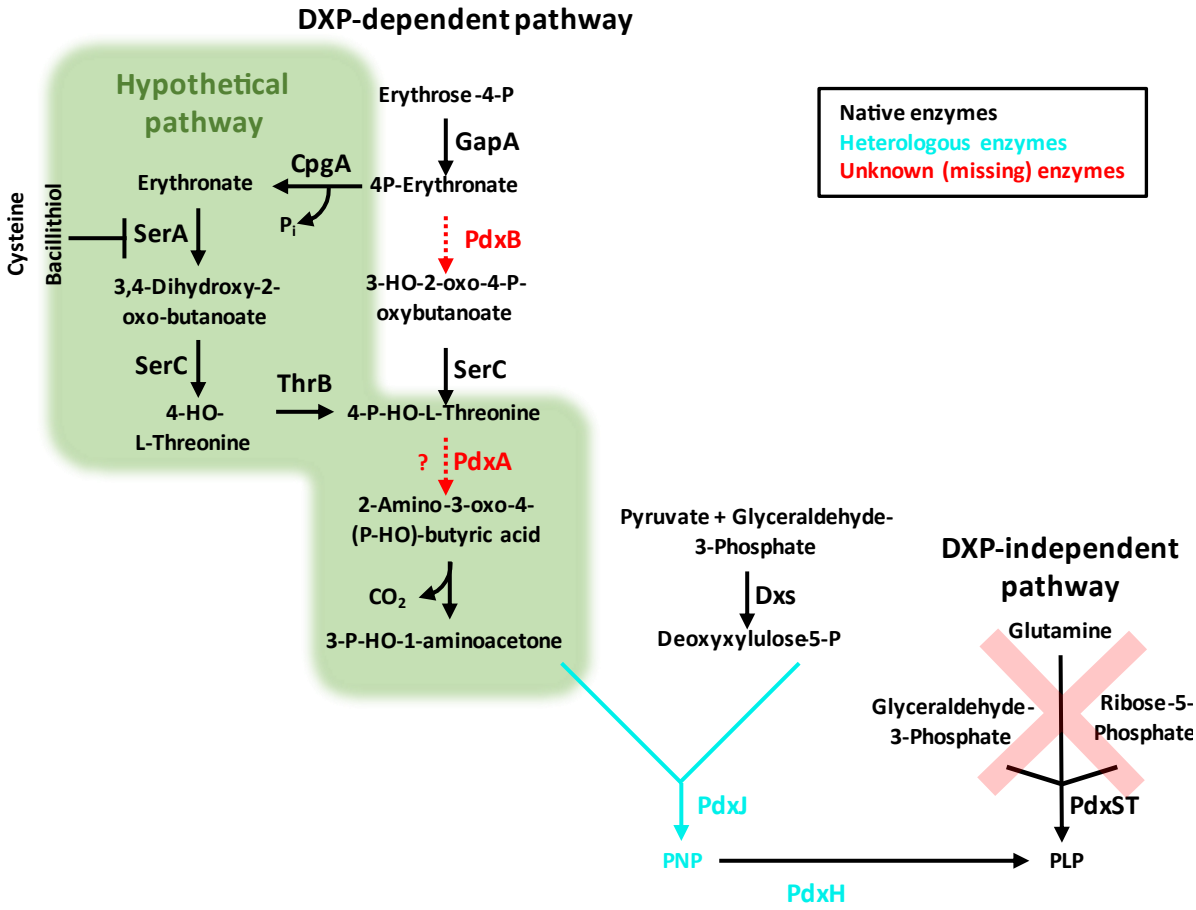


Figure 7 Putative serendipitous pathways for vitamin B6 synthesis in *B. subtilis*.
GapA, glyceraldehyde 3-phosphate dehydrogenase; CpgA, phosphatase; SerA, phosphoglycerate dehydrogenase; SerC, 3-phosphoserine aminotransferase; ThrB, homoserine kinase; PdxJ, pyridoxine 5′-phosphate synthase; Dxs, 1-deoxyxylulose 5-phosphate synthase; PdxH, pyridoxine 5'-phosphate oxidase; PdxS (PLP synthase subunit) and PdxT (glutaminase subunit) form the PdxST PLP synthase complex; YtoQ, a protein of unknown function (adapted from *Richts & Commichau*, 2021)

As described above, overexpression of the PdxST complex in *E. coli* yielded in low level production of PLP as it is slower than the DXP-independent pathway (*J. Rosenberg et al.*, 2020; *Yocum et al.*, 2005). Therefore, it is reasonable to use the DXP-dependent pathway of *E.coli* for production of vitamin B6. It was tried to evolve a *B. subtilis* strain harboring only a partial heterologous pathway of *E. coli* (*J. Rosenberg et al.*, 2018; *J. Rosenberg & Commichau*, 2018). To accomplish that, genes of the DXP-

dependent pathway were integrated step by step into a B6 auxotrophic *B. subtilis* strain (Δ*pdxST*), starting with the last gene *pdxH*. Interestingly, a strain expressing only PdxH and PdxJ showed poor growth on minimal medium without vitamin B6 supplementation and formed suppressor mutants after prolonged incubation (*J. Rosenberg et al.*, 2018). Sequencing revealed mutations inactivating genes required for the synthesis of bacillithiol, which is a Low-Molecular-Weight (LMW) thiol involved in oxidative stress response, redox buffering and in fosfomycin resistance (*Gaballa et al.*, 2010; *Newton et al.*, 2012). Since the suppressor mutant showed only slow growth, it was further evolved in minimal medium and reached a growth speed, comparable to that of the wild type strain. Whole genome sequencing showed an upregulation of the *ytoQ* gene and plasmid based overexpression of *ytoQ* in a Δ*pdxST pdxJH ΔbshC* mutant could suppress the loss of the *pdxST* genes (*J. Rosenberg et al.*, 2018). The integration of only two foreign genes was already enough to establish an underground metabolism pathway in *B. subtilis* for the production of PLP. We therefore compared the enzymes of the known underground metabolism pathways with the *B. subtilis* proteome and were able to identify homologous enzymes (see Figure 7) (*Richts & Commichau*, 2021).

When PdxJ and PdxH are present in *B. subtilis* only two enzymes are missing to have the full DXP-independent pathway: PdxA and PdxB. GapA, SerC and Dxs can catalyze the steps oxidizing E4P, converting 3-hydroxy-2-oxo-4-phosphooxybutanoate to 4-P-HO-L-threonine and producing DXP, respectively. It has been shown that *B. subtilis* CpgA is able to dephosphorylate 4PE to erythonate (*Sachla & Helmann*, 2019). Besides that, also enzymes of the serine pathway could be involved, as erythronate can be converted by the promiscuous activities of the 3PG dehydrogenase SerA, the serine aminotransferase SerC and the homoserine kinase ThrB to 4-P-HO-L-thronine. It was already confirmed that *B. subtilis* SerC and ThrB are active in a complete non-native DXP-dependent vitamin B6 synthesis pathway (*Commichau et al.*, 2014, 2015). As the function of PdxB could be detoured, only the promiscuous enzyme replacing PdxA remains unclear. Nevertheless, the function of the unknown protein YtoQ remains to be clarified as the protein is required for PLP production (*J. Rosenberg et al.*, 2018).

1.8 **Nitrogen metabolism**

B. subtilis harbors a complex regulatory system to maintain the balance of nitrogen and carbon metabolism as both cycles are interconnected. *α*-ketoglutarate is formed during the tricarboxylic acid (TCA) cycle and builds the link to the nitrogen metabolism as it is used by the glutamate synthase (GOGAT) to form glutamate (see Figure 8A) (*Belitsky*, 2001; *Commichau et al.*, 2006). The necessary ammonium derives from glutamine, which is transaminated by the GOGAT using NADPH. Thus, two molecules of glutamate are produced of, which one molecule can be used for further metabolism and

one stays in the cycle (*Belitsky*, 2001). The back reaction is also possible. Glutamate reacts back to *α*-ketoglutarate with the help of the glutamate dehydrogenases (GDH), which reduces NAD^+ to NADH and releases ammonium. By that glutamate can be used as a source of carbon as *α*-ketoglutarate can enter the TCA cycle (*Belitsky & Sonenshein*, 1998; *Gunka et al.*, 2013). In contrast to the GDHs of other organisms like *E. coli*, the *B. subtilis* GDH has a low affinity for ammonium, which makes an anabolic reaction to glutamate impossible under normal conditions (*Commichau et al*., 2008). Therefore, in *B subtilis* the integration of ammonium into the nitrogen cycle can only be achieved in the last step: the amination of glutamate by the glutamine synthetase GlnA. GlnA utilizes glutamate and free ammonium to form glutamine in an ATP-dependent reaction (*Belitsky*, 2001).

The most efficient nitrogen source for *B. subtilis* is glutamine but in absence of it, also ammonium is preferably taken up by the cells (*Fisher & Débarbouillé*, 2001; *Hu et al.*, 1999). The state of ammonium is an equilibrium between the gaseous form ammonia (NH_3) and the ionic form ammonium (NH_4^+), which depends on the pH value. At high pH the balance is shifted towards ammonia (NH_3), which is able to diffuse through the cell membrane. For ammonium the transporter NrgA is needed (*Detsch & Stülke*, 2003). To keep the nitrogen cycle in balance, it needs to be highly regulated. In a mutant screen the TnrA protein was identified as a regulator of the *nrgAB* operon, which codes for the ammonium channel NrgA and the PII-protein NrgB and it could be shown that TnrA activates its expression and by that helps channeling ammonium into the cells (*Gunka & Commichau*, 2012; *L V Wray et al.*, 1996). The P_{II} protein NrgB is co-localized with NrgA at the cell membrane in absence of ATP and is necessary for a full expression of the *nrgAB* operon (*Detsch & Stülke*, 2003; *Hauf et al.*, 2016; *Heinrich et al.*, 2006). P_{II} proteins are often involved in sensing the nitrogen status of a cells and building a monitor system of nitrogen availability (*Atkinson & Fisher*, 1991; *Wray et al.*, 1994).

TnrA is a global nitrogen regulator and binds to at least 84 genes in *B. subtilis* and at least 42 DNA regions (*Mirouze et al.*, 2015; *Zhu & Stülke*, 2018). It is a positive regulator of its own expression and, in absence of a good nitrogen source, TnrA mostly upregulates genes, which are involved in the uptake of glutamine and ammonium or in the assimilation of nitrate und nitrite. The glutamine synthetase and the glutamate synthase are downregulated under these conditions (*S H Fisher*, 1999; *Wray et al.*, 1996; *Yoshida et al.*, 2003). Together with GlnR, which is also a regulator of nitrogen metabolism, it belongs to the group of the TnrA/GlnR family of transcription regulators (*Schumacher et al.*, 2015). The glutamine synthase GlnA also plays a regulatory role besides its enzymatic function as the *glnR* and the *tnrA* gene is expressed constitutively when *glnA* is deleted (*Fisher & Wray*, 2008). These kind of proteins, which have a catalytic activity and also play a role in gene regulation are called trigger enzymes (*Commichau & Stülke*, 2015).

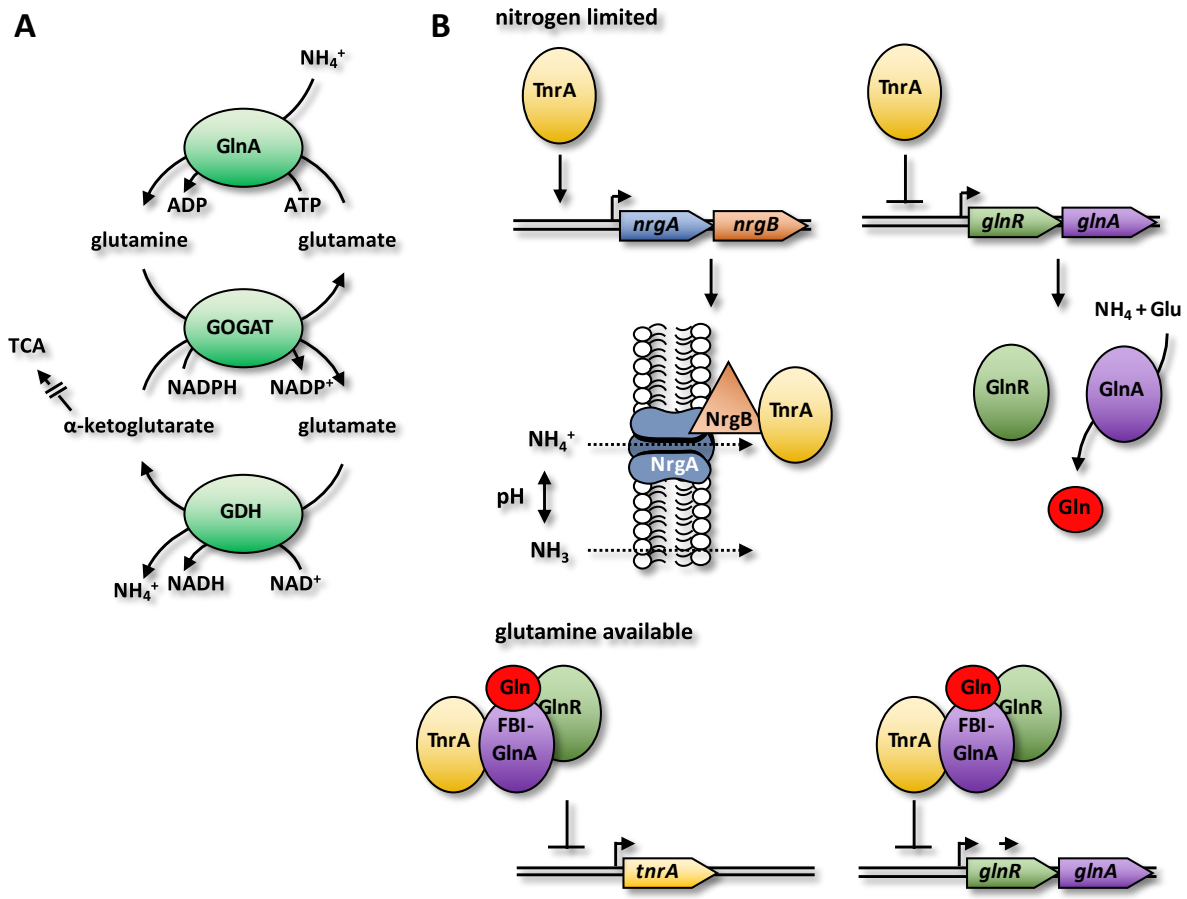


Figure 8 Regulation of nitrogen metabolism in *Bacillus subtilis*.
A: In *B. subtilis* free ammonium can be transferred to glutamate by GlnA. The formed glutamine can transfer this amino group to α-ketoglutarate via the GOGAT. By that two molecules of glutamate are formed from glutamine and α-ketoglutarate. The glutamate dehydrogenase cleaves off ammonium from glutamate resulting in free ammonium and α-ketoglutarate. GlnA: glutamine synthetase, GOGAT: glutamate synthase, GDH: glutamate dehydrogenase. **B**: If nitrogen is limited the global nitrogen regulator TnrA promotes the expression of *nrgAB* coding for the ammonium channel NrgA and the PII protein NrgB. Ammonium can enter the cell and as the nitrogen levels elevate, expression of the regulator GlnR and GlnA is no longer repressed by TnrA. By that ammonium can be utilized by the glutamine synthetase GlnA. Rising glutamine levels lead to feedback inhibition of GlnA (FBI-GlnA). FBI-GlnA can bind TnrA and GlnR. Thus, transcription of *tnrA* and of *glnRA* is blocked.

Excess of nitrogen leads to high amounts of glutamine by GlnA. At a certain point the glutamine synthase is feedback inhibited by glutamine (FBI-GlnA) (*Hauf et al.*, 2016; *Murray et al.*, 2013) and TnrA can bind to the FBI-GlnA. Consequently the oligomerization state of GlnA changes from its dodecameric form to a tetramer, which inactivates the glutamine synthesis capacities (*Murray et al.*, 2013;W*ray et al.*, 1996, 2001). In this bound state, TnrA cannot bind to the DNA and the *nrgAB* is not expressed (*Fisher & Wray*, 2008, 2009; *Wray et al.*, 2001; *Wray & Fisher*, 2010). For the binding of TnrA to GlnA, the last six amino acids of C-terminal domain are important. If they are not present, GlnA and TnrA cannot interact with each other (*Kayumov et al.*, 2011; *Shin et al.*, 2000; *Wray & Fisher*, 2007). The binding site to GlnA is located near the active site (*Fisher et al.*, 2002; *Fisher & Wray*, 2006, 2008; *Wray & Fisher*, 2005). TnrA as well as GlnR bind to GlnA under high nitrogen availability. Thereby, TnrA dimerizes and the GlnR-DNA complex is stabilized. Thus, expression of *glnRA* and *tnrA* can be repressed

(*S. W. Brown & Sonenshein*, 1996; *Fisher*, 1999; *Fisher & Wray*, 2008; *Schumacher et al.*, 2015; *Wray et al.*, 2001).

During growth under nitrogen limitation, the cellular glutamine levels are low and GlnA is not feedback inhibited but in an ATP bound state. TnrA is released from the GlnA-TnrA complex and it promotes binding to NrgB at the membrane. Therefore, its dimeric form is stabilized, which is necessary for activating *nrgAB* and ammonium can be taken up until a threshold is reached, where NrgA is blocked for new ammonium (*Hauf et al.*, 2016; *Heinrich et al.*, 2006; *Kayumov et al.*, 2011; *Schumacher et al.*, 2015). As GlnR can also bind only to the FBI-GlnA, it is released upon nitrogen limitation. In the unbound status it cannot bind to the DNA anymore, as it has an autoinhibitory function. Besides that, GlnA retains its dodecameric status and in its catalytically active state the enzyme produces new glutamine (*Hauf et al.*, 2016).

In a *nrgA* mutant, TnrA is bound to the cytoplasmic NrgB. If *nrgB* is deleted, TnrA does not bind to FBI-GlnA and lower its activity, which leads to an accumulation and secretion of ammonium into the medium (*Fedorova et al.*, 2013; *Kayumov et al.*, 2011).

TnrA also affects the expression of the *gltAB* genes, which code for the GOGAT. When nitrogen is limited, TnrA binds downstream of the *gltAB* promoter and blocks expression of the GOGAT (*Belitsky et al.*, 2000). When ammonium and *α*-ketoglutarate are present, *gltAB* is expressed because TnrA is bound to GlnA and cannot access the regulatory region of *gltAB* (*Belitsky et al.*, 2000; *Murray et al.*, 2013). Under these conditions, the LysR-type transcriptional activator GltC binds to the promoter region and activates the transcription of the *gltAB* genes (*Bohannon & Sonenshein*, 1989). A detailed regulation of the full nitrogen cycle is described in (*Gunka & Commichau*, 2012).

1.9 Bacillithiol (oxidative stress response)

As a soil living bacterium, *B. subtilis* has to cope all kinds of environmental stresses, among them oxidative stress, which is mostly dealt by reactive oxygen species (ROS) as hydrogen peroxide (H_2O_2), superoxides (O_2^-) hydroxyl radicals ($HO\cdot$) or reactive nitrogen species (NOS) as nitric oxide (NO). A reaction with these compounds leads to a primary oxidative stress response (*Antelmann & Helmann*, 2011). ROS can also actively be produced as anti-bacterial compounds to compete against other bacteria, which live in the same habitats. If the organism is stressed for longer period of time, this can have macromolecular damage as a consequence. Following this, increased mutation rates and subsequently cell death can be the consequences (*Dukan & Nyström*, 1998; *Imlay*, 2003). Oxygen is a very reactive molecule as it has two unpaired electrons in its outer shell, which can easily oxidize chemicals like metals or different carbohydrates. Reduction of oxygen can only occur sequentially, leading to the formation of reactive single radicals (*Imlay*, 2003, 2008). Those reactive oxygen species

can furthermore derive from respiratory chain, mainly from oxidation by reduced menaquinone, which can donate electrons to oxygen and thus form superoxides and H_2O_2. They can further take electrons from any molecule with low redox potential, mainly reduced metals, to form hydroxyl radicals (*Imlay & Linn*, 1988). An oxidative stress response can also be induced by general stressor exposure like heat, salt, acids or antibiotics, which is therefore entitled as secondary oxidative stress response. (*Kohanski et al.*, 2007; *Mols et al.*, 2009; *Mols & Abee*, 2011)

ROS and RNS can oxidize thiols, mostly cysteine and homocysteine residues of proteins. A reaction with free cysteine is too slow to cause any damage (*Di Simplicio et al.*, 2003; *J. W. Lee et al.*, 2007; *Winterbourn & Hampton*, 2008). If thiol residues in proteins are oxidized, this can limit the functionality of the enzymes or even lead to inactivation, as cysteine residues are often located in active sites of many enzymes. These cysteines can form disulfide bounds upon ROS treatment (*Hochgräfe et al.*, 2005, 2007; *Hondorp & Matthews*, 2004; *Winterbourn & Hampton*, 2008).

Nitric oxide can oxidize iron containing proteins as the cytochrome oxidase, which is necessary for the electron transport chain. Furthermore, it alters tyrosine or cysteine residues (*G. C. Brown et al.*, 1997; *Di Simplicio et al.*, 2003; *Rogers & Ding*, 2001; *Spiro*, 2007). As NO is a radical, it is highly reactive with oxygen or ROS forming different RNS. These are able to further attack cysteine residues but can also react with FeS-clusters and inhibit their function (*Di Simplicio et al.*, 2003; *Winterbourn & Hampton*, 2008). It was shown that addition of cysteine can rescue damaged FeS-clusters by releasing the dinitrosyl iron complexes into the cytoplasm and by that facilitate new iron-sulfur cluster formation (*Rogers & Ding*, 2001). Moreover, RNSs promote NADH and flavin^{red} formation, which forms hydroxyl radicals and reduce the amount of free iron (*Winterbourn & Hampton*, 2008)

Iron-containing enzymes are susceptible to oxidation. $[4Fe\text{-}4S]^+$ iron sulfur clusters are attacked by the ROS leading to the loss of one of the iron atoms $[3Fe\text{-}4S]^+$ and thereby to inactivity of the enzyme (*Imlay*, 2006; *Kiley & Beinert*, 2003). Fe^{3+} cations are released, which can be reduced by cysteine or flavin^{red} to Fe^{2+}. H_2O_2 can again oxidize the iron cation to form toxic hydroxylperoxide radicals being able to oxidize amino acid residues
(*Imlay*, 2006; *Rogers & Ding*, 2001; *Spiro*, 2007; *Woodmansee & Imlay*, 2003). As a protection, FeS clusters can be shielded from reactive oxygen species by the substrates of the enzymes or by locating the clusters at the inside of the enzyme where ROS cannot enter (*Varghese et al.*, 2007). Furthermore, the Fur protein is a master regulator of iron homeostasis and regulates 65 genes of iron uptake and metabolism (*Zhu & Stülke*, 2018). The protein can be easily oxidized as it associated with iron (*Varghese et al.*, 2007). By that, the DNA binding ability is lost and a deregulation of iron uptake is the consequence, which can lead to even higher ROS formation and damage the DNA (*Bsat et al.*, 1998; *Imlay & Linn*, 1988; *Varghese et al.*, 2007).

B. subtilis harbors a general response to oxidative stress, which is controlled by the σ^B factor and regulation by PerR and OhrR (*Hecker et al.*, 2007; *Helmann et al.*, 2003). Upon oxidative stress or when entering the stationary phase, SigB can induce gene expression of detoxification systems as the superoxide dismutase (SodA), catalase (KatE) or organic hydroperoxide reductase (OhrB) (*Antelmann et al.*, 1997; *Engelmann et al.*, 1995; *Hecker et al.*, 2007). PerR senses the peroxide levels in the cell with its associated Fe^{2+} and activates expression of genes protecting the DNA from Fe-based ROS formation (Fenton reaction), detoxifying ROS (catalase, alkylhydoperoxide reductase) and synthesizing heme. Mn^{2+} can also associate with PerR and thereby strongly decreases the sensitivity towards H_2O_2 (*Herbig & Helmann*, 2001). As manganese is more resistant to ROS, it can take over if the ROS levels are too high for iron-based sensing and by that maintain regulation under harsh conditions (*Imlay*, 2008).

The OhrR regulator belongs to the multiple antibiotic resistance regulators (MarR-type) and controls expression of the hydroperoxide reductase *ohrA*. A second hydroperoxide reductase *ohrB* is controlled by the sigma factor B (*Hecker et al.*, 2007). The binding ability of OhrR is controlled by the oxidation status of a conserved cysteine residue (Cys15), making it to a thiol-based sensor. This cysteine residue can also be target of bacillithiolation (see below) (*Chi et al.*, 2011; *J. W. Lee et al.*, 2007; *Soonsanga et al.*, 2007, 2008).

The last regulator is Spx, which is conserved among Gram-positive bacteria and belongs to the group of the ArsC (arsenate reductase) protein family as it harbors the typical CXXC redox motif to control its activity. It is a regulator, which directly binds to the α-subunit of the RNA polymerase (RNAP) (*Choi et al.*, 2006; *Nakano et al.*, 2003, 2005). Deletion of *spx* in *B. subtilis* leads to sensitivity for thiol specific oxidants as diamide and other thiol-depleting agents (*Nakano & Küster-Schöck, et al.*, 2003; *Zuber*, 2009).To execute its role as a regulator, the CXXC motif has to be in its oxidized form. Thus, addition of a reductant as DTT blocks transcriptional activation as Spx cannot bind to the RNAP anymore (*Nakano et al.*, 2005). The binding happens through the C-terminal part of the α-subunit of the RNA polymerase, which possesses an important tyrosine residue in one of its α-helices and is relevant for binding (*Newberry et al.*, 2005). When bound to the polymerase, Spx can hinder other RNAP binding activators from interacting and thereby inhibit their regulatory function. This can cause toxic effects (*Nakano et al.*, 2003). The *spx* gene lies in an operon with *yjbC* and can be expressed from four different promoters involving at least the sigma factors A,B and M (*Antelmann et al.*, 2000; *Min Cao et al.*, 2002; *Eiamphungporn & Helmann*, 2008; *Leelakriangsak et al.*, 2007). Thus, Spx can be formed under different stress conditions as cell envelope stress (sigma M), thiol-reactive electrophiles (YodB) or ROS stress (PerP) (*Min Cao et al.*, 2002; *Eiamphungporn & Helmann*, 2008; *Helmann et al.*, 2003). The Spx levels are controlled by the ATP-dependent protease ClpXP, which prevents Spx from

accumulation (*Nakano et al.*, 2003). YjbC is an adapter protein for ClpXP and modulates its Spx degradation ability (*Garg et al.*, 2009). Spx mainly activates genes involved in thiol homeostasis as *trxA* and *trxB*, cysteine biosynthesis as *yrrT*, *cysK* and *trxA*, detoxification or NADPH production but also genes involved in the formation of the low-molecular weight thiol bacillithiol (*Choi et al.*, 2006; *Gaballa et al.*, 2013; *Nakano & Küster-Schöck, et al.*, 2003). Genes involved in genetic competence formation or metabolic pathways are repressed in presence of Spx (*Nakano & Küster-Schöck, et al.*, 2003).

LMW thiols are important to maintain proteins in their functional states, especially when experiencing oxidative or antibiotic stress (*Chandrangsu et al.*, 2018; *Laer et al.*, 2013; *Van Loi et al.*, 2015). In most eukaryotes and Gram-negative bacteria, but also in few Gram-positive bacteria as *L. monocytogenes*, the main LMW thiol is glutathione (GSH) (*Potter et al.*, 2012; *Van Loi et al.*, 2015). In Gram-positive bacteria mostly use other LMW thiols as for example mycothiol in high-GC containing *Actinomycetes* (*Newton et al.*, 2008; *Reyes et al.*, 2018). BSH was discovered not long ago in *Bacillus anthracis* but is also present in *B. subtilis* and other closely related low GC species as *S. aureus* (*Gaballa et al.*, 2010; *Newton et al.*, 2012) (see Figure 9). BSH together with its derivatives are supposed to be the most widely distributed LMW thiols (*Hiras et al.*, 2018) . If BSH synthesis is compromised, some bacteria can even use coenzyme A (CoA) as a replacement (*Boylan et al.*, 2006; *DelCardayré & Davies*, 1998; *Van Loi et al.*, 2015).

BSH is produced from Uridinediphosphate-N-acetylglucosamin (UDP-GlcNAc) and malic acid in a reaction involving three enzymes. The glycosyltransferase BshA catalyzes the reaction of UDP-GlcNAc and malic acid to N-acetylglucosamine malate (GlcNAc-Mal), which is deacetylated by the N-acetylglucosamine-malate deacetylase BshB. Resulting N-glucoseamine malate (GlcN-Mal) is fused with cysteine by the putative cysteine ligase BhsC (*Chandrangsu et al.*, 2018; *Gaballa et al.*, 2010). The genes of *bshA* and *bshB1* are located in one operon together with methylglyoxal synthase *mgsA* and *birA*, the biotin ligase. The expression of the operon is under control of Spx and thereby sensitive to thiol stress (*Hecker et al.*, 2007; *Zhu & Stülke*, 2018; *Zuber*, 2009). The cysteine ligase gene *bshC* is also under the regulation of Spx but is located at a different position in the genome, together with *ylbQ*, which is needed for the synthesis of acetyl-CoA (*Gaballa et al.*, 2013; *Zhu & Stülke*, 2018). In *B. subtilis* there is a second homolog of BshB1, which is called BshB2 but it has only small impact on BSH synthesis (*Fang et al.*, 2013).

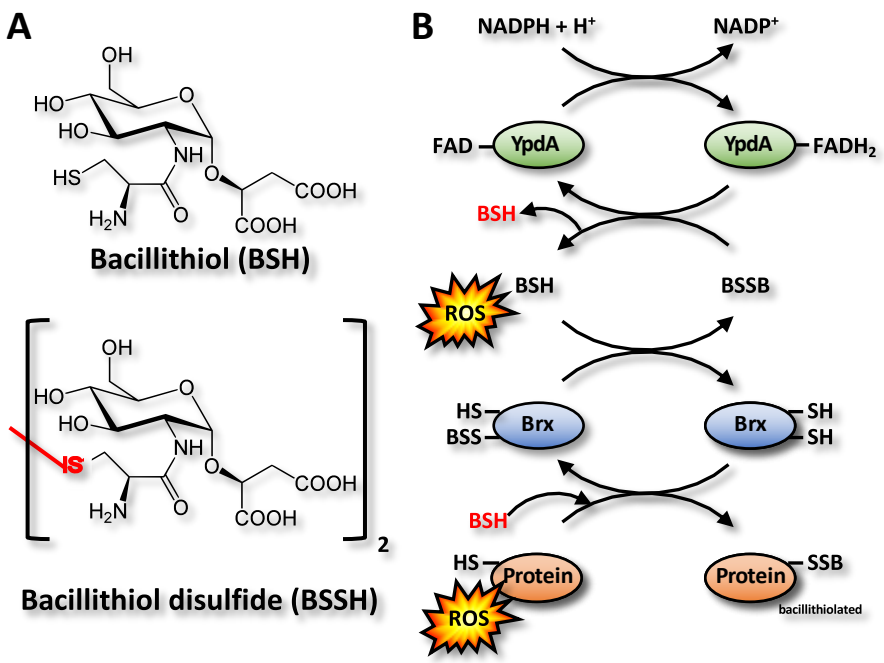


Figure 9 Bacillithiolation cycle.
A: chemical structures of bacillithiol (BSH) and its oxidized form bacillithiol disulfide (BSSH). **B:** Upon ROS formation, bacillithiol can be either oxidized to bacillithiol disulfide (BSSB) or can be used for S-bacilithiolation of proteins. The latter ones can be debacillithiolated by bacilliredoxin Brx. BSSB can be regenerated by the NADPH-dependent YpdA. Figure adapted from (*Hammerstad et al.*, 2020)

In case ROS are present in the cells, cysteine residues of oxidation prone proteins can be S-bacillithiolated and thereby protected. Bacillithiolation is a post-translational modification, which oxidizes thiol moieties and forms mixed disulfides with BSH (see Figure 9) (*J. W. Lee et al.*, 2007). In order to reverse this step, the bacilliredoxins BrxA and BrxB (Bxs) are able to transfer the BSH onto a cysteine in its active site and thereby releasing the de-modified protein. Under oxidative stress BSH can acts as a reductant by donating electrons. By that is oxidized and disulfide bond between two molecules of BSH is formed. Furthermore, the BSH from Brx can be transferred to BSH forming BSSB (*Gaballa et al.*, 2014; *Sharma et al.*, 2013). BSSB can be recycled by the NADPH-dependent flavin oxidoreductase YpdA (Bdr in *B. subtilis*) (*Hammerstad et al.*, 2020; *Linzner et al.*, 2019; *Mikheyeva et al.*, 2019; *Zhu & Stülke*, 2018). Although BSH is formed especially under peroxide stress, S-bacillithiolation of proteins could not detected under these conditions (*Chi et al.*, 2011, 2013). Nevertheless, bacillithiolation does not only protect the proteins, it can also serve as an inactivator for enzymes or transcriptional regulators (*Chi et al.*, 2011, 2013).

When BSH synthesis is disrupted, *B. subtilis* mutants are more sensitive to ROS, diamides, hypochloride, methylglyoxal osmotic stress and the antibiotic fosfomycin (*Chandrangsu et al.*, 2018; *Chi et al.*, 2011; *Gaballa et al.*, 2010). BSH is a cofactor for the epoxide hydrolase FosB, which is a detoxifying protein for fosfomycin. Without BSH, the antibiotic cannot be degraded and its toxic effect increases (*Gaballa et al.*, 2010; *Heaton et al.*, 1988; *Lamers et al.*, 2012). Following this, activity of iron-sulfur cluster containing enzymes is reduced in BSH-deficient mutants, also lowering the intracellular amounts of branched amino acids leucine and isoleucine (*Chandrangsu et al.*, 2018; *Fang & Dos Santos*,

2015; *Rosario-Cruz et al.*, 2015). BSH is involved in iron homeostasis and BSH mutants have a delayed growth in minimal medium if casamino acids (CAA) or iron is not supplemented (*Fang & Dos Santos*, 2015).

BSH can protect the cells also from reactive electrophile species (RES) and methylglyoxal (*Ma et al.*, 2014; *Newton et al.*, 2011; *Perera et al.*, 2014; *Rajan et al.*, 2004). It can binds to methylglyoxal and forms BSH-hemithioacetal, which can further react to lactate by the GlxA and GlxB glyoxalases (*Chandrangsu et al.*, 2014). S-lactoyl-BSH, a metabolite of this reaction pathway also acts as an activator for the potassium/proton antiporter KhtSTU and thereby promoting proton import and cytosol acidification, which protects from nucleophilic damage (*Chandrangsu et al.*, 2014). To detoxify electrophiles or other toxins, BSH is conjugated with the toxin by the BSH-S-transferase BstA. The emerging conjugates are then deacetylated by BshB2 leaving CysS-conjugates and GlcN-Mal, which can be recycled to produce new BSH. The Cys-S-conjugate can be exported as a derivative of mercapturic acid (*Chandrangsu et al.*, 2018).

Lastly, BSH is a metal iron buffer and can take part in formaldehyde detoxification (*Chandrangsu et al.*, 2018; *Harms et al.*, 1996; *Huyen et al.*, 2009; *Kay et al.*, 2016; *Ma et al.*, 2014). It was shown to control sulfide homeostasis in *S. aureus* under nitroxyl and H_2S stress (*Peng, et al.*, 2017a, 2017b).

1.10 **The MiniBacillus project**

The *MiniBacillus* project aims to generate a genome-reduced *B. subtilis* strain harboring only a defined set of genes (*Reuß et al.*, 2017). As every gene should have an assigned function, this can give further information about the genes that are necessary to establish life. Furthermore, genome minimized organisms can be suitable as special production hosts, since genes interfering with the production may be deleted (*Van Tilburg et al.*, 2020). The genomic deletions were made in a top-down approach in multiple steps. Therefore, a blueprint of the final minimized cell was made, based on the data of Kooh et al. and Kobayashi *et al*. who generated *B. subtilis* deletion library and depicted all genes, which are essential in the complex medium supplemented with glucose and growth at 37°C. This data has been intensively studied to build the blueprint with all important genes, either being essential or providing necessary metabolic features (*Commichau et al.*, 2013; *Juhas et al.*, 2014; *Kobayashi et al.*, 2003; *Koo et al.*, 2017; *Reuß et al.*, 2016).

All in all, 257 essential genes were identified and added to the blueprint. Furthermore, genes involved in competence and genomic stability were kept to guarantee successful deletion processes (*Reuß et al.*, 2016). The conditions for the genome minimization were also defined to the usage of LB-Glc medium and cultivation at 37°C. Therefore, amino acids are already present in the medium and amino acid transporter genes were favored in the blueprint in contrast to the synthesis genes. Glucose served

as the main carbon source allowing limitation of the carbon metabolism to glycolysis and pentose phosphate pathway. Thus, the citric acid cycle can be deleted, which was already tried in the *B. subtilis* wild type (http://hdl.handle.net/11858/00-1735-0000-002E-E5F8-F).

The *MiniBacillus* project was established by the group of Josef Altenbuchner. They started the genome minimization in the Δ6 strain, which already had deletions of all prophage genes (see Figure 10) (*Wenzel & Altenbuchner*, 2015). The deletion process in Göttingen started with the strain IIG-Bs27-47-24 with a genome reduction of 33%. After further deletion of 0.1 Mbps, the resulting strain PG10 was subjected to multi omics analysis, showing changes in the transcriptome, proteome and metabolome profiles (*Reuß et al.*, 2017). At the point of the PG18 (2.67 Mb left), the deletion process stumbled as it came to a dead end in PG21 (2.62 Mb left). The deletions lead to a loss of genetic competence, as the *nrnA* gene was deleted, which encodes for a nanoRNase NrnA and it was previously shown that deletion of phosphatases can reduce the genetic competence (*Reuß et al.*, 2017; *Luttinger et al.*, 1996). Therefore, a different deletion route was taken, with the PG18 as the starting point but resulted again in a dead end in PG28. This strain lost the chromosome integrity and thereby genetic stability (*Reuß et al.*, 2017). Again, PG18 served as a starting point and further deletions could be successfully made, reaching a final genome reduction of 40.5% in PG39. Phenotypic analysis showed no big differences compared to the wild type and also growth behavior of the strain was similar to the wild type in LB-Glc medium (http://hdl.handle.net/11858/00-1735-0000-002E-E5F8-F).

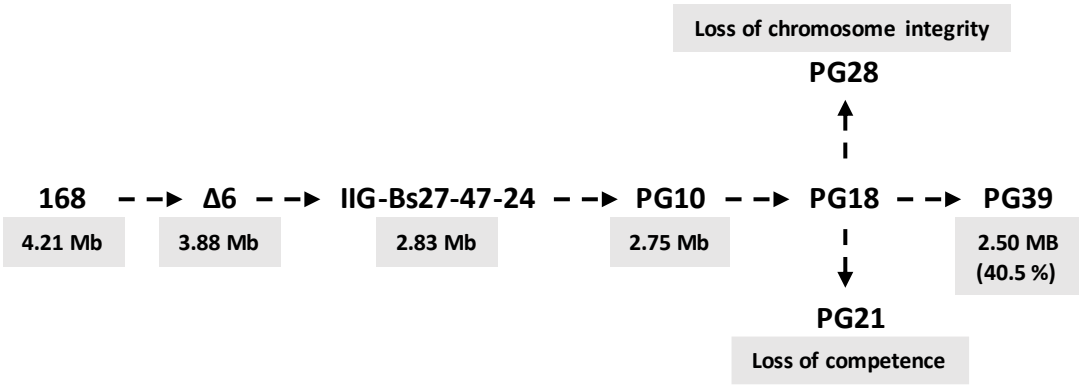


Figure 10 Deletion progress of the *MiniBacillus* project.
Deletions were made in multiple steps. The scheme only shows the the most important strains. Gey boxes represent the remaining number of base pairs. During the deletion process, two dead ends happened, where further deletions were not possible anymore and deletions needed to be carried out from an earlier strain (PG18).

The procedure of the project aims to display all necessary gene functions. Therefore, a final genome reduced strain, should be objected to transposon mutagenesis to find out, which of the last present genes can further be deleted (*Juhas et al.*, 2014). Additionally, the genome reduced strain should be

evolved to improve cultivation behavior and get new insights how genome reduced strains can adapt to their surroundings.

1.11 Aims of the work

To further minimize the genome of the *MiniBacillus,* it is necessary to understand all metabolic pathways to potentially adapt the blueprint. As the regulatory network of the vitamin B6 synthesis is only barely known in *B. subtilis,* this work focuses on the DXP-independent pathway including the PdxST complex. Since the PdxS synthase subunit can produce vitamin B6 without the glutaminase subunit PdxT, the relation between PdxS and PdxT is investigated. Therefore, the vitamin B6 sythesis is streamlined by an evolutionary experiment of a Δ*pdxT* deletion mutant to produce wild type-like levels of PLP in absence of PdxT. Furthermore, transporter candidate genes are deleted to identify the vitamin B6 transport mechanism in *B. subtilis.* This would enable the deletion of the vitamin B6 synthesis pathway in the *MiniBacillus* strain, as its blueprint prefers transport of metabolites over synthesis.

Since the latest genome reduced strain PG39 lost its genetic competence, an evolutionary experiment is applied to enhance general fitness and re-establish transformability. By that, the deletion process can be continued.

Besides, a vitamin B6 auxotroph *B. subtilis* strain, carrying only the last two gene of the *E. coli* DXP-dependent vitamin B6 synthesis pathway, was found to produce PLP when the bacillithiol synthesis gene *bshC* is deleted and the *ytoQ* gene is overexpressed. In this thesis the role of YtoQ and Bacillithiol in the heterologous vitamin B6 synthesis pathway is assessed.

2. Materials and Methods

2.1 Materials

All used chemicals, antibodies, utilities, equipment, commercial systems, proteins, enzymes and oligonucleotides are listed in the appendix. Strains and plasmids are also mentioned in the appendix.

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in the appendix.

Growth Media

Media were made with deionized water and autoclaved for 20 min at 121°C. Chemicals, which are not thermostable were dissolved and filtered. In order to obtain agar plates, the media were supplemented with agar to a final concentration of 1.5% (w/v).

LB-medium	10 g	Trypton
	5 g	Yeast extract
	10 g	NaCl
	Ad	H_2O
	1000 ml	
SP-medium	8 g	Nutrient broth
	0.25 g	$MgSO_4 \times 7\ H_2O$
	1 g	KCl
	Ad	H_2O
	1000 ml	
		After autoclaving add
	1 ml	$CaCl_2$ (0.5 M)
	1 ml	$MnCl_2$ (10 mM)
	2 ml	CAF (2.2 mg/ml)
10xMN medium	136 g	$K_2HPO_4 \times 3\ H_2O$
	60 g	KH_2PO_4
	10 g	Sodium-citrate × 2 H_2O
	Ad	H_2O
	1000 ml	
1xMNGE	1 ml	10xMn medium
	400 µl	Glucose (50% (w/v))
	50 µl	Potassium Glutamate (40% (w/v))
	50 µl	CAF (2.2 mg/ml)
	100 µl	Tryptophane (5 mg/ml)
	30 µl	$MgSO_4$ (1 M)
	(100 µl)	(CAA 10% (w/v))
	Ad 10 ml	H_2O
III' salts	0.232 g	$MnSO_4 \times 4\ H_2O$
	12.3 g	$MgSO_4 \times 7\ H_2O$
	Ad	H_2O
	1000 ml	

5x C salts	20 g	KH_2PO_4
	80 g	$K_2HPO_4 \times 3\ H_2O$
	16.5 g	$(NH_4)_2SO_4$
	Ad 1000 ml	H_2O
C medium	20 ml	5x C salts
	1 ml	Tryptophane (5 mg/ml)
	1 ml	CAF (2.2 mg/ml)
	1 ml	III' salts
	1 ml	Glucose (50%)
		For CE/CSE: glucose can be replaced with 2 ml potassium glutamate (40%) / 2 ml sodium succinate (30%) and 2 ml potassium glutamate (40%)
	Ad 100 ml	H_2O
5x MSSM medium stock	41 g	$Na_2HPO_4 \times 3\ H_2O$
	18.3 g	$NaH_2PO_4 \times 1\ H_2O$
	5 g	Na-citrate
	1 g	$MgSO_4 \times 7\ H_2O$
	Ad 1000 ml	
MSSM medium	200 ml	5x MSSM medium stock
	10 ml	100x Trace elements
	10 ml	100x Iron citrate
	5 ml	KCl (1M)
	10 ml	Glucose (50%)
	10 ml	Glutamine (2%)
	X	Ammonium chloride was added as described
		Adjust the pH to 6.5
	Ad 1000 ml	H_2O
100x Trace elements	0.735 g	$CaCl_2 \cdot 2\ H_2O$
	0.12 g	$MnCl_2 \cdot 6\ H_2O$
	0.17 g	$ZnCl_2$
	0.033 g	$CuCl_2 \cdot 2\ H_2O$
	0.06 g	$CoCl_2 \cdot 6\ H_2O$
	0.051 g	$Na_2MoO_4 \cdot 2\ H_2O$
	Ad 1000 ml	H_2O
100x Iron-Citrate	0.0135 g	$FeCl_3 \times 6\ H_2O$
	0.1 g	Na_3-citrate $\times 2\ H_2O$
	Ad 100 ml	H_2O
	Ad 1000 ml	H_2O
5x SM base	175 g	K_2HPO_4
	75	KH_2PO_4
	12.5	Na_3-Citrate $\times 2\ H_2O$
	2.5	$MgSO_4 \times 7\ H_2O$
	Ad 2000 ml	H_2O

SM medium	200 ml	5x SM base
	10 ml	100x Trace elements
	10 ml	100x Fe-Citrate
	10 ml	Glucose (50%)
	10 ml	Tryptophan (% mg / ml)
	10 ml	$(NH_4)_2\ SO_4$ (20%)
	Ad 1000 ml	H_2O
TSS	100 ml	10x Tris (0.5M, pH 7.5)
	1 ml	1000x Iron chloride (for TSS)
	1 ml	1000x Trace elements (for TSS)
	10 ml	Ammoniumchloride (20%)
	10 ml	Glucose (50%)
	10 ml	Glutamine (2%)
	Ad 1000 ml	H_2O
10x Tris (0.5M, pH 7.5)	60,57 g	Tris
		Adjust the pH 7.5
	Ad 1000 ml	H_2O
1000x Iron chloride (TSS)	40 g	$FeCl_3 \times 6\ H_2O$
	Ad 1000 ml	H_2O
1000x Trace elements (TSS)	570.55 g	$K_2HPO_4 \times 3\ H_2O$
	40 g	Na_3-Citrate $\times 2\ H_2O$
	0.2	$MgSO_4 \times 7\ H_2O$
	Ad 1000	H_2O

Antibiotics

In order to select for bacterial strains or plasmids, antibiotics were prepared in 1000-fold stock solutions, sterile filtrated (0.22 µm pore size) and stored at -20°C. After cooling down the autoclaved medium to ~50°C, the antibiotics were added.

Antibiotic	**Solvent**	**Stock (mg/ml)**	**Working concentration (µg/ml)**	
			E. coli	*B. subtilis*
Ampicillin	H_2O	100	100	-
Kanamycin	H_2O	10	50	10
Lincomycin[1]	H_2O	25	-	25
Erythromycin[1]	70% EtOH	2	5	2
Spectinomycin	H_2O	150	-	150
Tetracycline	70% EtOH	12.5	-	12.5
Zeocin (ThermoFisher)	-	100		30

[1] A mixture of erythromycin and lincomycin is used for selection on *ermC* (*Griffith et al.*, 1965).

Other buffers and solutions

Saline	0.9%	NaCl in water
Lysis Buffer	50 mg	Lysozyme
	50 µl	Tris-HCL pH 8.0 (1 M)
	10 µl	$Na_2EDTA \times 2\ H_2O$ (0.5 M)
	Ad 2.5 ml	H_2O
RNase A	20 mg/ml	
	Prior usage, heat for 5 min at 80°C	
50x TAE buffer	242 g	Tris-Base (2 M)
	57.1 ml	Acetic Acid (100%)
	100 ml	$Na_2EDTA \times 2\ H_2O$ pH 8 (0.5 M)
	Ad 1000 ml	H_2O
5x DNA loading dye	5 ml	Glycerol (100%)
	4.8 ml	H_2O
	0.2 ml	TAE (50x)
	10 mg	Bromphenol blue
Agarose	1%	In 1xTAE buffer
6x SDS loading dye	3.15 ml	Tris-HCl pH 6.8 (1M)
	600 µl	*β*-mercaptoethanol
	1.2 g	SDS
	6 ml	Glycerol
	6 mg	Bromphenol blue
	0.25 ml	H_2O
10x ZAP	60.57 g	Tris-base
	116.9 g	NaCl
	Adjust pH to 7.5 with HCl	
	Ad 1000 ml	H_2O
10x Buffer W	121.14 g	Tris-base
	87.7 g	NaCl
	3.72 g	$Na_2EDTA \times 2\ H_2O$
	Adjust the pH to 8 with HCl	
	Ad 1000 ml	H_2O
Buffer E	0.027 g	D-Desthiobiotin
	50 ml	Buffer W (1x)
10x Buffer R	121.14 g	Tris-base
	87.7 g	NaCl
	3.72 g	$Na_2EDTA \times 2\ H_2O$
	2.42 g	HABA
	Adjust the pH to 8 with HCl	
	Ad 1000 ml	H_2O
10x PBS	80 g	NaCl
	2 g	KCl
	14.24 g	Na_2HPO_4
	2.72 g	KH_2PO_4
	Adjust the pH to 6.5 with HCl	
	Ad 1000 ml	H_2O

PFA 4%	4 g	PFA in 1x PBS
10x PAGE Buffer	144 g	Glycine
	30.3 g	Tris-base
	10 g	SDS
	pH should be 8.3 without adjustment	
	Ad 1000 ml	H_2O
Fixation solution	10%	Acetic acid
	50%	Methanol
	Ad 1000 ml	H_2O
Staining solution	5 g	Coomassie Brilliant blue R-250
	10%	Acetic Acid
	45%	Methanol
De-stain solution	5%	Acetic acid
	20%	Ethanol
Silver-Fixation	50 %	Methanol
	12%	Acetic acid
	100 µl	Formaldehyde (37%)
Silver-Developer	6 g	Na_2CO_3
	2 ml	Thiosulfate solution
	50 µl	Formaldehyde (37%)
	Ad 100 ml	H_2O
Silver-Impregnator	0.2 g	$AgNO_3$
	37 µl	Formaldehyde
	Ad 100 ml	H_2O
Thiosulfate solution	20 mg	$Na_2S_2O_3 \times 5\ H_2O$
	Ad 100 ml	H_2O
Silver-Stop solution	18.612 g	$Na_2EDTA \times 2\ H_2O$
	pH should be 8.0 without adjustment	
	Ad 1000 ml	H_2O
Depurinization buffer	82.72 ml	HCl (37.5%)
	Ad 1000 ml	H_2O
Denaturation buffer	43.83 g	NaCl
	10 g	NaOH
	Ad 500 ml	H_2O
Neutralization buffer	121.14 g	Tris-base
	87.66 g	NaCl
	Ad 1000 ml	H_2O (adjust to pH 7.4)
20x SSPE	175.3 g	NaCl
	26.6 g	$NaH_2PO_4 \times 2\ H_2O$
	Ad 800 ml	H_2O
	7.4 g	Na_2EDTA
	Ad 100 ml	H_2O (adjust to pH 8-9)
	Merge both solutions and ad 1000 ml	H_2O (adjust pH to 7.4)
P1	100 ml	20x SSPE
	10 ml	SDS (10%)
	Ad 1000 ml	H_2O

5x DigP1	58.04 g	Maleic Acid
	43.83 g	NaCl
	36 g	NaOH
	Ad 1000 ml	H_2O (adjust to pH 7.5)
P2	5 ml	20x SSPE
	10 ml	SDS (10%)
	Ad 1000 ml	H_2O
P3	12.1 g	Tris-base
	5.8 g	NaCl2
	Ad 1000 ml	H_2O
Blocking solution	5g	Blocking reagent
	Ad 50 ml	1x DigP1
Pre-Hybridization solution	7.5 ml	20x SSPE
	3 ml	Blocking solution
	300 µl	N-Laurosylsarcosine (10%)
	60 µl	SDS solution (10%)
	Ad 30 ml	H_2O

2.2 Methods

The basic methods were adapted from the method collection of Sambrook, Fritsch, and Maniatis 1989.

Cultivation of Bacteria

If not differently remarked, *E. coli* and *B. subtilis* strains were cultivated in LB medium at 37°C with agitation (220 rpm). Therefore, a fresh colony from plate or material from a cryo-culture were used for inoculation.

In order to analyze the growth of bacteria, first a colony was taken to inoculate 4 ml LB medium supplemented with appropriate antibiotics. After incubation overnight, a pre-culture in the desired medium was set up and grown until the OD_{600} was ~0.5 – 0.8. The cell material was washed twice and used to inoculate 100 µl of fresh medium. The growth was monitored in the 96-well plate reader at 37°C with medium orbital shaking at 237 cpm (4 mm) in an Epoch 2 Microplate Sepctrophotometer, equipped with the Gen5 software (02.09.2001; BioTek)

To assess growth on plates, overnight cultures were centrifuged at 10,000 × g and resuspended in saline solution. After a washing step, the OD_{600} was set to 1 and cell material was propagated on the plates. Alternatively, bacterial strains were streaked on fresh plates and cell material was taken from the plates and resuspended in saline solution, washed and plated as described before.

The evaluation of growth on plates was made with a drop dilution assay. Therefore, the tested strains were grown over night in LB medium. The cultures were washed 2 times in saline and the OD was set to 1. A dilution series was made in 1:10 steps until a dilution of 10^{-7}. 10 µl of the dilutions were dropped onto the agar plate and the plates were incubated for up to two days at 37°C.

Pictures of bacterial strains were either taken with the GelDoc™ XR+ station or with smartphone cameras (Huawei P9 Pro/ Huawei P30 Pro).

For vitamin B6 auxotrophic strains PL was added to the medium to a final concentration of 100 µM. Tryptophan auxotrophic strains were cultivated with the addition of tryptophan (5µg/ml).

Storage of bacteria

For longtime storage 900 µl liquid bacterial culture were supplemented with 100 µl DMSO and stored at -80°C. Since *B. subtilis* produces spores, it can also be stored on SP agar at RT. *E. coli* can be stored up to 4 weeks on agar plates at 4°C.

Fluorescence microscopy

Pictures of *B. subtilis* strains grown on agar plates were taken with a stereo fluorescence microscope Lumar.V12 (Zeiss, Jana) equipped with the ZEN lite 2011 (blue edition) software. The applied filter sets were Lumar 38 for GFP detection, respectively (Zeiss, Jena). Images were taken at room temperature.

Biofilm assay

The effect of suppressor mutations on biofilm formation was tested by cultivating different suppressor and reference strains on in 4 ml MSSM medium with 30 mM NH4 and PL over night at 37°C. This culture was used to inoculate 2 ml of medium to an OD_{600} of 0.1. The cells were incubated at 37°C with agitation until the OD_{600} was 0.5. 10 µl of the cell suspension was dropped on MSSM agar plates containing 20 mM glutamine as sole nitrogen source. In addition, these plates were supplemented with PL (0.1 µM). As described in *Romero et al.*, 2010; *Shemesh & Chaia*, 2013, 1% glycerol, 100 µM MnCl2, 40 ng/ml Coomassie Brilliant Blue G-250 and 20 ng/ml mM congo red stain were supplemented to the plates to induce biofilm formation, strain protein structures and exopolysaccharides. Complex colony formation was documented after 4 days by using a stereo fluorescence microscope Lumar.V12 (Zeiss, Jana) equipped with the ZEN lite 2011 (blue edition) software. The exposure time was set to 1s and the magnification to 9.6 x.

Transformation of *B. subtilis*

Preparation of competent cells

An LB overnight culture of the desired *B. subtilis* strain was used to inoculate 10 ml MNGE medium, supplemented with 0.1% CAA, to an OD_{600} of ~0.1 and grown until the OD_{600} was ~1.3. The culture was diluted 1:1 with 10 ml MNGE (without CAA) in order to start a nutritional starvation step and by that activate competence gene expression in *B. subtilis* (*Hamoen et al.*, 2003). After 1 h of incubation at 37°C, the cells could be used either for transformation or were prepared for long time storage.

Therefore, 15 ml of the cells were harvested by centrifugation for 5 min at 5000 rpm and resuspended with 1.8 ml of the supernatant and 1.2 ml glycerol (50%). The competent cells were stored in 300 µl aliquots at -80°C.

Transformation of *B. subtilis*

One 300 µl aliquot of competent cells was thawed on ice and mixed with 1.7 ml MN-medium (1x) supplemented with 43 µl glucose (20% (w/v)) and 34 µl $MgSO_4$ (1 M). 0.1 - 1 µg genomic DNA or 2 µg plasmid DNA were added to 400 µl of the competent cell mixture and incubated for 30 min at 37°C. Afterwards, 100 µl expression mix were added and the cells were incubated at 37°C with agitation for 1 h until they were plated on SP plates containing appropriate antibiotics.

Transformation of *E. coli*

Preparation of competent cells

Competent *E.* coli cells were prepared by the $CaCl_2$ method. Therefore, 10 ml LB medium were inoculated with an LB overnight culture to an OD_{600} of ~0.1 and grown at 37°C to an OD_{600} of ~0.3. After harvesting the cells at 5000 rpm at 4°C, they were resuspended in 5 ml of ice cold $CaCl_2$ solution (50 mM) and incubated for 30 min on ice. The cells were harvested at 4°C 5000 rpm and resuspended in 1 ml of ice cold $CaCl_2$ solution. Afterwards the cells were ready for transformation.

Transformation of *E. coli*

100 µl of competent *E. coli* cells were incubated with 5 ng of DNA on ice for 30 min. The cells were then transferred to 42°C for 90 s and subsequently kept on ice for 5 min in order to guarantee heat shock induced DNA uptake. 500 µl of LB medium were added to the cells and they were incubated for 1 h at 37°C with agitation before being plated on LB-agar plates containing appropriate antibiotics.

Isolation of chromosomal and plasmid DNA from B. subtilis and E. coli

Preparation of plasmid DNA from *E. coli*

Plasmid DNA was prepared by using the NucleoSpin® Plasmid Kit by Machery-Nagel. Therefore, 4 - 15 ml of a bacterial overnight culture were harvested at 13000 rpm and treated as described in the manufacturers' manual. The plasmid was eluted by using deionized water instead of elution buffer.

Isolation of genomic DNA from *B. subtilis*

4 ml of an overnight culture of the target strain were harvested at 13000 rpm for 2 min and resuspended in 100 µl lysis buffer mixed with 100 µl TE Buffer. The mixture was incubated at 37°C for 30 min and centrifuged for 5 min at 4000 x g^{-1}. The pellet was resuspended in 300 µl DNA lysis Buffer

T supplemented with 20 µl Proteinase K solution and 15 ml RNase A (20 mg/ml) and incubated for 30 min at 70°C. The following steps can be consulted in the manufacturers' manual (peqGOLD Bacterial DNA Kit from PEQLAB)

Determining DNA concentrations (Nanodrop)

In order to determine the DNA concentration of a sample the optical density can be measured by using a UV-Vis-Spectrophotometer, which needs only very low volumes of 1 - 2 µl. The device measures the OD_{260} of the sample in relation to the solution the sample is stored in and the software calculates the amount of DNA.

Agarose gel electrophoresis

Agarose Gel electrophoresis is a method used to sort DNA and RNA molecules by size. Agarose is a polysaccharide consisting of the monomers D-Galactose and 3,6-Anhydro-L-Galactose, which polymerize and form a gel, acting like a molecular sieve for the nucleic acids. The sample is loaded into small pockets and, when a voltage is applied, the negatively charged nucleic acid molecules wander through the gel to reach the positive pol. Thereby, small molecules move faster, which separates the molecules by size. The concentration of the agarose gel is important for the resolution. If not differently stated 1% (w/v) agarose gels were used, which can separate molecules from 0.2 to 10,000 kbp size. Agarose was solved in TAE-buffer by boiling shortly, supplemented with HDGreen™ Plus DNA stain (Intas) and poured into a gel chamber. When the agarose is cooling down, hydrogen bridge bonds built up between the agarose monomers and the liquid becomes a gel. By inserting a comb into the gel, little pockets are created in which a mixture of sample and loading dye can be filled. In order to be able to have insights into the size of the DNA fragments a DNA ladder marker (λ marker) was also loaded, which consists of λ-DNA digested with *Eco*RI and *Hind*III

The polymerized gel was covered with 1x TAE buffer and a voltage of120 V was applied, so that the nucleic acid molecules started to move. After the samples went far enough, the gel was imaged in a GelDoc™ XR (Biorad). Since the HDGreen™ Plus DNA stain in the gel binds to the nucleic acid molecules, they can be detected by excitation with UV light (254 nm).

Polymerase chain reaction (PCR)

The polymerase chain reaction is a method used to exponentially duplicate DNA-fragments within a short amount of time. Therefore, two primers are needed, which set the replication area. The reaction itself consists of three basic steps, which proceed at different temperatures in a so-called thermocycler. At the denaturation step the DNA strands are separated from each other since the

hydrogen bridge bonds between the bases are cleaved. The necessary temperature for this step is between 95 and 98°C. Due to a cooling step to approximately 60°C the primers are able to bind to the DNA strand (annealing). The specific temperature depends on the designed primers, especially from the G-C content and the length. Basically, an annealing temperature is chosen, which is T_m- 5°C. When the primers are bound, the temperature is increased to 72°C so that the polymerase can start the synthesis of the complementary strand (elongation). These three steps are repeated until the desired amount of product is reached. Three additional steps are added to the PCR program in order to enhance its function: an initial denaturation step, which melts the chromosomal DNA, the final elongation, which ensures that the DNA polymerase can complete the elongation and the "hold" step, which stops the reaction and protects the product from degradation.

For cloning purpose, it is important to guarantee, that the PCR product is identical to the template DNA and by that, no mutations occur. Therefore, the Phusion®-polymerase (ThermoScientific) is used, which originates from *Pyrococcus furiosus* and is modified so that it has a up to 50-fold lower error rate than the *Taq*-polymerase (ThermoScientific). For check PCRs usage of the *Taq*-polymerase is sufficient. The products were purified by using the PCR Purification KIT (Qiagen).

Table 1: Pipetting scheme of a Phusion®-PCR

Volume [µl]	Compound
20	5x Phusion®HF Buffer
4	dNTPs (12.5 µmol/ml)
4	Fwd primer (5 pmol)
4	Rev primer (5 pmol)
2	Template DNA (2ng)
0.5	DNA Phusion® Polymerase (2 U/µl)
65.5	H_2O

Table 2: Cycler program Pfusion® PCR

Step	Temp. [°C]	Time [s]	
Initial denaturation	98	180	
Denaturation	98	30	30x
Annealing	Primer Tm – 5°C	35	
Elongation	72	30 / kbp	
Final elongation	72	600	
Hold	8	∞	

Table 3:Pipetting scheme for a 100 µl *Taq*-PCR

Volume [µl]	Compound
10	10x Taq-Buffer
4	dNTPs (12.5 µmol/ml)
4	Fwd primer (5 pmol)
4	Rev primer (5 pmol)
2	Template DNA (2ng)
0.5	*Taq*-Polymerase (5 U/µl)
75.5	H_2O

Table 4: Cycler program *Taq*-PCR

Step	Temp. [°C]	Time [s]	
Initial denaturation	95	180	
Denaturation	95	30	30x
Annealing	Primer Tm – 5°C	35	
Elongation	72	60 / kbp	
Final elongation	72	600	
Hold	8	∞	

Long-flanking homology PCR (LFH-PCR)

The LFH-PCR is a method to generate gene mutations or gene deletions, which can then be introduced into *B. subtilis*. The method originates from cloning in *Saccharomyces cerevisiae* and was used to amplify deletion cassettes, which generate a gene knock out by homologous recombination (*Wach*, 1996). In order to delete a gene, it is replaced by a deletion cassette, which harbors an antibiotic resistance marker. To do so, basically two steps are completed: First the deletion cassette and up-/ downstream fragments are amplified from a plasmid or chromosomal DNA, respectively. The up-/ and downstream fragments are around 1 kbp long, flanking regions of the gene of interest and by that allowing the bacterium double homologous recombination.

It is mandatory that the inner primers of the up-/ downstream fragments have a complementary region with the ends of the deletion cassette of 25 bp, so that in a second PCR all three fragments align to each other and the parts are fused together. To do so, the LFH-PCR mixture was prepared but the oligos were added in a short break, which allows a better mixture and pre-annealing of the fragments. After checking the product by gel electrophoresis, it was used for transformation in *B. subtilis.*

Table 5: Pipetting scheme for a 100 µl LFH PCR

Volume [µl]	Compound
20	5x Phusion ® HF Buffer
4	dNTPs (12.5 µmol/ml)
8	Fwd primer (5 pmol)[2]
8	Rev primer (5 pmol)
x	100 ng upstream fragment
x	100 ng downstream fragment
x	150 ng deletion cassette
2	DNA Phusion® Polymerase (2 U/µl)
Ad 100	H_2O

[2] Do not add until hold step

Table 6: Cycler program of a LFH PCR

Step	Temp. [°C]	Time [s]	
Initial denaturation	98	180	
Denaturation	98	30	
Annealing	Primer Tm – 5°C	35	10x
Elongation	72	30 / kbp	
Hold	15	∞	
Addition of oligos			
Denaturation	98	30	
Annealing	Primer Tm – 5°C	35	25x
Elongation	72	30 / kbp	
Final elongation	72	600	
Hold	8	∞	

Restriction digestion of DNA

The digestion of the DNA is done by FastDigest restriction endonucleases (ThermoFischer), which recognize a 4 - 10 bp palindromic sequence and cut it in a specific manner. The emerging ends can be then ligated with a vector backbone, cut with the same enzymes. For the reaction, buffers and concentrations were used as mentioned in the manufacturers' manual.

Table 7: pipetting scheme for DNA digestion

Volume [µl]	Compound
4	10x FD buffer
4	Enzyme 1
4	Enzyme 2
x	DNA (1 µg)
Ad 40	H_2O

Ligation of DNA

In order to prevent the digested DNA from re-ligation, prior to the ligation, the larger fragment has to be dephosphorylated at the 5'-prime end of the DNA, which is done by using FastAP (alkaline phosphatase) (ThermoFischer). 0.5 µl of the enzyme (1 U/µl} was added to the digested DNA and incubated for 5 min at 37°C. Afterwards, the DNA fragments were purified using a PCR purification Kit (Qiagen) and ligated using the T -DNA ligase (ThermoFischer). After ligating the fragments for two hours at room temperature or overnight on ice, the whole sample was used for transformation of the competent bacteria.

Table 8: pipetting scheme for a ligation

Volume [µl]	Compound
1	T4 DNA ligase (5 U/µl)
2	10x ligation buffer
X	150 ng insert
X	50 ng plasmid
Ad 20	H_2O

DNA sequencing

DNA sequencing is a method, which allows to determine the base sequence of a DNA strain. This is outsourced and done by SeqLab (Göttingen, Germany). There, the DNA sequence is detected by the chain termination method (*Sanger et al.*, 1992).

Table 9: pipetting scheme for sequencing sample

Volume[µl]	Compound
3	Primer
x	Plasmid (0.5 – 1.2 ng)[3]
Ad 15	H_2O

Southern blotting

For Southern blot analyses 300 µg/ml of chromosomal DNA were digested with 3 µl of restriction endonucleases according to the manufacturer's instructions (Thermo Scientific (Germany). The incubation time at 37°C was prolonged to 5 hours. Digests of chromosomal DNA were separated using 1% agarose gels and transferred onto a positively charged nylon membrane (Roche Diagnostics) using the VacuGene™ XL (GE Healthcare) blotting device ((*Sambrook et al.*, 1989)). The agarose gel was blotted as described in Table 10, dried and crosslinked by treating it for 90s with UV light. It was probed with Digoxygenin labelled riboprobes generated by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated fragments as templates. The primer pair BR165/BR166 was used to amplify the *pdxS* gene fragment. The reverse primers contained the T7 RNA polymerase recognition sequence. *In vitro* RNA labelling, was carried out according to the manufacturers' instructions (DIG RNA labelling Kit). For the hybridization process the nylon membrane was incubated for 1 h at 68°C in 25 ml prehybridization buffer. The probe was diluted (15 µl probe in 500 µl prehybridization buffer) and incubated at 95°C. After 10 minutes it was mixed with 4.5 ml prehybridization buffer. The membrane is removed from the hybridization buffer, the diluted probe is added and both were incubated overnight at 68°C. Afterwards, the membrane is treated with washing steps as described in

Table 11.

[3] For PCR product (18 ng / 100 bases) is recommended

Table 10: Southern blotting gel treatment

Buffer	Time	Pressure
Depurinization buffer	15 min	60 mbar
Denaturation buffer	20 min	60 mbar
Neutralization buffer	20 min	60 mbar
20 x SSPE	Min 2 h	80 mbar

Table 11: Southern blotting membrane preparation

Volume	Buffer	Time	Temperature
15 ml	P1	2x 10min	RT
15 ml	P2	2x 15 min	68°C
15 ml	1x DigP1	5 min	RT
5 ml	Blocking solution	30 min	
45 ml	1x DigP1		
5 ml	Blocking solution	30 min	
45 ml	1xDigP1		
5 µl	Anti-dioxigenin AP Fav fragments		
15 ml	DigP1	3x 10 min	
10 min	P3		

To start the reaction of the alkaline phosphatase, 1 ml CDP-Star was added to the membrane (5 µl diluted in 1 ml buffer P3). Images were taken with the ChemoCam Imager (INTAS).

Genome sequencing

To identify the mutations in suppressor mutants, chromosomal DNAs were subjected to sequencing. The Göttingen Genomics Laboratory (Göttingen, Germany) performed library preparation and sequencing on Illumina instruments. If not stated otherwise, the reads were mapped onto the *B. subtilis* SP1 reference genome as previously described (Richts et al., 2020) using the Geneious software package (Geneious Prime 2021.0.3.(https://www.geneious.com)). Single nucleotide polymorphisms (SNPs) were considered as significant when the total coverage depth exceeded 30 reads with a frequency variance of > 90%. Single nucleotide polymorphisms were verified by Sanger sequencing. The amplification level of a genomic region was determined by dividing the average coverage of the amplified region by the average coverage of the entire genome. Gene amplification was verified by Southern blotting.

Overexpression of recombinant proteins

Expression in *E. coli*

In order to produce proteins in large scale, expression plasmids were introduced into suitable *E. coli* strains. For this work three different vector backbones were used: pWH844 for N-terminally His-tag fusion, pGP172 for N-Terminally Strep-tag fusion and pETSUMOadapt for expression of a protein fused with a cleavable SUMO-Tag (6x His). All plasmids are under the control of a *lac* inducible promoter, which prevents the cells from expressing the proteins in absence of the artificial inductor IPTG. Thereby, the cells can focus on sufficient growth first and after they have reached a suitable cell density, they switch to protein expression. Since the accumulation of the expressed protein can be toxic for the cells, the inducible expression leads to better expression conditions.

After introduction of the expression plasmid into the *E. coli* strain, 80 ml LB medium supplemented with appropriate antibiotics were inoculated with a fresh colony and cultivated at 37°C overnight. At the next day 500 ml to 1 l of preheated LB-medium were inoculated with the pre-culture to an OD_{600} of 0.1 and incubated until the culture reached an OD_{600} of 0.8 – 1. IPTG with a final concentration of 1 mM was added and the protein expression started. After 2 – 3 hours, the culture was harvested at 5000 rpm for 10 min, washed shortly with Buffer W or 1xZAP, for Strep- and His-tagged proteins respectively, and pelleted by centrifugation (8500 rpm 10 min). The pellets were stored at -20°C until further processed.

Expression in *Bacillus subtilis*

To overproduce proteins in bacillus, expression plasmids based on pGP380 were used. The expression of the protein is under a constitutive P_{degQ}-Promoter (*Herzberg et al.*, 2007) and leads to a permanent protein production.

The expression plasmid was transformed into the desired *B. subtilis* strain and a pre-culture was made. After inoculation of the main culture, the bacteria were grown until an OD_{600} of 1, harvested at 5000 rpm for 10 min, washed wit Buffer W and stored at -20°C.

Cell Disruption (French press)

The proteins have to be separated from insoluble compounds of the bacteria. Therefore, the cells were lysed by using a French pressure cell consisting of a press (G. Heinemann) in which a hollow cylinder is mounted (Thermo). The cells were resuspended in the appropriate buffer and poured into the precooled cylinder, which is sealed on the top with a moveable piston. On the bottom side there is a narrow hole with a valve through, which the cell suspension is pressed. Thereby, a pressure of approximately 18000 psi is reached and when the valve is opened, the cells disrupt since shear forces

occur due to the smaller release opening. The cell lysate is collected and the process is repeated at least once for Gram negative and twice for Gram positive bacteria.
The lysed bacteria were centrifuged with 8500 rpm for 10 min at 4°C first and the supernatant for 35 min with 40000 rpm at 4°C afterwards.

Affinity chromatography

The purification of proteins was done with by using affinity-tags, which are amino acid sequences having a binding specificity for certain materials. They are fused to the target proteins and can be purified since the bacterial crude extract is applied to an affinity column, which has the affine molecules in its immobile phase. Only those proteins, which harbor the specific amino acid sequence, bind to the column; the other ones stay in the liquid phase and are washed away. By adding a ligand with a higher affinity to the stationary phase, the protein is eluted and can be collected.

Affinity chromatography for His-tagged proteins

For purification of proteins fused with the 6x His-tag a matrix consisting of Ni-NTA® is used, which has a high affinity for six consecutive histidine residues. For the washing and elution steps the concentration of the natural ligand imidazole is gradually increased from 10 mM up to 500 mM end concentration to prevent unspecific binding.
2.5 ml of Ni-NTA® (IBA Lifescience) matrix per 1 l cell culture were filled into the affinity column (Bio-Rad Poly-Prep® Chromatography Column) and equilibrated with 12.5 ml of 1xZAP buffer containing 10 mM imidazole. Afterwards, the crude extract was loaded in the column and the flow through was collected. The column was washed by adding 10 ml of 10 mM imidazole solution. The elution steps can be seen in the table below.

Table 12: Elution steps of a 6x His-tag purification

Volume [ml]	Mol Imidazol [mM]
8	10
8	50
8	75
8	100
4	500
4	500

Affinity chromatography for Strep-tagged proteins (Strep-tag II)

The Strep-tag harbors an amino acid sequence (WSHPQFEL), which has an affinity for streptavidin. When the Strep-tag II is fused by translational fusion to the target protein, it can be purified with an affinity column. The natural ligand for streptavidin is biotin but a biotin derivative, desthiobiotin, is used for elution of the protein. To improve the purification Strep-Tactin® is used instead of streptavidin

since it has, according to the manufacturer, a 100-fold higher affinity and is more resistant to SDS and proteases. The affinity column was filled with 1 ml of 50% Strep-Tactin® (IBA Lifescience) suspension for 1 l of cell culture and equilibrated with 5 ml of buffer W. The crude extract, gathered after the cell lysis, was loaded onto the column and the flow-through was collected. The column was washed five times with 2 ml buffer W before the elution started by adding first 160 µl buffer E and for the following elutions 250 µl.

Strep-protein interaction experiment (SPINE)

To detect protein-protein interactions, the SPINE experiment is an appropriate method in which a bait protein is tagged with a Strep-tag and expressed under conditions it is assumed to interact with the prey-protein. The cross-linker *para*-formaldehyde links the bait protein with its nearby interaction partner. By using a Strep-Tactin® column the cross-linked proteins can be purified and analyzed by loading them in an SDS-Gel. Since the samples were cooked for a short time in 6x PAP, the cross-links are resolved and the proteins occur as singe bands in the gel, which then can be analyzed by western blotting or mass spectrometry.

Expression plasmids containing a Strep-fusion to the expressed genes were introduced into a *B. subtilis* strain and a pre-culture was set up, preparing the bacteria for the growth medium. 1 l of medium, supplemented with appropriate antibiotics, was inoculated to an OD_{600} of ~0.1 and grown at 37°C with agitation to an OD_{600} of 1. Afterwards, the culture was split into 2x 500 ml and to one half PFA (4%) was added to 0.6% final concentration and incubated for additional 20 min at 37°C. The cultures were harvested at 5000 rpm for 10 min, washed with 1x PBS or to remove the PFA and pelleted by centrifugation (8500 rpm, 10 min). The cells were stored at -20°C until they were lysed by using the French press and purified by Strep-purification system (see above). After the purification, the cross-links were resolved by boiling the sample for 30 min in 6xPAP buffer at 95°C. In order to see even weak interactions, it is recommended to stain the SDS-gel by silver staining

Protein-Pulldown experiment

The protein-pulldown experiment is an in vitro interaction study of protein-protein interactions. Therefore, crude extract containing one of the proteins is applied to the affinity column and washed until no more proteins were measurable in 30 µl by Bradford assay (see 0). Next either crude extract of a bacillus strain overexpressing the potential interaction partner or the purified and dialyzed protein was added to the affinity column and washed as described in the respective purification protocol. Therefore, it is essential, that both proteins have either different purification tags fused to exclude tag-based interaction or the second protein has no tag at all. After elution, the different elution fractions were checked using an SDS gel with subsequent silver staining or Western blotting.

Determination of protein concentration (Bradford Assay)

One fast and simple possibility to measure the concentration of proteins is the Bradford assay. The Bradford reagent can be used for solutions and the mechanism relies on the formation of a complex between Coomassie brilliant blue G-250 and the proteins (*Bradford*, 1976). The absorption increases with increasing protein amount and vice versa. This assay can be used for determination of the protein concentration ranging from 0.1 – 1.4 mg.

1 ml of Bradford reagent (1x) was mixed with 2 – 20 µl of protein solution and the OD_{595} was measured in relation to a blank containing only Bradford reagent and elution buffer / dialysis buffer.

A calibration curve showed the linear relation between amount of protein and absorption at OD_{595}. The calibration curve hast the slope of 0.0536, which is taken as standard value for further measurements.

To calculate the amounts of protein in the sample the following equation is used:

$$c = \frac{OD_{595}}{V \times 0.0536}$$

Whereas *V* is the volume of protein solution in µl used for the measurement and *c* the protein concentration in the tested solution. The consequent unit is µg /µl.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a method developed to separate proteins by their molecular weight. A discontinued polyacrylamide gel serves as a separating agent, which is made of two gel phases: a stacking gel and a running gel. Both consist of acrylamide, water, Tris-HCl buffer, APS and TEMED. By addition of the radical starters APS and TEMED the radical polymerization starts and the acrylamide is crosslinked to the N,N'-Methylenbisacrylamid, which is supplemented in the acrylamide solution with a ratio of 37,5 : 1. Similar to the agarose gels, also the percentage of the gels define the size of the proteins, which are separated. For this work only 12% gels were used for separation of proteins between 10 and 180 kDa.

The SDS-PAGE is a special cariant of the PAGE, which uses SDS, an anionic, negatively charged tenside, to wrap around the proteins and by that, linearize the proteins (*Laemmli*, 1970). The size of the protein determines the size of the resulting micelle and the mobility. Additionally, it replaces the charge of the amino acids with negative charge, so that the proteins run towards the positive pole, if a voltage is applied. The amplitude of the voltage defines the speed, the proteins run through the gel. Since bigger proteins stuck more frequently in the gel matrix, they need longer and are separated from the smaller proteins. The stacking gel has a lower percentage and a pH value of nearly 7. After applying

the voltage, the proteins run through the stacking geld until they reach the running gel, which has a higher pH of 8.8. The pH difference leads to a different running behavior since the mobility of the glycine, which is part of the running PAGE buffer, is pH dependent. The proteins firstly stack at the border and start to run through the running gel at the same time. After pouring the running gel in a Mini-PROTEAN® (Bio-Rad) system with 1 mm thickness, it is covered with isopropanol, which excludes the excess of oxygen. After polymerization the isopropanol was removed and the stacking gel was applied. Inserting a comb led to pockets of ~ 30 µl volume. The gel was placed in the gel chamber and covered with PAGE-buffer. The comb was removed and the samples, mixed with 6x PAP buffer, were given into the pockets. Prestained Protein Marker (PageRuler^{TM})(ThermoFischer) was added as a size standard. By applying a voltage of approximately 160 V, the proteins needed around an hour to run through the gel. The blue band of the bromphenol blue indicates when the smallest proteins have reached the end of the gel.

Table 13: Pipetting scheme of a 12% SDS running gel

Volume [ml]	Compound
3.3	H_2O
4	Acrylamide
2.5	Tris-HCL pH 8.8 (1.5 M)
0.1	SDS (10%)
0.10	APS (10%)
0.010	TEMED

Table 14: Pipetting scheme of a separation gel

Volume [ml]	Compound
6.83	H_2O
1.5	Acrylamide
0.87	Tris-HCL pH 8.8 (1.5 M)
0.1	SDS (10%)
0.10	APS (10%)
0.010	TEMED

Coomassie staining for protein gels

In order to visualize the separated proteins, the gel is incubated for at least 10 min in fixing solution. After fixation, the proteins can be stained with Coomassie Brilliant Blue staining in which the gel is stored for 10 – 20 min. In order to de-stain the background of the SDS gel, it is incubated overnight in destaining solution.

Silver staining for protein gels

Since silver staining is a very sensitive technique, even small amounts of proteins, which occur in for example pull-downs or protein-protein interaction experiments, can be detected. The amino acid residues glutamate, aspartate and cysteine build complexes with the silver ions and by that, are

reduced to silver. Silver staining cannot be used for quantitative determination of proteins since the level of staining strongly depends on the amount of those residues (*Winkler et al.*, 2007). The procedure of the silver staining is according to the method of Nesterenko, Tilley and Upton (1994).

Table 15: Protocol of silver protein staining

Step	Solution	Time	
Fixing	Fixation solution	1 – 24 h	
Washing	EtOH (50%)	20 min	3x
Reduction	Thiosulfate solution	1.5 min	
Washing	Deion. H_2O	20 s	3x
Staining	Impregnator	15 – 25 min	
Washing	Deion. H_2O	20 s	3x
Development	Developer	Until sufficient stained	
Washing	Deion. H_2O	20 s	2x
Stop	Stop solution	5 min	

Dialysis

Proteins, which are purified using affinity tags, have to be eluted from the affinity column. In case of His-tagged proteins, this is done with Imidazol and for Strep-tagged proteins with D-desthiobiotin. These small molecules are also present in the elution fractions and can interfere in enzymatic reactions or other processes. Therefore, getting rid of all unnecessary molecules and salts is beneficial for further experiments.

For the dialysis the elution fraction was poured into a dialysis tube (Serva MEMBRA-CEL® 22mm diameter), a semi-permeable membrane, which is only open for smaller molecules and ions. This tube was cooked in deion. water for 10 min, before the protein solution was placed inside and sealed with clips from each side. The tube is then placed in 1000 fold dialysis buffer, which basically is the buffer in which the further reaction should take place. Small molecules diffuse through the membrane so that an equilibration occurs. After one hour the buffer is renewed and the dialysis process is repeated overnight. Afterwards, the protein is ready for further studies.

β-Galactosidase assay

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as described previously (*Stannek et al.*, 2015). Single colonies were taken to inoculate overnight cultures containing MSSM minimal medium supplemented with 30 mM ammonium chloride. Main cultures were inoculated with the overnight cultures to an OD_{600} of 0.1 and the cells were harvested at an OD_{600} of 0.6 - 0.8. *β*-galactosidase activities were determined as described previously (*Kunst & Rapoport*, 1995).

3. Results

3.1 The role of YtoQ in a heterologous vitamin B6 synthesis pathway

The inactivation of the *ytoQ* gene does not affect growth of the *B. subtilis* wild type

The role of YtoQ was firstly described in an artificial genetic background. Its overexpression could establish vitamin B6 production in a mutant lacking the PdxST PLP synthase complex but harboring only the *pdxHJ* genes from *E. coli* and a deletion of *bshC* (*J. Rosenberg et al.*, 2018). As nothing was known about its function outside of this special context, the influence of YtoQ on growth of the *B. subtilis* wild type was analyzed. Therefore, a *ytoQ* deletion mutant was constructed by transforming the SP1 wild type with genomic DNA of the strain BKK29850, deriving from the *Bacillus Genetic Stock Center* (BGSC). In addition, the *ytoQ* overexpression plasmid pBP639 and the empty plasmid pBQ200 were introduced into the SP1 strain. The influence of *ytoQ* overexpression and deletion of the *ytoQ* gene was tested by propagating the strains on LB medium plates supplemented with the required antibiotics and resuspending emerging cell material in saline solution. After washing twice, the cell suspensions were set to OD_{600} of 1 and 10 µl were spread on C-Glc, CSE-Glc, CE-Glc, C-Glc-Cysteine (0.5 mM), SP and LB agar plates. To assess if a PL-dependent phenotype exists, also agar plates supplemented with PL were used (Figure 11A). A difference in growth between *ytoQ* mutant, overexpression strain and wild type, carrying the empty plasmid, could not be observed. All strains grew to a high density on complex media and to a lesser extend also on C-Glc minimal medium. On CE plates the growth ability of all three strains was decreased. This effect could be complemented, when succinate was added. For the C-Glc plates supplemented with 0.5 mM cysteine difference in growth could not be observed and supplementation with PL also did not change growth of the strains.

The positive effect of *ytoQ* overexpression in the heterologous pathway only comes to place when bacillithiol synthesis is blocked (*J. Rosenberg et al.*, 2018). It could be shown that bacillithiol synthesis mutants show slow growth in SM minimal medium, which can be compromised by the addition of CAA or Fe^{2+} (*Fang & Dos Santos*, 2015). To assess whether YtoQ takes over the function of bacillithiol, growth of the Δ*bshC* mutant (BP977), the Δ*ytoQ* mutant (BP1101) and the Δ*ytoQ* Δ*bshC* double mutant (BP1104) was monitored in either SM minimal medium or medium supplemented with 0.5% CAA or 50µmol $FeCl_2$. The strains BP977 and BP1104 were transformed with the *ytoQ* overexpression plasmid (pBP639) and empty plasmid (pBQ200), respectively. After inoculation in SM-CAA medium from LB overnight culture, the strains were incubated until the OD_{600} was 0.5 -0.8. The cultures were washed twice in saline and 100 µl medium was inoculated from the precultures to OD_{600} of 0.05. Growth was measured in the plate reader at 37°C.

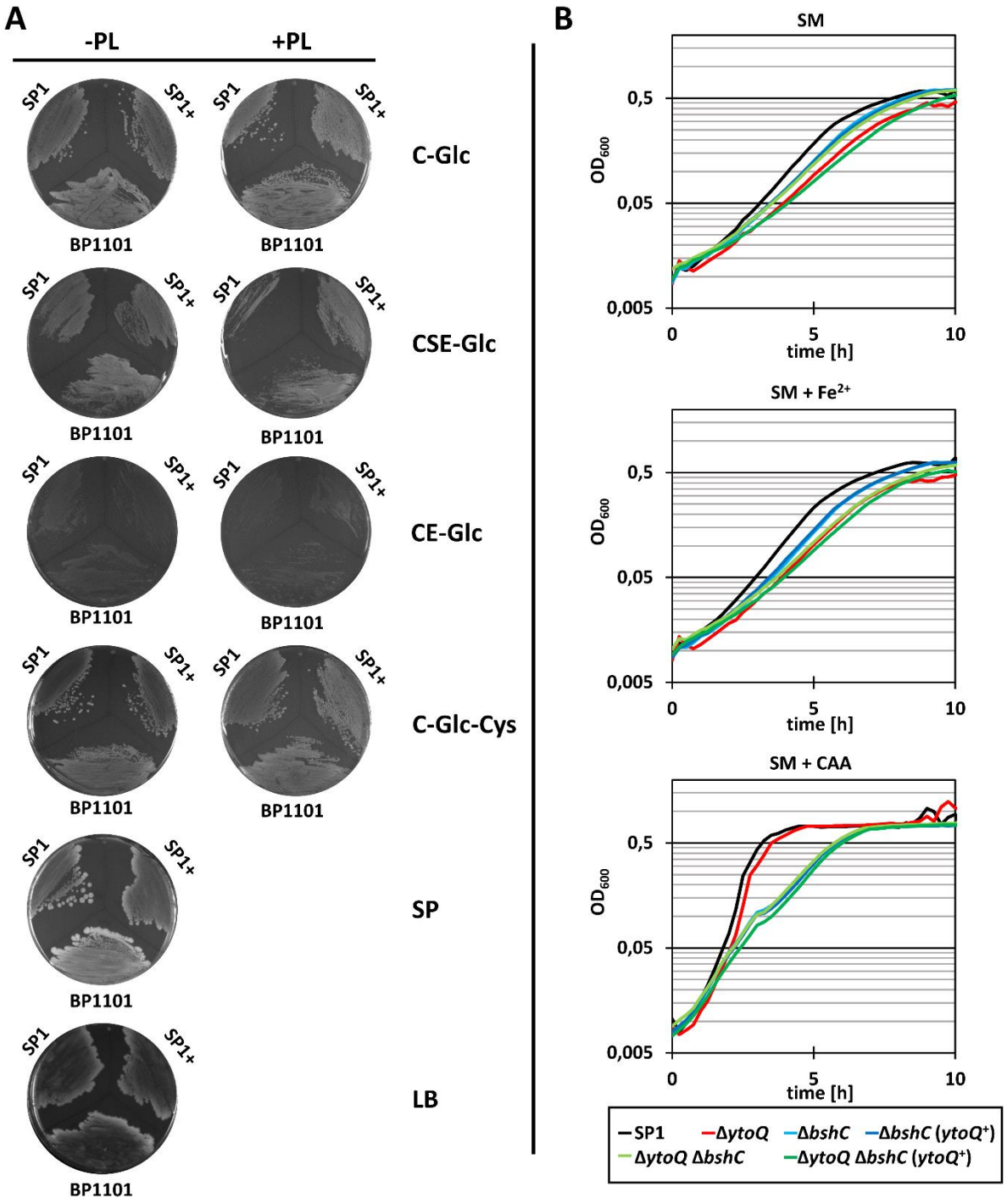


Figure 11 Growth behavior of different *ytoQ* deletion and overexpression mutants.
A: PL-dependent growth of *ytoQ* knockout strain (BP1101), *ytoQ* overexpression strain (SP1+) and wild type Strain (SP1) on different media agar plates. Cell material was taken from LB plate, washed 2x in saline solution and set to an OD_{600} of 1. 10 µl were streaked on complex or minimal medium agar plates supplemented with PL or without PL addition. **B**: *B. subtilis* strains were grown in SM medium or medium supplemented with either CAA or $FeCl_2$ to an OD_{600} of 0.5 – 0.8. The precultures were washed 1x in saline, set to OD1 and were used to inoculate 100 µl fresh medium. Growth was monitored at 37°C in the plate reader. Black: SP1 (WT), Red: BP1101 (Δ*ytoQ*), light green: BP977 (Δ*bhsC*) + pBQ200 (empty plasmid), dark green BP977 + pBP639 (*ytoQ*$^+$), light blue BP1104 (Δ*bshC* Δ*ytoQ*) + pBQ200, dark blue BP1104 + pBP639. Experiment was done in at least two biological replicates.

As seen in Figure 11B, all strains reached about the same optical density in SM medium without supplementation. The growth of the Δ*bshC* mutant BP977 was a little bit delayed compared to the wild type and did not change upon *ytoQ* overexpression (BP977 + pBP639) or *ytoQ* deletion (BP1104). Nevertheless, the growth rate was very close to the wild type strain. The lag phase of the Δ*ytoQ* single mutant BP1101 and the Δ*ytoQ* Δ*bshC* double mutant, overexpressing *ytoQ* (BP1104 + pBP639) was the longest and also the growth rate of both strains was lower compared to the other strains. The addition of Fe^{2+} to the medium, did not affect the growth of the Δ*bshC*, as it was described in Fang and Dos Santos (2015). Again, all strains produced the same amount of biomass. Interestingly, the Δ*bshC* single mutants (BP977), together with the Δ*ytoQ* singe mutant (BP1101) had the lowest growth rate. The Δ*ytoQ* Δ*bshC* double mutant BP1104 showed a similar growth rate as the wild type but with a longer lag phase. The overexpression of *ytoQ* did not lead to a change in growth. Moreover, supplementation with CAA indeed showed a phenotype for the mutants harboring a deletion of the Δ*bshC* gene. The wild type SP1 and the Δ*ytoQ* single mutant BP1101 had an increased growth rate in SM-CAA medium compared to the minimal medium without CAA supplementation, which is not the case for all of the Δ*bshC* mutants. Their growth rate improved slightly compared to the base minimal medium but to a lower extend than the other strains. Again, overexpression of *ytoQ* showed no difference, indicating that YtoQ was not able to take over the function of bacillithiol at least in this context. The growth experiment was also conducted in MSSM medium under the same conditions, resulting in the same growth behavior (data not shown).

Loss of YtoQ does not affect susceptibility to fosfomycin or peroxide stress

Previously, it was shown that bacillithiol is involved in oxidative stress response and fosfomycin resistance (*Fang & Dos Santos*, 2015; *Gaballa et al.*, 2010). It is possible that the cells suffer from the lack of bacillithiol and YtoQ could recover the negative effect for example of peroxide production in the last steps of PLP synthesis by PdxH (*Zhao & Winkler*, 1995). To determine the effect of different stressors, a disc diffusion assay was made (see Figure 12).

The *B. subtilis* strains SP1 (wild type), BP1100 (Δ*pdxST*), BP1101 (Δ*ytoQ*), BP977 (Δ*bshC*), BP1104 (Δ*bshC* Δ*ytoQ*), BP1246 (Δ*pdxST* Δ*bshC* Δ*ytoQ*), BP965 (Δ*pdxST pdxJH*), BP1102 (Δ*pdxST pdxJH* Δ*ytoQ*), BP1103 Δ*pdxST pdxJH* Δ*ytoQ* Δ*bshC*) and GP3153 (Δ*ohrAB*) were streaked on LB-PL plates and incubated over night at 37°C. Cell material was taken off the plates, resuspended in saline solution and washed two times. The OD_{600} was set to 1 and 100 µl of the cells were distributed evenly on LB-PL plates. A filter paper was placed on top and 10 µl of the stressors were added. The zone of inhibition was measured of three independent replicates and standard deviation was calculated. The zone of inhibition was defined as distinct, cell free area.

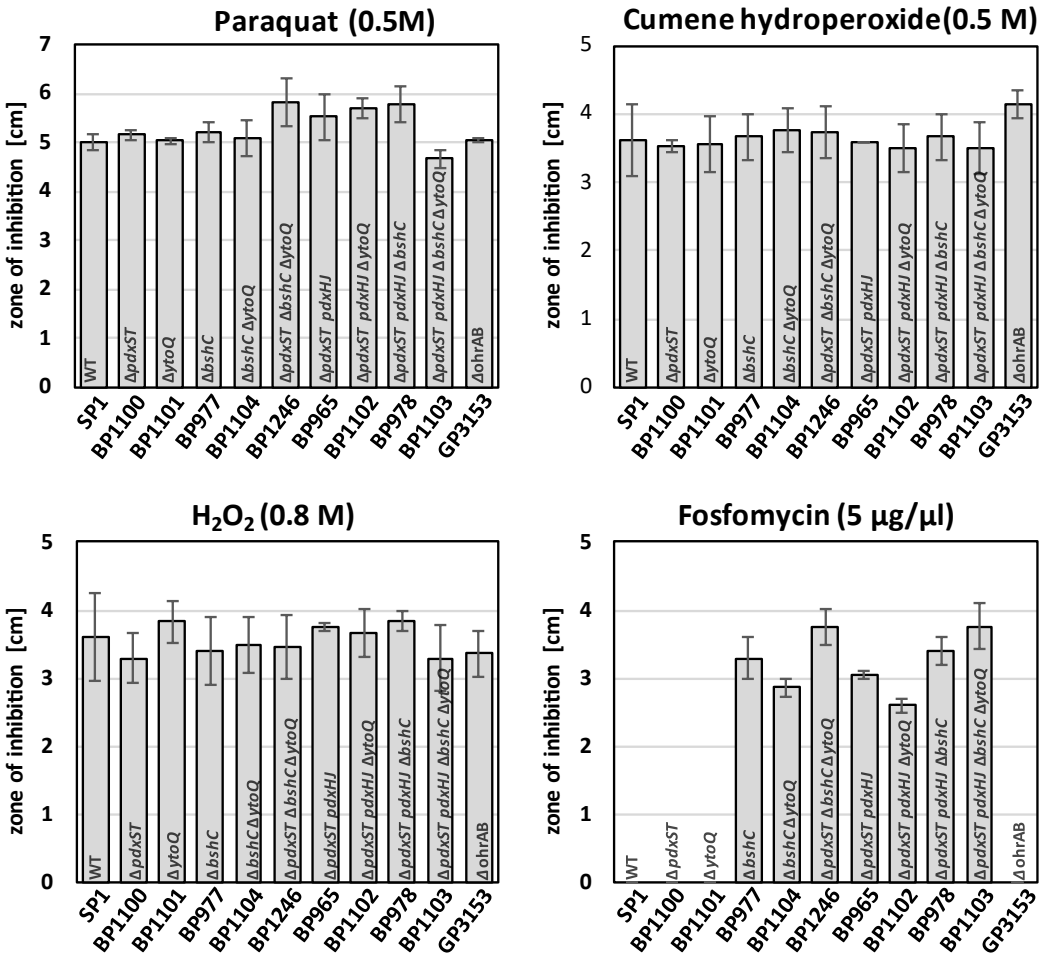


Figure 12 Stress test of pathway mutants.
Bacteria were streaked out on LB agar plates supplemented with PL and respective antibiotics and incubated over night at 37°C. Cell material was taken from the plates and resuspended in 1 ml sterile saline solution, washed two times by centrifugation at 10,000 × g and subsequent resuspension in saline solution. The OD_{600} was set to 1 and 100 µl of the cell suspension was plated on LB-PL agar plates. A filter paper was placed in the middle of the plate and 10 µl of the following stressors were added. The bars represent the average diameter of the zone of inhibition of three independent biological replicates. The error bars represent the standard deviation. SP1: wild type, BP1100: Δ*pdxST*, BP1101: Δ*ytoQ*, BP977: Δ*bshC*, BP1104: Δ*ytoQ* Δ*bshC,* BP1246: Δ*pdxST* Δ*ytoQ* Δ*bshC*, BP965: Δ*pdxST PdxHJ*, BP1102: Δ*pdxST pdxHJ* Δ*ytoQ,* BP1103: Δ*pdxST pdxHJ* Δ*bshC* Δ*ytoQ*, GP3153: *trpC2* Δ*ohrAB*

The strain GP3153 (Δ*ohrAB*) dealt as control strain for organic peroxide susceptibility (*Fuangthong et al.*, 2001). For paraquat, cumene hydroperoxide and H_2O_2 no significant difference was detectable between the different strains. Only BP1103 showed a slight decrease in diameter for paraquat. For fosfomycin the wild type SP1, the Δ*pdxST* mutant BP1100, the Δ*ytoQ* mutant and the control strain GP3153 (Δ*ohrAB*) showed no distinct zone of inhibition (Suppl. Figure 1). Thus, the diameter was set as 0 cm. Fosfomycin inhibited growth in all strains lacking the *bshC* gene. Surprisingly, also BP965 (Δ*pdxST pdxHJ*) showed a zone of inhibition without a deletion of *bshC*. The measured diameter did not differ significantly for the strains and only a tendency of BP1102 (Δ*pdxST pdxJH* Δ*ytoQ*) was perceived towards a decreased zone of inhibition. For *tert*-butyl hydroperoxide all strains tended to

form suppressors within the zone of inhibition, except for the control GP3153 whose plate showed no growth (see Suppl. Figure 2). Thus, the diameters were not definable.

Deletion of *bshC* and *ytoQ* in a heterologous pathway mutant abolishes genetic competence

When transforming the heterologous pathway mutants with different expression plasmids, we recognized various numbers of transformants on the selection plates and saw even loss of genetic competence for one of the strains. To clarify the role of the different mutations in genetic competence, we performed a competence test for the heterologous pathway mutants (see Figure 13). Therefore, the strains SP1 (wild type), BP1100 (Δ*pdxST*), BP1101 (Δ*ytoQ*), BP977 (Δ*bshC*), BP1104 (Δ*bshC* Δ*ytoQ*), BP1246 (Δ*pdxST* Δ*bshC* Δ*ytoQ*), BP965 (Δ*pdxST pdxJH*), BP1102 (Δ*pdxST pdxJH* Δ*ytoQ*) and BP1103 Δ*pdxST pdxJH* Δ*ytoQ* Δ*bshC*) were inoculated in LB medium supplemented with PL and incubated overnight. The cultures were used to inoculate MNGE-CAA-PL medium. After reaching OD_{600} of approximately 1, the cells were diluted with MNGE-PL medium in a 1:1 manner and incubated another hour at 37°C. Hence OD_{600} was set to 1 by centrifugation and subsequent resuspension with the supernatant. 400 µl cell suspension was transformed with 1 µg of the plasmid pBQ200. 1/5 of the total volume and the concentrated remain were plated on LB-PL plates supplemented with EL as selective antibiotic. The cells of two independent replicates were counted for both cell dilutions, extrapolated to match the full cell number and the mean and the standard deviation were computed.

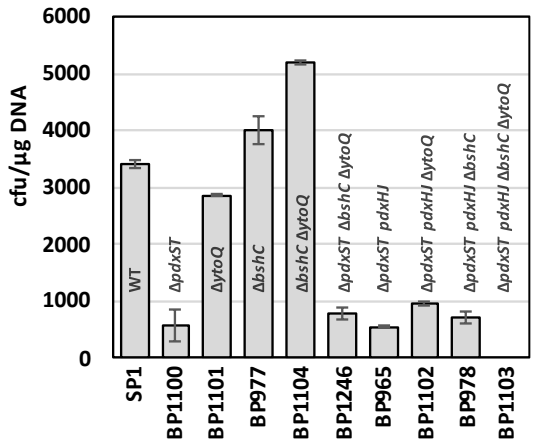


Figure 13 Competence test of different heterologous pathway mutants.
Bacterial strains were inoculated from LB culture to MNGE+CAA minimal medium and incubated at 37°C until OD_{600} was 1. The cells were diluted with fresh MNGE medium an incubated for 1 h. The cells were centrifuged at 10,000 × g and the supernatant was used to set the OD_{600} to 1. 1 µg of pBQ200 plasmid DNA was used to transform the competent cells. 4/5 of the cells were applied to a LB-PL plate and the cfu was calculated after one day of incubation at 37°C. The bars represent the average cfu/µg DNA of two biological replicates. The error bars indicate the standard deviation. SP1: wild type, BP1100: Δ*pdxST*, BP1101: Δ*ytoQ*, BP977: Δ*bshC*, BP1104: Δ*ytoQ* Δ*bshC,* BP1246: Δ*pdxST* Δ*ytoQ* Δ*bshC*, BP965: Δ*pdxST PdxHJ*, BP1103: Δ*pdxST pdxHJ* Δ*bshC* Δ*ytoQ.*

The *B. subtilis* wild type strain, the Δ*ytoQ* and the Δ*bshC* single mutant showed a similar colony forming unit (cfu)/µg DNA. The Δ*bshC* Δ*ytoQ* double mutant exhibits a slight increase in cfu. For all the strains carrying a deletion of the *pdxST* gene, the genetic competence was strongly reduced, independent of the other mutations. Only for the strain BP1103 (Δ*pdxST pdxHJ* Δ*bshC* Δ*ytoQ)* no colonies were visible on the transformation plates (see Suppl. Figure 2).

Cysteine negatively affects PLP synthesis *via* a non-native vitamin B6 synthesis pathway

Bacillithiol is a low-molecular-weight thiol keeping cytosolic proteins in a reduced state and protecting cells from reactive oxygen species, antibiotics and heavy metals (*Chandrangsu et al.*, 2018). Under oxidative stress, it can act as a redox switch by S-bacillithiolation of regulatory proteins. In the same way cysteine can act as a redox switch by S-cysteinylation (*J. W. Lee et al.*, 2007; *Newton et al.*, 2012). Therefore, we suggested that the loss of bacillithiol could be complemented by the addition of cysteine, which is also a LMW thiol and important in oxidative stress response. Furthermore, former studies showed that toxic levels of PL can be complemented by mutations upregulating biotin synthesis genes and that PL inhibits the expression of the *bio* operon (http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B). The supplementation with biotin could thereby be a growth improving setup for the heterologous pathway. We tested the impact of cysteine and biotin in a drop dilution assay with mutants carrying different stages of the pathway.

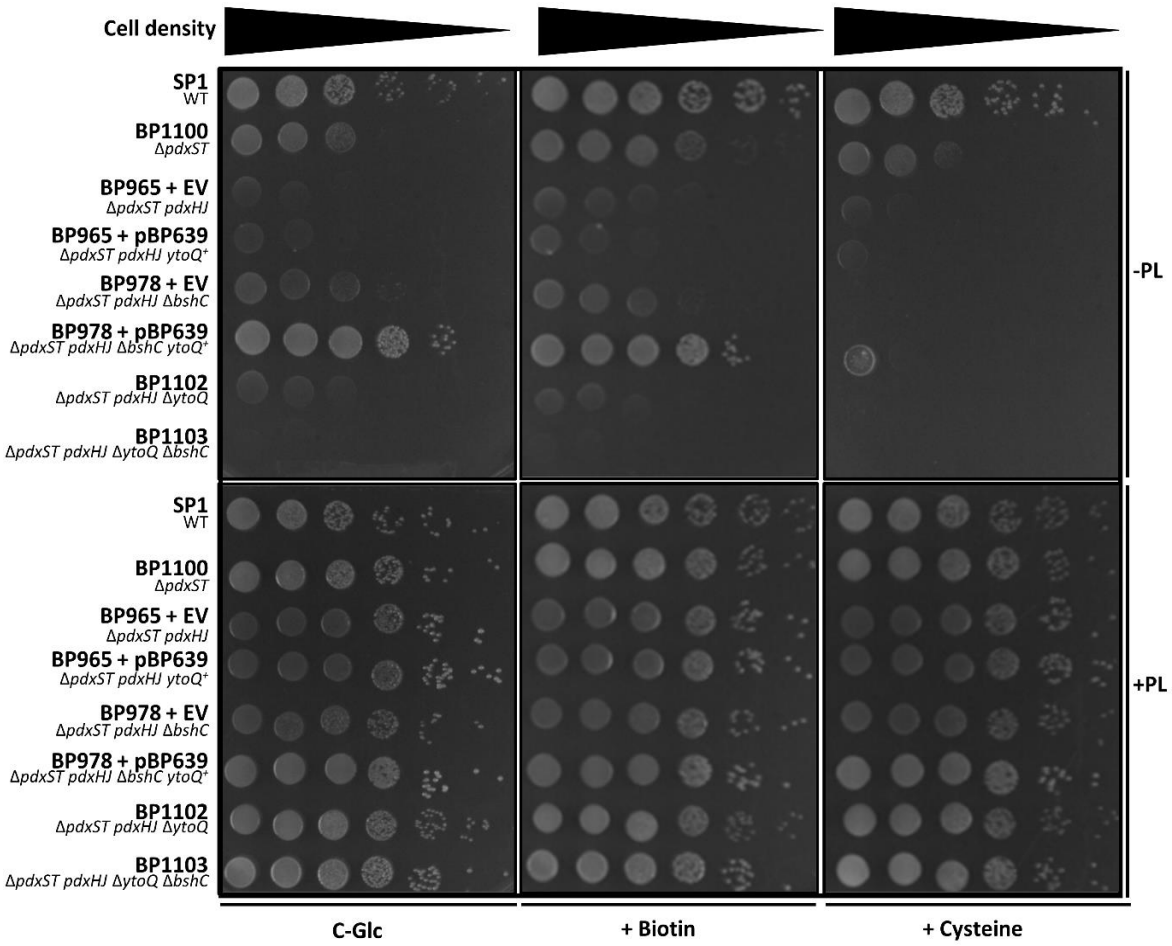


Figure 14 Drop dilution assay of heterologous pathway mutants.
Cell material of *B. subtilis* strains incubated in C-Glc minimal medium supplemented with respective antibiotics and PL. 1 ml of the grown culture was centrifuged at 10,000 × g and the pellet was resuspended in 1 ml saline. This step was repeated two times and the OD_{600} was set to 1. Serial dilution of the cells in steps of 1:10 was performed and 5 µl of the suspensions ranging from 10^{-1}-10^{-6} were spotted on C-Glc agar plates either with supplementation of biotin (5 µM), cysteine (0.5 mM) or without supplementation. SP1: wild type, BP1100: Δ*pdxST,* BP965: Δ*pdxST pdxJ pdxH,* BP978: Δ*pdxST pdxJ pdxH* Δ*bshc,* BP1102: Δ*pdxST pdxJ pdxH* Δ*ytoQ,* BP1103: Δ*pdxST pdxJ pdxH* Δ*ytoQ* Δ*bshc*

Hence, the *ytoQ* overexpression plasmid pBP639 and empty plasmid were introduced into the *B. subtilis* strains BP965 (Δ*pdxST pdxHJ*) and BP978 (Δ*pdxST pdxHJ* Δ*bshC*). Together with the wild type strain SP1, the Δ*pdxST* mutant BP1100 and the two mutants BP1102 (Δ*pdxST pdxHJ* Δ*ytoQ*) and BP1103 (Δ*pdxST pdxHJ* Δ*ytoQ* Δ*bshC*) the strains were inoculated in LB-PL medium overnight. After washing, the OD_{600} was set to 1 and a 1:10 dilution series was set up. 10 µl of the dilutions ranging from 10^{-1} – 10^{-6} were dropped on C-Glc and C-Glc-PL plates supplemented with cysteine (0.5 mM), biotin (5 µM) or without further supplementation. Growth of the strains was monitored after two days of incubation at 37°C (see Figure 14).

All strains were able to grew in the presence of PL but only the wild type strain SP1 and BP978 overexpressing *ytoQ* grew without exogenous PL. BP1100, BP978 + empty plasmid and BP1102, which were dropped in close proximity to the strains, also showed growth but had a directional growth behavior, increasing at the sites at which they are pointing at the well growing strains. As produced PL is secreted, it can be taken up by auxotrophic strains (see Figure 32). BP965 showed poor growth and overexpression of *ytoQ* did not enhance growth. BP1103 lacking *ytoQ* did not grew at all. When biotin was in the medium, the strains reached a higher cell density but growth patterns stayed the same. The strains on the plate supplemented with cysteine showed a similar growth behavior as on the C-Glc plate without cysteine with the exception of BP978 overexpressing *ytoQ*, which lost its growth advantage. As the strain was not able to grow, PL was not secreted into the medium. Thus, also BP978 did not grow any more. The negative effect of cysteine did not show up in the presence of PL indicating interference in the PL synthesis.

Deletion mutants of the putative underground pathway can still produce PL

To shed light on possible underground metabolism pathway routes, knockout mutants of *cpgA*; *serA*, *serS* and *thrB* were constructed in either wild type or in BP1036 background. BP1036 is a suppressor mutant harboring the partial heterologous pathway, a knockout of the *bshC* gene and a promoter-up mutation for *ytoQ* (Δ*pdxST pdxJ pdxH* Δ*bsh*-$^{\Delta A464}$ P_{ytoQ}*) and is therefore able to produce PL in minimal medium. SP1 and BP1036 were transformed with PCR products containing homologues regions of the respective genes, fused to an antibiotic resistance cassette resulting in the strains BP1272/BP1276 (Δ*cpgA*), BP1273/BP1277 (Δ*serA*), BP1274/BP1278 (Δ*serS*) and BP1275/BP1279 (Δ*thrB*) for SP1 and BP1036, respectively. If *B. subtilis* uses the expected underground metabolism route, knockout of one of the participating genes should block PL synthesis in the BP1036 background and convey PL auxotrophy. The single and double mutants, the wild type SP1 and BP965 (Δ*pdxST pdxHJ*) were checked for PL dependent growth and were firstly inoculated in SP liquid medium with PL and respective antibiotics and incubated overnight at 37°C. The cultures were centrifuged at 10,000 × g and the cell

pellets were washed two times in saline. The OD was set to 1 and 10 µl spots of each strain were plated on G-Glc- and G-Glc-PL plates supplemented with serine (0.008%) and threonine (0.004%). As a deletion of *cpgA* leads to severe growth defect when glucose or other carbon sources are present, which feed into the pentose phosphate pathway (*Sachla & Helmann*, 2019), CSE plates were used in addition. As seen in Figure 15, on the C-Glc plate supplemented with PL all strains were able to grow except for BP1272 and BP1276 carrying the knockout of *cpgA*. Interestingly, all strains but BP965 and the *cpgA* mutants grew without B6, indicating that SerA, SerS and ThrB did not take part in the underground metabolism route or that another route exists in addition. The *cpgA* mutants could grow on the CSE plates, independent of PL. The other strains showed the same growth behavior as on the C-Glc plate.

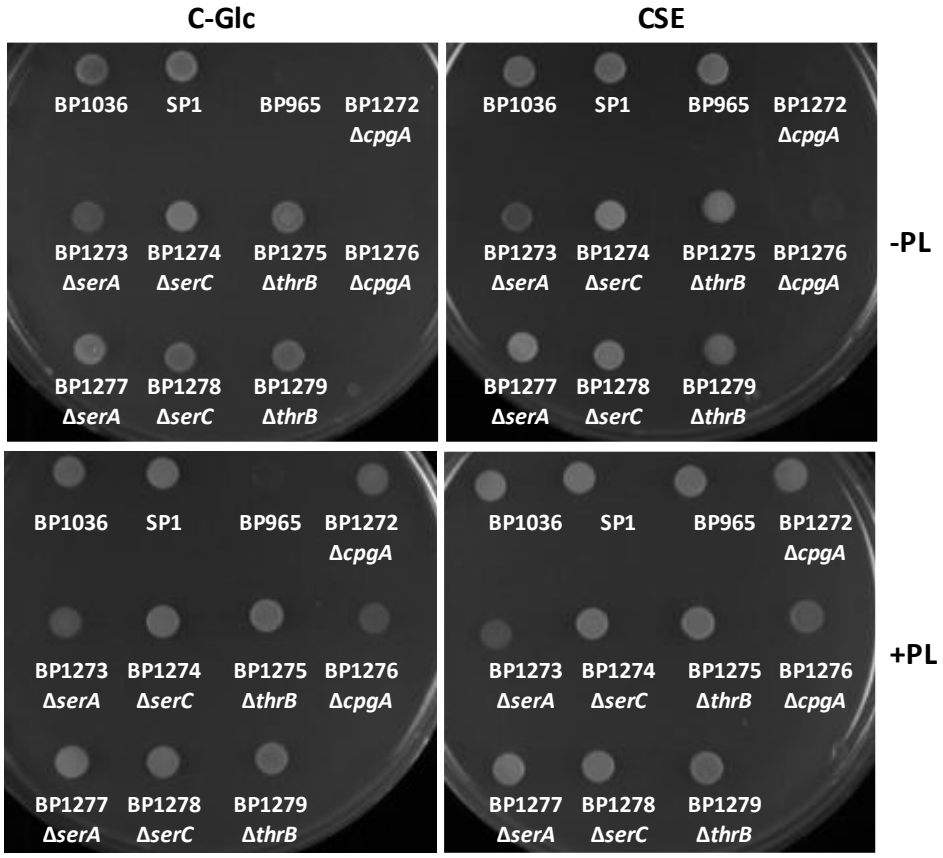


Figure 15 Influence of CpgA, SerA, SerC and ThrB on the heterologous B6 synthesis pathway.
B. subtilis strains were grown in liquid SP medium supplemented with PL overnight. The cultures were centrifuged and the cell pellet washed 2 times in saline. After setting the OD_{600} to 1, 5 µl were dropped on either C-Glc or CSE plates and plates supplemented with PL. Growth was monitored after 1 day of incubation at 37°C.

The role of YtoQ and Bacillithiol in the underground metabolism pathway

Since the proposed underground metabolism pathway could not be confirmed, we wanted to assess at which position YtoQ and bacillithiol feed into the pathway.

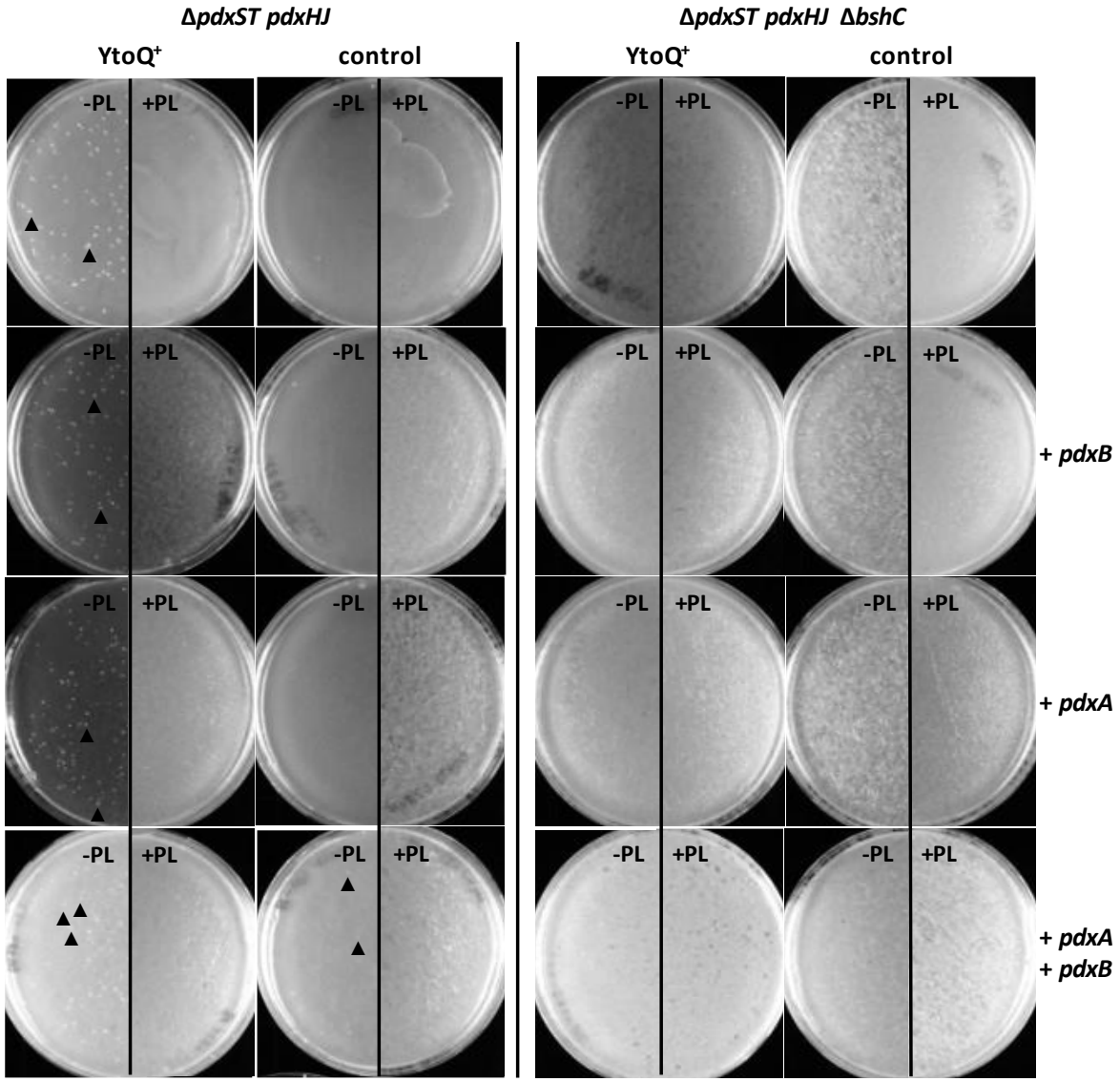


Figure 16 Feeding of YtoQ and BSH into the pathway.
pdxA, *pdxB* or a combination of both were introduced into the strains BP965 (Δ*pdxST pdxJH*) and BP978 (Δ*pdxST pdxJH* Δ*bshC*). Growth was analyzed with YtoQ overexpression plasmid and empty vector control on C-Glc minimal medium plates and plates supplemented with PL. Plates were incubated for 2days at 37°C. Black arrows indicate suppressor mutants.

Therefore, we introduced the *pdxA* and *pdxB* genes and a combination of both into the strains BP965 (Δ*pdxST pdxJ pdxH*) and BP978 (Δ*pdxST pdxJ pdxH* Δ*bshC*). The resulting strains BP1280 (Δ*pdxST pdxJ pdxH pdxB*), BP1281 (Δ*pdxST pdxJ pdxH* Δ*bshC pdxB*), BP1282 (Δ*pdxST pdxJ pdxH pdxA*), BP1283 (Δ*pdxST pdxJ pdxH* Δ*bshC pdxB*) BP1284 (Δ*pdxST pdxJ pdxH pdxA pdxB*) and BP1285 (Δ*pdxST pdxJ pdxH* Δ*bshC pdxA pdxB*) were transformed with empty plasmid pBQ200 and the *ytoQ* overexpression

plasmid pBP639 and propagated on either C-Glc and G-Glc-PL minimal medium plates and incubated fur 2 days at 37°C (see Figure 16).

When PL was added to the medium, all strains could grow and formed a bacterial lawn. The growth behavior was highly dependent on the presence of YtoQ and of PdxA + PdxB without supplementation with PL. In the background of BP965 (Δ*pdxST pdxJ pdxH*) growth was neither possible with *pdxA* nor with *pdxB*. Interestingly, when both genes were present, the cells grew slightly but formed suppressor mutants, indicating that the genes are functionally expressed. Similar can be seen when YtoQ was overexpressed in this genetic background. This time, suppressor mutants formed independently of the presence of absence of the *pdxA* and *pdxB* genes, as it was observed before on C-Glc minimal medium (*J. Rosenberg et al.*, 2018). In the background of BP978 (Δ*pdxST pdxJ pdxH* Δ*bshC*) overexpression of *ytoQ* enabled growth under all conditions but also without further *ytoQ* overexpression the cells grew but to a lesser extent.

Identification of potential interaction partners of YtoQ

YtoQ is a protein of unknown function. As it enables feeding into the heterologous pathway, it can be assumed that it has either enzymatic or regulatory activity on genome or protein level. As unspecific DNA binding of YtoQ could not be proven, we checked if YtoQ has interaction partners in *B. subtilis* with pulldown and in *vivo* crosslinking experiments. Therefore, the YtoQ expression plasmids pBP641 and pBP642, adding a N-terminal and C-terminal Strep-tag, respectively and the empty plasmid pBQ200 were introduced into *E. coli* Rosetta (DE3). LB medium was inoculated with the transformed strains and cultivated until OD_{600} reached 0.8 and protein expression was induced by the addition of IPTG. After incubation for 3 h at 37°C the cells were harvested by centrifugation. In parallel, *B. subtilis* wild type strain SP1 and the suppressor mutant BP1036 harboring a functional heterologous pathway, were grown in C-Glc medium until OD_{600} of 1 and also harvested. Cell pellets of *E. coli* and *B. subtilis* were resuspended in Strep-tag purification buffer W and lysed by using the French press. The crude extracts of the *E. coli* cells carrying empty vector and the expression plasmids were split and each half was added to a separate purification column. The matrix was washed intensively with buffer W and a sample was collected (W). The *B. subtilis* crude extract of either SP1 (A) or BP1036 (B) were applied to one of the columns containing the empty plasmid, the expression plasmid and to a column containing the Strep-Tactin matrix without bound protein to check for unspecific binding. After another washing step bound proteins were eluted with buffer E. Fractions were analyzed with SDS-PAGE and subsequent silver staining. As shown in Figure 17 A, N-terminally tagged YtoQ was easily purified and thick bands occurred at the corresponding height of approximately 19 kDa in the washing fraction, as well as in all elution fractions containing the expression plasmid. When crude extract of SP1 was added

no additional bands co-eluted with YtoQ. The same was true for the C-terminally tagged YtoQ expressed by pBP642 (see Suppl. Figure 3). As no difference between both constructs were detectable, further experiments were conducted only with the N-terminally tagged construct expressed by pBP641. The context in which YtoQ plays a role is highly artificial as the *pdxH* and *pdxJ* genes from *E. coli* were introduced into *B. subtilis*. Thus, it cannot be ruled out that YtoQ is only active when PdxJ and PdxJ are present. The pulldown was repeated using crude extract of the *B. subtilis* strain BP1036, which carries the partial heterologous pathway, the deletion of *bshC* and a mutation upregulating *ytoQ* gene expression (see Figure 17 B). The elution fractions 2 – 4 were analyzed and again, high amounts of YtoQ could be purified, especially in the later elutions. For E3 bands were present for the YtoQ expression plasmid, which are not in the empty vector control. These corresponding proteins had a molecular weight of approximately 17 kDa and 45 kDa, respectively and were co-eluted from the *E. coli* cell extract. Addition of BP1036 crude extract did not yield in additional bands, which do not also occured in the controls. Nevertheless, the gels were sent for MS analysis to Dr. Elke Hammer (University of Greifswald).

To rule out the possibility of transient interaction, we conducted an in *vivo* crosslinking experiment in different genetic backgrounds of *B. subtilis*. Accordingly, bacterial strains BKE29850 (Δ*ytoQ*), BP1102 (Δ*ytoQ* Δ*bshC*), BP1246 (Δ*pdxST* Δ*ytoQ* Δ*bshC*) and BP978 (Δ*pdxST* Δ*bshC pdxHJ*) were transformed with the SPINE plasmid pBP770 and the empty plasmid pGP382. The cells were cultivated in C-Glc medium supplemented with PL and respective antibiotics. When reached an OD_{600} of 1 the cultures were split and one half was treated with PFA for 20 min. After centrifugation, the cells were washed, lysed with the French press and purified by Strep-Tactin-affinity chromatography. The fractions were collected, boiled at 95°C for 30 min to release crosslinking bonds and subsequently analyzed by SDS-PAGE and silver staining (see Figure 18). YtoQ was expressed and eluted in E2- E5 with increasing amount. In all genetic backgrounds, co-elution of interaction partners could be detected on the gels. Also crosslinking with PFA did not show additional bands. Together with the pulldown gels, the whole gel of the BP978 background was sent for MS analysis to Greifswald.

Table 16 MS analysis of pulldown candidates

Candidate Protein	Function	Size [kDa]	Number of detected spectra[3]			
			EV	YtoQ	EV+ SP1	YtoQ + SP1
RpoC[2]	RNA polymerase beta'subuni	134	-	4	-	-
YrkL[2]	Similar to NAD(P)H oxidoreductase	20	-	-	-	10
YtoQ[2]	unknown	17	15	519	71	406
RpoC[1]	RNA-polymerase beta'subunit	155	-	8	-	6
RpoB[1]	RNA-polymerase beta subunit	151	-	2	-	3

MetH[1]	Homocysteine-N5-methyltetrahydrofolat transmethylase	136	-	30	-	38
CarB[1]	Carbamoyl-phosphate synthase	118	-	18	-	12
AcrB[1]	Multidrug efflux protein	114	-	4	-	5
MutS[1]	DNA mismatch repair protein	95	-	2	-	2
Pta[1]	Phosphate acetyltransferase	77	-	5	-	7
SecD[1]	Protein translocase	67	-	11	-	14
PtsI[1]	Phosphoenolpyruvate-protein phosphotransferase	64	-	7	-	6
Yjjk[1]	Predicted transporter of ABS superfamily	62	-	11	-	14
FumA[1]	Fumerate hydratase	60	-	3	-	10
PyrG[1]	CTP synthase	60	-	3	-	3
SucC[1]	Succinyl-CoA ligase	41	-	4	-	4
RlpA[1]	Minor lipoprotein	38	-	5	-	2
OmpR[1]	DNA-binding response regulator	27	-	2	-	4
Ftn[1]	Ferritin iron storage protein	19	-	2	-	2

1 proteins identified when mapped against *E. coli* database

2 proteins identified when mapped against *B. subtilis* database

3 detected proteins with at least two spectra and with significantly less spectra in the EV controls

The results of the pulldown analysis can be seen in Table 16. Since the protein was purified in *E. coli* and cell extract of *B. subtilis* was added, the spectra were mapped against the *B. subtilis* and the *E. coli* database. As promising candidates all proteins were selected for which at least two spectra were found and for which significantly lower spectra were detected in the empty vector control compared to the protein sample. Only three spectra were found, matching proteins in *B. subtilis,* the bait protein YtoQ, the RNA-polymerase beta' subunit RpoC and YrkL, which is similar to a NAD(P)H oxidoreductase. Interestingly, for YrkL only spectra were found in the fraction containing the bait protein and crude extract of *B. subtilis*, suggesting a specific interaction. YrkL is similar to a NAD(P)H oxidoreductase. When mapping the spectra against the *E. coli* database, 16 hits were found. Again, RNA polymerase subunits beta and beta' were identified in both fractions containing YtoQ. In addition, MutS (DNA missmach repair), PyrG (CTP synthase) and OmpR (DNA binding response regulator) also belong to the proteins involved in DNA homeostasis. From the *E. coli* candidates most spectra were found for MetH, a cobalamin-dependent homocysteine-N5-methyltetrahydrofolat transmethylase, involved in the synthesis of methionine from homoserine. Additional identified proteins are involved in central metabolism as CarB (carbamoyl-phosphate synthase), Pta (phosphate-acetyl transferase), PtsI (phosphoenolpyruvate-protein phosphotransferase), FumA (fumerate hydratase) and SucC (succinyl-

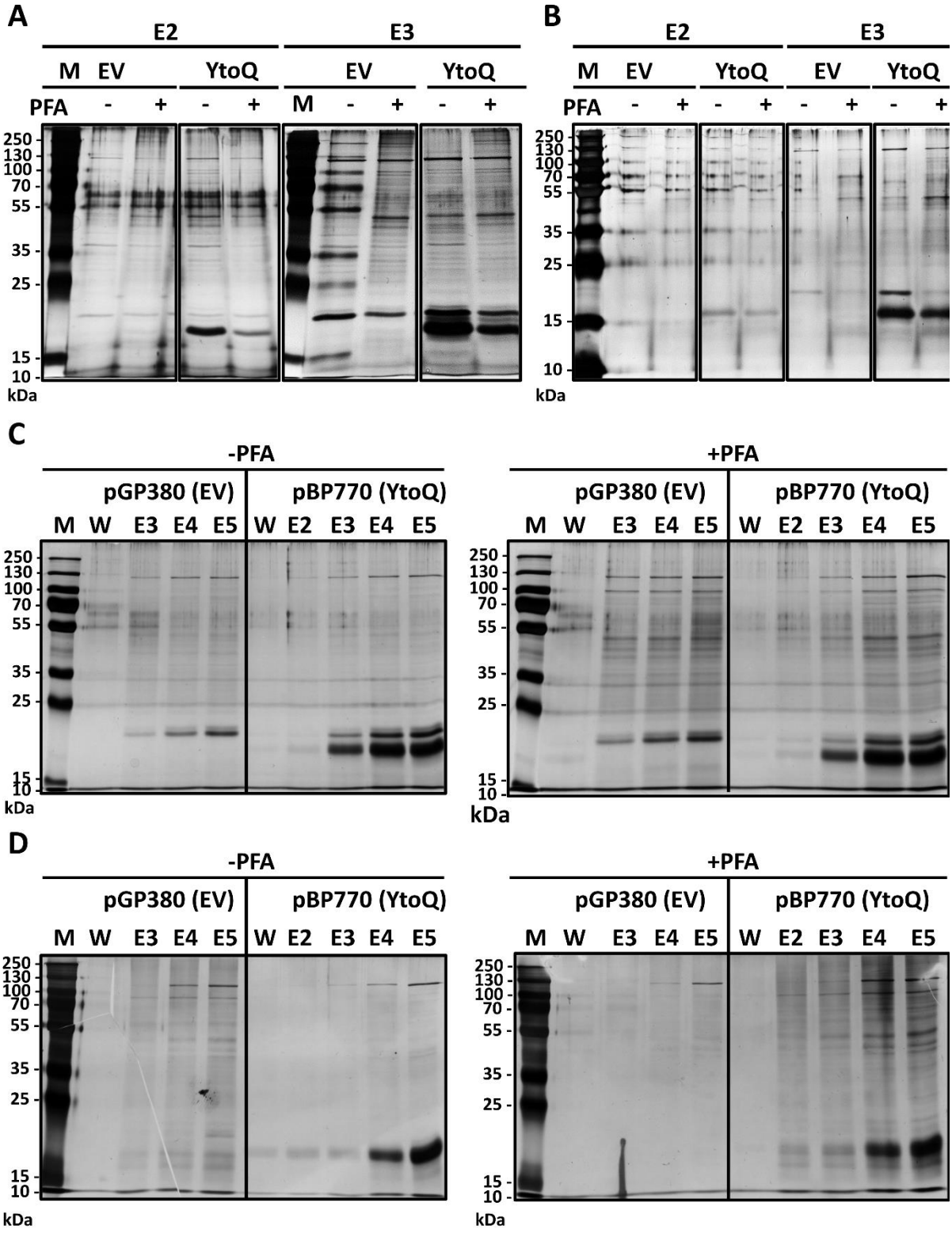


Figure 17 Pulldown with Strep-YtoQ.

Strep-YtoQ (pBP641), expressed by Rosetta (DE3) was coupled to a Strep-Tactin matrix. The matrix was washed with 15 ml buffer and bound proteins were either directly eluted or crude extract of *B. subtilis* wild type (**A**) or BP1036 (**B**) was added beforehand. Elution fractions were analyzed by SDS –PAGE and silver staining. M: protein size marker, W: fraction after washing step, E_x: elution fraction 1 - x of bound Strep-YtoQ, E+SP1/BP1036: elution fraction of bound STREP-YtoQ saturated with *B. subtilis* crude extract, SP1/BP1036: crude extract of SP1 or BP1036 added to the Strep-Tactin matrix. Arrows indicate bands, which are only present in elution fractions together with YtoQ

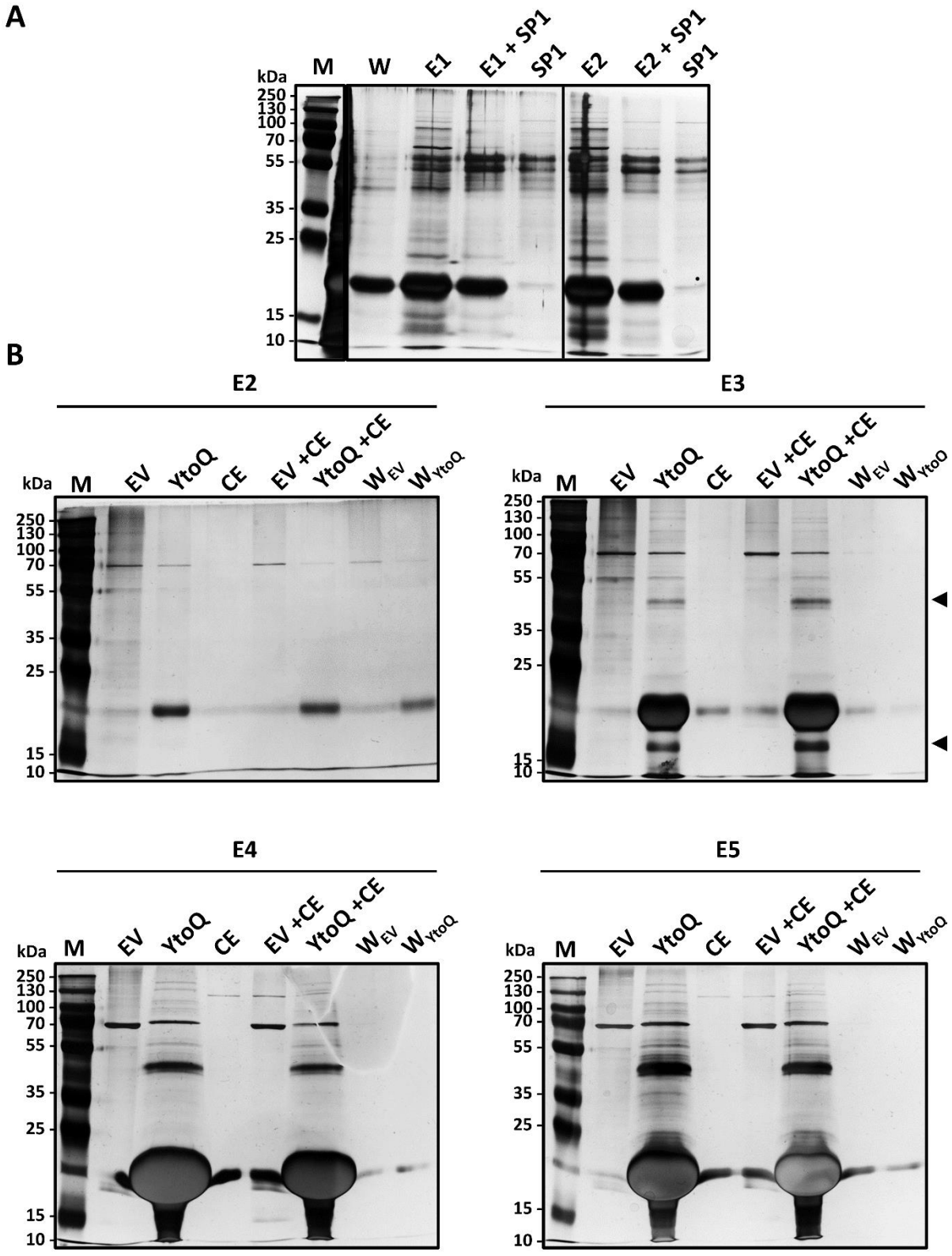


Figure 18 SPINE with YtoQ in different genetic backgrounds.
Plasmid pBP770 (pGP380::ytoQ) for expression of N-terminally Strep-tagged ytoQ and empty vector (pBP380) were introduced into different strains and cultivated in C-Glc minimal medium. At an OD between 0.6 and 1 the cultures were split and one half was treated with para-formaldehyde (PFA). STREP-YtoQ was purified and different elution fractions (E2-E5) were analysed by SDS-PAGE and subsequent silver staining. M: protein size marker. A: BKE29850 (Δ*ytoQ*) B: BP1102 (Δ*ytoQ* Δ*bshC*) C: BP1246 (Δ*pdxST* Δ*ytoQ* Δ*bshC* D: BP978 (Δ*pdxST* Δ*bshC pdxHJ).*

CoA ligase). Moreover, AcrB (Multidrug-efflux protein), SecD (protein translocase) and Yjjk (predicted transporter of ABS superfamily) belong to the group of transport proteins. The two remaining proteins were RlpA (Minor lipoprotein) and Ftn (ferritin-iron storage protein). Interestingly, for Ftn and YrkL C-terminal peptides were identified in the YtoQ band, although the proteins do not share sequence homology. This could indicate a binding mechanism of YtoQ.

Multiple interaction partner candidates for YtoQ could be identified by the pulldown assay. The SPINE data was not completely analyzed at the timepoint of writing this thesis. Nevertheless, the interaction of YtoQ with the interesting candidates needs to be confirmed by different experiments in the future.

3.2 A *B. subtilis* Δ*pdxT* mutant suppresses vitamin B6 limitation by acquiring mutations enhancing *pdxS* gene dosage and ammonium assimilation

PLP synthesis in a Δ*pdxT* mutant depends on the extracellular ammonium

B. subtilis synthesizes vitamin B6 via the PdxST enzyme complex. Previously it was shown that PdxS can produce PLP with high amounts of ammonium in a mutant lacking the glutaminase PdxT (*Belitsky*, 2004b; *Itagaki et al.*, 2013; *Sakai et al.*, 2002). We constructed the Δ*pdxT* mutant BP1105 in the background of the *B. subtilis* wild type strain SP1 and tested for ammonium dependent growth together with the PLP auxotrophic strain BP1100 (Δ*pdxST*) and SP1. Therefore, the strains were cultivated in MSSM minimal medium containing 0.5% glucose and 0.02% glutamine (w/v) as carbon/ nitrogen sources. By adding increasing amounts of ammonium chloride, ranging from 1 to 120 mM, ammonium dependent growth was monitored (see Figure 19). Besides PdxT, also the high-affinity glutaminase Ylam and the low-affinity glutaminase GlsA are expressed in *B. subtilis* (*G. Brown et al.*, 2008). Hence, some ammonium could derive from cleaved glutamine.

The growth rate of the wild type strain is constant among all tested conditions, only the biomass was decreased for 1 mM ammonium, indicating a depletion of ammonium at a later stage of growth. The Δ*pdxST* mutant BP1100 was not able to grow at all, confirming its inability to form PLP. On the other hand, BP1105 (Δ*pdxT*) showed an ammonium-depended growth behavior with increasing growth rate and biomass formation at higher ammonium concentrations.

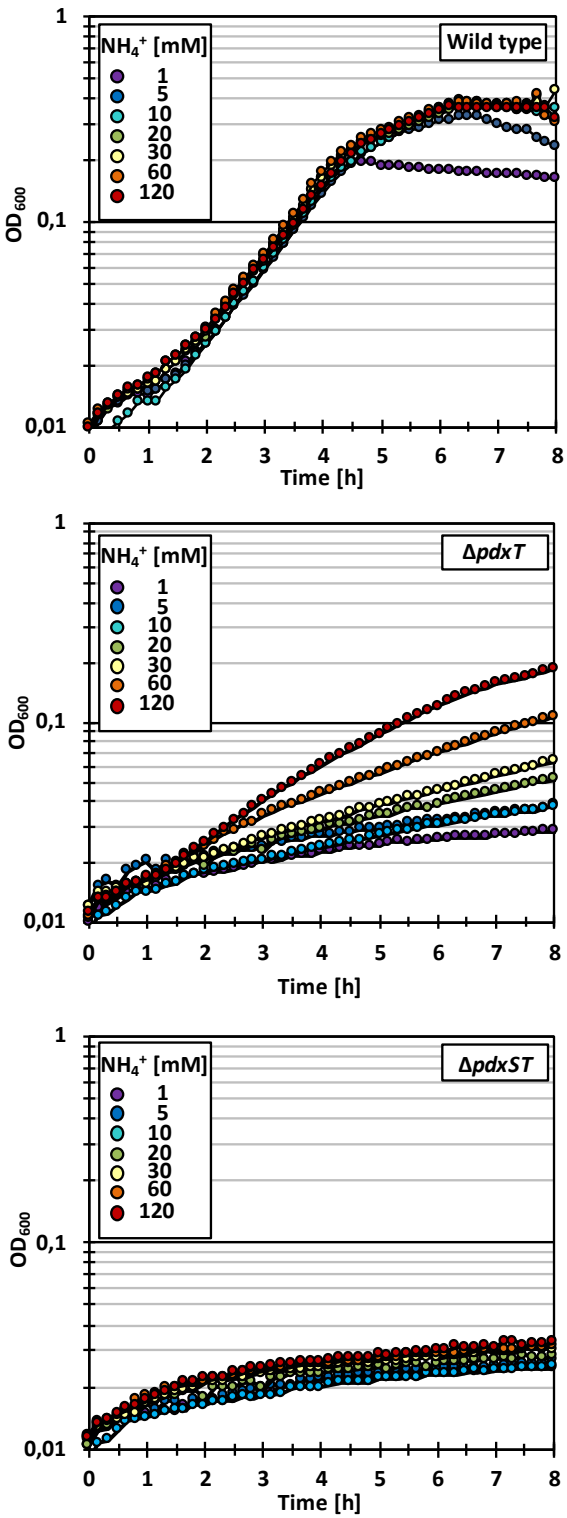


Figure 19 The dependency of a *B. subtilis pdxT* mutant on ammonium.
The wild type strain SP1 and the *pdxT* and *pdxST* mutant strains were cultivated in MSSM minimal medium supplemented with increasing amounts of ammonium. The bacteria were cultivated in a 96 well plate and growth was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}) using a multi-well plate reader. Figure and legend derived from (*Richts et al.*, 2021).

Characterization of Δ*pdxT* mutants with decreased demand on extracellular ammonium

The *pdxT* deletion mutant showed strongly reduced growth in medium containing only 10 mM and 20 mM ammonium. Thus, we assumed that the mutant could be adapted to the low ammonium concentrations to gather beneficial mutations, enhancing the PLP synthesis. We propagated the Δ*pdxT* mutant on MSSM agar plates containing either 10 mM or 20 mM ammonium and incubated the plates

for 48 h at 37°C. As seen in Figure 20A, suppressor mutants arose under both conditions. Six different suppressor mutants were isolated from the 10 mM plate and 13 more from the 20 mM plate.

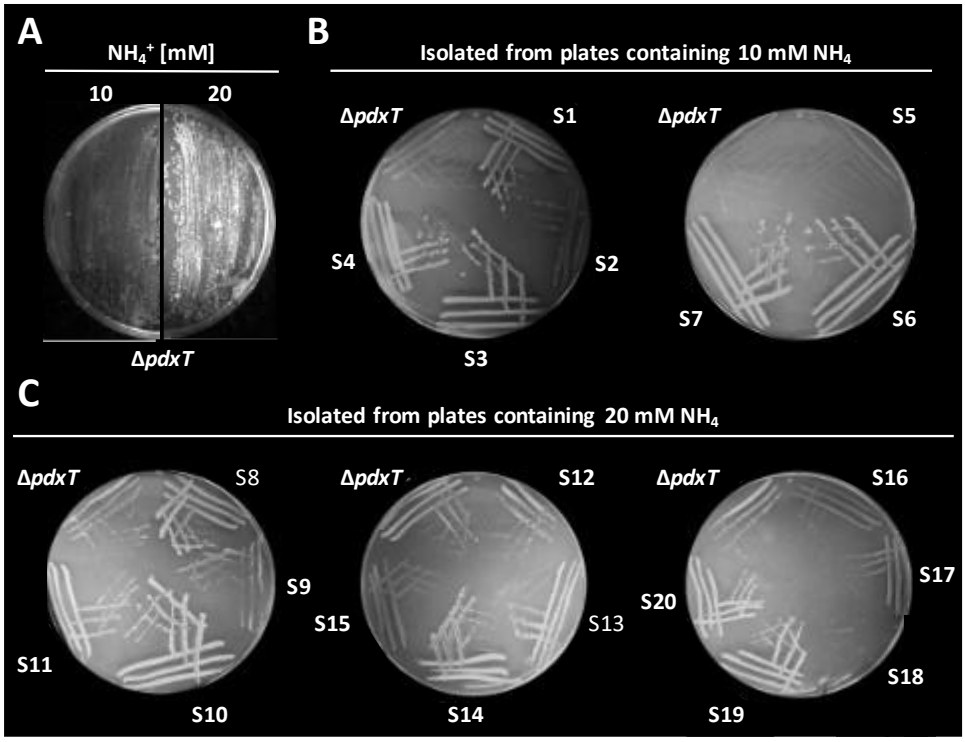


Figure 20 Isolation of Δ*pdxT* suppressor mutants with decreased dependency on extracellular ammonium.
A. Formation of suppressor mutants by the strain BP1105 (Δ*pdxT*) on MSSM agar plates supplemented with either 10 mM or 20 mM ammonium. The plates were incubated for 48°C at 37°C. **B**. and **C**. Evaluation of growth of the *pdxT* suppressor mutants on MSSM agar plates supplemented with 10 mM and 20 mM ammonium. The plates were incubated for 48 h at 37°C. S, suppressor mutant. Figure and legend derived from (*Richts et al.*, 2021).

To check if the mutants are stable, they were passaged twice on the respective MSSM medium and once on SP-rich medium plates. The suppressors were streaked on a plate together with the Δ*pdxT* mutant BP1105 to compare growth behavior at low ammonium. Five of the seven suppressors (S1, S3, S4, S6 and S7), which appeared on the 10 mM plate could grow better compared to the parental strain BP1105. The suppressor isolated on 20 mM ammonium, S8, S10, S11, S13, S14, S19 and S20 grew slightly better than the parental strain (see Figure 20C). Furthermore, we characterized the growth behavior of S1, S4 and S14 in liquid MSSM minimal medium. Consequently, MSSM medium containing 30 mM ammonium and PL was inoculated with the parental strain BP1105 and the suppressor mutants and incubated overnight at 37°C. Precultures were inoculated from the cell suspensions, grown until OD_{600} of 0.5 – 0.8, washed 1 x in MSSM and used for inoculation of 100 µl MSSM medium containing 30 mM ammonium. Growth was monitored in the plate reader.

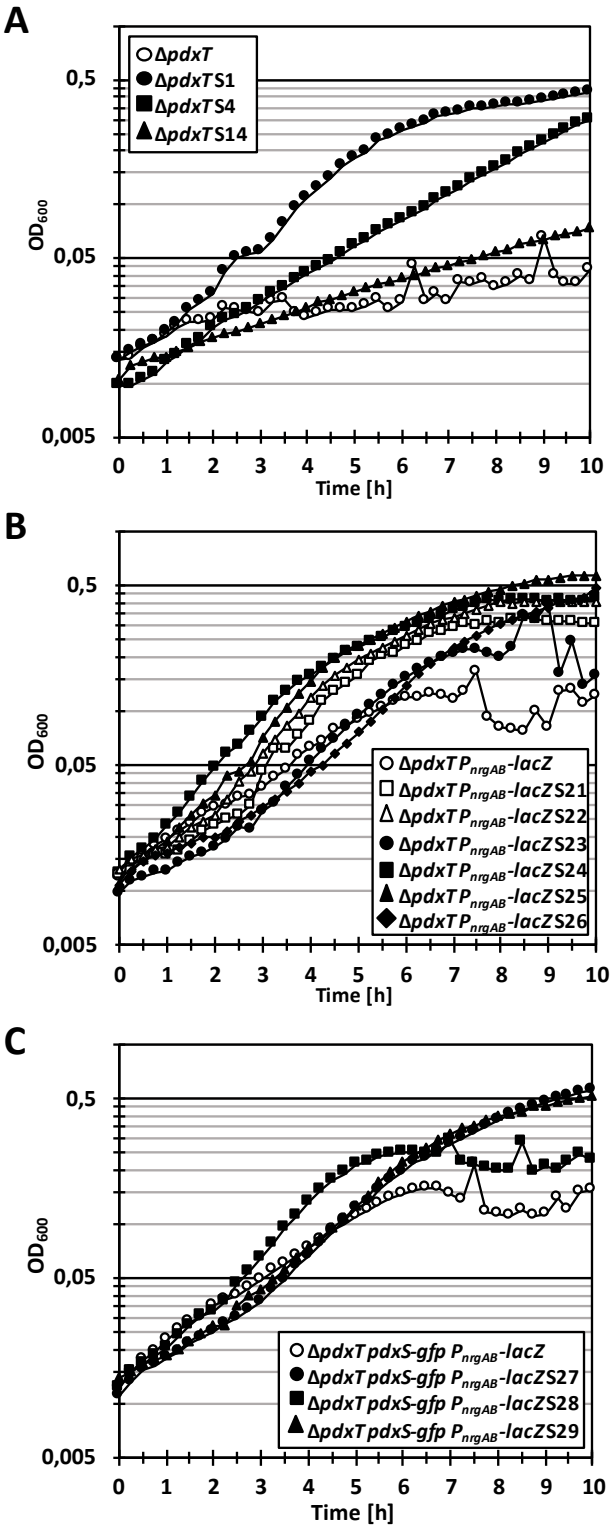


Figure 21 Characterization of pdxT suppressor mutants with decreased dependency on extracellular ammonium.
A. Growth of the Δ*pdxT* suppressors S1, S4 and S14 that are derived from the strain BP1105 (Δ*pdxT*). **B**. Growth of the Δ*pdxT* suppressors S21 - S26 that are derived from the strain BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*). **C**. Growth of the Δ*pdxT* suppressors S27 - S29 that are derived from the strain BP1182 (Δ*pdxT-gfp* P_{nrgAB}-*lacZ*). The bacteria were cultivated in MSSM minimal medium supplemented with 30 mM ammonium. The bacteria were cultivated in a 96 well plate and growth was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}) using a multi-well plate reader. Figure and legend derived from (*Richts et al.*, 2021).

All strains showed an improved growth compared to the parental strain but S1 and S4 to a higher extend than S14 as shown in Figure 21A. To assess the genetic changes allowing growth at limiting ammonium concentrations, we sequenced the genomes of the three suppressors. For a detailed list of mutations see Table 17.

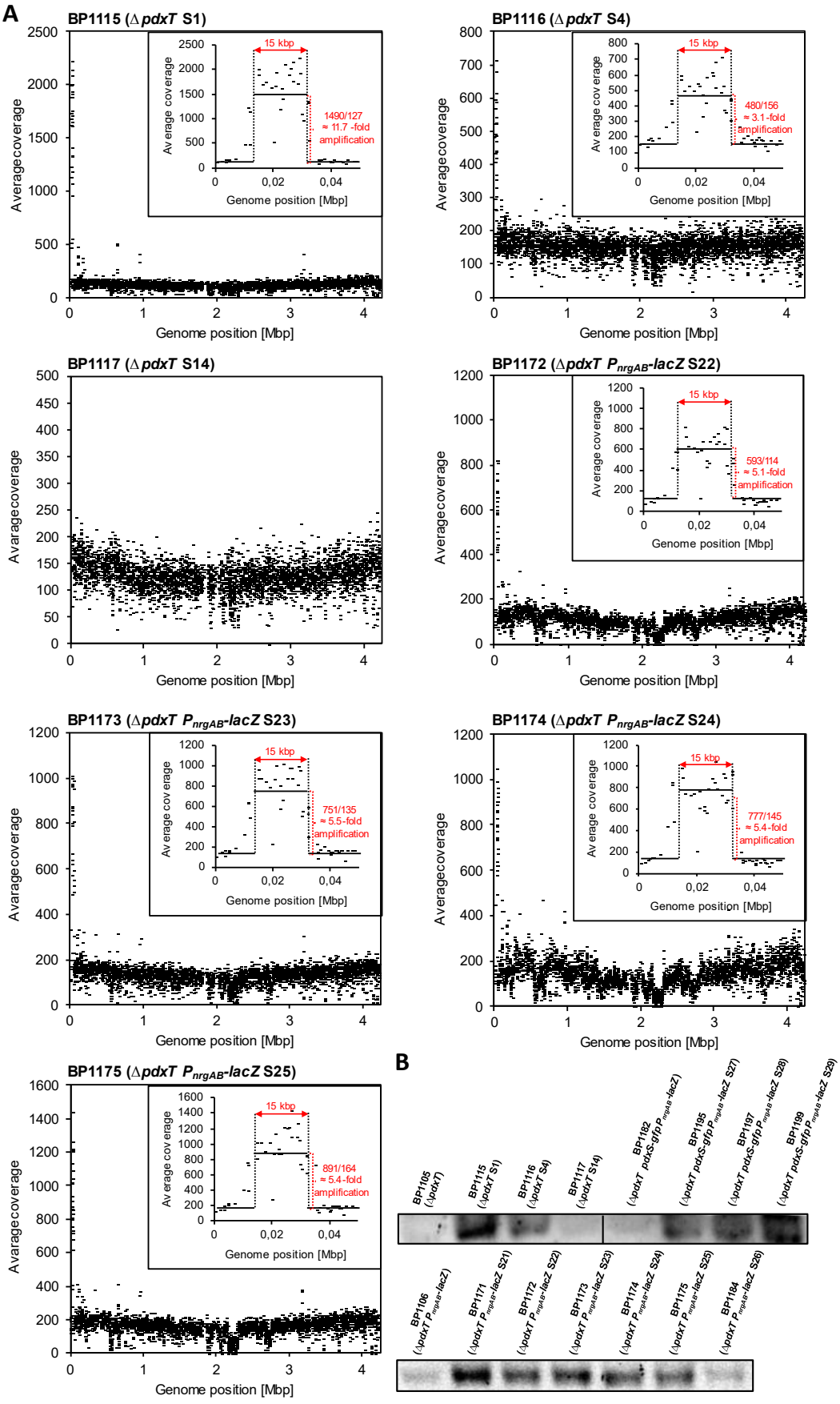

A
BP1115 (Δ pdxT S1)
BP1116 (Δ pdxT S4)
BP1117 (Δ pdxT S14)
BP1172 (Δ pdxT PnrgAB-lacZ S22)
BP1173 (Δ pdxT PnrgAB-lacZ S23)
BP1174 (Δ pdxT PnrgAB-lacZ S24)
BP1175 (Δ pdxT PnrgAB-lacZ S25)
Averagecoverage
Genome position [Mbp]
Average coverage
15 kbp
1490/127
≈ 11.7 -fold
amplification
480/156
≈ 3.1-fold
amplification
593/114
≈ 5.1-fold
amplification
751/135
≈ 5.5-fold
amplification
777/145
≈ 5.4-fold
amplification
891/164
≈ 5.4-fold
amplification
B
BP1105 (ΔpdxT)
BP1115 (ΔpdxT S1)
BP1116 (ΔpdxT S4)
BP1117 (ΔpdxT S14)
BP1182 (ΔpdxT pdxS-gfp PnrgAB-lacZ)
BP1195 (ΔpdxT pdxS-gfp PnrgAB-lacZ S27)
BP1197 (ΔpdxT pdxS-gfp PnrgAB-lacZ S26)
BP1199 (ΔpdxT pdxS-gfp PnrgAB-lacZ S25)
BP1106 (ΔpdxT PnrgAB-lacZ)
BP1171 (ΔpdxT PnrgAB-lacZ S24)
BP1172 (ΔpdxT PnrgAB-lacZ S22)
BP1173 (ΔpdxT PnrgAB-lacZ S23)
BP1174 (ΔpdxT PnrgAB-lacZ S24)
BP1175 (ΔpdxT PnrgAB-lacZ S25)
BP1184 (ΔpdxT PnrgAB-lacZ S26)

Figure 22 Genome sequencing and Southern blotting analyses to assess the extent of *pdxS* amplification in the *pdxT* suppressors. A. Read coverage (average) along the chromosome of the *pdxT* suppressors. As shown in the figure inlays, based on these values the copy numbers of chromosomal segment containing the *pdxS* gene can be inferred. In all suppressors that were subjected to Illumina sequencing, the amplified is 15 kbp long and is located between two rRNA genes (rrnO-23S - rrnA16S, coordinates 11,709 – 31,832). **B**. Southern blotting analysis to detect the amplified chromosomal segment containing the *pdxS* gene. The parent strains BP1105, BP1106 and BP1182 served as controls. Chromosomal DNAs were digested with *Sac*I and *Hind*III and the blots were hybridized with a *pdxS*-specific probe. Figure and legend derived from (*Richts et al.*, 2021).

S1 had a mutation in *pbpA*, which codes for the penicillin binding protein PBP2A. It controls the cell wall diameter and participates in cell wall biosynthesis (http://subtiwiki.uni-goettingen.de) (*Zhu & Stülke*, 2018). Also in S4 and S14 singe nucleotide polymorphisms (SNPs) emerged in *ispD* and *pgpH,* respectively. IspD is a 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, catalyzing a step in the isoprenoid synthesis pathway. The exchange of guanosine to thymine at position 574 in the *pgpH* gene leads to a stop codon truncating the protein after 192 of 711 amino acids. We furthermore recognized a genomic amplification of a 15 kbp long region when we had a look at the Illumina sequencing read depth. This region is bordered by the rrnO-16S/rrnO-23 and the rrnA16S/rrnA-23S genes and contains 21 genes in total (see Suppl. Table 1). For S1 the amplification factor is about 11-fold and for S4 about 3-fold (see Figure 22A). S14 does not amplify this region and only has the mutation in *pgpH*. Interestingly, the *pdxS* gene lies within in the amplified genome area. We confirmed the amplification by Southern blot analysis using a *pdxS*-specific probe. Due to the amplification, higher copy numbers of the probe binding site are available leading to a higher signal emission. Compared to the parental strain BP1105, the signal strength of the suppressor mutants was elevated, confirming the whole-genome sequencing data (see Figure 22B). In conclusion, the conditional pyridoxal auxotroph Δ*pdxT* mutant can recover its ability to produce sufficient amounts of PL by amplification of the *pdxS* gene. As PBP2A and IspD act in different cellular processes, it is unlikely that they participate in PLP synthesis and mutations accumulated as secondary effects. The role of PgpH is discussed in the next section.

Table 17 Mutations identified in the ΔpdxT suppressor mutants.

Strain	Parent strain (genotype)	Locus	Coordinates[c]	Protein, Function	Type of mutation
BP1115 (S1)[a]	BP1105 (Δ*pdxT*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	11.7-fold amplification
		pbpA	2,582,084	Penicillin-binding protein, cell wall biosynthesis	C1844T (A616V)
BP1116 (S4)[a]	BP1105 (Δ*pdxT*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	3.1-fold amplification
		ispD	109,813	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, isoprenoid biosynthesis	G25A (A9T)
BP1117 (S14)[a]	BP1105 (Δ*pdxT*)	*pgpH*	2,613,850	c-di-AMP-specific phosphodiesterase, osmoregulation	C574A (Stop)

BP1171 (S21)[b, d]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*pdxS*	Unknown	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	Amplification
		glnA	1,878,506 1,879,443	Glutamine synthetase, nitrogen metabolism	G79A (D27N) C1016A (P339Q)
BP1172 (S22)[a]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	5.1-fold amplification
		rpoE	3,813,083	RNA polymerase delta subunit, transcription	C → T (14 bp upstream of the start codon)
		tnrA	1,397,468	Transcriptional pleiotropic regulator, nitrogen metabolism	Insertion 280T (93 of 111 amino acids and 9 different amino acids at the C terminus)
		yknV	1,502,282	ATP-binding protein of unknown ABC exporter, unknown function	Deletion A622 (lack of 207 of 604 amino acids and 10 different amino acids at the C terminus)
BP1173 (S23)[a]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	5.5-fold amplification
		glnA	1,878,607	Glutamine synthetase, nitrogen metabolism	C180A (F60L)
		thrS T-box RNA	2,961,374	Threonyl-tRNA synthetase, protein synthesis	G → A (179 bp upstream of the start codon)
BP1174 (S24)[a, c]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	5.4-fold amplification
		dhbF	3,285,033	Involved in 2,3-dihydroxybenzoate biosynthesis, bacillibactin biosynthesis	G2629A (H877Y)
		nhaK	3,429,304	Cation/H^+ antiporter, metal ion homeostasis	G1046A (P349L)
		rpoE	3,812,989	RNA polymerase delta subunit, transcription	Deletion T82 (lack of 27 of 173 amino acids and 10 different amino acids at the C terminus)
		rpoE	3,812,984	RNA polymerase delta subunit, transcription	T87A (K27N)
		rpsJ	135,234	Ribosomal protein, translation	G → A 130 bp upstream of the start codon
		ahpA	1,492,176	Alkyl hydroperoxide reductase, protection against peroxide stress	C → T (88 bp upstream of the start codon and 2 bp downstream of -10 region)
BP1175 (S25)[a]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	5.4-fold amplification
		alaS	2,799,351	Alanyl-tRNA synthetase, protein synthesis	C1466T (S489N)
		pgpH	2,613,916	c-di-AMP-specific phosphodiesterase, osmoregulation	Deletion C508 (lack of 169 of 711 amino acids) and 6 different amino acids at the C-terminus)
		pgpH	2,613,912	c-di-AMP-specific phosphodiesterase, osmoregulation	A510C (Stop509E)

		pksN	1,847,873	Polyketide synthase, biosynthesis of antibacterial compounds	G13462A (E4488K)
BP1184 (S26)[b]	BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)	*glnA*	1,878,506	Glutamine synthetase, nitrogen metabolism	G79A (D27N)
BP1195 (S27)[b, d]	BP1182 (Δ*pdxT::cat amyE::*(P_{nrgAB}-*lacZ aphA3*) *serS::(serS-*P_{alf4}*-gfp ermC)*)	*pdxS*	Unknown	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	Amplification
		glnA	1,878,506	Glutamine synthetase, nitrogen metabolism	G79A (D27N)
BP1197 (S28)[b, d]	BP1182 (Δ*pdxT::cat amyE::*(P_{nrgAB}-*lacZ aphA3*) *serS::(serS-*P_{alf4}*-gfp ermC)*	*pdxS*	Unknown	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	Amplification
		glnA	1,878,607	Glutamine synthetase, nitrogen metabolism	C180A (F60L)
BP1199 (S29)[b, d]	BP1182 (Δ*pdxT::cat amyE::*(P_{nrgAB}-*lacZ aphA3*) *serS::(serS-*P_{alf4}*-gfp ermC)*)	*pdxS*	Unknown	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	Amplification
		glnA	1,878,607	Glutamine synthetase, nitrogen metabolism	C180A (F60L)
BP1148[a]	BP1147 (Δ*pdxT::cat amyE::*(P_{nrgAB}-*lacZ aphA3*) *serS::(serS-*P_{alf4}*-gfp ermC) recA::aad9*)	*pdxS*	11,709 – 31,832	Pyridoxal 5'-phosphate synthase domain, vitamin B6 biosynthesis	2-fold amplification
		yonO	2,222,914	SPβ DNA-dependent RNA polymerase	G337T (Stop113E)
		yqjM	2,476,713	NADPH-dependent flavin oxidoreductase	C864T (no effect)
		patA	1,472,749	PLP dependent biosynthesis of lysine and peptidoglycan	G293A (A98V)
		ymfD	1,756,606	exporter for the siderophore bacillibactin	G976A (A326T)

Table derived from (*Richts et al.*, 2021)
[a]Strains were subjected to whole genome sequencing.
[b]Mutations in the *glnA* gene was identified by PCR using the primer pairs CD03/CD24.
[c]Coordinates refer to the position in the genome sequence (GenBank: CP058242.1) (Richts et al., 2020) of the *B. subtilis* SP1 strain.
[d]The amplification of a genomic region containing the *pdxS* gene was verified only by Southern blotting.

Characterization of Δ*pdxT* mutants carrying mutations in ammonium assimilation genes

When growing with ammonium as only nitrogen source, the glutamine synthetase (GS), encoded by *glnA*, utilizes glutamate and ammonium to form glutamine (see Figure 23A) (*Atkinson & Fisher*, 1991; *Gunka & Commichau*, 2012). Hence, the global nitrogen regulator TnrA activates expression of the *nrgAB* genes, which code for the ammonium channel NrgA (see Figure 23A) (*Detsch & Stülke*, 2003; *Mirouze et al.*, 2015;*Wray et al.*, 1994, 1996; *Yoshida et al.*, 2003). Produced glutamine inhibits the GS (FBI-GS), enables its ability to bind to TnrA and thereby blocks the DNA binding activity (Figure 23A) (*Hauf et al.*, 2016; *Wray et al.*, 2001). Moreover, the FBI-GS can bind and inhibit the transcription factor GlnR, which is an activator of its own expression (*Fisher & Wray*, 2008). With this regulatory network

B. subtilis can adapt the ammonium assimilation system to the available N-sources (*Commichau et al.*, 2008; *Gunka & Commichau*, 2012).

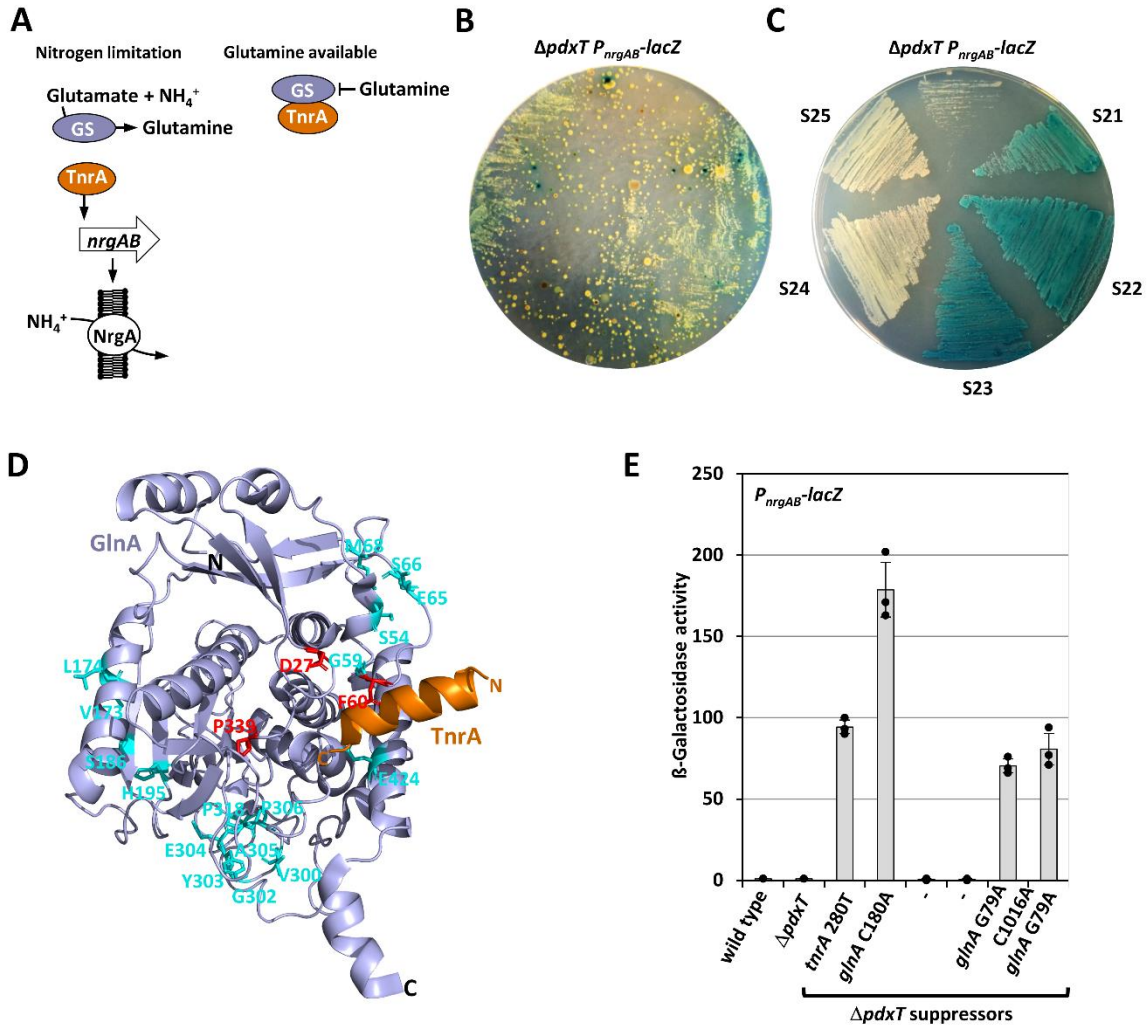


Figure 23 Isolation of *pdxT* mutants carrying mutations in ammonium assimilation genes and their impact on the regulation of the P_{nrgA} promoter. A. Control of ammonium assimilation in *B. subtilis*. When glutamine is limiting, TnrA activates the expression of the *nrgAB* genes encoding the ammonium transporter NrgA and the P_{II} protein NrgB (Detsch and Stülke, 2003). The GS GlnA converts glutamate and the incoming ammonium to glutamine. Under conditions of excess nitrogen, FBI-GS binds to and inhibits TnrA. The *nrgAB* genes are not expressed. **B**. Formation of suppressor mutants by the strain BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*) on MSSM agar plates supplemented with 20 mM ammonium and X-Gal. The plates were incubated for 48 h at 37°C. **C**. Evaluation of growth of the *pdxT* suppressor mutants on MSSM agar plates supplemented with 20 mM ammonium. The plates were incubated for 48°C at 37°C. S, suppressor mutant. **D**. Localization of the amino acid exchanges in GlnA that affect the interaction with TnrA. Amino acid exchanges that have been previously described (Fisher et al., 2002; Wray and Fisher, 2005, 2010; Fisher and Wray, 2006, 2009) and identified in the present study are indicated in turquoise and red, respectively, in a structure model of the GlnA-TnrA complex (PDBid 4S0R; Schumacher et al., 2015). **E**. β-galactosidase activity assays to monitor the effect of the mutations in the *tnrA* and *glnA* genes on the activity of the P_{nrgAB} promoter. The assay was performed with the *B. subtilis* wild type BP1124 (P_{nrgAB}-*lacZ*), the *pdxT* mutant BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*) and with the *pdxT* suppressors BP1171 (Δ*pdxT glnA* $^{G79A\ C1016A}$ P_{nrgAB}-*lacZ*), BP1172 (Δ*pdxT tnrA*$^{\Delta T280}$ P_{nrgAB}-*lacZ*), BP1173 (Δ*pdxT glnA* ^{C180A} P_{nrgAB}-*lacZ*) BP1174 (Δ*pdxT* P_{nrgAB}-*lacZ*), BP1175 (Δ*pdxT* P_{nrgAB}-*lacZ*) and BP1184 (Δ*pdxT glnA* ^{G79A} P_{nrgAB}-*lacZ*). It should be noted that the strains BP1172, BP1173, BP1174 and BP1175 carry additional mutations (see Table 1). The bacteria were cultivated in MSSM medium supplemented with 1.3 mM glutamine and 30 mM ammonium. Data points represent biologically independent replicates. Bars indicate means of replicates and the standard deviations are shown. β-Galactosidase activities are given as units per milligram of protein. Figure and legend derived from (*Richts et al.*, 2021)

As the suppressors of the *pdxT* mutant were isolated on MSSM medium containing small amounts of glutamine, the GS is feedback inhibited and binds the global DNA-binding transcription factor of nitrogen metabolism TnrA, so that the transcription of the ammonium transporter gene *nrgA* is blocked (*Wray et al.*, 2001). The uptake of ammonium is mandatory for the cells to produce PLP so we supposed that suppressor mutants could come up, which upregulate the ammonium uptake and thereby suppress the partial PL auxotrophy. To screen for such mutants, we integrated a translational P_{nrgAB}-promoter-*lacZ* construct into the Δ*pdxT* mutant by transformation with the plasmid pGP168, resulting in the strain BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*). We propagated the strain on MSSM plates containing 20 mM ammonium and X-Gal to screen for upregulation of the P_{nrgA} promoter. After two days of incubation blue and white suppressor mutants formed, whereas the number of white suppressors was clearly higher (see Figure 23B). Four blue (S21-S23 and S26) and two white colonies (S24-S25) were isolated on plates containing X-Gal and growth was tested in MSSM medium supplemented with 30 mM ammonium (see Figure 21B-C). All strains produced more biomass compared to the parental strain but grew with different speed. S21, S22, S24, S25 had a similar, but lower doubling time as the wild type. The doubling time of S23 and S26 was close to the one of the parental strain BP1106. Therefore, two blue and two white suppressors were subjected to whole-genome sequencing showing indeed mutations in *tnrA* and *glnA*. For the other blue suppressors these *loci* had been sequenced by sanger sequencing (Table 17). In S21, S23 and S26 mutations led to amino acid changes in GlnA, respectively to D27N/P339Q, F60L and D27N. These amino acids are in close proximity the binding site of TnrA (see Figure 21D. Thus, binding ability of the C-terminus of TnrA to the GS could be lowered and DNA binding favored, which would activate *nrgAB* expression. The found mutations have not been described before, although many known mutations in *glnA* were already described (*Fisher et al.*, 2002;. *Fisher & Wray*, 2006, 2009; *Wray & Fisher*, 2005, 2010). In S22 we found a base deletion in the *tnrA* gene resulting in C-terminal truncation of 18 amino acids and a change of the last 9 amino acids (see Table 17). The C-terminus of TnrA is responsible for the binding to the FBI-GS (see Figure 21D) (*Schumacher et al.*, 2015; *Shin et al.*, 2000; *Wray et al.*, 2001). Hence the variant is probably not able to bind to the FBI-GS and again, activates the P_{nrgAB} promoter. We tested the effect on the *nrgAB* promoter by measuring the *β*-galactosidase activities of the white and blue suppressors, the parental strain BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*) and the wild type strain BP1124 (P_{nrgAB}-*lacZ*). The strains were cultivated in MSSM medium containing 30 mM ammonium.

The promoters of the wild type (BP1124), the parental strain BP1106 and of the white suppressors S24 and S25 showed no activity (see Figure 21E). The promoters of the blue suppressors were highly active. The *glnA* C180A mutation had the biggest effect on activity. The *tnrA* truncation and the other mutations in *glnA* showed lower activity but reached approximately the same level.

As we also analyzed the Illumina depth and Southern blot probe binding, we found that the *pdxS* gene was amplified also in the suppressors S21-S25 (Figure 22A and B). To conclude, the mutations affecting the ammonium assimilation increased the expression of the ammonium channel *nrgA* and thereby elevate ammonium uptake. Nevertheless, overproduction of the PLP synthase PdxS suppressed the loss of the glutaminase domain most effectively.

Besides the amplification of *pdxS* genetic region and the mutations in *glnA* and *tnrA,* also additional mutations were acquired (see Table 17). The ribosomal binding site and the open reading frame of the *rpoE* gene was mutated in S22 and S25, respectively, probably leading to a deregulation of the RNA polymerase δ-subunit. Indeed, the activity of the *rpoE** promoter of the suppressor mutant was 3-fold lower than the wild type version (Suppl. Figure 6) and the mutation in S24 led to a truncation of 27 amino acids and 10 different amino acids at the C-terminus (see Table 17). Thereby, the protein was probably not functional. The suppressor 25 carried a mutation in the *pgpH* gene, similar to the mutation of suppressor 14, which truncated the PgpH protein by 202 amino acids. The effect of a loss of *pgpH* and *rpoE* was tested in terms of the bachelor thesis of Melvin Bönninger. He created *pgpH* and *rpoE* knockouts in the background of the Δ*pdxT* mutant and compared the growth behavior of the double mutants with the Δ*pdxT* mutant. As a control for Δ*pgpH* he also knocked out the second c-di-AMP specific phosphodiesterase GdpP. To assess whether a possible positive effect is still present when *pdxS* is amplified, the double mutants as well as the Δ*pdxT* singe mutant, were transformed with the empty plasmid pBQ200 and the *pdxS* overexpression plasmid pBP775, which was constructed beforehand. Growth was tested in MSSM minimal medium containing ammonium ranging from 10 mM – 120 mM (Suppl. Figure 4). In presence of 120 mM ammonium all strains reached about the same biomass, independently of *pdxS* overexpression. Only the Δ*rpoE* mutant with the empty plasmid had a slight growth defect. In 10 mM the difference between overexpression of *pdxS* and empty plasmid was clearer. As shown before, strains carrying the overexpression plasmid reached higher biomass and growth rate. Interestingly, without additional *pdxS* dosage, the Δ*pgpH* Δ*pgpH* double mutant showed a growth advantage in contrast to the Δ*gdpP* mutant. To confirm this effect, the Δ*pdxT* mutant was transformed with constructs for xylose inducible expression of *rpoE*, *pgpH*, and *gdpP*. The growth experiment was repeated with the same ammonium concentrations but growth was monitored also in presence 0.8% xylose (Suppl. Figure 4B). As expected, overexpression of *pgpH* caused a growth defect in 10 mM ammonium, confirming its role in suppressing the PL auxotrophy by *pdxT* deletion. Knockout or inducible expression of *rpoE* did not yield a positive effect, indicating its secondary effect, probably due to the amplified region.

Suppressor S23, S24 and S25 also showed mutations in other genes, which are not involved in vitamin B6 metabolism (Table 17) (*Richts et al.*, 2019). In conclusion, loss of *pdxT* can be complemented by

addition of exogenous ammonium. If ammonium is limited, amplification of the *pdxS* gene and mutations regarding ammonium uptake accumulate.

pdxS gene amplification does not facilitate the evolution of a PdxS variant with enhanced enzyme activity

Previous studies showed, that the replication of larger chromosomes, are more energy consuming. Thus, amplification of a genomic region can also have negative consequences for the cell (*Reuß et al.*, 2019). A possibility to escape this burden, is the rise of mutations, which directly compete with the limiting factor and provoke a de-amplification of the amplified region. We assumed that this could be also be applicable for the amplification of the Δ*pdxT* mutant, as further evolution could lead to the acquisition of beneficial mutations. As appropriate, de-amplification of the 15 kbp region could be the consequence. To monitor the de-amplification, we integrated a genetic cassette containing the green-fluorescent protein (GFP) and an erythromycin resistance cassette under the expression of a constitutive promoter downstream of the *pdxS* genetic region in the Δ*pdxT* mutant BP1106 resulting in the strain BP1182 (Δ*pdxT* P_{nrgAB}-*lacZ pdxS-gfp*) (see Suppl. Figure 5). Successful application of the used cassette was recently proven by Reuß et al. 2019 who visualized selective amplification and de-amplification of the *parEC* genes being able to replace the essential *topA* gene if overexpressed (*Reuß et al.*, 2019).

The screening strain BP1182 was applied to MSSM minimal medium plates supplemented with 20 mM ammonium. The emerging suppressors were checked for an increase in GFP signal and the mutants BP1191 and BP1192 were isolated (see Suppl. Figure 5). We passaged the mutants 20 times in liquid MSSM medium and plated the cells after each passage on MSSM plates to analyze GFP signaling but were not able to see a decrease of amplification (see Suppl. Figure 5). Nevertheless, we isolated three more suppressors of the screening strain BP1182 and characterized them. The suppressor mutants S27-29 showed a growth advantage in MSSM medium compared to the parental strain (see Figure 21, Figure 22C) proved that the strains also carried the amplified region containing the *pdxS* gene. Besides, the suppressors also had mutations in *glnA* (G79A, C180A), which was checked by sanger sequencing (Table 17).

To prevent amplification and select for mutants acquiring mutations in the *pdxS* gene itself, we deleted the *recA* gene coding for RecA a multifunctional recombination protein, resulting in the strain BP1147 (Δ*pdxT* P_{nrgAB}-*lacZ pdxS-gfp* Δ*recA).* We isolated one suppressor BP1148 of the strain and analyzed it by whole-genome sequencing (Table 17). Interestingly, the suppressor was still able to amplify the *pdxS* gene region but only by factor 2. This was also confirmed as the suppressor still emitted fluorescence (data not shown). Furthermore, the suppressor had mutations in the genes *patA*

(N-acetyl-$_{L,L}$-diaminopimelate aminotransferase), *yqjM* (NADPH-dependent flavin oxidoreductase) and *yonO* (SP*β* RNA-dependent DNA polymerase). All of the enzymes do not seem to be directly involved in PLP metabolism except for PatA, which belongs to the PLP-dependent proteins (*Richts et al.*, 2019). To conclude, mutations regarding the *pdxS* gene or its gene dosage cannot be achieved by evolution under the tested conditions.

PdxS variants with higher enzymatic activity can be visualized by a PL-sensitive screening system

As mutations regarding the *pdxS* gene could not be obtained by evolution experiments, we supposed that directed evolution of *pdxS* gene by random mutagenesis could lead to mutations in *pdxS* resulting in higher enzymatic activity. To test *pdxS* variants, activities need to be measured to shed light on the enzymatic activity, which implies purification of the variants and subsequent enzymatic assays. To prevent this time-consuming step, we decided to develop a screening system for *pdxS* variants to screen for higher activity in *vivo*. We planned to use plasmid-based mutagenesis in the *E. coli* strain XL1-Red, which lacks important DNA repair genes and thereby inserting random mutations during DNA replication. It was successfully used in former studies to generate randomly mutagenized gene variants (*Dormeyer et al.*, 2019).The emerging plasmids were used to transform the *B. subtilis* screening strain and promising plasmid candidates could be detected directly on agar plate. For the screening system we were looking for a possibility to display varying amounts of produced PLP by promoting expression of the *lacZ* gene.

PLP synthesis in *B. subtilis* is not feedback regulated on gene level as it is in other organisms (*Belitsky*, 2014). But for example, the expression of the GabT, a *γ*-aminobutyric acid aminotransferase is controlled by the presence of PLP. As this system needs a combination of GABA, the substrate of GabT, and PLP for an activation of the *gabTD* promoter, the system was not ideal for the screening system as it was also dependent on the presence of GABA. The *thi* riboswitch in *Arabidopsis thaliana* changes it conformation upon PLP binding and blocks the polymerase to access the Shine-Dalgarno sequence (*Han et al.*, 2020) and was a possibility for the screening system. Nevertheless, we decided to use a PLP-dependent expression system deriving from the human pathogen *L. monocytogenes* in which the expression of the *pdxST* genes is regulated in a PL-dependent manner (*Belitsky*, 2014). The promoter of the *pdxST* complex is bidirectional, driving the expression of the *pdxST* and *pdxR* genes, of which the latter gene encodes a regulator of the promoter. As seen in Figure 24A, under low PLP conditions, the promoter of *pdxR* is active leading to the expression of PdxR. The *pdxST* promoter is inactive as it needs PdxR binding to allow the RNA polymerase to be active. When PdxR is synthesized it binds to the

bidirectional promoter, activates *pdxST* expression but blocks its own transcription. PdxST can form PLP from ribose-5-phosphate, glyceraldehyde-3-phosphate and glutamine.

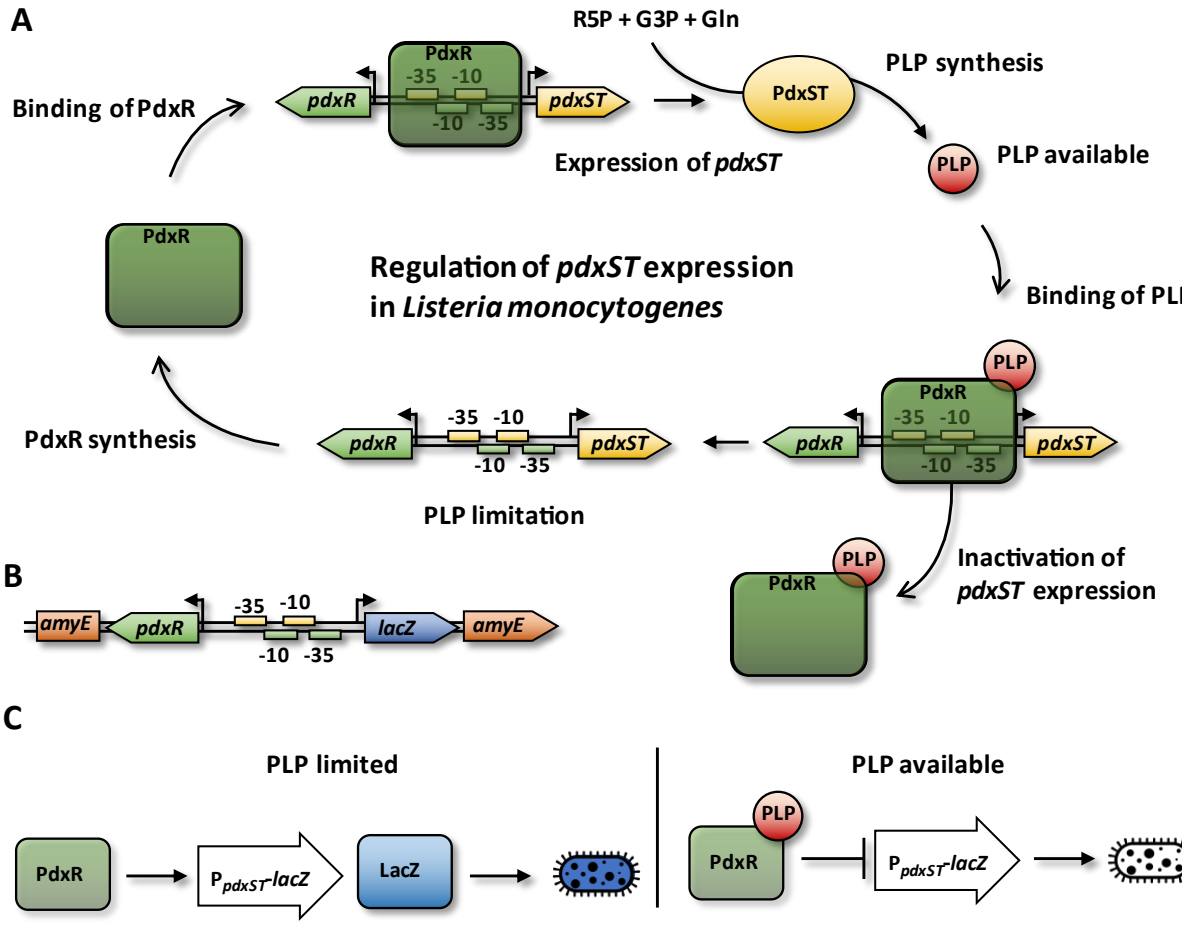


Figure 24 Screening system to measure PLP levels in *vivo*.
A: Upon PLP limitation the bidirectional promoter P_{pdx}R-pdxST is active for the expression of *pdxR* and inactive for *pdxST* expression. Synthesized PdxR binds to the promoter region and facilitates conformational changes. Thereby, the expression of *pdxR* is blocked and the *pdxST* genes can be transcribed. PLP is synthesized from ribose-5-phosphate (R5P), glyceraldehyde-3-phosphate (G3P) and glutamine (Gln) by the PdxST complex. Free PLP can bind to PdxR and causes a conformational change, leading to a depositioning on the DNA by, which the expression of *pdxST* is blocked. **B**: Construct of the screening system. The bidirectional promoter of *L. monocytogenes* was amplified and introduced into the pAC7 vector, which integrates into the *amyE* locus of *B. subtilis* and allows promoter-driven expression of the *lacZ* gene. **C**: When B6 is a limiting factor, PdxR is build and activates the bidirectional promoter. Thus, the *lacZ* gene is expressed. With the addition of X-Gal to the medium, cells expressing *lacZ* turn blue. On the other hand, when PLP is available, it binds to PdxR and prevents it from activating the promoter. As a consequence, the cells stay white.

With rising levels, more and more PLP binds to PdxR and thereby leading to a conformational change, a repositioning on the DNA and finally a detachment. The expression of *pdxST* is blocked again and the *pdxR* is promoter active. To establish our PLP sensitive screening system, we amplified the *pdxR* gene together with the bi-directional promoter from *L. monocytogenes* chromosomal DNA and cloned using the vector pAC7 resulting in the plasmid pBP781 (*pdxR-*P_{pdxST}^{Lmo}*-lacZ*), which integrates into the *amyE* locus in *B. subtilis* and promotes *lacZ* expression directed from the inserted promoter (see Figure 24B). As also the open reading frame of *pdxR* is included, PdxR is expressed in the absence of PLP and binds

to its promoter (see Figure 24C). According to this, *lacZ* is expressed and converts X-Gal in the medium, so that the cells turn blue. If PLP is available, it binds PdxR and the promoter is inactive. Since *lacZ* is not expressed the cells remain white.

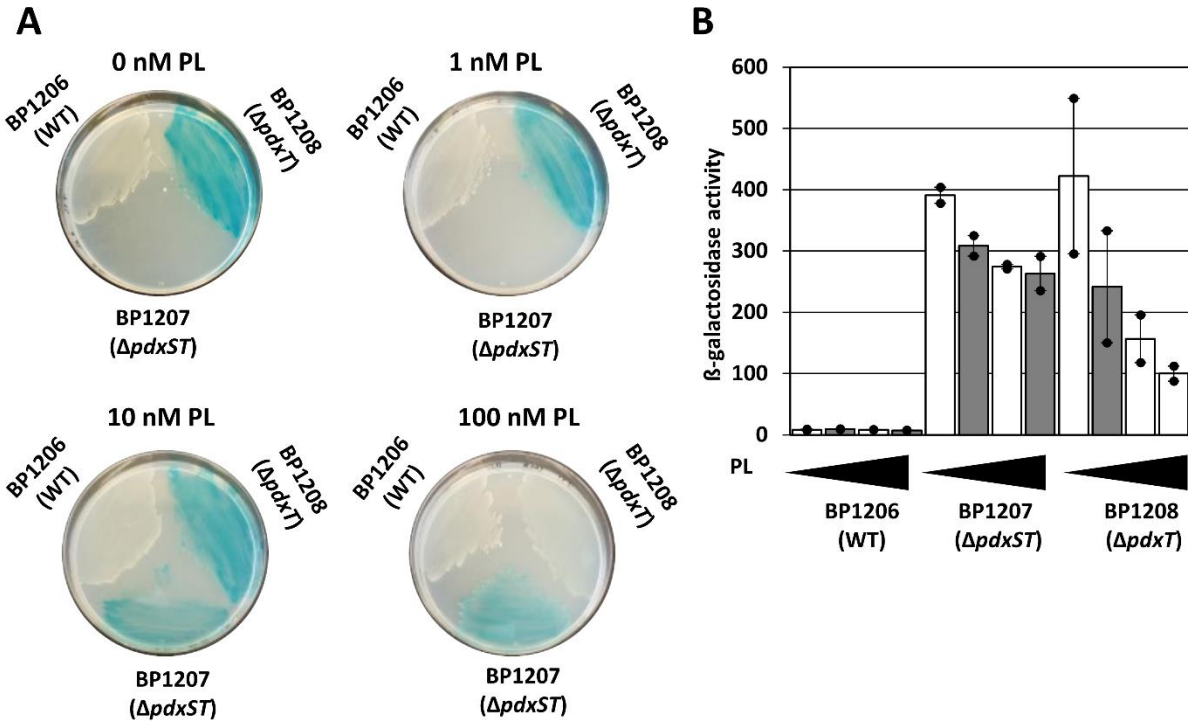


Figure 25 Test of the screening system.
Plasmid pBP781 was introduced into *B. subtilis* wild type SP1, Δ*pdxT* mutant BP1105 and Δ*pdxST* mutant BP1100, resulting in the strains BP1206, BP1207 and BP1208, respectively. **A**: The strains were grown over night in LB medium supplemented with PL. The cell cultures were washed 2x in saline solution, the OD_{600} was adapted to 1 and 10 µl cell suspension were spread on TSS medium plate containing X-Gal and different PL concentrations. **B**: Screening strains were inoculated in MSSM minimal medium containing 30 mM NH_4 and PL and were incubated over night at 37°C. The cultures were used to inoculate fresh minimal medium supplemented with either 100 nM, 500 nM, 900 nM or 1300 nM PL. When reached OD_{600} between 0.5 and 0.8 cells were harvested by centrifugation and the resulting cell pellets were used to measure the LacZ activity. Bars indicate means of replicates and the standard deviations are shown. β-galactosidase activities are given as units per milligram of protein.

To test the screening construct, we introduced the plasmid pBP781 into the wild type strains SP1 (BP1206), the Δ*pdxST* mutant (BP1207) and the Δ*pdxT* mutant (BP1208) to have three different situations of PLP availability. The wild type can produce PLP *via* PdxST without limitation. As PdxS is able to synthesize PLP from ammonium in the absence of PdxT but with a lower extend, PLP availability is lower than in the wild type. The *pdxST* mutant cannot produce PL at all and relies on PLP uptake from the medium. The strains were propagated on TSS medium plates containing X-Gal and different concentrations of PL ranging from 0 nM – 100 nM and incubated over night at 37°C. As depicted in Figure 25A, the Δ*pdxST* mutant BP1207 can only grow in presence of PL starting at 10 nM. The wild type mutant BP1206 and the Δ*pdxST* mutant are able to grow on all plates. As the wild type produces vitamin B6 without limitation, the cells will remain white under all tested conditions. The Δ*pdxT* mutant

only produces small amounts of PL as the glutaminase domain is missing. Thereby, without supplementation with exogenous PL the cells turn blue. With rising PL amounts a color change was detectible, reaching wild type-like color at 100 nM PL. Since the Δ*pdxST* mutant cannot produce additional PL, it remained blue even at 100 nM PL.

To quantify the screening capabilities of the screening system, we assessed the activity of LacZ crude extracts obtained from the three strains. Accordingly, we cultivated the strains in MSSM medium supplemented with 30 mM ammonium and varying PL concentrations ranging from 100 nM to 1300 nM (Figure 25B). Since growth in liquid TSS medium was not possible, we decided to conduct the experiment in MSSM medium. As seen on plates, in the wild type background no *β*-galactosidase activity was measurable. The differences between the Δ*pdxST* and the Δ*pdxT* background was not as prominent as on solid medium but the latter reached lower minimum LacZ activity as the auxotrophic mutant. The *lacZ* expression seemed to be not in a linear relationship to the used PL amounts but more in an exponential decrease, which stands in agreement with the PdxR based regulation of the promoter. The present PLP affects both directions of the promoter at once and thereby limits the expression of *lacZ* and increased the expression of *pdxR*. Consequently, it can buffer away the free PLP and re-establish a situation in which unbound PdxR is present again.

Next, we were looking for a suitable plasmid for the random mutagenesis in the *E. coli* XL1 Red strain. We aimed to transform the Δ*pdxST* mutant (BP1207) with the generated plasmid pool, so that the emerging strain should have the same PLP-sensitive screening behavior as BP1208 (Δ*pdxT*). In this strain differences in PLP levels could be indicated by a different colony color. We constructed the *pdxS* overexpression plasmid pBP775 by inserting the open reading frame of the *pdxS* gene into the pBQ200 plasmid. The plasmid was introduced into BP1207 and the cells were plated on TSS agar plates containing X-Gal and rising concentrations of PL. As shown in Figure 26A, independent of the PL amount of the plates, the colonies remained white. As the plasmid pBQ200 is a high copy plasmid and expresses genes under the strong P_{degQ*}promoter (*Martin-Verstraete et al.*, 1994; *Msadek et al.*, 1991), the emerging gene dosage was too high. Hence, we excised the strong promoter and inserted the *pdxS* gene under the control of the P_{alf4} promoter (pBP788) and under control of the native P_{pdxS} promoter (pBP789). When introduced into the screening strain, the strain carrying pBP789 was white even without exogenous PL addition, making the plasmid inappropriate. For 10 mM and 100 mM no colonies were on the plate due to the overall lower competence of the *pdxST* mutant as described in the competence test (see Figure 13). Colonies carrying pBP788 appeared blue on the plate without PL supplementation and turned white with increasing PL concentrations and were therefore suitable for random mutagenesis. Higher active PdxS variants produce more PL, which is signaled by whitish colonies when plated on a plate without exogenous PL supplementation. Furthermore, we analyzed

the range in, which the screening strain, carrying the *pdxS* expression plasmid, can sense differences in PL, by measuring *β*-galactosidase activity, as described before. Even small differences in exogenous PL amounts had a big effect of *lacZ* expression (see Figure 26B). Although, the difference between 0- and 1 mM PL was not significant, a strong decrease in *β*-galactosidase activity was seen when 10 nM were added, leading to a decrease of about 70%. The intracellular vitamin B6 concentration in *E. coli* is about 0.3 nmol per mg cell material (*Matsuura et al.*, 1983). As one cell has a weight of about 10^{-9} mg and a volume of 10^{-6} liter (*Sundararaj et al.*, 2004), we can calculate the intracellular amount of PL by dividing the weight per cell by the PL concentration. 3.33×10^{-9} nmol of PL(P) are present in one cell. To get the molarity, we can divide by the volume of one cell and get 0,033 nmol or 33 pmol as the intracellular amount of PL. Therefore, even a slight increase in *pdxS* activity should be detectable.

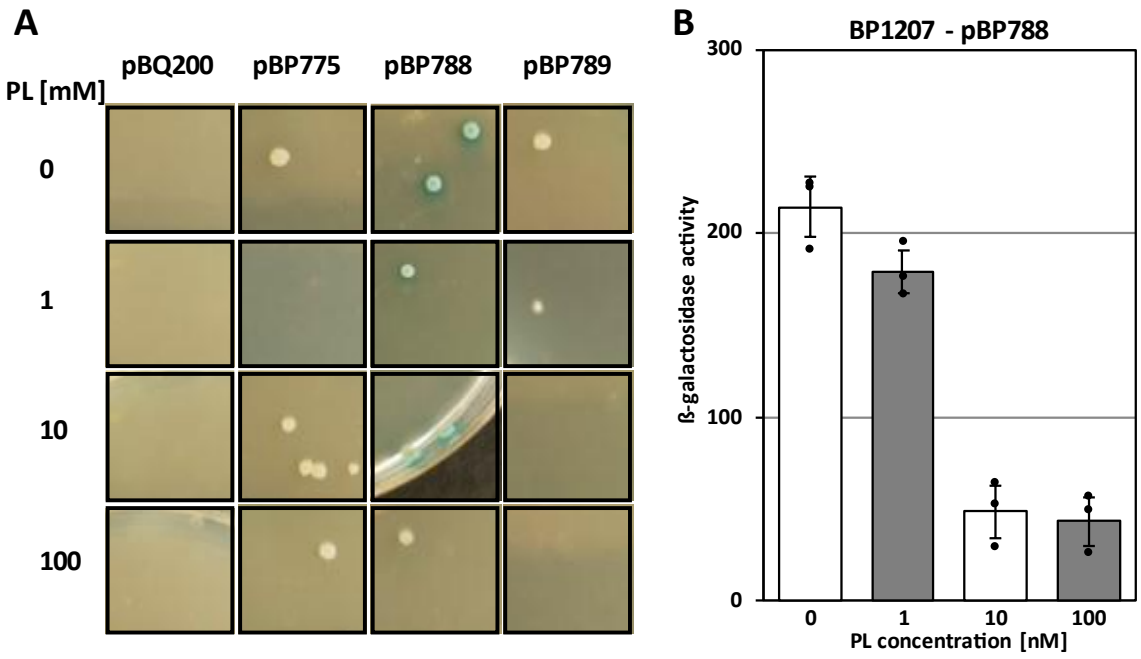


Figure 26 PL levels generated by different *pdxS*-expressing plasmids.
A: Expression plasmids pBP775 (P_{degQ*}-*pdxS*), pBP788 (P_{alf4}-*pdxS*), pBP789 (P_{pdxS}-*pdxS*) and empty plasmid pBQ200 were introduced into the screening strain BP1207 and plated on TSS agar plates containing X-Gal. **B**: *β*-galactosidase activity assay was tested to check the sensitivity of the screening strain BP1207 containing the plasmid pBP788. The strain was cultivated in MSSM medium supplemented with 1.3 mM glutamine and 30 mM ammonium. Data points represent biologically independent replicates. Bars indicate means of replicates and the standard deviations are shown. *β*-galactosidase activities are given as units per milligram of protein.

On that score, we introduced the plasmid pBP788 into *E. coli* XL1 Red and generated plasmid pools as it was described in Dormeyer et al. (2019) and transformed the plasmid pools into BP1207. After multiple attempts, we were not able to successfully transform the strain, neither with the plasmid pools, nor with the original plasmid and decided to stop the experiment.

Overproduction of PdxS is sufficient to relieve vitamin B6 auxotrophy of a Δ*pdxT* mutant

Most of our analyzed suppressor mutants showed an amplification of the 15 kbp long region including the *pdxS* gene (see Table 17). All genes, except for *pdxS*, cannot directly be related to vitamin B6 or ammonium metabolism and thereby explain the suppression mechanism (Suppl. Table 1). When counting the suppressors, which have an enhanced ammonium uptake and the other suppressors, we came up with a ratio of 0.3% for the blue suppressors. Thus, we concluded, that increasing the *pdxS* gene dose could be sufficient to suppress the loss of *pdxT*. To check this hypothesis, we introduced the *pdxS* overexpression plasmid pBP775 and the empty plasmid pBQ200 into the Δ*pdxT* mutant BP1105 and into the Δ*pdxT* mutant carrying the P_{nrgAB}-*lacZ* fusion (BP1106) and analyzed the emergence of suppressor mutants on MSSM minimal medium plates containing 20 mM ammonium. Therefore, cell material was taken from an LB medium plate containing PL and washed in saline. Equal amounts of cell material were propagated on the plates. When carrying the empty vector, both strains form suppressor mutants under the selective pressure (see Figure 27). For BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*), also a blue colony was found, indicating mutations probably in the nitrogen assimilation system (*glnA* or *tnrA*). Moreover, the strains formed a bacterial lawn when they were transformed with the *pdxS* overexpression plasmid pBP775, demonstrating that overproduction of the PdxS PLP synthase subunit was sufficient to suppress the deletion of *pdxT* under the selective conditions.

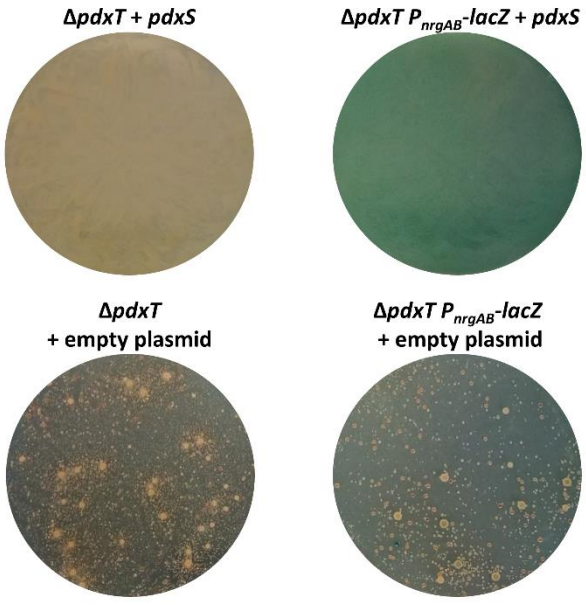


Figure 27 Overproduction of PdxS is sufficient to relieve vitamin B6 auxotrophy of a *pdxT* mutant.
The cells of the strains BP1105 (Δ*pdxT*) and BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*) that were transformed with the plasmid pBQ200 (empty plasmid) and pBP775 (*pdxS*) were propagated on MSSM agar plates supplemented with 20 mM ammonium and X-Gal. The plates were incubated for 48 h at 37°C. Figure and legend derived from (*Richts et al.*, 2021).

In addition, we wanted to relate the role of the mutations in *glnA* and *tnrA* we found in the suppressor mutants to the growth. As the suppressors carried additional mutations, which can be responsible for a difference in growth, we amplified the $tnrA^{\Delta T280}$, $glnA^{G79A}$, $glnA^{C180A}$ and $glnA^{G79A\ C1016A}$ alleles by PCR, fused them to an antibiotic resistance gene and transformed the strain BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*) with

it. The insertion of the mutations was checked by PCR. To assess the effect of the mutations, the emerging strains were transformed with the *pdxS* overexpression plasmid pBP775 and the empty vector pBQ200 and growth was monitored in MSSM minimal medium containing 1-, 10-, 30- and 120 mM ammonium). The strain carrying the wild type *tnrA* and *glnA* gene served as a control.

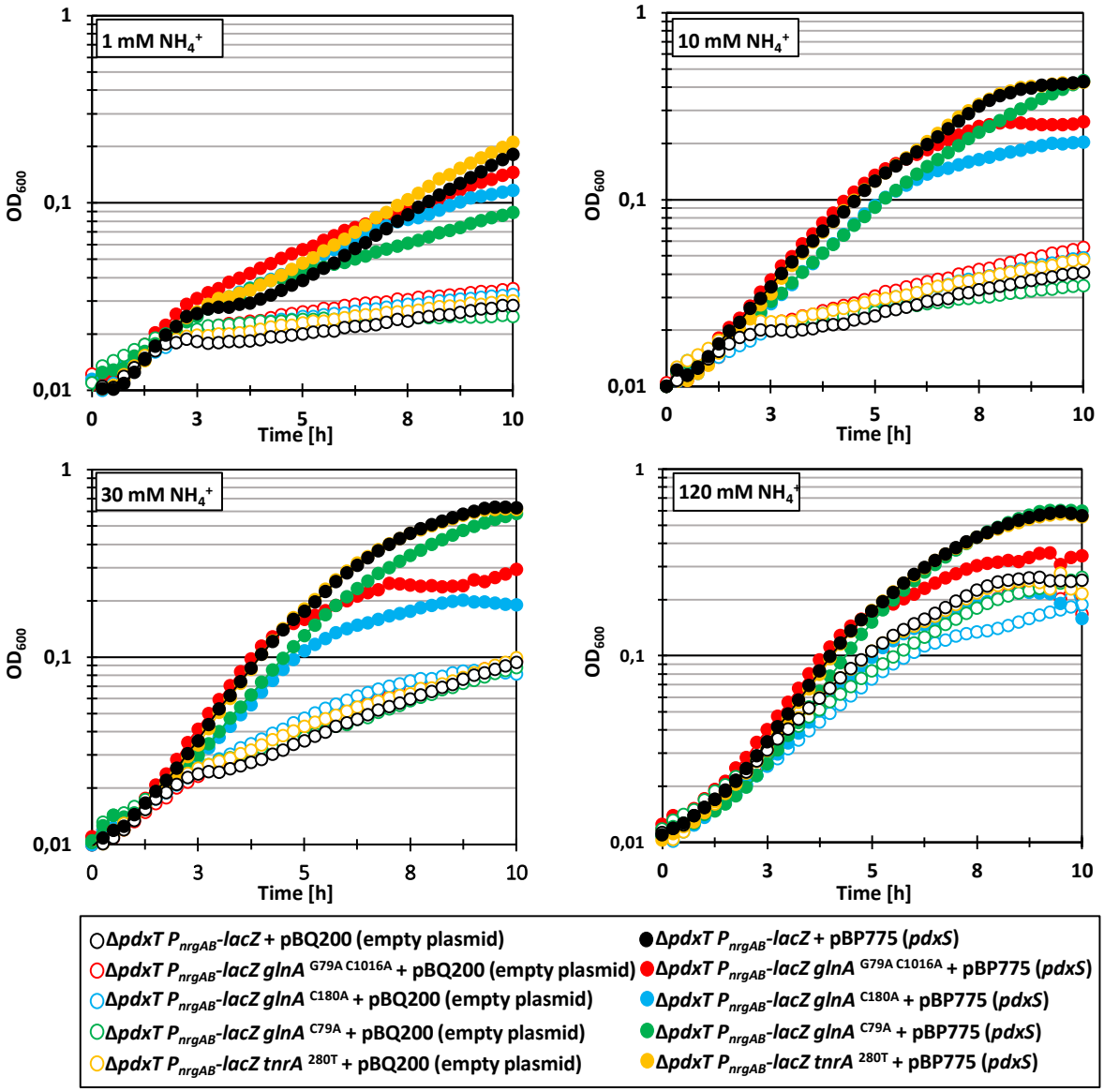


Figure 28 Effect of pdxS overexpression and increased ammonium uptake on PL synthesis.
4 ml MSSM 30mM NH_4 medium supplemented with PL were inoculated with single colonies of the *B. subtilis* strains BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)-pBQ200, BP1209 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{G79A\ C1016A}$)-pBQ200, BP1210 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{C180A}$)-pBQ200, BP1211 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{C79A}$)-pBQ200, BP1212 (Δ*pdxT* P_{nrgAB}-*lacZ* $tnrA^{\Delta T280}$)-pBQ200, BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*)-pBP775, BP1209 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{G79A\ C1016A}$)-pBP775, BP1210 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{C180A}$)-pBP775, BP1211 (Δ*pdxT* P_{nrgAB}-*lacZ* $glnA^{C79A}$)-pBP775 and BP1212 (Δ*pdxT* P_{nrgAB}-*lacZ* $tnrA^{\Delta T280}$)-pBP775 and the precultures were incubated overnight at 37°C and 220 rpm. The overnight cultures were used to inoculate precultures in the same medium. When reaching an OD_{600} of 0.5 – 0.8 the cultures were used to inoculate a 96 well plate that was supplemented with 100 µl MSSM medium containing increasing amounts of ammonium chloride. The growth was monitored with an Epoch 2 Microplate Spectrophotometer. Figure and legend derived from (*Richts et al.*, 2021).

All strains overproducing *pdxS* had a better growth than the strains carrying the empty plasmid in medium supplemented with 1 – 30 mM ammonium (see **Fehler! Verweisquelle konnte nicht gefunden werden.**). Nevertheless, the mutations *glnA*^{C180A} and *glnA*$^{G79A\ C1016A}$ produced less biomass compared to the wild type strain in the medium containing 30- or 120 mM ammonium. In the latter, the strain carrying *glnA*^{C180A} mutation even growed worse than the strains transformed with the empty plasmid. This mutation showed the highest increase of P_{nrgAB} promoter activity in Figure 23E. Accordingly, uncontrolled uptake of ammonium from the medium was toxic for the cells, as the regulatory network between GS and TnrA is disturbed. Without overproduction of PdxS, the growth difference between the mutants and the parental strain was only small and more prominent in medium containing less ammonium. Same as before, high concentrations of ammonium became toxic and the growth rate was diminished. Taking this into consideration, the overexpression of *pdxS* is dominant over the mutations in *tnrA* and *glnA*.

PdxS positively affects complex colony formation

When we evaluated the merge of suppressor mutants, we recognized that the colony morphology differs between the suppressors and the parental strains BP1105 (Δ*pdxT*) and BP1106 (Δ*pdxT* P_{nrgAB}-*lacZ*). We analyzed the influence of PdxS on complex colony formation by conducting a biofilm assay. Hence, the parental strains BP1105 and BP1106 and the suppressor mutants S1, S4, S14, S21-S26 were propagated on MSSM medium plates containing 30 mM ammonium and PL. To assess also the effect of *pdxS* overexpression, the parental strain BP1105 transformed with the empty plasmid (BP1105-pBQ200) and the *pdxS* overexpression plasmid pBP775 (BP1105-pBP775) was tested together with the control strains SP1, 168, GP922 and DK1042. GP922 served as a negative control, as the *ymdB* gene was knocked out, which codes for a phosphodiesterase important for biofilm formation (*Diethmaier et al.*, 2011). In contrast to the laboratory strain 168, the DK1042, a descendent of the "wild-wild type" NCIB3610 was able to form complex colonies (*Konkol et al.*, 2013). The 168 strain carries mutated version of the genes *rapP, sfp, epsC, swrA, and degQ* and is therefore not able to form biofilms (*McLoon et al.*, 2011). Why the tryptophane prototrophic wild type strain SP1 forms biofilms, although it harbors the same mutations, remains to be elucidated.

The laboratory wild type 168, GP922 (Δ*ymdB*) and the Δ*pdxT* mutants (BP1105, BP1105 – pBQ200, BP1106) did not form a biofilm (see Figure 29). However, the suppressors S1 and S21 - S26, the wild type strains SP1 and DK1042 and the Δ*pdxT* mutant carrying the *pdxS* overexpression plasmid (BP1105-pBP775) formed complex colonies. Only the suppressors S4, S14 and S26 didnot form complex colonies. S14 and S26 did not harbor the amplification of the *pdxS* gene according to the Illumina read depth and Southern blotting analysis (see Figure 22). S4 carried a mutation in the *ispD* gene, which is

essential for isoprenoid biosynthesis. According to this, loss of *pdxT* leads to a defect in biofilm formation, which can be relieved by overexpression of *pdxS*.

Figure 29 Influence of PLP on complex colony formation.
The *B. subtilis* strains were cultivated overnight in MSSM minimal medium with 30 mM NH_4 and PL supplementation. The culture was used to inoculate 2 ml of the medium to an OD_{600} of 0.1 and incubated at 37°C until OD_{600} of 0.5. 10 µl of the cell suspensions were spotted onto MSSM minimal medium agar plates supplemented with 20 mM glutamine, 0.1 µM PL, 1% glycerol, 100µM $MnCl_2$, 40 ng/ml Coomassie Brilliant Blue G-250 and 20 ng/ml mM congo red stain. The plates were incubated for 4 days at 37°C and the images of the complex colonies were obtained with a stereo fluorescence microscope Lumar.V12, 9,6-fold magnification. Figure and legend derived from (*Richts et al.*, 2021).

Activity of the PdxS is negatively regulated in the presence of NH_4^+

The PdxS PLP synthase domain can form PLP even in absence of the PdxT glutaminase subunit by utilizing ammonium instead of glutamine. The growth defect of a Δ*pdxT* mutant in minimal medium supplemented with low ammonium can be complemented by the overexpression of *pdxS* or to a lower extend by increasing the ammonium uptake from the growth medium. Therefore, it is reasonable that free ammonium can be a limiting factor for PdxS to form PLP. We speculated that an increase of free ammonium could elevate the PLP production by PdxS.

PdxT and PdxS were purified from the plasmids pBP766 (petSUMOadapt::*pdxS*) and BP767 (petSUMOadapt::*pdxT*) (Dennis Wicke, internship 2019). These plasmids express *pdxS* and *pdxT* under the control the T7 promoter in *E. coli*, adding a 6 x His-tag, which can be cleaved off with the addition of SUMO protease. The SDS gels in Figure 30A show the purified proteins after cleavage with the

protease. As the tag is cleaved, the untagged protein is in the flow-through fraction of a Ni-NTA purification column and the uncut protein together with the SUMO protease in the elution fraction (E1). Although PdxT was not cleaved completely both proteins were purified without major impurities in the flow-through (FT) as indicated by the red rectangles.

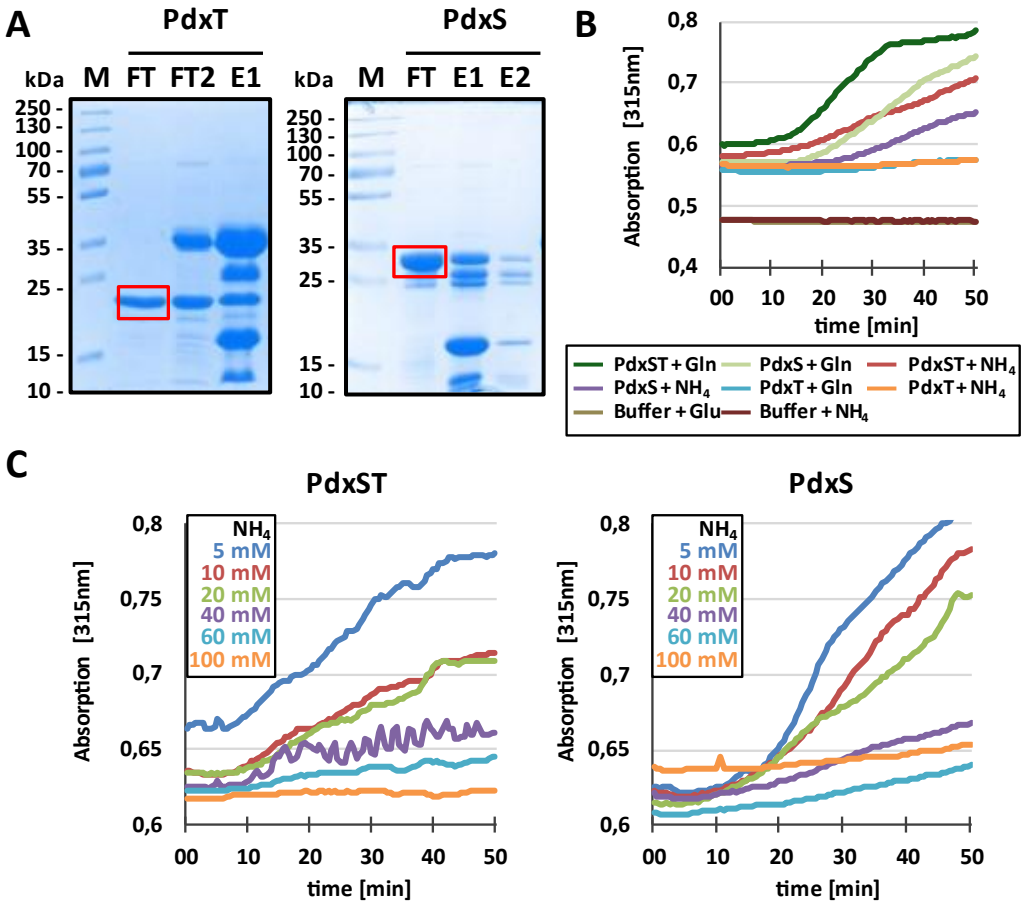


Figure 30 Ammonium dependent activity of PdxS.
PdxS and PdxT were expressed from pBP766 and pBP767 in *E. coli* Rosetta strain and purified *via* 6xHis-tag. The tag was cleaved off using SUMO protease. **A**: Coomassie stained SDS-PAGE of PdxT and PdxS elution fractions after cleavage with SUMO protease. tag-free protein is present in the flow-through fraction (FT/ FT2), SUMO protease and uncleaved protein in the elution fractions (E). For PdxT the flow-through was split in half and collected separately. Red boxes indicate protein, which was used for the enzyme assay. **B**: Functionality test of purified PdxS and PdxT was done in a reaction volume of 100 µl containing 25µM PdxS, 25 µM PdxT, 50 mM Tris-HCL, pH 7.9, 20 mM glutamine, 10 mM Ribose-5-phosphate. The synthesis of chromophore I2 was determined in a multi-well plate reader at 315 nm. Different combinations of subunits and substrates were tested for activity. **C**: Influence of ammonium on PdxS and PdxST activity. Instead of glutamine, different amounts of ammonium were used as substrate for the assay.

Next, we tested the proteins for functionality and conducted an enzymatic assay sensing the formation of the chromophore I2 at 315 nm as it was described by Smith et al. (2015). We carried out the reaction in 100 µl volume containing 50 mM Tris-HCl, pH 7.9, 10 mM ribose-5-phosphate and either 20 mM glutamine or 20 mM ammonium. The reaction was started by adding 25 µM PdxS, 25 µM PdxT or a combination of both. As seen in Figure 30B, both of the reactions PdxS + ammonium (PdxS + NH_4^+) and PdxS + glutamine (PdxS + Gln) showed enzymatic activity, whereas the reaction with glutamine was

faster. When PdxT was added to the reaction the turnover speed increased for both glutamine and ammonium utilization. Thereby, the reaction of PdxST with ammonium was slower than the reaction of PdxS with glutamine, which is unexpected as glutamine cannot be used by PdxS. Glutamine can easily degrade to glutamate and ammonium after short period of storage (Sigma-Aldrich technical bulletin glutamine) so that NH_4^+ is free for the reaction. As the same amounts of ammonium and glutamine were used for the assay and not all of the glutamine degraded to glutamate (PdxST + glutamine still exhibits the best activity), it did not explain the faster reaction. Both subunits of the PdxST complex were fully functional and we tested the influence of different ammonium levels on enzyme activity of the PdxST complex and only the PLP synthase subunit PdxS to form chromophore I2. The assay was conducted with the same substrate concentrations as before but we used rising concentrations of ammonium. Against our expectation, increasing concentrations of ammonium did not speed up the turnover time but led to a decrease of chromophore I2 formation for the PdxST complex and also for PdxS alone (see Figure 30C). Although, this seems to be counterintuitive it could explain why PdxS + glutamine could reach faster turnover speed as with ammonium supplementation. For both substrates 20 mM was set into the reaction. 20 mM of ammonium led to a drastic reduction of enzyme activity. As only few amounts of glutamine were probably degraded to ammonium, the ammonium concentration was rather low, giving a higher activity.

In vivo-crosslinking of the PdxST complex revealed possible interaction partners

PLP is a co-factor involved in almost 4% of all known enzymatic reactions (*Percudani & Peracchi*, 2003, 2009). In *B. subtilis* there are 65 PLP-dependent proteins of, which at least 61 are *bona fide* (*Richts et al.*, 2019). As PLP serves as a co-factor also for essential cellular functions, it is indispensable for the cells (*Percudani & Peracchi*, 2009; *Zhu & Stülke*, 2018). Moreover, high amounts of PL are toxic for the cells as the 4-aldehyde moiety is very reactive and can form covalent adducts even with PLP-independent proteins (*W. M. Lee et al.*, 2005; *Mizushina et al.*, 2003; *Vermeersh et al.*, 2004). Therefore, PLP metabolism has to be tightly regulated to keep the PLP levels in balance to prevent intoxication and allow its use as a co-factor. In contrast to other organisms, *pdxST* expression is not regulated on genome level as in other organism (*Belitsky*, 2014; *Liao et al.*, 2015). Thus, we came up with the hypothesis, that the PdxST complex is probably feedback inhibited by PLP but may be able to interact with PLP-dependent proteins and thereby, provide the cofactor directly into needed reactions. For this reason, we conducted an in *vivo* crosslinking experiment with PdxST. Firstly, we cloned the *pdxST* gene region into the vector pGP382, which adds a C-terminal Strep-tag. The resulting plasmid pBP772 and the empty vector were introduced into the PL auxotrophic mutant BP1100 (Δ*pdxST*) and the

functionality of the plasmid was checked by plating both strains on C-Glc and C-Glc plates supplemented with PL (see Figure 31A).

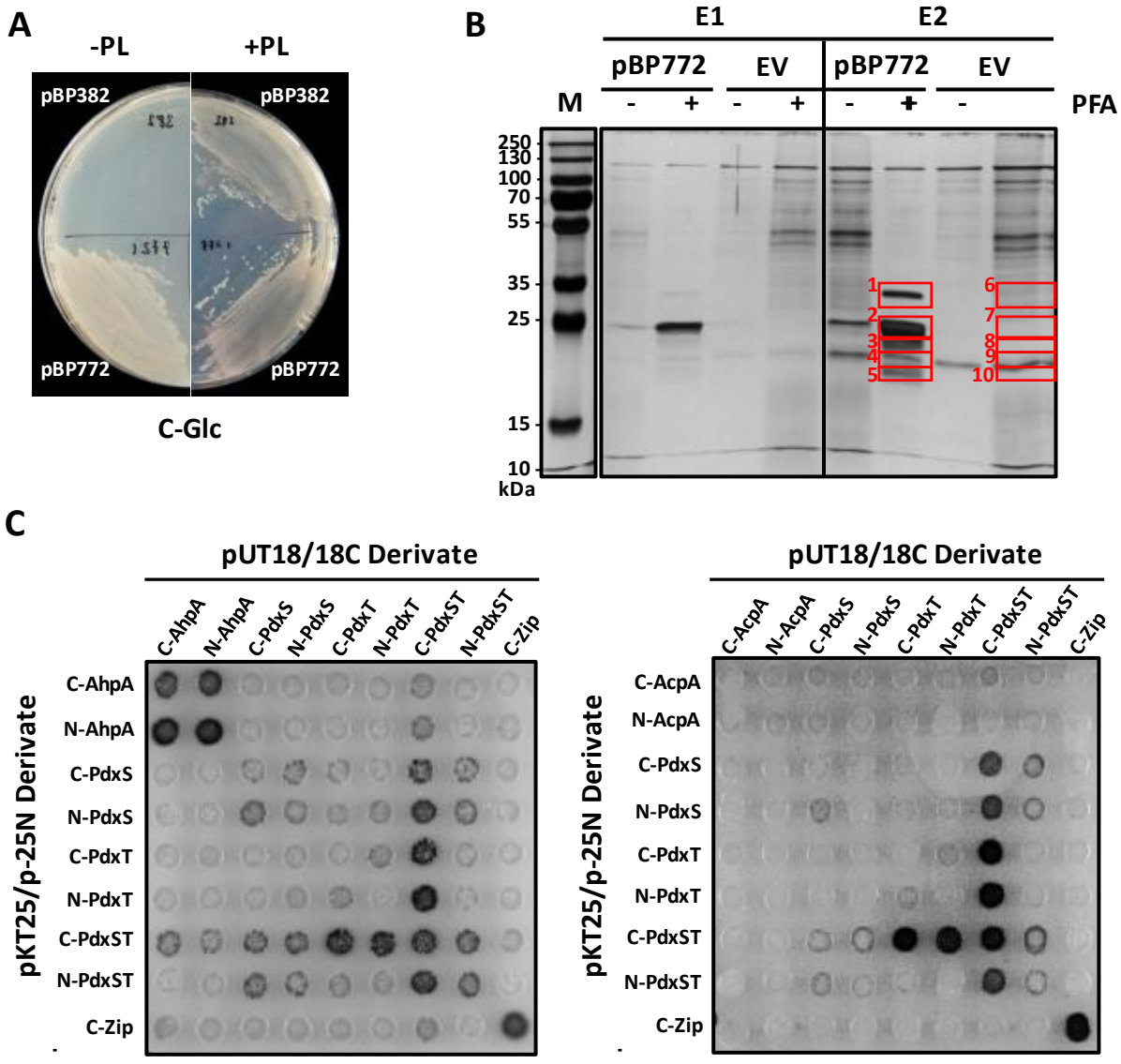


Figure 31 Interaction partners of the PdxST complex.
A: Functionality check of the SPINE plasmids. BP1100 carrying the SPINE plasmid pBP772 (*pdxST*) or the empty plasmid pBP382) were streaked on C-Glc or C-Glc-PL agar plates. **B**: SPINE PdxST. Plasmid pBP772 (pGP382::*pdxST*) for expression of C-terminally Strep-tagged PdxST and empty plasmid (EV, pBP382) were introduced into BP1100 (Δ*pdxST*) and cultivated in C-Glc minimal medium. At an OD between 0.6 -1 the culture was split and one half was treated with para-formaldehyde (+/- PFA). PdxST-Strep was purified and different elution fractions (E1/ E2) were analyzed by SDS-PAGE and subsequent silver staining. M: protein size marker. Red squares indicate bands, which were cut out and sent for mass spectrometry analysis **C**: Different combinations of bacterial two-hybrid plasmids were introduced into *E. coli* strain BTH01. After incubation 5 µl cell suspension were dropped on LB agar plates containing respective antibiotics, IPTG and X-Gal. The plates were incubated at 28°C for 48 hours.

In presence of PL, both strains grew well on minimal medium. But without exogenous PL only the strain could grow carrying the *pdxST* expression plasmid showing that a functional PdxST complex was formed. Next, we cultivated the stains in 1 l C-Glc medium supplemented with PL until they reached and OD of 1.1. The cultures were split and one half was treated with PFA for 15 min at 37°C. After

washing with Buffer W, the cells they were lysed in the French press and applied to a Strep-Tactin matrix. The bound protein was rinsed extensively with 30 ml Buffer W so that no protein could be detected in 30 µl washing fraction. Elution Buffer E was added and the samples were analyzed on a silver stained SDS-PAGE gel (see Figure 31B). In all elution fractions of the expression plasmid and the empty plasmids bands were present at heights of 20 kDa and 130 kDa. As these bands came up independently of *pdxST* expression, they probably belong to the biotinylated proteins AccB (17 kDA) and PycA (127 kDa), respectively. A prominent band appeared in the elution fractions of the expression plasmid at a height of 25 kDa, which was not present in the empty plasmid control, matching the size of PdxT-Strep (PdxT 17 kDa). As the co-expressed PdxS (31 kDa) was not tagged, it was co-purified with PdxT but in a lower fashion and appears at a height below 35 kDa. The amount of the eluted subunits was higher when PFA was added and increased even more in E2. In the fraction of pBP772 treated with PFA we could identify additional bands, which were not present in the EV control. Therefore, we cut out the most prominent bands (Red rectangles) and gave them for MS analysis to Dr. Elke Hammer (University of Greifswald). It could be confirmed that sample one contained mainly PdxS protein, which was not present in the empty vector control. In addition, the presence of PdxT in sample 2 could be confirmed as it was not detected in the control. In sample 3 only few hits were found but these indicated the presence of YkuU (AhpA) an alkyl hydroperoxide reductase. Probe 4 did not show any hits, although maximal injection volume was used. Thus, no further information could be gathered. Besides, in lane 5 only 3 hits were found for AcpA whereas one also in the control. AcpA is an essential acyl carrier protein, active in fatty acid biosynthesis. Although only few hits were found, interaction of AhpA and AcpA with the PdxST complex were checked by bacterial two-hybrid assay. Accordingly, two-hybrid plasmids were constructed (pBP800 – pBP819) fusing PdxS, PdxT, PdxST, AhpA, AcpA both N-terminally and C-terminally with either T18 or T25 fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase. If these fragments come together, enzymatic activity is reconstituted and cAMP is formed. cAMP activates the expression of the lactose operon, giving rise to blue colonies on plates supplemented with X-Gal. Different combinations of the plasmids were transformed into the *E. coli* strain BTH101. Plasmids containing the C-Zip domain served as a control. As seen in Figure 31C, C- and N-terminally fused AhpA strongly interacted with itself as it was already shown for a dimeric human peroxiredoxin-1 mutant sharing 48% identity with AhpA (*Cho et al.*, 2015; *Zhu & Stülke*, 2018). Furthermore, both AhpA constructs showed a weaker interaction with the C-terminally tagged PdxST complex but the latter also interacted slightly with the C-Zip control, suggesting unspecific binding. As expected, PdxS interacted with itself and the whole PdxST complex. PdxT only weakly interacted with itself and PdxS, but gave a strong signal when the complete C-terminally fused complex was expressed. For AcpA no interaction was found. As AcpA forms a hetero-hexamer together with AcpS, it could be

that an intact complex is necessary for interaction with PdxST (*Parris et al.*, 2000). Intriguingly, in the PdxT suppressors S24 a mutation was found in the intergenic region of *ahpA*, 88 bp upstream of the start codon and 2 bp downstream of the putative -10 region (see Table 17). Therefore, promoter activity tests were made for the wild type and mutant promoter of *ahpA* but no chage in activity could be measured (data not shown).

3.3 Vitamin B6 transporter

Assessment of a role of *nucleobase:cation symporter* family proteins in PL uptake

To date, it is still not much known about the im- or export of the B6 vitamers. Only few transporters have been identified such as Pup1 in *Arabidopsis thaliana*, Bsu1 in *Schizosaccharomyces pombe*, Tpn1p in *Saccharomyces cerevisiae* or PdxU2 (HmtP) in *Lactobacillus brevis* (*Stolz et al.*, 2005; *Stolz & Vielreicher*, 2003; *Szydlowski et al.*, 2013; *T. Wang et al.*, 2015). As a BLASTp search with all four proteins against the *B. subtilis* proteome did not lead to the identification of any promising candidates, we decided for a more general approach by checking the properties of the known transporters. Tpn1p belongs to the group of purine-cytosine permeases (2.A.39) and to the family of nucleobase:cation symporter-1 (ncs1) according to UniProt (https://www.uniprot.org/uniprot/C7GX08). Since Pup1 of *A. thaliana* also belongs to the purine nucleobase transporters (*Bürkle et al.*, 2003), we in checked *B. subtilis* for members of this protein group and identified the purine-cytosine permease-like YxlA and the allantoin permease PucI. Besides the nucleobase:cation symporter-1, there is a second group the nucleobase:cation symporter-2 (NCS2) (TC 2.A.40) in *B. subtilis* with the members PbuX (xanthine permease), PucJK (uric acid permease), PyrP (uracil permease) and YwdJ (unknown function). PdxU2 is the S-component of the energy coupling factor (ECF) transporter consisting of the S-component, which binds the substrate, two ATPases (A-components) and the transmembrane domain (T-component), which links A- and C-component (*Eitinger et al.*, 2011; *Slotboom*, 2014; *P. Zhang*, 2013). In *B. subtilis* micronutrients are transported *via* the ECF transporter system, encoded by *ybxA* (A-component1), *ybaE* (A-component2) and *ybaF* (T-component) (*Zhu & Stülke*, 2018).

To assess if one of the candidate genes is able to im- or export PL, we created knockout mutants in the wild type background and in the background of the PL auxotroph mutant BP1100 (Δ*pdxST*). As the auxotroph strain relies on the uptake of PL, construction of the Δ*pdxST* transporter double mutant should not be possible, unless there are multiple transport systems. On that score, we first constructed the single mutants by transforming the *B. subtilis* wild type strain SP1 with PCR products. The chromosomal DNA of the mutants was extracted and used to transform the *pdxST* mutant BP1100. As a positive control for the transformation process, genomic DNA of GP550 (*amyE::cat*) was transformed carrying a construct with the same resistance cassette. As seen in Figure 32A, double mutants in the

background of the auxotrophic strain were possible for all candidates. Consequently, the strains were given the following names BP1148 (Δ*ybxA* Δ*ybaEF*), BP1256 (Δ*pbuX*), BP1257 (Δ*pucJK*), BP1258 (Δ*pyrP*), BP1259 (Δ*ywdJ*), BP1260 (Δ*ylxA),* BP1261 (Δ*pucI*), BP1198 (Δ*pdxST* Δ*ybxA* Δ*ybaEF*), BP1266 (Δ*pdxST* Δ*pbuX*), BP1267 (Δ*pdxST* Δ*pucJK*), BP1268 (Δ*pdxST* Δ*pyrP*), BP1269 (Δ*pdxST* Δ*ywdJ*), BP1270 (Δ*pdxST* Δ*ylxA),* BP1271 (Δ*pdxST* Δ*pucI*).

Next, we quantified the growth of single and double mutants in C-Glc minimal medium containing PL. For this purpose, the strains were cultivated in a C-Glc preculture until they reached an OD_{600} of 0.5 – 0.8. The cells were washed in saline solution and used to inoculate 100 µl of C-Glc-PL medium. Growth was monitored in the plate reader at 37°C for 9 hours. As depicted in Figure 32B, all single mutants grew at wild type level, except for the Δ*pyrP* mutant, which produced the same biomass but with a reduced growth rate. However, this difference was not present in the double mutants, which all showed same growth behaviour as the wild type or the Δ*pdxST* mutant.

Since differences in import of PL could not be shown for the mutants, we tested if export of PL was compromised. Accordingly, we cultivated the single and double mutants together with the wild type strain SP1 and the PL auxotroph strain BP1100 in SP medium overnight, washed the cells 2 x in saline and dropped 10 µl of a OD_{600} 1 cell suspension on C-Glc screening plates, containing the PL-sensitive screening strain BP1207 (see above). Because this strain is auxotroph for PL, it can only grow if PL is secreted by the bacteria growing on top. When PL is taken up by the strain, the PL sensitive promoter is active and expresses X-Gal in a PL-dependent manner (see Figure 24). If the strains are able to secrete PL, a halo will form around the bacteria being white at high PL and blue at low PL levels. The Δ*pdxST* mutant BP1100 was not able to grow since the agar plates did not contain PL. Thus, there was no halo in contrast to the positive control (SP1). Furthermore, all double mutants (BP1198, BP1267-BP1271) did not grow as expected. The single mutants (BP1148, BP1257-BP1261) also secreted PL, showing that the proteins were not solely responsible for PL export.

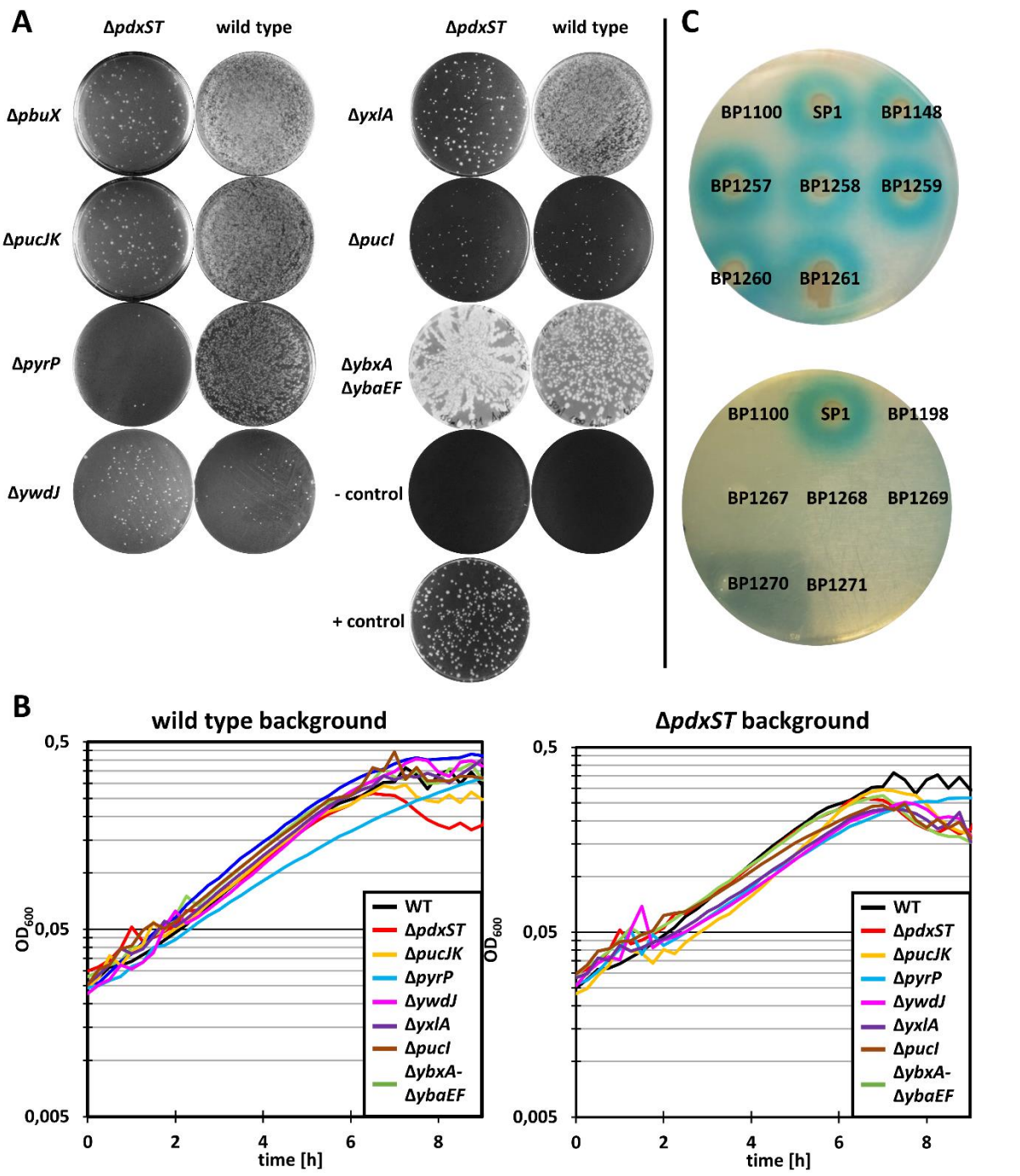


Figure 32 Characterization of candidate transporter.
A: Knockout mutants of possible transporter genes were created by transforming the *B. subtilis* wild type strain with a PCR product. Genomic DNA of the single mutants BP1148 (Δ*ybxA* Δ*ybaEF*), BP1256 (Δ*pbuX*), BP1257 (Δ*pucJK*), BP1258 (Δ*pyrP*), BP1259 (Δ*ywdJ*), BP1260 (Δ*ylxA),* BP1261 (Δ*pucI*) was used to transform the PL auxotrophic strain BP1100 (Δ*pdxST*). Transformability was tested by transformation with gDNA of GP550, harboring the same antibiotic resistance cassette (+control). To exclude weak DNA quality, the gDNA was also introduced into the wild type strain SP1. **B**: The growth of transporter candidate mutants was monitored in a 96-well platereader. Therefore, singe mutants BP1148 (Δ*ybxA* Δ*ybaEF*), BP1256 (Δ*pbuX*), BP1257 (Δ*pucJK*), BP1258 (Δ*pyrP*), BP1259 (Δ*ywdJ*), BP1260 (Δ*ylxA),* BP1261 (Δ*pucI*), double mutants BP1198 (Δ*pdxST* Δ*ybxA* Δ*ybaEF*), BP1266 (Δ*pdxST* Δ*pbuX*), BP1267 (Δ*pdxST* Δ*pucJK*), BP1268 (Δ*pdxST* Δ*pyrP*), BP1269 (Δ*pdxST* Δ*ywdJ*), BP1270 (Δ*pdxST* Δ*ylxA),* BP1271 (Δ*pdxST* Δ*pucI*), the Δ*pdxST* mutant BP1100 and wild type strain SP1 were used to inoculate C-Glc medium supplemented with PL. When reached an OD_{600} of 0.5 -0.8 the cells were washed with saline and used to inoculate 100 µl of the same medium. **C**: Export capability of transporter candidate mutants. Strains were grown over night in LB medium supplemented with PL. The cell suspension was washed two times with saline solution and the OD_{600} was set to 1. 10 µl were spotted on C-Glc screening plate containing the *B. subtilis* PL-sensitive strain BP1207 (for further information see above).

Evolution under low PL conditions or with toxic B6 analogues does not trigger suppressor formation

The deletion of candidate genes could exclude a role as B6 transporting proteins. Therefore, we decided to go a step back and try out evolutionary approaches. We firstly plated the B6-auxotrophic strain BP1100 (Δ*pdxST*) and an auxotrophic mutant BP1103 (Δ*pdxST pdxJ pdxH* Δ*ytoQ* Δ*bshC*) carrying the *pdxH* gene on C-Glc minimal medium plates and added a filter paper at one site containing either 10 µl of a PL stock (100 µM) or a PN stock solution (100 mM). Although the cells only grew in a small radius around the filter paper, we were not able to find suppressor mutants at the border region, where the vitamer concentration was limited (data not shown). We hoped to find mutants, which either overexpress the transporter or carry mutations enhancing uptake capabilities for the vitamers. Since we were not able to find suppressor mutants, we changed the approach and tried to select for mutants by evolving *B. subtilis* in the presence of the vitamers and antivitamins, which share a similar structure with the vitamins but are known to be toxic. Two of these substances are ginkgotoxin and 4-deoxypyridoxine. As seen in Figure 33A the antivitamins only differ in their 4' headgroup from the vitamers so that it is very likely that they are taken up by the same transport system. Due to the similar structure both substances inhibit the pyridoxal kinase PdxK and thereby prevent that taken up B6 is phosphorylated and converted to the active compound PLP (*Leistner & Drewke*, 2010; *Mooney et al.*, 2009; *Woolley*, 1963) .

We tested the growth of the *B. subtilis* wild type SP1, a mutant BP965 (Δ*pdxST pdxHJ*) carrying the *pdxH* gene and by that being able to convert PNP to PLP and the two auxotrophic strains BP1100 (Δ*pdxST*) and BP1207 (Δ*pdxST pdxR*-P_{pdxST}^{Lmo}-*lacZ*) for growth on C-Glc minimal medium supplemented with each of the three vitamers pyridoxal (100 nM), pyridoxine (100 mM) and pyridoxamine (100 mM). A plate without vitamer addition served as a control. As seen in Figure 33B, only the wild type strain grew on the minimal medium plate without further vitamer supplementation. All strains grew with addition of PL or PM and only wild type SP1 and the mutant carrying *pdxJ* grew upon PN supplementation, as expected. Moreover, we analyzed the inhibitory effect of ginkgotoxin and 4-deoxypyridoxine. Consequently, the auxotrophic strain BP1103 (Δ*pdxST pdxJ pdxH* Δ*ytoQ* Δ*bshC*) carrying the *pdxH* gene was equally spread on C-Glc medium plates. Filter papers were added containing either 10 µl of one of the respective vitamer or antivitamer stock solutions. For ginkgotoxin, 10 mg ginkgotoxin (Sigma-Aldrich) were solved in 50 µl sterile water and 900 mg 4-deoxypyridoxine (Sigma-Aldrich) were solved in 6 ml 70°C sterile water for the second antivitamin solution. The control plate contained only a filter with the respective vitamer. To create a gradient between vitamer and antivitamer, two filter papers were placed on the plates, one containing the vitamer and one either ginkgotoxin or 4-deoxypyridoxine (see Figure 33C).

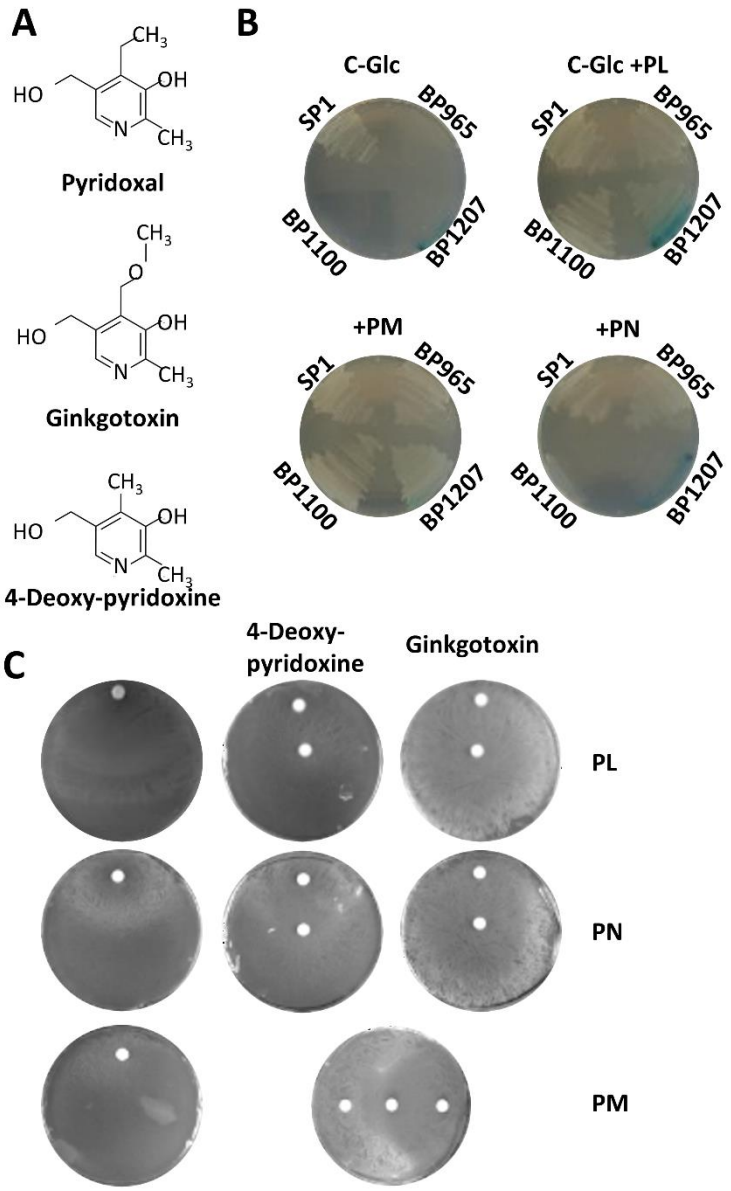


Figure 33 Antivitamin-driven evolution of *B. subtilis.*
A: Chemical Structures of Pyridoxal and the two antivitamins ginkgotoxin and 4-Deoxypyridoxine. **B**: Strains SP1 (wild type) BP965 (Δ*pdxST pdxHJ*), BP1100 (Δ*pdxST*) and BP1207 (Δ*pdxST amyE::pdxR-P*$_{pdxS}^{Lmo}$*-lacZ*) were taken from LB agar plates, washed 2 x with saline solution and set to OD_{600} of 1. 10 µl were streaked out on C-Glc plates supplemented with the vitamers PL, PM or PN, respectively. **C**: Cell material of BP1103 (Δ*pdxST pdxJH* Δ*bshC* Δ*ytoQ*) was taken from LB plate, washed 2 x in saline and set to OD_{600} of 1. 100 µl of cell suspension was equally distributed on C-Glc plates. Filter papers were added with 10 µl of vitamer solution (upper filter plate) or 10 µl vitamer solution (center filter plate). For PM the vitamer was added to the center, 4-deoxypyridoxine to the left and ginkgotoxin to the bottom filter, respectively.

As the filter papers were placed with a distance in between, gradients for both substances formed, intercepting at one point. We speculated that as the vitamer competes with the antivitamin for PdxK binding, the bacteria could evolve to either increase vitamer transport or mutate the transporter to only transport the vitamers but not the antimetabolites. On the control plates only containing the vitamer filter paper, growth was seen in an area around the filter for all vitamers. For 4-deoxypyridoxine no inhibitory effect was detected on the plates with PL or PN. When ginkgotoxin was added, directly around the filter paper a faint zone of decreased growth could be observed but to the outside of the plate, the diameter of grown cells was strongly increased compared to the control plate. For the PM plate the vitamer was put on top of the middle filter paper and 4-deoxypyridoxine (left)

and ginkgotoxin (right) were added besides. Again, we saw that the amount of cell material was strongly increased and that in the surrounding of the ginkgotoxin a faint zone of inhibition occurred. We also checked the *E. coli* wild type 3110 but it showed the same outcome (data not shown). Following this, we were wondering why ginkgotoxin elevated the total amount of cell material on minimal medium plates even without vitamers added (data not shown). Ginkgotoxin is produced in the ginkgo tree *Ginkgo biloba* by a pathway firstly producing PLP by its PdxST analogue and subsequent modification of the head group by yet unknown reactions (*Leistner & Drewke*, 2010). As the manufacturer of the ginkgotoxin has given no information whether it is extracted from the tree or synthesized and only gave the information that it is >98% pure. We analyzed the ginkgotoxin *via* MS to check for the presence of PL and found out that indeed about 1% PL was present in the ginkgotoxin (data not shown). Since we used high concentrations of 0.2 mg/µl, which corresponds to 200 g/l, 1% PL makes a 2 g/l stock, which is about 1000 times higher than the stock that is normally used. As a consequence, a slight inhibitory effect could be observed for ginkgotoxin but the toxin:PL ratio was too low to raise evolutionary pressure.

Mutations affecting the *sigA* operon and the *arsR* regulator promote PL detoxification

Next, we assessed the ability of *B. subtilis* and *E. coli* to adapt to toxic concentrations of PL. For *B. subtilis*, experiments with toxic amounts of PL were already conducted by Jonathan Rosenberg (http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B). He evolved *B. subtilis* on minimal medium plates supplemented with 3 mM PL and could identify a connection between biotin availability and tolerated PL amounts. We were curious if we can find additional mutations preventing PL intoxication, for example in the uptake/ secretion system of PL. As shown by Wicke et al. 2019, evolutionary adaptive mechanisms can differ between organisms. For instance, the evolution of *B. subtilis* and *E. coli* in the presence of the herbicide glyphosate revealed that *B. subtilis* acquired mutations in the *gltT* glutamate transporter gene, thereby reducing herbicide uptake, *and E. coli* acquired mutations in the *aroA* gene encoding the 3-phosphoshikimate 1-carboxyvinyltransferase AroA, which is targeted by the herbicide. Therefore, we tested PL tolerance of *E. coli* wild type W3110, a *B. subtilis* wild type derivate BP1124 (P_{nrgAB}-*lacZ*) and the PL auxotrophic screening strain BP1207 (Δ*pdxST pdxR*-P_{pdxST}^{Lmo}-*lacZ*) in C-Glc medium containing PL ranging from 100 µM to 5 mM. As depicted in Figure 34A, the *E. coli* wild type W3110 growed with up to 1 mM PL without constraint. When 2 mM were added, the cells reached the same optical density but the growth rate was already slightly compromised. Further increase to 3 mM of PL led to a strong growth defect and a complete inhibition of growth in 4 mM and 5 mM. As expected, the PL auxotroph *B. subtilis* mutant BP1207 had a higher PL tolerance compared to the wild type derivate, as it is not able to synthesize additional PL. Besides of that, growth behavior was very

similar. In 100 µM PL the cells showed the best growth and further PL supplementation led to a decrease in growth rate and also lower biomass. In contrast to *E. coli*, the negative effect of PL was in a linear manner. The more PL was added, the more growth compromised were the cells. Since the growth was still possible in 2 mM PL for *E. coli* and in 4 mM PL for *B. subtilis*, the three strains were plated on C-Glc plates, containing either 2 mM PL or 4 mM PL and incubated for up to seven days at 37°C. For the *B. subtilis* strains suppressor mutants emerged on the plates containing 4 mM PL but not on the plate with 2 mM PL. Contrary, *E. coli* cells were able to grow fine on agar plates supplemented with 2 mM PL but no colonies appeared on the plate with 4 mM PL. We isolated multiple suppressors of BP1207 from the 4 mM PL plate and tested their growth behavior in C-Glc medium with 4 mM PL compared to the parental strain BP1207. As seen in Figure 34C, both suppressor 7 (BP1252) and suppressor 11 (BP1254) showed a growth advantage compared to the parental strain BP1207, whereas suppressor 11 reached the higher biomass of both. Consequently, both suppressor mutants were analyzed by whole-genome sequencing (see Table 18).

Table 18 Mutations identified in the PL tolerating suppressor mutants.

Strain	Parent strain (genotype)	Locus	Coordinates[a]	Function	Type of mutation
BP1252 (S7)	Δ*pdxST pdxR*-P_{pdxST}^{Lmo}-*lacZ*	*arsR*	334,279	transcription repressor (ArsR family)	190-A (33 different amino acids, truncation of 58 aa)
BP1254 (S11)	Δ*pdxST pdxR*-P_{pdxST}^{Lmo}-*lacZ*	*yqeT*	2,624,733	similar to ribosomal protein methyltransferase)	G34A (H12Y)
		mutS2	2,922,754	Putative DNA repair protein	547T (3 different aa, truncation of 600 aa)
		yqxD	2,604,034	Unknown	A->T 161 bp upstream of the start codon (putative - 35 region)
		dnaG	2,604,034	DNA primase	
		sigA	2,604,034	Sigma factor A	

[a]Coordinates refer to the position in the genome sequence (GenBank: CP058242.1) (Richts et al., 2020) of the *B. subtilis* SP1 strain.

For S7, we found a mutation in ArsR, a repressor of the *ars* operon. The mutation led to an early stop and thereby, probably to a loss of function. ArsR represses the transcription of an operon, containing *yqcK*, *arsB and arsC.* YqcK is a protein of unknown function, sharing 64.7% similarity with FosB from *Bacillus licheniformis*, which refers resistance to fosfomycin (*Zhu & Stülke*, 2018). ArsB is an arsenate exporter and ArsC an arsenate reductase. As these genes are probably upregulated in the suppressor mutant, higher yields can be expected. It remains to be elucidated, which of the proteins is responsible for the higher PL tolerance. Interestingly, ArsB is a transporter, which could hint for a PL exporter and YqcK is involved in fosfomycin resistance. In the heterologous pathway mutant it could be shown, that bacillithiol and PL synthesis are connected with each other (*J. Rosenberg et al.*, 2018). Since deletion

of the bacillithiol synthesis creates a fosfomycin sensitive phenotype a connection between metabolism of vitamin B6 and bacillithiol remains elucidated.

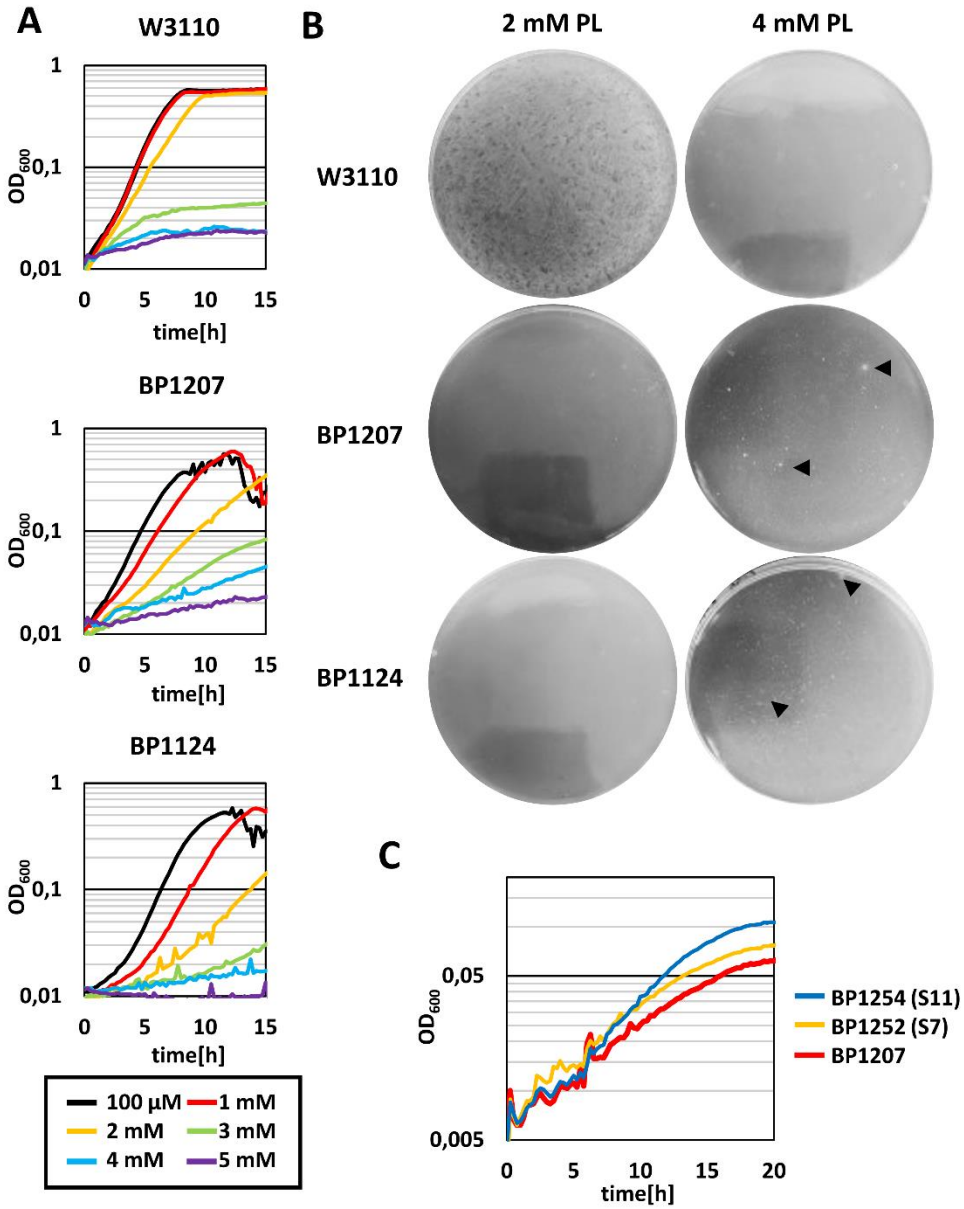


Figure 34 Toxic concentrations of PL lead to suppressor formation.
A: PL tolerance of *E. coli* wild type strain W3110 and *B. subtilis* strains BP1207 (Δ*pdxST amyE::(pdxR-P*$_{pdxS}$*)*^{Imo}*-lacZ*) and BP1124 (*P*$_{nrgA}$*-lacZ*) were tested. The strains were inoculated from C-Glc-PL overnight culture in fresh C-Glc-Pl medium and incubated until OD_{600} of 0.8 to 1 was reached. The cells were washed in saline, adapted to OD_{600} 0.5 and were used to inoculate 100 µl of C-Glc medium supplemented with different concentrations of PL. Growth was monitored in a multi-well-plate reader at 37°C. **B**: Cell material of *B. subtilis* and *E. coli* strains was taken off an LB agar plate, washed 2x in saline solution and set to the same OD. 100 µl cell suspension was spread equally on C-Glc agar plates containing either 2- or 4 mM pyridoxal. The plates were incubated for 5 days at 37°C. Black arrows indicate suppressor mutants. **C**: Growth comparison of BP1207 and its suppressor mutants BP1252 and BP1254 in C-Glc medium containing 4 mM PL.

Suppressor 11 carried a SNP in the *yqeT* gene, which codes for a protein similar to ribosomal protein methyltransferase. Furthermore, a frameshift in *mutS2* led to a truncated protein missing 600 aa and having the last 3 amino acids exchanged. Lastly, the putative -35 region of an operon was mutated and

thereby its expression is probably changed. The operon consists of the genes *yqxD*, *dnaG* and *sigA* coding for an unknown protein, the DNA primase DnaG and the sigma factor A SigA, respectively. Since the expression of the sigma factor was changed, the regulation of more than 1000 genes was disturbed, which makes it hard to elucidate the involved mechanism in PL resistance (*Zhu & Stülke*, 2018). Nevertheless, the impact of the mutations on gene expression in both S7 and S11 remains to be proven experimentally.

3.4 Evolution of a genome-reduced organism

Improvement of growth of genome-reduced *B. subtilis* strains by adaptive laboratory evolution

Genome-reduced organisms are taking big parts in modern science as they can be used to shed light on essential gene functions, to address questions in evolution biology and they may serve as production hosts (*Breuer et al.*, 2019; *Juhas et al.*, 2014; *Reuß et al.*, 2016; *Van Tilburg et al.*, 2020). The *MiniBacillus* project aims at generating genome-reduced *B. subtilis* strains harbouring only a defined set of genes. Therefore, a blueprint was made to identify all essential gene functions, which are needed to remain in the final organism (*Reuß et al.*, 2016). In theory, growth of the strain should not be compromised, as all important metabolites can either be taken up or synthesized (*Reuß et al.*, 2016).

We were able to delete approximately 40% of the *B. subtilis* genome in the Strain PG39 but came to a dead end, though, as further genome reduction was not possible. Instead of taking some steps back and using a different deletion route as it was done before (*Reuß et al.*, 2017), we decided to conduct an evolutionary approach to enhance general fitness of the minimal organisms. Thus, we cultivated the strains PG10 (genome reduction 34.6% (*Reuß et al.*, 2017) and the strain PG39 (genome reduction 40.2 % http://hdl.handle.net/11858/00-1735-0000-002E-E5F8-F) in 10 ml LB-Glc medium and diluted the culture every day 1:200. After 5 dilutions, the strains were stored in cryo cultures and used to start the serial dilution again. We cultivated the cells for 30 dilution cycles and compared the growth of the evolved strains with the parental strains. As seen in Figure 35 Growth of evolved, genome-reduced strainsthe evolved PG10 strain ($PG10_{evo}$) reached a higher biomass than the parental strain PG10 and also showed slight enhance in growth speed. The growth speed of the evolved $PG39_{evo}$ did not differ from the parental strain but the strain reached a higher final OD_{600}. Moreover, the evolved strain remained stable and kept it amount of biomass, whereas the parental strain lysed after prolonged incubation time.

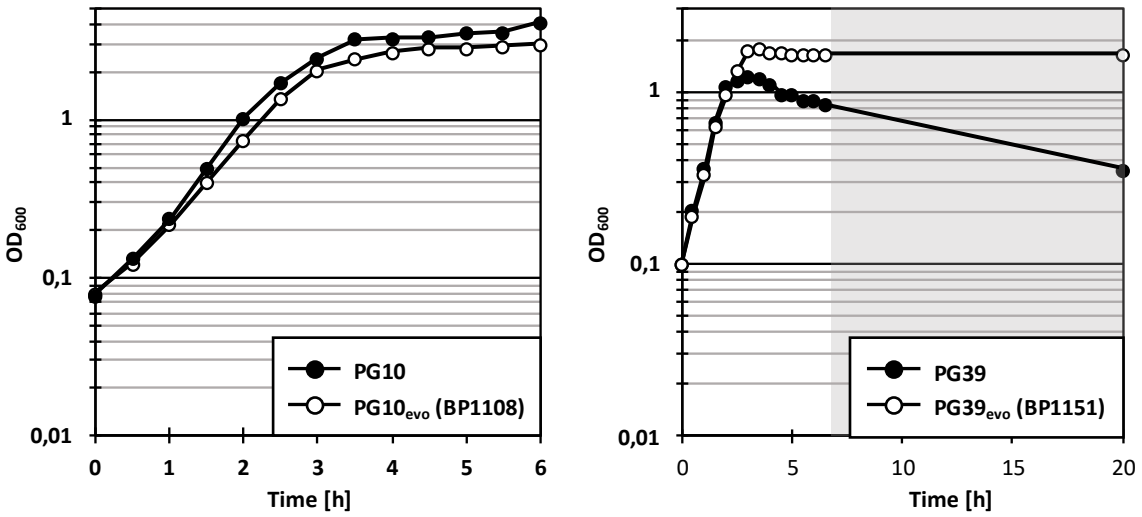


Figure 35 Growth of evolved, genome-reduced strains.
Genome reduced *B. subtilis* strains PG10 and PG39 were evolved in LB-Glc medium for 6 weeks by serial dilution, resulting in the strains BP1108 and BP1151, respectively. Growth of the evolved strains was compared to the parental strains. Therefore, precultures of the strains were inoculated from cryo cultures and incubated overnight. Fresh LB-Glc medium was inoculated from the overnight culture and incubated until the OD_{600} was ~0.5. The growth was monitored in 100 ml baffled flasks containing 20 ml LB-Glc medium every 30 minutes by measuring the OD_{600} in 1 ml micro cuvettes. The grey box indicates the timespan where no measurements were made. The resulting curve is an estimation and can differ.

We sequenced the genomes of the strains and mapped the reads to a modified *B. subtilis* 168 sequences considering all respective deletions of PG10 and PG39. We were able to identify differences between parental strains and evolved strains as depicted in Table 19.

In the evolved PG10 strain, we found 25 mutations of which 7 were silent mutations. We further categorized the mutations and classified the gene as uncharacterized, amino acid metabolism, stress adaptation, nitrogen metabolism, nucleotide utilization, co-factor synthesis, information processing and lifestyles (see Figure 36). Five mutations were found in genes involved in amino acid metabolism. MtrA is a methylthioribose transporter, taking up methylthioribose a precursor of methionine and other deriving amino acids. The *lysC* gene codes for an aspartokinase II, which is involved in lysine biosynthesis. RocA is a 3-hydroxy-1-pyrroline-5-carboxylate dehydrogenase synthesizing a step in the arginine pathway. Thus, also ornithine and citrulline utilization may be affected. The mutation in *asnH* was silent and had no effect on the amino acid composition. AsnH is an asparagine synthase, which forms L-asparagine and L-glutamate from L-aspartate and L-glutamine. GabT is active in utilization of gamma-amino butyric acid (GABA) and acts as a gamma-aminobutyrate transaminase transferring the amino group from 4-aminobutanoate or (S)-3-amino-2-methylpropanoate to 2-oxoglutarate to form glutamine (*Zhu & Stülke*, 2018).

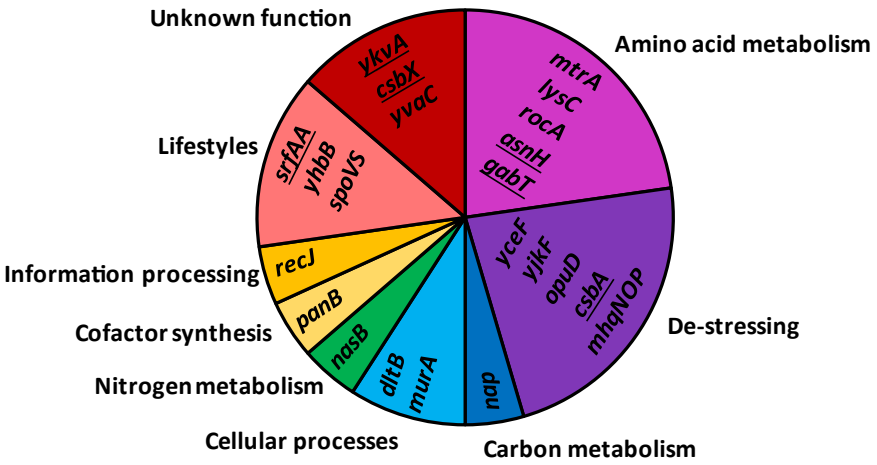


Figure 36 Classification of PG10 mutations.
Mutated genes found in the evolved PG10 strain were categorized by function. Underlined genes symbolize that only silent mutations occurred in the gene. Size of the pieces correlate with the amount of the found mutations.

The next proteins belong to the de-stressing class, which involves cellular processes that help the cells to overcome stress situations. Ycef is a general stress protein referring resistance to Mn^{2+} intoxification. MhqN is a protein of unknown function but similar to a NAD(P)H nitroreductase. MhqO and MhqP are a hydroquinone-specific dioxygenase, conferring resistance to methyl-hydroxyquinone and a protein of unknown function probably also involved in methyl-hydroquinone resistance. The function of YfkF is also unknown but it is similar to the multidrug efflux transporters. The compatible solute transporter OpuD mainly transports glycine betaine and arsenobetaine after osmotic pressure. Furthermore, CsbA is a general stress protein, required for protection against paraquat stress but the mutation in the evolved strain is silent and does not affect the amino acid sequence. Moreover, one gene coded for a protein, acting in carbon metabolism, Nap a carboxylesterase cleaving lipid esters to alcohols and carboxylate. To the group of cellular processes belongs DltB, which participates in teichoic acid synthesis and is therefore important for the biosynthesis of cell wall components and MurA the UDP-N-acetylglucosamine 1-carboxyvinyltransferase, forming precursors of peptidoglycan. Besides the genes taking part in amino acid metabolism, also *nasB* a gene involved in the electron transfer subunit of the nitrate reductase was mutated. Together with NasC it reduces nitrate to nitrite using NADH + H^+ as a cofactor. With the mutation in *panB* biosynthesis of coenzyme A could be altered and co-factor synthesis would be affected. RecJ belongs to the group of proteins operating in the field of information processing. It is a single-strand specific DNA exonuclease, which acts together with RecQ in replication fork maintenance and is crucial for RecA-dependent acquisition of homologous genes from related species by natural transformation. *srfAA*, *yhbB* and *spoVS* belong to the group of genes defining the lifestyle of *B. subtilis* and play a role in surfactin biosynthesis, which is important for swarming, in spore coating and in spore core dehydration, respectively. However, the mutation in srfAA is again a silent

mutation. The last group defined all the genes with an unknown function: *ykvA*, *csbX* and *yvac* of which only the latter carried a mutation leading to a change in amino acid sequence.

For the evolved PG39 strain we also found differences compared to the parental strain but to a lesser extent. The strain carried mutations in the 5'-UTR of *spoIIE,* which controls SigF activity and is required for normal formation of the asymmetric septum, in *cysE* involved in biosynthesis of cysteine, in *desK* a coding for a sensor kinase, regulating the cold shock expression of *des*, in the intergenic region between *pgi* and *ptsG* of the inserted glycolytic cassette and in *yuxN* coding for a transcriptional repressor controlling the Spx stability. We also checked the parental strain for the mutations and found that the mutation in the intergenic region of *pgi* and *ptsG* already existed in PG10.

Table 19 Mutations identified in the evolved *MiniBacillus* strains.

Strain	Parent strain (genotype)	Locus	Coordinates[a]	Function	Type of mutation
BP1108 ($PG10_{evo}$)	PG10	*murR*	191,828	regulation of muramic acid utilization	C210T (M70I)
		yceF	272,726	resistance to Mn^{2+} intoxication	G208A (G70S)
		nasB	319,589	utilization of nitrate	C1663T (A555T)
		srfAA	342,355	surfactin synthetase	C6894T no effects
		srfAA	342,358		C6897G no effect
		gabT	378,231	utilization of gamma-amino butyric acid	T230A (R77E)
		nap	507,920	lipid degradation	Substitution in 5'UTR T->G
		mhqNOP	512,207	highly upregulated operon in PG10, which was therefore deleted in further deletion strain	Substitution in 5'UTR. T-C
		mtrA	608,763	uptake of methylthioribose	C379A (A127S)
		yfkF	666,536	similar to multidrug-efflux transporter	C802T (D268N)
		yhbB	731,330	spore coat protein, amidase	G9T no effect
		yhbB	731,335		G14T (G5V)
		ykvA	1,037,991	unknown	C42T no effect
		spoVS	1,294,123	spore coat assembly	G51T no effect
		spoVS	1,294,125		G53C (G18A)
		panB	1,469,042	biosynthesis of coenzyme A	A239G (V80A)
		recJ	1,819,931	Replication fork maintenance	C2352G (R784S)
		csbX	1,834,707	unknown	C73T no effect
		lysC	1,907,408	biosynthesis of lysine	Substitution in 5'UTR. G->T
		opuD	2,053,475	compatible solute transport	374 +C, frame shift:52 altered aa, 336 aa missing
		yvaC	2,312,382	unknown	C1462A (V488F)
		csbA	2,383,252	protection against paraquat stress	C186G no effect
		rocA	2,587,207	arginine, ornithine and citrulline utilization	C578A (G193V)

		dltB	2,638,964	biosynthesis of teichoic acid	G191T (G64V)
		asnH	2,692,699	biosynthesis of asparagine	G1167V no effect
BP1151 (PG39$_{evo}$)	PG39	*spoIIE*	70,355	control of SigF activity, required for normal formation of the asymmetric septum	Substitution in 5'UTR C->A
		cysE	113,214	biosynthesis of cysteine	416+GTTCTATTACGG (tandem repeat) (139+GSIT)
		desK	1,256,695	regulation of cold shock expression of des	T1036C (F139L)
		yuxN	2,034,999	control of Spx stability	473+T frameshift 112 aa missing

[a]Coordinates refer to the position in the genome sequence of *B. subtilis* 168. All genome deletions were manually synchronized in the sequence.

The highly upregulated *mhqNOP* operon is downregulated in the evolved PG10 strain

Taken together, several mutations were identified in the evolved strains, which could be responsible for a higher general fitness compared to the parental strain. Nevertheless, the mutation in the promoter of the *mhqNOP* operon was of our special interest because transcriptomic data revealed that this operon is highly upregulated in PG10. This is due to a deletion of its repressor MhqR in a former strain and connected to a uncontrolled expression of the operon, leading to a waste of energy since the expressed proteins are not used by the *B. subtilis* strain under the cultivation conditions (*Reuß et al.*, 2017). Therefore, the *mhqNOP* operon was deleted in a further strain to cope this energy waste. We were curious, if the mutation in the *mhqNOP* promoter region could be the evolutionary answer of the strain to lower the expression of the operon and thereby confirming our idea to delete it. We constructed plasmids by cloning the mutant (pBP760) and the wild type (pBP761) 5'-UTR of *mhqNOP* into the pAC7 vector. As the promoter region is fused to the *lacZ* gene, promoter activities can be measured. We introduced the plasmids pBP760 and pBP761 into the *B. subtilis* PG10 strain, resulting in BP1113 and BP1114, respectively. The strains were cultivated in LB-Glc medium until the OD_{600} was about 0.5 and the *β*-galactosidase activity was measured as described. As seen in Figure 37, the wild type promoter had an activity of approximately 60 units per milligram of protein. In contrast to that, the mutant promoter only turned over 1 unit per milligram of protein. We wondered in which time frame the mutation in the *mhqNOP* promoter came up and also sequenced the promoter region in the strains we stored after 5, 10, 15, 20 and 25 cycles of cultivation. Consequently, we amplified the *mhqNOP* 5'-UTR with PCR, analyzed the fragment by Sanger sequencing and found out that the mutation occurred between cycle 25 and 30.

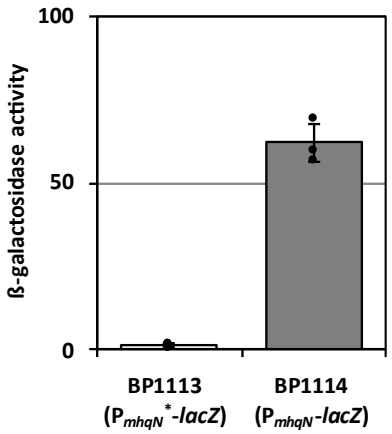


Figure 37 β-galactosidase activity of wild type and mutant *mhqNOP* promoter. Wild type and mutant *mhqNOP* promoter activities were measured. The strain BP1113 and BP1114, harboring the mutant and the wild type promoter, respectively, were cultivated in LB-Glc medium and harvested when the OD_{600} reached 0.4 – 0.6. Data points represent biologically independent replicates. Bars indicate means of replicates and the standard deviations are shown. β-galactosidase activities are given as units per milligram of protein.

4. Discussion

4.1 The role of YtoQ and BSH in the heterologous pathway

The phenotypes of *ytoQ* and *bshC* mutants

A PL-auxotroph *B. subtilis* mutant harboring only the last two genes of the DXP-dependent pathway *pdxJ* and *pdxH*, forms suppressor mutants, which delete the bacillithiol synthesis gene *bshC* and upregulate the *ytoQ* gene. To assess the function of YtoQ, we deleted and overexpressed the *ytoQ* gene in the *B. subtilis* wild type and analyzed the growth (see Figure 11). Although a growth effect on solid medium could not be detected, the *ytoQ* mutant showed a slightly reduced growth compared to the wild type, when grown in liquid SM minimal medium. We furthermore tested growth of the *bshC* single mutant and of the *ytoQ bshC* double mutant in presence and in absence of *ytoQ* overexpression. Besides the *ytoQ* mutant, also the *bshC* and the *ytoQ bshC* double mutant had an impaired growth compared to the wild type, but the defect of the *ytoQ* mutant was bigger than of the *bshC* mutant. The double mutant showed the same growth behavior as the *bshC* mutant. Thus, the negative effects did neither stack nor were reversed when both genes were deleted. Moreover, overexpression of *ytoQ* had no significant effect on growth in the *bshC* or in the *bshC ytoQ* double mutant. As the growth did not improve upon complementation with *ytoQ*$^{+}$, this renders out that the deletion of *bshC* could be superior compared to the *ytoQ* deletion. Otherwise, as stated by Fang & Dos Santos (2015), supplementation with CAA or Fe^{2+} could not rescue the phenotype of the *bshC* mutant. They showed that addition of especially glutamine, glutamate, leucine and isoleucine were able to overcome the growth defect. These amino acids need Fe-S enzymes in their synthesis routes. Therefore, BSH could be important for the synthesis of the amino acids as it is needed to maintain high activity of Fe-S containing enzymes (*Chandrangsu et al.*, 2018; *Fang & Dos Santos*, 2015; *Rosario-Cruz et al.*, 2015). When supplemented with iron, the growth curves are mostly identical to the ones without supplementation (see Figure 11). Since Fe^{2+} is oxidation-prone, the experiment was repeated with fresh $FeCl_2$ and also in MSSM minimal medium but showed the same results (data not shown). Upon CAA supplementation, the wild type reached the stationary phase faster. Interestingly, the *ytoQ* single mutants showed wild type-like growth behavior when CAA was added to the medium. This was not the case for the other mutants, regardless of the overexpression of *ytoQ.* It would have been interesting to test if overexpression of *bshC* shows any beneficial effects in the *ytoQ* deletion mutant. Besides that, the growth of the *ytoQ* mutant with different amino acids could be checked to assess if the beneficial effect of CAA derives from glutamine, glutamate, leucine and isoleucine or from different amino acids.

We furthermore tested the susceptibility of different heterologous pathway mutants to oxidative stress and the antibiotic fosfomycin. Although no differences could be seen in size of the zone of inhibition for the oxidative stressors, suppressor mutants arose on the H_2O_2 plates for the *ytoQ* mutant, the *bshC* mutant the *ytoQ bshC* double mutant and for the *pdxST ytoQ bshC* triple mutant (see Suppl. Figure 1C). As the suppressor formation was observed only in two replicates, the mutants have not been isolated. However, it would be interesting to repeat the experiment and to analyze potential suppressor mutants.

For the fosfomycin test, only the strains carrying a deletion in *bshC* were susceptible, as it was described before (*Cao et al.*, 2001). The only exception is BP965 (Δ*pdxST pdxJ pdxH*), which also showed sensitivity to fosfomycin. Fosfomycin is an epoxide-antibiotic and inhibits the UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA (*Kahan et al.*, 1974). *B. subtilis* is naturally fosfomycin resistant as its FosB protein can transfer BSH to the antibiotic and thereby open the epoxide-ring. This inactivates the antibiotic and MurA is no longer inhibited (*Roberts et al.*, 2013). Deletion of BSH synthesis therefore leads to an increased fosfomycin susceptibility, which matches the outcome of the experiment (*Gaballa et al.*, 2010). Since the *pdxST* mutant (BP1100) is fosfomycin resistant, the presence of PdxJ or PdxH must play a role in the susceptibility of BP965 to fosfomycin, but how needs to be further analyzed. YtoQ seemed to have no significant influence on fosfomycin susceptibility as its presence did not change the sensitivity towards fosfomycin significantly.

Moreover, we tested genetic competence for the pathway mutants. YtoQ an BSH did not seem to have a direct influence on genetic competence (see Figure 13). The deletion of *pdxST* lead to a strong loss of competence, although PL was present in the medium. It could be, that for some PLP-dependent proteins it is important to receive PLP from the synthesis complex. Thus, the complex could be directly involved in competence formation by sequestering PLP to the target proteins. Similar could be observed for biofilm formation, discussed below. Two PLP-dependent proteins NifZ and YrvO play a role in tRNA modification. If the PLP transfer to the proteins is diminished, this could lead to a lack of specific tRNAs and thereby to a deregulation of DNA metabolism, leading to decrease competence efficiency. The competence levels among the other mutants harboring the *pdxST* deletion was about the same, independent of additional genetic changes. Interestingly, the competence in the Δ*pdxST pdxHJ* Δ*bshC* Δ*ytoQ* mutant (BP1103) was completely abolished. As neither the Δ*pdxST pdxHJ* (BP965) nor the Δ*pdxST* Δ*bshC* Δ*ytoQ* (BP1246) mutant showed a difference in competence compared to the *pdxST* mutant, the loss of competence is probably a secondary effect. As described above, the *ytoQ* mutant showed a growth defect in minimal medium. Although the competence test was conducted on complex medium, the additional deletion of *pdxST* or the other genetic changes could lead to growth defects and thus to a lower general fitness. This could be tested by conducting growth experiments of

the used strains also in complex medium and check for growth differences between the *ytoQ* singe mutant and the other stains.

Identification of potential interaction partners of YtoQ

In the pulldown experiment multiple potential interaction partners of YtoQ were identified from *E. coli* and *B. subtilis* (see Table 16. One candidate, which was found exclusively when *B. subtilis* cell extract was added to the bait protein, is YrkL. Similar to YtoQ, YrkL is highly expressed under thiol depletion and in *E. coli* It has a well described homolog KefF (*Zhu & Stülke*, 2018). KefF is a glutathione-regulated potassium efflux system, which can efficiently detoxify electrophiles (*Lyngberg et al.*, 2011). Besides that, it can activate the potassium efflux channel KefC, thus leading to efflux of potassium and import of protons. By that, the intracellular pH is lowered and reducing environment is generated (*Lyngberg et al.*, 2011). The Kef system is exclusively present in Gram-negative bacteria but a similar mechanism could be also present in *B. subtilis* maybe involving BSH as replacement for glutathione (*Ferguson et al.*, 1993). The spectra found for RpoC occurred in the search against the *E. coli* and the *B. subtilis* database. The homologs share 68.9% similarity (*Zhu & Stülke*, 2018). As spectra were found in the fractions with and without SP1 cell extract, it is likely that the RNA polymerase subunit derived from *E. coli*. However, the interaction with the RNA polymerase beta and beta' subunit from *E. coli* could give a hint that YtoQ indeed has a regulatory function and possesses DNA binding activity by its predicted helix-turn helix motif at the C-terminus (http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B).

A connection to oxidative stress response was found by the putative interaction with MetH, which is a cobalamin-dependent enzyme involved in methionine synthesis. MetH is not present in *B. subtilis* but a similar protein MetE catalyzes the same step but cobalamin-independent (*Zhu & Stülke*, 2018). Upon oxidative stress, MetE is S-bacillithiolated and S-cysteinylated, signaling a relation between YtoQ and BSH (*Chi et al.*, 2011; *Hochgräfe et al.*, 2007). Furthermore, Pta catalyzes the conversion of acetyl-CoA and acetyl phosphate in *E. coli* (*Dittrich et al.*, 2005). As CoA can act as an oxidative stress reducing agent interaction with YtoQ could alter the equilibrium of the reaction to cope with oxidative stress (*Newton et al.*, 2008). Lastly, several enzymes involved in central carbon metabolism were identified as potential interaction partners of YtoQ, namely Pta (phosphate-acetyl transferase), PtsI (phosphoenolpyruvate-protein phosphotransferase), FumA (fumerate hydratase) and SucC (succinyl-CoA ligase). All of these candidates also have homologs in *B. subtilis* and it would be interesting to test, if YtoQ plays a role in central carbon metabolism. Since PLP is produced from carbon backbones, deriving from glycolysis and pentose phosphate pathway, YtoQ could regulate the

availability of substrates for the synthesis of B6. Since the number of spectra was quite low in general, possible interactions need to be confirmed by additional experiments.

To roles of YtoQ and BSH in the heterologous vitamin B6 synthesis pathway

As BSH can be partially replaced by cysteine in *vivo*, we tested the growth of the heterologous pathway mutants in presence of cysteine. Interestingly, the beneficial effect of *bshC* deletion and *ytoQ* overexpression was lost. The presence of cysteine had a negative effect on growth in the heterologous pathway mutant. This indicates, that the removal of BSH probably has something to do with the oxidative stress response, in which cysteine can take over.

Overexpression of *ytoQ* in the *bshC* background did not cure the growth defect in minimal medium but was beneficial in the heterologous pathway mutant. We tried to further evolve a strain lacking *ytoQ* (Δ*pdxST pdxJH* Δ*bshC* Δ*ytoQ*) to screen for different suppression mechanisms, but growth was not possible in this strain on minimal medium without PL addition (data not shown). As *ytoQ* needs to be overexpressed to establish wild type-like growth, the cellular levels seem to be too low to maintain its function in the pathway. Both *ytoQ* and *bshC* are expressed under control of the DNA-binding regulator Spx and thus have a similar expression pattern (*Zhu & Stülke*, 2018; *Zuber et al.*, 2011; http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B). Spx is required to be in its oxidized form to regulate gene expression (*Nakano et al.*, 2005). Accordingly, the upregulation of *ytoQ* expression makes it independent of the oxidative levels. Since BSH is missing in the mutant, the cells are prone to oxidative stress, which could also have an effect on the oxidative state of Spx and could explain why *ytoQ* expression needs to be upregulated (*Imber et al.*, 2019). Nevertheless, an increased susceptibility to oxidative stress could not be seen in the heterologous pathway mutants (see above). Moreover, vitamin B6 was shown to be active as an antioxidant being able to scavenge up to eight hydroxy radicals (*Matxain et al.*, 2006, 2009). As the oxidative stress potential is increased by the absence of BSH, PLP could also be used as a protectant, which would result in decreased levels of available PLP. Thus, it would be interesting to measure the oxidation state of the heterologous pathway mutant, the wild type and different *ytoQ* / *bshC* deletion mutants and overexpression strains. Furthermore, measuring the PLP pools in the wild type and the heterologous pathway mutant could give a hint about the synthesis quality of the heterologous pathway and of how much PLP is available for the reaction.

We firstly supposed, that BSH and cysteine could act on SerA, since they can to bind at the active center under thiol depletion and lead to a and an inhibition of the enzyme (*Chi et al.*, 2011, 2013; *Hochgräfe et al.*, 2007). This would have confirmed the presence of our suggested underground metabolism pathway route consisting of GapA, CpgA, SerA, SerC and ThrB. Thus, we deleted the nonessential of those genes *cpgA*, *serA*, *serC* and *thrB* in the background of a fully functional

heterologous pathway mutant and checked for PL-dependent growth. As GapA is essential in *B.* subtilis, knockout is not possible and a participation of Gap in the pathway was not checked. As deletion of the other genes had no effect on growth on minimal medium without PL supplementation, we can exclude the presence of the genes in the underground metabolism pathway (see Figure 15). Next, we introduced *pdxA*, *pdxB* and a combination of both into the Δ*pdxST pdxJH* (BP965) and the Δ*pdxST pdxJH* Δ*bshC* (BP978) mutant to assess, whether the mutants are still dependent on the deletion of *bshC* or the overexpression of *ytoQ*. By that, we tried to find out if the underground metabolism pathway is similar to the DXP-independent pathway or follows a completely different route. For the single integration no growth difference was seen, indicating that the function of neither PdxA nor PdxB is complemented by the overexpression of *ytoQ* and the loss of BSH and the pathway follows a different route. When *pdxA* and *pdxB* were expressed together, the Δ*pdxST pdxJH* and Δ*pdxST pdxJH* Δ*bshC* mutant were able to grow without addition of PL. Suppressor mutants occurred in presence of *bshC*, though. As these depend on the presence of the *bshC* gene, it is likely that they have mutated BSH synthesis related genes. Thus, although in theory all necessary vitamin B6 synthesis genes are present in the mutant, the absence of BSH still seems to be important. Therefore, it is probably involved either upstream or downstream of this branch for example at GapA. GapA is a homolog of the *E. coli* Epd and is known to have a redox sensitive active site, which can be oxidized to intramolecular disulfides (*Chi et al.*, 2011; *Liebeke et al.*, 2008). Although GapA is not S-bacillithiolated, BSH is involved in maintaining the redox state of oxidized thiol-groups and maybe GapA is a target of BSH and thus is a candidate for the underground metabolism pathway. It is also possible that by integration of *pdxA* and *pdxB* another novel route including SerC is formed, which is not the underground metabolism pathway YtoQ acts in. Overexpression of *ytoQ* is not necessary when *pdxA* and *pdxB* are expressed. All in all, the data suggest, that an underground metabolism pathway exists, which could detour PdxA and PdxB. As this experiment was conducted only once, it needs to be repeated to confirm the roles of YtoQ and BSH, though.

Perspective for the underground metabolism pathway

The DXP-dependent vitamin B6 synthesis pathway is limited to *α*- and *γ*-proteobacteria and the evolutionary younger pathway (*Mittenhuber*, 2001). It was stated that the last common ancestor first lost the PdxST complex, as it lived in a B6-rich surrounding and later on needed to acquire a new way to synthesize PLP (*Fitzpatrick et al.*, 2007). As the salvage pathway still was present, the pathway could have evolved from mostly already existing enzymes (*Mittenhuber*, 2001; *Schoenlein et al.*, 1989). One exception is PdxJ, which has high similarity with PdxS in terms of structure and mechanism. Thus, PdxJ could have evolved convergently or was taken up from the surrounding (*Fitzpatrick et al.*, 2007).

It was also shown that a point mutation in *pdxJ* can suppress the loss of *pdxH*. If this mutation mimics the PdxS function, or just takes over the PdxH mechanism still needs to be elucidated (*Man et al.*, 1996). Besides that, ThiG was identified as a protein with high similarity to the *B. subtilis* PdxS in a computational screen and overexpression could recover the loss of *pdxB* in *E. coli* (*Oberhardt et al.*, 2016). In addition, SerA is a homolog of PdxB and both share a common ancestor (*Schoenlein et al.*, 1989). The remaining reactions of the pathway mostly depend on proteins acting in serine metabolism (SerC) and deoxyxylulose synthesis (Dxs), which are also common in organisms harboring the DXP-independent pathway. All in all, the chance, that a heterologous synthesis pathway can form in *B. subtilis* when only *pdxH* and *pdxJ* are introduced as additional genes is, expectably high. Since the DXP-independent pathway emerged from a last common ancestor harboring the DXP-independent pathway, the fundamental set of needed enzymes is already present. PdxJ and PdxH seem to be the only enzymes, which are not related with additional metabolic tasks and have to be added to the genome to establish the DXP-independent pathway. It would be interesting to test if the point mutation in PdxJ can also complement the absence of PdxH in the background of the heterologous vitamin B6 synthesis pathway in *B. subtilis* (*Man et al.*, 1996).

Nevertheless, CpgA, SerA, SerS and ThrB can be excluded from the theoretical route of the underground metabolism pathway. Thus, it could be tested, if the pathway involves the deoxyxylulose producing branch, or if PdxJ, PdxH and YtoQ for example directly synthesizing PdxS maybe together with other proteins like ThiG. As the deoxyxylulose synthase Dxs is essential in *B. subtilis* it cannot be deleted. For this reason, it could be introduced into the genome under an inducible promoter and deleted afterwards to knock down the gene. Thus, if a PL-dependent phenotype could be observed at low levels of Dxs, this could demonstrate that Dxs is used for the heterologous pathway. The second substrate of PdxJ APA seems to be absent in in *B. subtilis*. Although similar metabolites are present in the cells, this could indicate a role of PdxJ and PdxH apart from the DXP-dependent pathway.

4.2 Deletion of *pdxT* can be complemented by overexpression of *pdxS*

Genomic amplification as the main suppression mechanism

Deletion of the *pdxT* gene leads to a growth defect in *B. subtilis* when cultivated in minimal medium (*Belitsky*, 2004b). We confirmed existing data, showing that the loss of the transaminase subunit of the PdxST synthesis complex can be complemented by ammonium supplementation (see Figure 19)(*Belitsky*, 2004b). We evolved the Δ*pdxT* mutant under limiting amounts of ammonium and found that some suppressors were able to reach the same biomass than the wild type (see Figure 19 and Figure 21). Most of the suppressors harbored a genomic amplification of a 15 kbp region including the

pdxS gene, which codes for the vitamin B6 synthase domain (see Table 17 and Figure 22). As described before, growth can be enhanced by increasing the dosage of beneficial genes by selective genomic amplification (*Dormeyer et al.*, 2018; *Elliott et al.*, 2013; *Reuß et al.*, 2019; *J. Rosenberg et al.*, 2018; *Wicke et al.*, 2019). Genetic amplifications occur in all kingdoms of life and are a common possibility to adapt to changing environmental conditions (*Andersson & Hughes*, 2009). Mostly, they are not very stable and get quickly lost if the selective pressure decreases. The stability depends on the size, the amplification factor and the location of the amplified region (*Pettersson et al.*, 2008; *Roth et al.*, 1996). Accordingly, it could be shown that amplification leads to a decrease in fitness and slower growth as in the parental strain without amplification (*Reams et al.*, 2010). Therefore, amplifications can be seen as a temporary solution before the enrichment of stable mutations since a duplication of a gene encoding a beneficial enzyme is more likely to occur than mutations enhancing the activity directly. Once one of the genes in the amplified genomic region has acquired a beneficial mutation, the gene dosage can decrease by selective de-amplification (*Andersson & Hughes*, 2009; *Sonti & Roth*, 1989; *Straus*, 1975, 1976). It was estimated that in about 10 % of a bacterial population, which was grown in medium without a selective pressure, a gene duplication is present somewhere in the genome (*Roth et al.*, 1996). These random amplifications may be beneficial to the cells when they are facing new selective pressures.

General steps of homologous recombination are conserved among all organisms in the basic mechanism and only the involved proteins differ. During replication an arrest of the DNA synthesis can occur and single stranded DNA (ssDNA) gaps can emerge. These gaps need to be repaired as double strand breaks are promoted, which can cause lethality (*Cox et al.*, 2000). Firstly, double stranded DNA ends are detected and processed. Next, recombinases as RecA are loaded onto the ssDNA and the complex pairs with intact homologous DNA fragment. A junction is formed and DNA is synthesized involving the 3'-OH end of the invading strand. Lastly, the crossover junction is resolved by endonucleases and the daughter strands are formed (*Friedberg et al.*, 2006; *Lenhart et al.*, 2012).

In *E. coli* DNA strands are processed by the RecFOR complex and thus, RecA can be translocated to the DNA by either the RecBCD- or the RecFOR complex of, which the former acts in the repair of double strand breaks (DSB) and the latter in single strand DNA (SSG) gap repair (*Arnold & Kowalczykowski*, 2000; *Clark*, 1973; *Hegde et al.*, 1996; *Korolev*, 2017; *T. C. Wang et al.*, 1993). Under normal conditions, binding of RecA to the ssDNA is inhibited by single strand DNA binding proteins (SSB) but the mediating RecBCD or RecFOR complexes detach the SSBs from the ssDNA and promote RecA binding at the gaps (*Korolev*, 2017; *Morimatsu et al.*, 2012; *Umezu et al.*, 1993; *Umezu & Kolodner*, 1994; *Webb et al.*, 1997). The RecA/ssDNA nucleoprotein filament facilitates the search for homologous regions and DNA synthesis (*Kreuzer*, 2013). Finally, the emerging Holliday junctions are

resolved by RuvABC and RecG (*A Kuzminov*, 1999; *Shinagawa & Iwasaki*, 1995; *West*, 1996). The complex pathway of homologous recombination in *E. coli* is reviewed in more details in *Dubnau* (1999), *Korolev* (2017) or *Sonenshein et al.* (1993).

In *B. subtilis* double strand ends are processed by the AddAB complex as the RecBCD complex does not exist. Both complexes have the same function but use different mechanisms for the processing of the DNA (*Lenhart et al.*, 2012; *Yeeles & Dillingham*, 2010). Among the Firmicutes the AddAB complex is widely conserved and deletion of the complex leads to severe defects in double strand break repair (*Alonso et al.*, 1993; *Cromie*, 2009; *Haijema et al.*, 1996; *Kooistra et al.*, 1997; *Meima et al.*, 1995). It could be shown that the RecJ, RecQ and RecS proteins can complement the loss of *addAB*, also indicating a role in strand processing (*Sanchez et al.*, 2006). After the DSB ends are processed, this enables RecA to bind to the resulting 3'ssDNA. Similar to the mechanism in *E. coli* the SSBs need to be removed by AddAB before RecA can bind at the gap position. For the translocation of RecA to the DNA the RecFOR complex is necessary. In contrast to the *E. coli* pathway, the DNA mediating AddAB complex could not be related with RecA-DNA binding (*Ayora et al.*, 2011; *Sanchez, Carrasco, Ayora, et al.*, 2007a, 2007b). It is still not clear if the RecFOR complex also takes part in the repair of double stranded breaks as the complex in *E. coli* and also how the RecA loading to the DNA is managed mechanistically (*Kidane & Graumann*, 2005; *Lenhart et al.*, 2012; *Manfredi et al.*, 2008; *Umezu & Kolodner*, 1994). The recruited RecA needs a homologous DNA template and ATP or desoxyATP (dATP) to start extending the DNA strand, whereby dATP is the favored nucleotide as it yields in a 10-fold higher activity of RecA (*Carrasco et al.*, 2008). Contrary to *E. coli*, *B. subtilis* possesses only the RuvA and RuvB proteins for the branch migration to resolve Holiday junctions. It is furthermore hypnotized, that RecU takes over the part of RuvC in *B. subtilis* and closes the gaps in the DNA (*Fernández et al.*, 1998; *McGregor et al.*, 2005). The RecG protein is also thought to facilitate the branch migration in addition to RuvAB (*Sanchez, Carrasco, Cozar, et al.*, 2007). More detailed information about the recombination process in *B. subtilis* can be found in *Ayora et al.* (2011) or *Sanchez et al.* (2007a).

We tried to further evolve the Δ*pdxT* suppressor mutants by using a green fluorescent protein-based screening system, which can display the amplification level of a suppressor and assumed that a de-amplification could occur and mutations regarding the *pdxS* gene or acquisition of promoter-up mutations could be the consequence as seen before (*Dormeyer et al.*, 2017; *Reuß et al.*, 2019; *J. Rosenberg*, 2016; *Zaprasis et al.*, 2014). We cultivated a Δ*pdxT* suppressor with high fluorescent signal for 20 generations in minimal medium but were not able to select a de-amplification mutant (see Suppl. Figure 5). We furthermore deleted the *recA* gene in the Δ*pdxT* mutant to force a different mechanism

of suppression but still obtained genomic amplification of the 15 kbp region in the suppressor mutant although only 2-fold.

For the amplification of a genetic region its surrounding is important (*Reams & Neidle*, 2004). To facilitate the amplification, repetitive sequences are mostly required, as insertion sequences, transposable elements, repetitive extragenic palindromic (REP) sequences or ribosomal operons, which are present among many bacteria (*Anderson & Roth*, 1981; *Haack & Roth*, 1995; *Jessop & Clugston*, 1985; *Lehner & Hill*, 1980; *Lin et al.*, 1984; *Shyamala et al.*, 1990). The amplified genetic region in the suppressors was guided by the rRNA genes rrnO-16S-rrnAO-23S and rrnA16S-rrnA23S. It has already been observed in other studies that amplified regions are bordered by rRNA genes, as described in *Anderson & Roth* (1981), *Benda et al.* (2020) or *Lehner & Hill* (1980). As these genes have a high sequence identity, they allow homologous recombination and by that multiplication of the genetic area.

Amplifications, which are bordered by direct repeats of at least 200 bps length could be related to the *recA* gene and *recA* deletion mutants show a at least ten-fold reduction in amplification and de-amplification frequency (*Petes & Hill*, 1988; *D Romero & Palacios*, 1997). Recombination events between direct repeats can be mediated also independent of RecA at a high frequency in *E. coli* but shorter homologous repeats with a length ranging from few groups of ten to several hundred nucleotides are required (*Bi & Liu*, 1994; *Dianov et al.*, 1991; *Dutra et al.*, 2007; *Jain et al.*, 2020; *Lovett et al.*, 1993, 1994). The RecA-independent rearrangement of DNA has an efficiency of about 25-30% of the RecA-dependent homologous recombination but depends on the distance between the homologous regions (*Bi & Liu*, 1994; *Chédin et al.*, 1994; *Lovett et al.*, 1993, 1994; *Morag et al.*, 1999). It is assumed that the repeats need to interact with the same replication fork to allow RecA-independent template switch (*Lovett*, 2017). Furthermore, RecA-independent recombination events are associated with crossover formation between sister chromosomes and the frequency increases if DNA replication proteins as *dnaC*, *dnaE*, *dnaQ*, *holC*, *ssb* or *priA* have a defect (*Lovett et al.*, 1993; *Saveson & Lovett*, 1997). This supposes a similar recombination event as performed by RecA, which can be explained by a template switch promoting a discontinued lagging strand. Holliday junction formation and further processing enable crossovers (*Lovett*, 2017). In this model, the recombination is not guided by strand invasion into duplex DNA as for RecA-dependent recombination but the nascent strand unwinds and generates singe stranded DNA. Deletion of *ruvAB* inhibits the migration of holiday junctions and increases RecA-independent crossover events as more ssDNA is free for annealing (*S T Lovett et al.*, 1993). In contrast to the RecFOR-mediated homologous recombination, this RecA-independent rearrangement of DNA is suggested to take place mostly in replication of sister chromosomes and not with separate homologs (*Lovett et al.*, 1993, 2017). When bordered by *rrnA*

genes, multiplication rates of the genetic region are unaffected by the deletion of *recA*, *recB*, *recF* or *recBF* in *E. coli*. Interestingly, a Δ*ruvC* Δ*recG* double mutants shows a 30-fold decreased amplification rate in that can be recovered if one of the genes (*recA, recB* or *recF*) is deleted in addition (*Andrei Kuzminov*, 2001; *Spies & Kowalczykowski*, 2004). Accordingly, deletion of *recA*, *recB* and *recF* presumably promotes accumulation of unrepaired DNA strand breaks, which can further anneal with *rrnA* loci from another sister chromosomes during replication and by that allow formation of amplifications (*Reams & Roth*, 2015). Furthermore, a Δ*recG* Δ*ruvC* double mutant does neither allow homologous recombination nor single strand annealing as emerging Holliday junctions cannot be resolved. Nevertheless, the RecFOR or RecBCD pathways can initially start the repair of DSS and SSG and therefore single strand ends, which are necessary for annealing are still processed but also lead to the accumulation of free ends. These can block the pathway and thereby recombination events (*Reams & Roth*, 2015). As *recA*, *recB* or *recF* are acting upstream of *recG* and *ruvC* and additional deletions of one of the genes facilitate that single strand ends cannot accumulate anymore and ends are free for alternative annealing (*Reams et al.*, 2014; *Reams & Roth*, 2015). Recently the *rarA* gene was related with RecA independent recombination (*Jain et al.*, 2020; *H. Romero et al.*, 2020). RarA is an AAA^+ ATPase with sequence similarities to the DNA clamp loader complex and is important to keep the genome stability but its mechanism was widely unknown (*Jain et al.*, 2020; *Page et al.*, 2011). It is hypothesized that RarA is involved in cross-over formation by but this could not be experimentally proven yet (*Jain et al.*, 2020).

A second model for RecA-independent recombination is based on misalignments of nascent DNA strand and with the template strand, which is defined as “replication slippage” (*Albertini et al.*, 1982; *Lovett et al.*, 1993, 2017; *Morag et al.*, 1999). This could be provoked by an unwinding of the nascent strand and a subsequent pairing with the downstream homologous region, which will cause a deletion or could also promote multiplication of the region (*Morag et al.*, 1999) This effect often happens when the adenine residues of the DNA are still hemimethylated as it is shortly after replication (*Bzymek et al.*, 1999; *Lovett & Feschenko*, 1996).

Deleted or amplified genetic regions can also lie within inverted repeat sequences (*Galas*, 1978; *Glickman & Ripley*, 1984). This mechanism is increased in RecA-deficient- or DNA Polymerase III mutants. It is assumed that replication gaps can foster the formation of a cruciform like structure of the double stranded DNA that is induced by the inverted repeats (*Bzymek & Lovett*, 2001). Two different routes were proposed for the further process, which are depend either on SbcCD or DnaK of, which the latter is also important for RecA-independent recombination, which does not imply inverted repeat sequences (*Goldfless et al.*, 2006; *Pinder et al.*, 1998; *Trinh & Sinden*, 1991).

Since the amplified region is bordered by the *rrn* genes, which allow RecA-independent recombination, the deletion of *recA* just decreased general fitness of the suppressor mutants and could not stop the amplification of the region containing *pdxS*. In contrast to the Δ*pdxT* mutant, the Δ*pdxT* Δ*recA* double mutant forms only few suppressor mutants of, which only one could be isolated and was viable after isolation. As the homologous *rrn* gene regions have a size of approximately 4500 bp, they enable both RecA-dependent and RecA-independent recombination events (*Dutra et al.*, 2007; *Jain et al.*, 2020; *Lovett*, 2017). When deleting *recA*, only RecA-independent recombination is possible, which is less efficient and depends on the distance between the homologous regions (*Bi & Liu*, 1994; *Chédin et al.*, 1994; *Lovett et al.*, 1993, 1994; *Morag et al.*, 1999). This could be the reason for the low amplification factor of the Δ*pdxT* Δ*recA* double mutant in comparison to the other suppressors (see Table 17, Figure 22 Genome sequencing and Southern blotting analyses to assess the extent of *pdxS* amplification in the *pdxT* suppressors. A. Read coverage (average) along the chromosome of the *pdxT* suppressors. As shown in the figure inlays, based on these values the copy numbers of chromosomal segment containing the *pdxS* gene can be inferred. In all suppressors that were subjected to Illumina sequencing, the amplified is 15 kbp long and is located between two rRNA genes (rrnO-23S - rrnA16S, coordinates 11,709 – 31,832). B. Southern blotting analysis to detect the amplified chromosomal segment containing the *pdxS* gene. The parent strains BP1105, BP1106 and BP1182 served as controls. Chromosomal DNAs were digested with *SacI* and *HindIII* and the blots were hybridized with a *pdxS-specific* probe.).

Promoter-up mutations and evolvability of PdxS

The fact that no promoter up-mutations were accumulated regarding the *pdxS* gene, as it has been observed in other studies, hints at a limited evolvability of the promoter (*Dormeyer et al.*, 2018; *Elliott et al.*, 2013; *Reuß et al.*, 2019; *J. Rosenberg et al.*, 2018; *Wicke et al.*, 2019). PdxST can be translated from two different transcripts. The first transcript, *dacA-pdxS-pdxT-serS* can be processed upstream of *dacA* by RNase Y and the second transcript includes *pdxS pdxT and serS* (*DeLoughery et al.*, 2018; *Nicolas et al.*, 2012). In general, *pdxST* is already highly expressed under most growth conditions as the transcript amounts are nearly equal to highly active P43 promoter (*P. Z. Wang & Doi*, 1984; *Zhu & Stülke*, 2018). Under sporulation-favored conditions, the expression of *pdxS* is lower compared to the constitutive P43 promoter, which can be explained by a regulation of Spo0A. Spo0A regulates sporulation and also represses *pdxS* transcription (*Molle, Fujita, et al.*, 2003; *Zhu & Stülke*, 2018). *dacA* and *serS* are also overproduced by the amplification and a toxic effect based on the accumulation of these operon members can be excluded. Otherwise, such toxic effects could also be resolved by one of the other genes, which lie in the amplified region and are also expressed at a higher

degree (see below). Furthermore, the evolvability of the promoter itself could be limited by its sequence and a combination of multiple mutations could be needed to enhance the activity of the promoter (*Richts et al.*, 2021). Following this, the quantity of free promoter regions could also be a limiting factor, if the amount of RNA polymerase exceeds the free binding sites on the DNA and a fast expression is blocked. In contrast to promoter up mutations, amplifications could thereby free more promoter sites for the RNA polymerase.

Since an evolutionary approach to promote mutations in the *pdxS* gene is not possible under the tested conditions, a directed mutagenesis of the *pdxS* gene seems to be a reasonable possibility. We constructed a PLP-sensitive screening system, which can monitor differences in the PLP levels of the cells and to detect *pdxS* variants with higher activity (see Figure 25). We did not finish the mutagenesis and screening process as we questioned the ability of *B. subtilis* to evolve the *pdxS* gene. It was not possible to force mutations regarding the *pdxS* gene or its promoter, although amplifications are energy costly and often accomplished by further mutations in the relevant gene (*Andersson & Hughes*, 2009; *Sonti & Roth*, 1989; *Straus*, 1975, 1976). Besides that, structural analysis of the PdxS protein revealed that targeted amino acid exchanges in the PdxS protein only at 1 of 14 sites lead to an increase of the initial synthesis speed of PLP (*Smith et al.*, 2015). Although these changes were biased as they were made to check for synthesis relevant sites in the active center of PdxS, this shows that a beneficial amino acid exchange in the active center is not likely to evolve. Interestingly, the *S. cerevisiae* PdxS homologs were shown to form only homohexamers instead of a homododecamers, due to a sequence insertion. Especially a single lysine residue could be identified as important for the formation of the hexameric structure. The residue is only found in the yeast and when it was integrated into the *B. subtilis* PdxS, it also formed a hexameric complex. Moreover, it was shown that this hexameric PLP synthase complex has a 4-fold higher activity than the bacterial counterpart (*Neuwirth et al.*, 2009). The advantage of having a dodecameric PdxS protein over the hexameric complex still needs to be elucidated.

Apart from that, the activity of PdxS was tested together with PdxT and our evolutionary approach aimed to find PdxS variants, which produce PLP independent of PdxT and in the presence of low amounts of ammonium. Therefore, suppressor mutants could on the one hand indeed increase PdxS activity to form PLP but on the other hand also enhance the channeling of ammonium to the active center of PdxS. In the full enzyme complex PdxT cleaves off ammonia from glutamine and channels it through a hydrophobic pore to the active site of PdxS, where it interacts with ribose-5-phosphate (*M. Strohmeier et al.*, 2006). In *B. subtilis* cells, nitrogen is mostly present as ammonium (NH_4^+). Since the pH in the cells is about 7.5, 2% of the nitrogen is available as hydrophobic ammonia (*Clément & Merlin*, 1995; *Detsch & Stülke*, 2003; *Padan et al.*, 2005). Amino acid changes in the PdxS

protein could thereby increase the ammonium/ammonia flux to the active center. As this potentially requires complex structural changes, an increase in PdxS concentrations seems to be an easier way to overcome the problems of ammonium channeling to the active center and increasing the enzymatic activity.

This could also provide an explanation why the PdxT protein exists, although it is not necessary for the PLP synthesis. Under limiting nitrogen concentrations, the ability of PdxS to shuttle ammonia to its active site is limited and the PLP synthesis capacity cannot compete with the production of the PdxST complex. When comparing the genetic background of bacteria using the DXP-independent pathway, it striking that the *pdxS* and the *pdxT* gene almost always occur together in the genome. Only few exceptions can be found, in which only the *pdxS* gene is present as for *Natranaerobius thermophilus*, *Clostridium novyi NT* or *Anaerococcus hydrogenalis* DSM 7454 (www.microbesonline.org). When comparing entries of *pdxS* and *pdxT* in the Database of Clusters of Orthologous Genes (Cogs), 461 of 1309 organisms are listed, which carry the *pdxT* gene and 420 of 1309 with the *pdxS* gene (*Galperin et al.*, 2021). Hence, PdxS variants exist, which seem to function independent of PdxT either by having an improved ammonium utilization or being supplied with ammonium by different glutaminases (*G. Brown et al.*, 2008). Furthermore, organisms, which live in an ammonium-rich environment could also be independent of PdxT, as we were able to see that the emergence of suppressor mutants highly depends on the pH value of the medium (data not shown). When the pH of the medium was 6.5 suppressors formed but at a pH of 7.5 the *pdxT* mutant grew like the wild type on minimal medium plates and low ammonium concentrations. At this pH the equilibrium shifts more towards ammonia, which can diffuse through the membrane and does not need an uptake system (*Clément & Merlin*, 1995; *Detsch & Stülke*, 2003). Nevertheless, it might be interesting to study the PdxS variants of these organisms to shed more light onto the relation between PdxS and PdxT and of how PLP synthesis can be enhanced in absence of PdxT. This might be also interesting for the design of a minimal organism (see below).

Moreover, also organisms exist, which carry multiple copies of the *pdxS* gene as *Syntrophobotulus glycolicus DSM 8271*. This could either lead to higher PLP synthesis but also to the emergence of a proteins with different functions. Plants have multiple PdxS homologs, which are also responsible for thiamine production (*Paxhia & Downs*, 2019).

Additional mutations in the *pdxT* suppressor mutants

In addition to the amplification of the *pdxS* gene, we identified mutations in the nitrogen assimilation genes *tnrA* and *glnA* (see Figure 20). TnrA is a master regulator of nitrogen metabolism and involved in the regulation of at least 30 genes (*Mirouze et al.*, 2015; *Wray et al.*, 1996; *Yoshida et al.*, 2003). To

establish the functional regulatory network, binding of GlnA and TnrA is crucial (*Gunka & Commichau*, 2012; *Wray et al.*, 1996). The C-terminus of TnrA binds to a cleft in the feedback inhibited GlnA. Thereby, DNA-binding capacity of TnrA alters and expression behavior changes. The mutations regarding GlnA were located in close proximity to the TnrA binding sites (see Figure 23D). Furthermore, the mutation in the *tnrA* gene led to a truncation of the C-terminus of TnrA. Both types of mutation inhibit binding of TnrA to GlnA and thus foster binding of TnrA to NrgB at the membrane. This activates TnrA and promotes transcription of the *nrgAB* promoter. In the wild type, TnrA is bound to NrgB only under ammonium limitation, which promotes the expression of *nrgA* (*Fedorova et al.*, 2013; *Kayumov et al.*, 2011). The promoter showed 100-fold higher activity for the TrnA truncation, the GlnA G79A C1016A and the GlnA G79A mutations and even a 175 times higher activity with the GlnA C180A mutation. These mutations yielded in a growth advantage in the Δ*pdxT* mutant, if the ammonium concentrations did not exceed 30 mM and were even detrimental if the 120 mM ammonium was supplemented or *pdxS* was overexpressed. An increase of ammonium uptake in *pdxS* overexpression mutants did only have a positive effect, if the available ammonium was lower equal 10 mM. Since the regulation of nitrogen assimilation is disturbed in these mutants, uncontrolled expression of the ammonium channel in combination with high ammonium in the medium, led to an accumulation of ammonium in the cells. This was also supported by the fact that the suppressor mutants had defects growing on complex medium (data not shown). Under normal conditions *B. subtilis* can tolerate high amounts of ammonium in the medium without growth disadvantages, but due to the deregulation of the nitrogen network, this harbors a detrimental effect (*Leejeerajumnean et al.*, 2000). Since TnrA cannot be in a GlnA-bound state, its own expression becomes constitutive. The same is true for *glnRA.* By that not only the glutamine synthesis is not controlled anymore on gene level; the GOGAT cannot be expressed in the presence of TnrA and by that the link between carbon- and nitrogen metabolism is disturbed (*Belitsky et al.*, 2000). The amplification of *pdxS* was shown to be the main suppression mechanism and deregulation of the ammonium uptake occurred only in few suppressor mutants and always together with the amplification. Therefore, the mutations upregulating the ammonium uptake could be a first mechanism to quickly boost the available amounts of ammonium for PdxS. The cultivation medium contains glutamine, which inhibits *tnrA* expression and leads to limited NrgA formation. The more the glutamine is catabolized, the more TnrA is produced and by that, ammonium channel is expressed. The low amounts of TnrA cannot be blocked by binding to GlnA in the mutants and thus, ammonium uptake can be increased more rapidly. Since ammonium probably can only hardly reach the active center of PdxS and it could come to a block of the active centers and thereby to a congestion of ammonium (see above). When the glutamine is fully catabolized in the *pdxT* mutant, PdxS levels could be the limiting factor for the PLP formation, which generates the evolutionary

pressure for the amplification. Furthermore, we tested the influence of the GlnA/TnrA mutations only in an artificial context not considering the other genes within the amplified region. These genes could have an effect on growth in the suppressors, which is not considered in the experiment.

Ammonia is thought to be the limiting substrate in PLP production as R5P and G3P are produced in high amounts during glycolysis and pentose phosphate pathway (*Smith et al.*, 2015). Thus, it is surprising that increasing concentrations of ammonium lower the turnover speed of PdxS and PdxST to form PLP (see Figure 30). To assess the inhibitory potential of ammonium on the PLP synthesis, more experiments must be done.

The amplified region containing *pdxS* does not contain any other gene, which can be directly related to vitamin B6 metabolism and we were able to show that overexpression of the *pdxS* gene in the Δ*pdxT* mutant re-establishes genetic stability and wild type-like growth behavior in minimal medium containing even moderate amounts of ammonium (see Figure 27, Figure 28). Several of the co-amplified genes affect DNA information processing as *guaB* (biosynthesis of GMP), *dck* (purine salvage)*, dgk* (purine salvage), *yaaJ* (tRNA modification)*, scr* (RNA-driven elongation arrest), *dnaX* (replisome)*, recR* (RecA mediated DNA repair) (*Zhu & Stülke*, 2018). By changing the copy numbers of these genes, de-regulation of these nucleotide involving processes can occur, probably harming the cells. In addition, also *bofA,* coding for an inhibitor of the sigma factor SigK, lies within the region. SigK is mainly responsible for the regulation of sporulation-specific genes in the mother cell and increased inhibition of SigK leads to a loss of sporulation (*Losick & Shapiro*, 1993; *Sun et al.*, 2021).

Besides the amplification, only two genes harbored mutations among multiple suppressors, rendering them as relevant. The deletion and downregulation of *rpoE* in two of the suppressor mutants could not be related with an increase of PLP production. Deletion of *rpoE* in the *pdxT* mutant does not show any growth advantage and even a detrimental effect under high ammonium conditions (see Suppl. Figure 4). The *rpoE* gene codes for the delta subunit of the RNA polymerase and is present in *B. subtilis* and some other Gram-positive bacteria at least at the same level or higher as the other components of the RNA polymerase (RNAP). The amount of RpoE increases when reaching the stationary phase (*Doherty et al.*, 2010; *López de Saro et al.*, 1995). Deletion of *rpoE* gene leads to an altered cell morphology and longer lag phases. RpoE is able to destabilize the binding of RNAP with weak or cryptic promoters. Thereby, deletion can upregulate such genes, which otherwise would not be expressed. By that, growth can be improved when exposed to stress (*López de Saro et al.*, 1999; *Xue et al.*, 2010, 2012). Furthermore, RpoE is involved in the recycling of RNAP and helps removing the sigma factors from the holoenzyme, and DNA and RNA from binary complexes. On that score, it is important to maintain constant RNAP levels in the cell (*Keller et al.*, 2014; *Williamson & Doi*, 1978). We therefore suggest, that lowering the RpoE levels in the cells counteracts the amplification of the other

genes. As amplifications are not stable and energy costly, lowering the overall amount of transcription could overcome detrimental effects of the amplification (*Andersson & Hughes*, 2009; *Reams et al.*, 2010). Besides that, also toxic accumulation of the other co-amplified proteins could be rescued by lowering the RNAP efficiency.

The second gene which was mutated in two suppressors is *pgpH* coding for the cyclic-di-AMP (c-di-AMP)-specific phosphodiesterase PgpH (*Zhu & Stülke*, 2018). Together with GdpP, PgpH catalyzes the degradation of the second messenger c-di-AMP, which takes part mainly in potassium- and osmo-homeostasis but could also recently be related with the stringent response and glutamate metabolism (*Gundlach et al.*, 2016; *Krüger et al.*, 2021a, 2021b; *Peterson et al.*, 2020). Thus, we tested the growth of the *pdxT* mutant, carrying either a deletion of *pgpH* or *gdpP* in addition (see Suppl. Figure 4). We observed that the deletion of *pgpH* leads to a growth advantage in medium containing low amounts of ammonium when the *pdxS* gene dosage were at wild type levels. This effect vanishes if *pdxS* was overexpressed or the ammonium concentration was high. When *pgpH* was overexpressed in the *pdxT* mutant, the opposite effect could be observed, indicating a functional relation between PgpH and vitamin B6 metabolism. This effect could not be observed for the second phosphodiesterase GdpP, which can be because PgpP is supposed to be the main phosphodiesterase and it is more abundant than GdpP (*Commichau et al.*, 2019). In *L. monocytogenes* deletion of *pgpH* increases the intracellular concentration of the alarmone guanosin-3',5'-bispyrophosphat ((p)ppGpp) (*S. Liu et al.*, 2006). The second messenger (p)ppGpp promotes entering the stringent response, in which growth promoting processes are stopped and important metabolites are synthesized. This cellular program is for example executed in situations of amino acid starvation (*Cashel & Gallant*, 1969; *K. Liu et al.*, 2015). Furthermore, c-di-AMP is directly related with (p)ppGpp signaling as it was recently shown (*Krüger et al.,* 2021a; *Peterson et al.*, 2020). When the c-di-AMP levels are low (p)ppGpp acquisition is stimulated and the stringent response is activated. Furthermore, (p)ppGpp binds to both phosphodiesterases and inhibits c-di-AMP degradation (*Corrigan et al.*, 2015; *Huynh et al.*, 2015; *Rao et al.*, 2010). Our suppressors emerged on minimal medium with 5 mM potassium. Under this condition the bacteria form high amounts of c-di-AMP (*Gundlach et al.*, 2017). Thus, (p)ppGpp synthesis is not further stimulated and the stringent response intensity is lowered. Since PgpH and GdpP are not blocked by (p)ppGpp, c-di-AMP is degraded. A detailed model of the connection between c-di-AMP and (p)ppGpp signaling was described in *Krüger et al.* (2021). The deletion of *pgpH* in the suppressors could thereby maintain high c-di-AMP levels in the cells. As c-di-AMP could be also related with nitrogen metabolism recently, elevated c-di-AMP concentrations could thereby also lead to an increases ammonium pool for supplying PdxS (*Krüger, et al.*, 2021a). In addition, one suppressor harbored a mutation in the cation-proton antiporter *nhaK* gene, which could be identified as a potassium exporter (*Krüger et al.*,

2021a). The described mutation decreased potassium export and maintained internal high potassium concentrations. The mutation in the *pdxT* mutant suppressor is in close proximity to the mutation, which was already described, indicating that potassium export could be also lowered in the mutant. This would also keep the c-di-AMP pool high. How exactly the elevated c-di-AMP pool supports PLP synthesis by PdxS needs to be further investigated but as the deletion of *pgpH* was the only mutation in one suppressor to overcome the loss of PdxT, a relation between c-di-AMP signaling and PLP synthesis by PdxS cannot be excluded.

The role of vitamin B6 in biofilm formation

Some of the *pdxT* suppressor mutants showed complex colony formation after longer incubation time. We therefore checked the ability of the parental strain and the suppressors to form complex colonies. Interestingly, first attempts to analyze complex colony formation on Msgg agar plates (see *Diego Romero et al.*, 2010) failed, since the strains showed no growth. A closer look at the *B. subtilis* expression data showed that *pdxS* is only weakly expressed in Msgg medium (*Zhu & Stülke*, 2018). Therefore, we induced biofilm formation by adding manganese and glycerol to the MSSM medium plates and added PL. The *B. subtilis* wild type strain SP1 shows complex colony formation in contrast to the laboratory wild type strain 168. This strain carries mutations in the genes *rapP, sfp, epsC, swrA, and degQ* and is therefore not able to form biofilms (*McLoon et al.*, 2011). The SP1 strain was genome sequenced and compared to the 168 sequence (*Richts et al.*, 2020). Although 25 genome modifications could be identified, none affects the genes mentioned above or can be directly related with biofilm formation. One mutation in the intergenic region of the penicillin binding protein A *pbpA* was found though, which was shown to be important for biofilm formation (*Kumar et al.*, 2012; *Miyamoto et al.*, 2020). In the future it might be interesting to test, which of the genetic differences can complement the diminished biofilm formation of the strain 168, as none of the genes described by *McLoon et al* is altered in the SP1 strain.

The *pdxT* mutant showed abolished complex colony formation, which could be rescued alone by overexpression of *pdxS*. As PL is supplemented to the medium in sufficient amounts, the vitamin B6 concentration cannot be solely responsible for the lack of biofilms in the *pdxT* mutant, indicating that the PdxST complex is somehow directly involved in the formation of biofilms. The EpsN UDP-2,6-dideoxy 2-acetamido 4-keto glucose aminotransferase and the SpeA arginine decarboxylase, are both PLP dependent proteins and involved in biofilm formation. Thus, depletion of PLP or deletion of *espN* or *speA* abolishes complex colony formation (*Burrell et al.*, 2010; *Kaundinya et al.*, 2018). It was shown though that strains with too high PL levels have a deficient effect on biofilm formation in *Streptococcus mutans* (*Liao et al.*, 2015). Recovery of biofilm formation was given, if the suppressors harbored the

amplification of the *pdxS* genetic region. It could be that PLP needs to be transported to the target proteins via PdxST. As discussed below, PLP was found to be bound at the PdxST complex and directly provided to the PLP-dependent proteins. By deletion of PdxT, the holding site for PLP could be damaged and the delivery to the targets interrupted. Since overexpression alone, restores biofilm formation, a positive effect of one of the other amplified genes can be excluded. Only three suppressors did not form a biofilm of which two (BP1117 and BP1184) carried no amplification of *pdxS*. BP1116 was the only suppressor with an amplification of *pdxS* and no complex colony formation. This can be explained by an additional mutation in the *ispD* gene, which is responsible for isoprenoid synthesis. A relation between isoprenoid synthesis and biofilm formation could be shown in *Thermotoga maritima* (*Pysz et al.*, 2004).

4.3 Vitamin B6 transport and detoxification

Until now only few vitamin B6 transport mechanisms could be identified. Examples are Pup1 in *Arabidopsis thaliana* or Tpn1p in the yeast *S. cerevisieae* (*Stolz & Vielreicher*, 2003; *Szydlowski et al.*, 2013). Furthermore, the ECF transporter system was related as a PN transporter (*Slotboom*, 2014; *T. Wang et al.*, 2015). We searched for Tpn1p similar proteins in *B. subtilis* and identified 6 candidate genes, belonging to the same group of nucleobase:cation symporters. We deleted these genes and the genes for the ECF transporter in *B. subtilis* in the wild type and in a vitamin B6 auxotrophic strain. Next, the mutants were tested for PL-dependent growth phenotypes and export capabilities. Deletion of all of the transporter candidates was possible in the auxotrophic *pdxST* mutant, indicating that none of the genes is solely responsible for vitamin B6 uptake (see Figure 32A). We also quantified growth of the single mutants and the *pdxST* double mutants but could not identify differences in between the strains. We also checked for export of PL but all mutants are still able to excrete PL into the medium (see Figure 32C). We conducted the experiment only in a qualitative fashion and could not determine differences in the exact amount of secreted PL between the different mutants and control strains. Recently, it could be shown, that a *yggS* mutant of *E. coli* secretes phosphorylated PL into the medium (*Vu et al.*, 2020). The fact that phosphorylated vitamers can be exported, indicates that im- and export are probably separated and conducted by different transporters, as growth of microorganisms could only be described with the unphosphorylated vitamers yet. One exception is *Salmonella enterica*. This organism harbors a periplasmic vitamin B6 phosphatases PhoN, dephosphorylating PLP in the medium to PL and thereby allowing the uptake (*Vu & Downs*, 2020).

Furthermore, we tried to evolve auxotrophic *B. subtilis* mutants under vitamin B6-limiting conditions but no suppressor mutants evolved, independent of the used vitamer. Moreover,

cultivation of *B. subtilis* with anti-vitamins did also not yield in mutations. The tested compounds ginkgotoxin and 4'-deoxypyridoxal have a similar structure with the vitamers and inhibit the pyridoxal kinase PdxK by competitive inhibition. Thereby, phosphorylation of imported vitamers is prevented (*Leistner & Drewke*, 2010; *Scott & Hockney*, 1979; *Woolley*, 1963). Due to the structural similarity, it is likely, that anti-vitamins and vitamers are taken up by the same transport system. Thus, we suggested that mutations in the transport system could emerge, preventing the toxins from import or changing the uptake specificity towards the vitamers. We used a doubled gradient approach, in which vitamin and anti-vitamin were added to the opposite poles of an agar plate. Thus, bacteria were exposed to crossing concentration gradients of vitamer and toxin. By that, we hoped to find a sweet spot where vitamers are taken up to promote growth and sufficient toxin is imported to establish an evolutionary pressure. Nevertheless, no mutations could be observed, probably due to contamination of the anti-vitamins with portions of vitamin B6, as we identified in the ginkgotoxin. Hence, the toxic effect of the anti-vitamins could be recovered by the presence of high amounts of vitamers, which remove the toxin from the active site of PdxK (*Wada & Haga*, 1997).

Since evolution under limiting vitamin B6 conditions was not successful, we cultivated bacteria under toxic PL amounts, as it was done before (http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B). We evolved *E. coli* and *B. subtilis* wild type derivatives under two selective conditions on solid medium plates (see Figure 34). For *E. coli* we were not able to isolate suppressors, as evolution at 2 mM PL concentration resulted in a bacterial lawn and at 4 mM the cell did not grow at all. However, for *B. subtilis* we isolated two suppressor mutants on the 4 mM plate, which showed an improved growth behavior compared to the parental strain and sequenced their genomes. The first suppressor harbored only one mutation in the *arsR* gene, truncating the protein by 58 aa and substituting the last 33 C-terminal aa. Thus, the ArsR regulator is likely to have lost its functionality. ArsR is a repressor of an operon which contains the *yqcK* gene of unknown function, the *arsB* arsenate exporter gene and the *arsC* arsenate reductase gene (see Table 18). YqcK shares a 64.7% identity with FosB from *Bacillus licheniformis*, which is a metallothiol transferase involved in fosfomycin resistance (*Zhu & Stülke*, 2018). Interestingly, a relation between vitamin B6 production in a heterologous pathway and the presence of the LMW thiol bacillithiol could be identified (*J. Rosenberg et al.*, 2018). As FosB requires bacillithiol as a thiol donor to confer fosfomycin resistance, more and more clues come up indicating that the LMW thiol and vitamin B6 are related. Anyways, a relation of *yqcK* and PLP detoxification needs to be confirmed experimentally. In addition, ArsB is an exporter of arsenate. As arsenate and PL do not share structural similarities, it is unlikely that PL is exported by ArsB. The last enzyme in the operon is ArsC, which catalyzes the reduction of arsenate to arsenite.

Enzymes using phosphoric monoesters as prosthetic groups can also use the unphosphorylated derivatives when arsenate is present (*Lagunas & Solis*, 1968). In contrast to phosphate, arsenate spontaneously form esters, as the central atom offers better access to a fifth ligand. It could be shown that arsenate and pyridoxal together form pyridoxal-arsenate and can be used by aspartate aminotransferase instead of PLP (*Ali & Dixon*, 1992). Since PL levels are high and enzymes can use PL in presence of arsenate, enzyme activity is independent on phosphorylation of PL. Furthermore, arsenate and PL form a covalent bound and thereby produce a similar structural property as in PLP. Thus, it should also have the same reactivity and toxicity. The deletion of ArsR results in higher expression of the arsenate detoxification system, removing arsenate from the cells. Although this would lower the arsenate levels in the cells and prevent unintended reactions of PL-arsenate, the cellular amounts of arsenate should be rather low. It is not supplemented in the growth medium and should not be present in the water used for the medium as it was deionized before.

The second suppressor harbored mutations regarding the *yqeT* and the *mutS2* gene, coding for a putative ribosomal protein methyltransferase and a putative DNA repair protein, respectively. In addition, a SNP was present in the putative -35 region of an operon coding for *yqxD*, *dnaG* and *sigA* and thus probably deregulating the operon. The function of *yqxD* is unknown and *dnaG* codes for the DNA primase. The sigma factor A is also deregulated. As it controls the expression of over 1000 genes, this makes an assumption about the detoxification mechanism impossible. In former studies biotin and CAA could be identified as a detoxifying agent, when the cells are exposed to high amounts of PL (http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B).

To find out more about the transport of vitamin B6, more experiments need to be done. To find new candidate genes, further bioinformatical studies could help, which for example could consider comparison to genome reduced strains as *M. pneumoniae* or the synthetic organism Syn3A. These organisms do not possess a vitamin B6 synthesis system and need to take up vitamin B6 from the surrounding. Comparing known transporter proteins of these organism with the *B. subtilis* transporters, could identify new candidates for vitamin B6 uptake. On the contrary, for *M. pneumoniae* only two proteins are annotated as PLP-dependent according to UniProt, the serine hydroxymethyltransferase GlyA and the cysteine sulfinate desulfinase CsdA. The functions of these proteins are predicted based on homology to homologous proteins and it is not confirmed experimentally. Since *M. pneumoniae* is a pathogen and draws its nutrients from the surrounding rather than producing them, it could be that these proteins function without PLP and PLP-dependent proteins do not exist or that the PLP-dependent reaction of the proteins is not relevant und import of PLP is not necessary anymore. Most reactions catalyzed by PLP belong to the amino acid biosynthesis or other metabolic processes (*Richts et al.*, 2019). As these are not needed in the pathogen, the dependency of PLP is highly reduced. Thus

To find out more about the shuttling of PLP to the target proteins, we crosslinked PdxST in *vivo* to find possible interaction partners (See Figure 31). Although prominent bands occurred in the SDS PAGE of the elution samples, only weak signals were detected in the MS screen for AhpA and AcpA. For AcpA no interaction could be observed and AhpA only showed a weak interaction for the C-terminally tagged sample and only if PdxS and PdxT were present. The PdxST complex also showed slight interaction with the Zip control, indicating an unspecific interaction. Thus, the interaction of AhpA and PdxST needs to be proven by different experiments. AhpA is an alkyl hydroperoxide reductase and acts in protection against peroxide stress. Interestingly, it possesses a thioredoxin domain.

Although we could not identify direct interaction partners of the PdxST complex, shuttling mechanisms of PLP to the targets are probably present in *B. subtilis*. It was thought that produced vitamin B6 is bound at the PdxST through in Schiff base and is thereby in a steady-state. When a PLP-dependent protein is in close proximity, PLP leaves the PdxST complex and interacts with the PLP-dependent protein (*Fitzpatrick et al.*, 2010). This could be for example shown for the aspartate aminotransferase AspB and such carrier proteins could explain the low intracellular PLP concentrations (*Fu et al.*, 2001; *Moccand et al.*, 2011). A similar mechanism was confirmed for *E. coli*. In PdxH and PdxK synthesized PLP is bound and carried to the apoenzymes (*Barile et al.*, 2019; *Ghatge et al.*, 2012, 2016). Furthermore, Yggs was identified as a PLP binding protein without enzymatic activity and it was suggested that it is a PLP carrier protein, involved in the control of the B6 salvage (*Ito et al.*, 2013, 2020). Moreover, of the approximately 188,000 vitamin B6 molecules in *E. coli* PNP and PMP together have an amount of 51% and PLP of 42%. Furthermore, 60% of the PLP is estimated to be bound by proteins (*Fu et al.*, 2001). This indicates that the amount free PLP is kept low in the cells to prevent toxic reactions. Since the DXP-dependent pathway is catalyzed by multiple enzymes, the pathway can be regulated at several steps during synthesis of PLP to prevent further toxic accumulation of vitamin B6 (*Tramonti et al.*, 2021). The synthesis of PLP via PdxST is not controlled on gene level (*Belitsky, 2014*) and thus it is likely that the synthesis of PLP is controlled by feed-back inhibition. The fact, that PLP can be hold by the complex until it is needed by PLP-dependent enzymes, supports this hypothesis but detailed enzyme kinetics still remain to be determined. Moreover, the slower synthesis speed of the PdxST complex, compared to the DXP-dependent pathway could be another possibility to overcome toxic PLP accumulation without the need of regulation.

4.4 *MiniBacillus*

The *MiniBacillus* project aims at creating a minimal *B. subtilis* strain, which only contains genes with assigned and necessary functions (*Reuß et al.*, 2016). On the one hand, this project sheds light on the question: "what is the requirement for life?" but on the other hand a genome-reduced organism is constructed, which can be the starting point for novel biotechnological production pathways (*Van Tilburg et al.*, 2020). In former studies a genome reduction of 40.51% was achieved in the PG39 strain (http://hdl.handle.net/11858/00-1735-0000-002E-E5F8-F). All of our attempts to further reduce the genome of the PG39 strain were not successful and we reached another dead end in the deletion progress. Thus, we decided evolve the reduced strain to enhance its general fitness in the cultivation medium. We conducted the evolutionary approach for the PG10 strain (34.6% reduction) and the PG39 strain and were able to isolate mutants with enhanced growth ability after six weeks of cultivation (see Figure 35). Genome sequencing revealed, that the PG10 strain contained 25 mutations compared to the parental strain, which can be assigned to different cellular processes, as lifestyle formation, information processing or carbon- and nitrogen metabolism. This indicates, that a genome-reduced strain can be optimized by evolution and further improved. Interestingly, the PG10 strain harbored a mutation in the promoter of the *mhqNOP* operon, which was shown to be highly upregulated in PG10 due to a deletion of its regulator MhqR (*Reuß et al.*, 2017). The mutation led to a downregulation of the promoter. This confirms the design of further deletion strains because the operon was deleted in a following strain as it did not fulfill a relevant function and the high expression resulted in a waste of energy (*Reuß et al.*, 2017). In contrast to PG10, PG39 carried only mutations in four genes, *spoIIE*, *cysE*, *desK* and *yuxN* (see Table 19). When comparing the growth of the parental strain with the evolved strain, the latter produced more biomass and maintained its biomass in the stationary phase in contrast to the parental strain (see Figure 35). As YuxN controls Spx stability, the truncation of YuxN probably alters the availability of Spx. Spx is a regulator involved in the oxidative stress response (see above). Thus, this could be an indication that the PG39 strains suffers from oxidative stress. Moreover, a tandem repeat was found in the cysteine biosynthesis gene *cysE*. A similar mechanism was identified in the glutamate dehydrogenase GudB (*B R Belitsky & Sonenshein*, 1998; *Gunka & Commichau*, 2012). Under normal growth conditions the main glutamate dehydrogenase is RocG active and GudB is inactive due to a tandem repeat in the *gudB* gene. When *rocG* is deleted, the *gudB* gene is decryptified and the active glutamate dehydrogenase GudB can fully replace RocG. Thus, the insertion of the tandem repeat in *cysE* could be a cryptification mechanism to either inhibit or promote its activity. CysE takes part in cysteine synthesis. As cysteine is an important LMW thiol, this again suggests an adaptation to oxidative stress. In the future it might be interesting to test the evolved PG39 strain for genetic competence. If the strain regained the ability to take up DNA, it could be used for proceeding

with the deletion process. Otherwise, an earlier strain could be used to generate a new linage in the deletion tree of *MiniBacillus*.

5. References

Acevedo-Rocha, C. G., Gronenberg, L. S., Mack, M., Commichau, F. M., & Genee, H. J. (2019). Microbial cell factories for the sustainable manufacturing of B vitamins. *Current Opinion in Biotechnology*, *56*, 18–29.

Agranat, I. (2009). Pharmaceutical substances: syntheses, patents and applications of the most relevant AIPs. In *Synthesis* (5th ed., Vol. 2009, Issue 12). Stuttgart: Thieme. https://doi.org/10.1055/s-0029-1216864

Al-Zyoud, W. A., Hynson, R. M. G., Ganuelas, L. A., Coster, A. C. F., Duff, A. P., Baker, M. A. B., Stewart, A. G., Giannoulatou, E., Ho, J. W. K., Gaus, K., Liu, D., Lee, L. K., & Böcking, T. (2016). Binding of transcription factor GabR to DNA requires recognition of DNA shape at a location distinct from its cognate binding site. *Nucleic Acids Research*, *44*(3), 1411–1420.

Albertini, A. M., Hofer, M., Calos, M. P., & Miller, J. H. (1982). On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. *Cell*, *29*(2), 319–328.

Ali, B. R. S., & Dixon, H. B. F. (1992). Pyridoxal arsenate as a prosthetic group for aspartate aminotransferase. *Biochemical Journal*, *284*(2), 349–352.

Alonso, J. C., Stiege, A. C., & Lüder, G. (1993). Genetic recombination in *Bacillus subtilis* 168: effect of *recN*, *recF*, *recH* and *addAB* mutations on DNA repair and recombination. *Molecular & General Genetics : MGG*, *239*(1–2), 129–136.

Amidani, D., Tramonti, A., Canosa, A. V., Campanini, B., Maggi, S., Milano, T., di Salvo, M. L., Pascarella, S., Contestabile, R., Bettati, S., & Rivetti, C. (2017). Study of DNA binding and bending by *Bacillus subtilis* GabR, a PLP-dependent transcription factor. *Biochimica et Biophysica Acta - General Subjects*, *1861*(1), 3474–3489.

Anderson, P., & Roth, J. (1981). Spontaneous tandem genetic duplications in *Salmonella typhimurium* arise by unequal recombination between rRNA (*rrn*) cistrons. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(5), 3113–3117.

Andersson, D. I., & Hughes, D. (2009). Gene amplification and adaptive evolution in bacteria. *Annual Review of Genetics*, *43*, 167–195.

Antelmann, H., Engelmann, S., Schmid, R., Sorokin, A., Lapidus, A., & Hecker, M. (1997). Expression of a stress- and starvation-induced *dps*/*pexB*-homologous gene is controlled by the alternative sigma factor *σ*(B) in *Bacillus subtilis*. *Journal of Bacteriology*, *179*(23), 7251–7256.

Antelmann, H., & Helmann, J. D. (2011). Thiol-based redox switches and gene regulation. *Antioxidants & Redox Signaling*, *14*(6), 1049–1063.

Antelmann, H., Scharf, C., & Hecker, M. (2000). Phosphate starvation-inducible proteins of *Bacillus subtilis*: Proteomics and transcriptional analysis. *Journal of Bacteriology*, *182*(16), 4478–4490.

Ari, R. D., & Casadesu, J. (1998). Underground metabolism. *BioEssays*, 181–186.

Arnold, D. A., & Kowalczykowski, S. C. (2000). Facilitated loading of RecA protein is essential to recombination by RecBCD enzyme. *The Journal of Biological Chemistry*, *275*(16), 12261–12265.

Atkinson, M. R., & Fisher, S. H. (1991). Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. *Journal of Bacteriology*, *173*(1), 23 LP – 27.

Ayora, S., Carrasco, B., Cárdenas, P. P., César, C. E., Cañas, C., Yadav, T., Marchisone, C., & Alonso, J. C. (2011). Double-strand break repair in bacteria: a view from *Bacillus subtilis*. *FEMS Microbiology Reviews*, *35*(6), 1055–1081.

Babitzke, P., Gollnick, P., & Yanofsky, C. (1992). The *mtrAB* operon of *Bacillus subtilis* encodes GTP cyclohydrolase I (MtrA), an enzyme involved in folic acid biosynthesis, and MtrB, a regulator of tryptophan biosynthesis. *Journal of Bacteriology*, *174*(7), 2059–2064.

Barb, A. W., Hekmatyar, S. K., Glushka, J. N., & Prestegard, J. H. (2013). Probing alanine transaminase catalysis with hyperpolarized 13CD3-pyruvate. *Journal of Magnetic Resonance (San Diego, Calif. : 1997)*, *228*, 59–65.

Barile, A., Tramonti, A., Di Salvo, M. L., Nogués, I., Nardella, C., Malatesta, F., & Contestabile, R. (2019). Allosteric feedback inhibition of pyridoxine 5'-phosphate oxidase from *Escherichia coli*. *Journal of Biological Chemistry*, *294*(43), 15593–15603.

Barrows, F. T., Gaylord, T. G., Sealey, W. M., Porter, L., & Smith, C. E. (2008). The effect of vitamin premix in extruded plant-based and fish meal based diets on growth efficiency and health of rainbow trout, Oncorhynchus mykiss. *Aquaculture*, *283*(1), 148–155.

Belitsky, B. R. (2001). Biosynthesis of amino acids of the glutamate and aspartate families, alanine, and polyamines. in *Bacillus subtilis and Its closest relatives* (pp. 203–231).

Belitsky, B. R. (2004a). *Bacillus subtilis* GabR, a protein with DNA-binding and aminotransferase domains, is a PLP-dependent transcriptional regulator. *Journal of Molecular Biology*, *340*(4), 655–664.

Belitsky, B. R. (2004b). Physical and enzymological interaction of *Bacillus subtilis* proteins required for *de novo* pyridoxal 5'-phosphate biosynthesis. *Journal of Bacteriology*, *186*(4), 1191–1196.

Belitsky, B. R. (2014). Role of PdxR in the activation of vitamin B6 biosynthesis in *Listeria monocytogenes*. *Molecular Microbiology*, *92*(5), 1113–1128.

Belitsky, B. R., Wray, L. V., Fisher, S. H., Bohannon, D. E. & Sonenshein, A. L. (2000). Role of TnrA in nitrogen source-dependent repression of *Bacillus subtilis* glutamate synthase gene expression. *mBio182*, (21), 5939–5947.

Belitsky, B. R., & Sonenshein, A. L. (1998). Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. *Journal of Bacteriology*, *180*(23), 6298–6305.

Belitsky, B. R., & Sonenshein, A. L. (2002). GabR, a member of a novel protein family, regulates the utilization of *γ*-aminobutyrate in *Bacillus subtilis*. *Molecular Microbiology*, *45*(2), 569–583.

Benda, M., Woelfel, S., Gunka, K., Klumpp, S., Poehlein, A., Kálalová, D., Šanderová, H., Daniel, R., Krásný, L., & Stülke, J. (2020). Quasi-essentiality of RNase Y in *Bacillus subtilis* is caused by its critical role in the control of mRNA homeostasis. *BioRxiv*, 2020.05.20.106237. https://doi.org/10.1101/2020.05.20.106237

Bergthorsson, U., Andersson, D. I., & Roth, J. R. (2007). Ohno's dilemma: evolution of new genes under continuous selection. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(43), 17004–17009.

Bi, X., & Liu, L. F. (1994). *recA*-independent and *recA*-dependent intramolecular plasmid recombination. Differential homology requirement and distance effect. *Journal of Molecular Biology*, *235*(2), 414–423.

Bilski, P., Li, M. Y., Ehrenshaft, M., Daub, M. E., & Chignell, C. F. (2007). Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochemistry and Photobiology*, *71*(2), 129–134.

Bohannon, D. E., & Sonenshein, A. L. (1989). Positive regulation of glutamate biosynthesis in *Bacillus subtilis*. *Journal of Bacteriology*, *171*(9), 4718–4727.

Boschi-Muller, S., Azza, S., Pollastro, D., Corbier, C., & Branlant, G. (1997). Comparative enzymatic properties of GapB-encoded erythrose-4-phosphate dehydrogenase of *Escherichia coli* and phosphorylating glyceraldehyde-3- phosphate dehydrogenase. *Journal of Biological Chemistry*, *272*(24), 15106–15112.

Boylan, J. A., Hummel, C. S., Benoit, S., Garcia-Lara, J., Treglown-Downey, J., Crane, E. J., & Gherardini, F. C. (2006). *Borrelia burgdorferi* bb0728 encodes a coenzyme A disulphide reductase whose function suggests a role in intracellular redox and the oxidative stress response. *Molecular Microbiology*, *59*(2), 475–486.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254.

Bramucci, E., Milano, T., & Pascarella, S. (2011). Genomic distribution and heterogeneity of MocR-like transcriptional factors containing a domain belonging to the superfamily of the pyridoxal-5'-phosphate dependent enzymes of fold type I. *Biochemical and Biophysical Research Communications*, *415*(1), 88–93.

Bretzel, W., Schurter, W., Ludwig, B., Kupfer, E., Doswald, S., Pfister, M., & van Loon, A. P. G. M. (1999). Commercial riboflavin production by recombinant *Bacillus subtilis*: down-stream processing and comparison of the composition of riboflavin produced by fermentation or chemical synthesis. *Journal of Industrial Microbiology and Biotechnology*, *22*(1), 19–26.

Breuer, M., Earnest, T. M., Merryman, C., Wise, K. S., Sun, L., Lynott, M. R., Hutchison, C. A., Smith, H. O., Lapek, J. D., Gonzalez, D. J., Crécy-Lagard, V. de, Haas, D., Hanson, A. D., Labhsetwar, P., Glass, J. I., & Luthey-Schulten, Z. (2019). Essential metabolism for a minimal cell. *ELife*, *8*, 1–75.

Brown, G. C., McBride, A. G., Fox, E. J., McNaught, K. S., & Borutaite, V. (1997). Nitric oxide and oxygen metabolism. *Biochemical Society Transactions*, *25*(3), 901–904.

Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimiak, A., Savchenko, A., & Yakunin, A. F. (2008). Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*. *Biochemistry*, *47*(21), 5724–5735.

Brown, S. W., & Sonenshein, A. L. (1996). Autogenous regulation of the *Bacillus subtilis glnRA* operon. *Journal of Bacteriology*, *178*(8), 2450–2454.

Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P., & Helmann, J. D. (1998). *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Molecular Microbiology*, *29*(1), 189–198.

Bull, H., Murray, P. G., Thomas, D., Fraser, A. M., & Nelson, P. N. (2002). Acid phosphatases. *Journal of Clinical Pathology - Molecular Pathology*, *55*(2), 65–72.

Burg, R. W., Rodwell, V. W., & Snell, E. E. (1960). Bacterial oxidation of vitamin B6. III. Metabolites of pyridoxamine. *The Journal of Biological Chemistry*, *235*(4), 1164–1169.

Burg, R. W., & Snell, E. E. (1969). The bacterial oxidation of vitamin b6: vi. pyridoxal dehydrogenase and 4-pyridoxolactonase. *Journal of Biological Chemistry*, *244*(10), 2585–2589.

Bürkle, L., Cedzich, A., Döpke, C., Stransky, H., Okumoto, S., Gillissen, B., Kühn, C., & Frommer, W. B. (2003). Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis*. *Plant Journal*, *34*(1), 13–26.

Burns, K. D., Pieper, P. A., Liu, H. W., & Stankovich, M. T. (1996). Studies of the redox properties of CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase (E1) and CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase reductase (E3): Two important enzymes involved in the biosynthesis of ascarylose. *Biochemistry*, *35*(24), 7879–7889.

Burns, K. E., Xiang, Y., Kinsland, C. L., McLafferty, F. W., & Begley, T. P. (2005). Reconstitution and biochemical characterization of a new pyridoxal-5'-phosphate biosynthetic pathway. *Journal of the American Chemical Society*, *127*(11), 3682–3683.

Burrell, M., Hanfrey, C. C., Murray, E. J., Stanley-Wall, N. R., & Michael, A. J. (2010). Evolution and multiplicity of arginine decarboxylases in polyamine biosynthesis and essential role in *Bacillus subtilis* biofilm formation. *Journal of Biological Chemistry*, *285*(50), 39224–39238.

Bzymek, M., & Lovett, S. T. (2001). Evidence for two mechanisms of palindrome-stimulated deletion in *Escherichia coli*: single-strand annealing and replication slipped mispairing. *Genetics*, *158*(2), 527–540.

Bzymek, M., Saveson, C. J., Feschenko, V. V, & Lovett, S. T. (1999). Slipped misalignment mechanisms of deletion formation: in *vivo* susceptibility to nucleases. *Journal of Bacteriology*, *181*(2), 477–482.

Callizot, N., & Poindron, P. (2008). Pyridoxine-induced peripheral neuropathy. In *Biovalley Monographs* (pp. 66–80).

Campbell, T. L., & Brown, E. D. (2008). Genetic interaction screens with ordered overexpression and deletion clone sets implicate the *Escherichia coli* GTPase YjeQ in late ribosome biogenesis. *Journal of Bacteriology*, *190*(7), 2537–2545.

Cane, D. E., Chow, C., Lillo, A., & Kang, I. (2001). Molecular cloning, expression and characterization of the first three genes in the mevalonate-independent isoprenoid pathway in *Streptomyces coelicolor*. *Bioorganic and Medicinal Chemistry*, *9*(6), 1467–1477.

Cane, D. E., Du, S., Robinson, J. K., Hsiung, Y., & Spenser, I. D. (1999). Biosynthesis of vitamin B6: Enzymatic conversion of 1-deoxy-D-xylulose- 5-phosphate to pyridoxol phosphate [14]. *Journal of the American Chemical Society*, *121*(33), 7722–7723.

Cao, Min., Bernat, B. A., Wang, Z., Armstrong, R. N., & Helmann, J. D. (2001). FosB, a cysteine-dependent fosfomycin resistance protein under the control of *σ*W, an extracytoplasmic-function *σ* factor in *Bacillus subtilis*. *Journal of Bacteriology*, *183*(7), 2380–2383.

Cao, Min, Wang, T., Ye, R., & Helmann, J. D. (2002). Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis σ*W and *σ*M regulons. *Molecular Microbiology*, *45*(5), 1267–1276.

Carrasco, B., Manfredi, C., Ayora, S., & Alonso, J. C. (2008). *Bacillus subtilis* SsbA and dATP regulate RecA nucleation onto single-stranded DNA. *DNA Repair*, *7*(6), 990–996.

Cashel, M., & Gallant, J. (1969). Two compounds implicated in the function of the RC gene of *Escherichia coli*. *Nature*, *221*(5183), 838–841.

Cena, H., & Calder, P. C. (2020). Defining a healthy diet: evidence for the role of contemporary dietary patterns in health and disease. *Nutrients*, *12*(2), 334.

Chandrangsu, P., Dusi, R., Hamilton, C. J., & Helmann, J. D. (2014). Methylglyoxal resistance in *Bacillus subtilis*: contributions of bacillithiol-dependent and independent pathways. *Molecular Microbiology*, *91*(4), 706–715.

Chandrangsu, P., Loi, V. Van, Antelmann, H., & Helmann, J. D. (2018). The role of bacillithiol in Gram-positive Firmicutes. *Antioxidants and Redox Signaling*, *28*(6), 445–462.

Chédin, F., Dervyn, E., Dervyn, R., Ehrlich, S. D., & Noirot, P. (1994). Frequency of deletion formation decreases exponentially with distance between short direct repeats. *Molecular Microbiology*, *12*(4), 561–569.

Chi, B. K., Gronau, K., Mäder, U., Hessling, B., Becher, D., & Antelmann, H. (2011). S-bacillithiolation protects against hypochlorite stress in *Bacillus subtilis* as revealed by transcriptomics and redox proteomics. *Molecular and Cellular Proteomics*, *10*(11), M111.009506. https://doi.org/10.1074/mcp.M111.009506

Chi, B. K., Roberts, A. A., Huyen, T. T. T., Bäsell, K., Becher, D., Albrecht, D., Hamilton, C. J., & Antelmann, H. (2013). S-bacillithiolation protects conserved and essential proteins against hypochlorite stress in Firmicutes bacteria. *Antioxidants and Redox Signaling*, *18*(11), 1273–1295.

Cho, K. J., Park, Y. H., Khan, T., Lee, J. H., Kim, S., Seok, J. H., Chung, Y. Bin, Cho, A. E., Choi, Y., Chang, T. S., & Kim, K. H. (2015). Crystal structure of dimeric human peroxiredoxin-1 C83S mutant. *Bulletin of the Korean Chemical Society*, *36*(5), 1543–1545.

Choi, S. Y., Reyes, D., Leelakriangsak, M., & Zuber, P. (2006). The global regulator Spx functions in the control of organosulfur metabolism in *Bacillus subtilis*. *Journal of Bacteriology*, *188*(16), 5741–5751.

Christen, P., & Mehta, P. K. (2001). From cofactor to enzymes. The molecular evolution of pyridoxal-s'-pliosphate-dependent enzymes. *Chemical Records*, *1*(6), 436–447.

Chu, W.-H. (2007). Optimization of extracellular alkaline protease production from species of *Bacillus*. *Journal of Industrial Microbiology & Biotechnology*, *34*(3), 241–245.

Clark, A. J. (1973). Recombination deficient mutants of *E. coli* and other bacteria. *Annual Review of Genetics*, *7*, 67–86.

Clément, B., & Merlin, G. (1995). The contribution of ammonia and alkalinity to landfill leachate toxicity to duckweed. *Science of the Total Environment*, *170*(1–2), 71–79.

Commichau, F. M., Alzinger, A., Sande, R., Bretzel, W., Meyer, F. M., Chevreux, B., Wyss, M., Hohmann, H.-P. P., & Prágai, Z. (2014). Overexpression of a non-native deoxyxylulose-dependent vitamin B6 pathway in *Bacillus subtilis* for the production of pyridoxine. *Metabolic Engineering*, *25*, 38–49.

Commichau, F. M., Alzinger, A., Sande, R., Bretzel, W., Reuß, D. R., Dormeyer, M., Chevreux, B., Schuldes, J., Daniel, R., Akeroyd, M., Wyss, M., Hohmann, H. P., & Prágai, Z. (2015). Engineering *Bacillus subtilis* for the conversion of the antimetabolite 4-hydroxy-l-threonine to pyridoxine. *Metabolic Engineering*, *29*, 196–207.

Commichau, F. M., Forchhammer, K., & Stülke, J. (2006). Regulatory links between carbon and nitrogen metabolism. *Current Opinion in Microbiology*, *9*(2), 167–172.

Commichau, F. M., Gunka, K., Landmann, J. J., & Stülke, J. (2008). Glutamate metabolism in *Bacillus subtilis*: Gene expression and enzyme activities evolved to avoid futile cycles and to allow rapid responses to perturbations of the system. *Journal of Bacteriology*, *190*(10), 3557–3564.

Commichau, F. M., Heidemann, J. L., Ficner, R., & Stülke, J. (2019). Making and breaking of an essential poison: the cyclases and phosphodiesterases that produce and degrade the essential second messenger cyclic di-AMP in bacteria. *Journal of Bacteriology*, *201*(1), e00462-18. https://doi.org/10.1128/JB.00462-18

Commichau, F. M., Herzberg, C., Tripal, P., Valerius, O., & Stülke, J. (2007). A regulatory protein-protein interaction governs glutamate biosynthesis in *Bacillus subtilis*: The glutamate dehydrogenase RocG moonlights in controlling the transcription factor GltC. *Molecular Microbiology*, *65*(3), 642–654.

Commichau, F. M., Pietack, N., & Stülke, J. (2013). Essential genes in *Bacillus subtilis*: A re-evaluation after ten years. *Molecular BioSystems*, *9*(6), 1068–1075.

Commichau, F. M., & Stülke, J. (2008). Trigger enzymes: bifunctional proteins active in metabolism and in controlling gene expression. *Molecular Microbiology*, *67*(4), 692–702.

Commichau, F. M., & Stülke, J. (2015). Trigger enzymes: Coordination of metabolism and virulence gene expression. *Metabolism and Bacterial Pathogenesis*, 105–127.

Cooper, T. F. (2010). Metabolism gets lucky. *Molecular Systems Biology*, *6*(1), 439. https://doi.org/https://doi.org/10.1038/msb.2010.94

Copley, S. D. (2012). Toward a systems biology perspective on enzyme evolution. *The Journal of Biological Chemistry*, *287*(1), 3–10.

Corrigan, R. M., Bowman, L., Willis, A. R., Kaever, V., & Gründling, A. (2015). Cross-talk between two nucleotide-signaling pathways in *Staphylococcus aureus*. *The Journal of Biological Chemistry*, *290*(9), 5826–5839.

Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., & Marians, K. J. (2000). The importance of repairing stalled replication forks. *Nature*, *404*(6773), 37–41.

Cromie, G. A. (2009). Phylogenetic ubiquity and shuffling of the bacterial RecBCD and AddAB recombination complexes. *Journal of Bacteriology*, *191*(16), 5076–5084.

Cupa, N., Schulte, D. M., Ahrens, M., Schreiber, S., & Laudes, M. (2015). Vitamin B6 intoxication after inappropriate supplementation with micronutrients following bariatric surgery. *European Journal of Clinical Nutrition*, *69*(7), 862–863.

Degering, C., Eggert, T., Puls, M., Bongaerts, J., Evers, S., Maurer, K. H., & Jaeger, K. E. (2010). Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologousand heterologous signal peptides. *Applied and Environmental Microbiology*, *76*(19), 6370–6376.

DelCardayré, S. B., & Davies, J. E. (1998). *Staphylococcus aureus* coenzyme A disulfide reductase, a new subfamily of pyridine nucleotide-disulfide oxidoreductase. Sequence, expression, and analysis of *cdr*. *Journal of Biological Chemistry*, *273*(10), 5752–5757.

DeLoughery, A., Lalanne, J. B., Losick, R., & Li, G. W. (2018). Maturation of polycistronic mRNAs by the endoribonuclease RNase Y and its associated Y-complex in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(24), E5585–E5594.

Detsch, C., & Stülke, J. (2003). Ammonium utilization in *Bacillus subtilis*: Transport and regulatory functions of NrgA and NrgB. *Microbiology*, *149*(11), 3289–3297.

Di Salvo, Martino Luigi., Safo, M. K., & Contestabile, R. (2012). Biomedical aspects of pyridoxal 5'-phosphate availability. *Frontiers in Bioscience - Elite*, *4 E*(3), 897–913.

Di Salvo, Martino Luigi, Contestabile, R., & Safo, M. K. (2011). Vitamin B6 salvage enzymes: Mechanism, structure and regulation. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1814*(11), 1597–1608.

Di Salvo, Martino Luigi, Nogués, I., Parroni, A., Tramonti, A., Milano, T., Pascarella, S., & Contestabile, R. (2015). On the mechanism of *Escherichia coli* pyridoxal kinase inhibition by pyridoxal and pyridoxal 5'-phosphate. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1854*(9), 1160–1166.

Di Simplicio, P., Franconi, F., Frosalí, S., & Di Giuseppe, D. (2003). Thiolation and nitrosation of cysteines in biological fluids and cells. *Amino Acids*, *25*(3–4), 323–339.

Dianov, G. L., Kuzminov, A. V, Mazin, A. V, & Salganik, R. I. (1991). Molecular mechanisms of deletion formation in *Escherichia coli* plasmids. I. Deletion formation mediated by long direct repeats. *Molecular & General Genetics : MGG*, *228*(1–2), 153–159.

Diethmaier, C., Pietack, N., Gunka, K., Wrede, C., Lehnik-Habrink, M., Herzberg, C., Hübner, S., & Stülke, J. (2011). A novel factor controlling bistability in *Bacillus subtilis*: The Ymdb protein affects flagellin expression and biofilm formation. *Journal of Bacteriology*, *193*(21), 5997–6007.

Dittrich, C. R., Bennett, G. N., & San, K.-Y. (2005). Characterization of the acetate-producing pathways in *Escherichia coli*. *Biotechnology Progress*, *21*(4), 1062–1067.

Doherty, G. P., Fogg, M. J., Wilkinson, A. J., & Lewis, P. J. (2010). Small subunits of RNA polymerase: localization, levels and implications for core enzyme composition. *Microbiology (Reading, England)*, *156*(Pt 12), 3532–3543.

Domke, A., Großklaus, R., Niemann, B., Przyrembel, H., Richter, K., Schmidt, E., Weißenborn, A., Wörner, B., Ziegenhagen, R., Bfr, I., & Domke, W. A. (2005). *Use of Vitamins in Foods Toxicological and nutritional-physiological aspects Part I*. Washington, DC: The National Academies Press. ISSN 1614 – 3795, ISBN 3-938163-10-0.0

Dong, Q., & Fromm, H. J. (1990). Chemical modification of adenylosuccinate synthetase from *Escherichia coli* by pyridoxal 5'-phosphate. Identification of an active site lysyl residue. *Journal of Biological Chemistry*, *265*(11), 6235–6240.

Dormeyer, M., Lentes, S., Ballin, P., Wilkens, M., Klumpp, S., Kohlheyer, D., Stannek, L., Grünberger, A., & Commichau, F. M. (2018). Visualization of tandem repeat mutagenesis in *Bacillus subtilis*. *DNA Repair*, *63*, 10–15.

Dormeyer, M., Lentes, S., Richts, B., Heermann, R., & Ischebeck, T. (2019). Variants of the *Bacillus subtilis* LysR-type regulator gltc with altered activator and repressor function. *10*(October), 1–16.

Dormeyer, M., Lübke, A. L., Müller, P., Lentes, S., Reuß, D. R., Thürmer, A., Stülke, J., Daniel, R., Brantl, S., & Commichau, F. M. (2017). Hierarchical mutational events compensate for glutamate auxotrophy of a *Bacillus subtilis gltC* mutant. *Environmental Microbiology Reports*, *9*(3), 279–289.

Drewke, C., Klein, M., Clade, D., Arenz, A., Müller, R., & Leistner, E. (1996). 4-O-Phosphoryl-L-threonine, a substrate of the *pdxC*(*serC*) gene product involved in vitamin B6 biosynthesis. *FEBS Letters*, *390*(2), 179–182.

Drewke, C., Notheis, C., Hansen, U., Leistner, E., Hemscheidt, T., Hill, R. E., & Spenser, I. D. (1993). Growth response to 4-hydroxy-l-threonine of *Escherichia coli* mutants blocked in vitamin B6 biosynthesis. *FEBS Letters*, *318*(2), 125–128.

Dubnau, D. (1999). DNA uptake in bacteria. *Annual Review of Microbiology*, *53*, 217–244.

Dukan, S., & Nyström, T. (1998). Bacterial senescence: Stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes and Development*, *12*(21), 3431–3441.

Dutra, B. E., Sutera, V. A., & Lovett, S. T. (2007). RecA-independent recombination is efficient but limited by exonucleases. *Proceedings of the National Academy of Sciences*, *104*(1), 216 – 221.

Edayathumangalam, R., Wu, R., Garcia, R., Wang, Y., Wang, W., Kreinbring, C. A., Bach, A., Liao, J., Stone, T. A., Terwilliger, T. C., Hoang, Q. Q., Belitsky, B. R., Petsko, G. A., Ringe, D., & Liu, D. (2013). Crystal structure of *Bacillus subtilis* GabR, an autorepressor and transcriptional activator of *gabT*. *Proceedings of the National Academy of Sciences*, *110*(44), 17820 – 17825.

Eggersdorfer, M., Adam, G., John, M., Hähnlein, W., Labler, L., Baldenius, K.-U., von dem Bussche-Hünnefeld, L., Hilgemann, E., Hoppe, P., Stürmer, R., Weber, F., Rüttimann, A., Moine, G., Hohmann, H.-P., Kurth, R., Paust, J., Hähnlein, W., Pauling, H., Weimann, B., … Blum, R. (2000). Vitamins. In *Ullmann's Encyclopedia of Industrial Chemistry*. https://doi.org/https://doi.org/10.1002/14356007.a27_443

Eggersdorfer, M., Laudert, D., Létinois, U., McClymont, T., Medlock, J., Netscher, T., & Bonrath, W. (2012). One hundred years of vitamins - A success story of the natural sciences. *Angewandte Chemie - International Edition*, *51*(52), 12960–12990.

Ehrenshaft, M., & Daub, M. E. (2001). Isolation of PDX2, a second novel gene in the pyridoxine biosynthesis pathway of eukaryotes, archaebacteria, and a subset of eubacteria. *Journal of Bacteriology*, *183*(11), 3383–3390.

Eiamphungporn, W., & Helmann, J. D. (2008). The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. *Molecular Microbiology*, *67*(4), 830–848.

Eitinger, T., Rodionov, D. A., Grote, M., & Schneider, E. (2011). Canonical and ECF-type ATP-binding cassette importers in prokaryotes: Diversity in modular organization and cellular functions. *FEMS Microbiology Reviews*, *35*(1), 3–67.

Eliot, A. C., & Kirsch, J. F. (2004). Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. *Annual Review of Biochemistry*, *73*(1), 383–415.

Elliott, K. T., Cuff, L. E., & Neidle, E. L. (2013). Copy number change: evolving views on gene amplification. *Future Microbiology*, *8*(7), 887–899.

Engelmann, S., Lindner, C., & Hecker, M. (1995). Cloning, nucleotide sequence, and regulation of *katE* encoding a *σ*(B)- dependent catalase in *Bacillus subtilis*. *Journal of Bacteriology*, *177*(19), 5598–5605.

Eymann, C., Dreisbach, A., Albrecht, D., Bernhardt, J., Becher, D., Gentner, S., Tam, L. T., Büttner, K., Buurman, G., Scharf, C., Venz, S., Völker, U., & Hecker, M. (2004). A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics*, *4*(10), 2849–2876.

Fang, Z., & Dos Santos, P. C. (2015). Protective role of bacillithiol in superoxide stress and Fe-S metabolism in *Bacillus subtilis*. *MicrobiologyOpen*, *4*(4), 616–631.

Fang, Z., Roberts, A. A., Weidman, K., Sharma, S. V., Claiborne, A., Hamilton, C. J., & Dos Santos, P. C. (2013). Cross-functionalities of *Bacillus* deacetylases involved in bacillithiol biosynthesis and bacillithiol-S-conjugate detoxification pathways. *Biochemical Journal*, *454*(2), 239–247.

Farrington, G. K., Kumar, A., Shames, S. L., Ewaskiewicz, J. I., Ash, D. E., & Wedler, F. C. (1993). Threonine synthase of *Escherichia coli*: Inhibition by classical and slow-binding analogues of homoserine phosphate. *Archives of Biochemistry and Biophysics*, *307*(1), 165–174.

Fedorova, K., Kayumov, A., Woyda, K., Ilinskaja, O., & Forchhammer, K. (2013). Transcription factor TnrA inhibits the biosynthetic activity of glutamine synthetase in *Bacillus subtilis*. *FEBS Letters*, *587*(9), 1293–1298.

Ferguson, G. P., Munro, A. W., Douglas, R. M., McLaggan, D., & Booth, I. R. (1993). Activation of potassium channels during metabolite detoxification in *Escherichia coli*. *Molecular Microbiology*, *9*(6), 1297–1303.

Fernández, S., Sorokin, A., & Alonso, J. C. (1998). Genetic recombination in *Bacillus subtilis* 168: effects of *recU* and *recS* mutations on DNA repair and homologous recombination. *Journal of Bacteriology*, *180*(13), 3405–3409.

Fisher, Susan H. (1999). Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la différence! *Molecular Microbiology*, *32*(2), 223–232.

Fisher, Susan H., Brandenburg, J. L., & Wray, L. V. (2002). Mutations in *Bacillus subtilis* glutamine synthetase that block its interaction with transcription factor TnrA. *Molecular Microbiology*, *45*(3), 627–635.

Fisher, Susan H., & Wray, L. V. (2006). Feedback-resistant mutations in *Bacillus subtilis* glutamine synthetase are clustered in the active site. *Journal of Bacteriology*, *188*(16), 5966–5974.

Fisher, Susan H., & Wray, L. V. (2008). *Bacillus subtilis* glutamine synthetase regulates its own synthesis by acting as a chaperone to stabilize GlnR-DNA complexes. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 1014–1019.

Fisher, Susan H., & Wray, L. V. (2009). Novel trans-acting *Bacillus subtilis glnA* mutations that derepress *glnRA* expression. *Journal of Bacteriology*, *191*(8), 2485–2492.

Fisher, Susan H, & Débarbouillé, M. (2001). Nitrogen source utilization and its regulation. In *Bacillus subtilis and Its Closest Relatives,* 181–191.

Fitzpatrick, T. B., Amrhein, N., Kappes, B., Macheroux, P., Tews, I., & Raschle, T. (2007). Two independent routes of *de novo* vitamin B6 biosynthesis: Not that different after all. *Biochemical Journal*, *407*(1), 1–13.

Fitzpatrick, T. B., Moccand, C., & Roux, C. (2010). Vitamin B6 biosynthesis: Charting the mechanistic landscape. *ChemBioChem*, *11*(9), 1185–1193.

Fonda, M. L. (1992). Purification and characterization of vitamin B6-phosphate phosphatase from human erythrocytes. *Journal of Biological Chemistry*, *267*(22), 15978–15983.

Fonda, Margaret L., & Zhang, Y. na. (1995). Kinetic mechanism and divalent metal activation of human erythrocyte pyridoxal phosphatase. *Archives of Biochemistry and Biophysics*, *320*(2), 345–352.

Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2006). DNA Repair and mutagenesis. In B. A. Montelone (Ed.), *The Quarterly Review of Biology* (2nd ed., Vol. 81, Issue 3). The University of Chicago Press. https://doi.org/10.1086/509407

Fu, T. F., Di Salvo, M., & Schirch, V. (2001). Distribution of B6 vitamers in *Escherichia coli* as determined by enzymatic assay. *Analytical Biochemistry*, *298*(2), 314–321.

Fuangthong, M., Atichartpongkul, S., Mongkolsuk, S., & Helmann, J. D. (2001). OhrR is a repressor of *ohrA*, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. *Journal of Bacteriology*, *183*(14), 4134–4141.

Gaballa, A., Antelmann, H., Hamilton, C. J., & Helmann, J. D. (2013). Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx. *Microbiology (United Kingdom)*, *159*(10), 2025–2035.

Gaballa, A., Chi, B. K., Roberts, A. A., Becher, D., Hamilton, C. J., Antelmann, H., & Helmann, J. D. (2014). Redox regulation in *Bacillus subtilis*: The bacilliredoxins BrxA (YphP) and BrxB (YqiW) function in de-bacillithiolation of s-bacillithiolated OhrR and MetE. *Antioxidants and Redox Signaling*, *21*(3), 357–367.

Gaballa, A., Newton, G. L., Antelmann, H., Parsonage, D., Upton, H., Rawat, M., Claiborne, A., Fahey, R. C., & Helmann, J. D. (2010). Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(14), 6482–6486.

Galas, D. J. (1978). An analysis of sequence repeats in the lacI gene of *Escherichia coli*. *Journal of Molecular Biology*, *126*(4), 858–863.

Galperin, M. Y., Wolf, Y. I., Makarova, K. S., Vera Alvarez, R., Landsman, D., & Koonin, E. V. (2021). COG database update: focus on microbial diversity, model organisms, and widespread pathogens. *Nucleic Acids Research*, *49*(D1), D274–D281.

Garg, S. K., Kommineni, S., Henslee, L., Zhang, Y., & Zuber, P. (2009). The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *Journal of Bacteriology*, *191*(4), 1268–1277.

Ghatge, M. S., Contestabile, R., di Salvo, M. L., Desai, J. V., Gandhi, A. K., Camara, C. M., Florio, R., González, I. N., Parroni, A., Schirch, V., & Safo, M. K. (2012). Pyridoxal 5'-phosphate is a slow tight binding inhibitor of *E. coli* pyridoxal kinase. *PLoS ONE*, *7*(7), e41680. https://doi.org/10.1371/journal.pone.0041680

Ghatge, M. S., Karve, S. S., David, T. M. S., Ahmed, M. H., Musayev, F. N., Cunningham, K., Schirch, V., & Safo, M. K. (2016). Inactive mutants of human pyridoxine 5'-phosphate oxidase: a possible role for a noncatalytic pyridoxal 5'-phosphate tight binding site. *FEBS Open Bio*, *6*(5), 398–408.

Glickman, B. W., & Ripley, L. S. (1984). Structural intermediates of deletion mutagenesis: a role for palindromic DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(2), 512–516.

Goldfless, S. J., Morag, A. S., Belisle, K. A., Sutera, V. A. J., & Lovett, S. T. (2006). DNA repeat rearrangements mediated by DnaK-dependent replication fork repair. *Molecular Cell*, *21*(5), 595–604.

González-Pastor, J. E., Hobbs, E. C., & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science (New York, N.Y.)*, *301*(5632), 510–513.

Griffith, L. J., Ostrander, W. E., Mullins, C. G., & Beswick, D. E. (1965). Drug antagonism between lincomycin and erythromycin. *Science*, *147*(3659), 746 – 747.

Guan, Z., Xue, D., Abdallah, I. I., Dijkshoorn, L., Setroikromo, R., Lv, G., & Quax, W. J. (2015). Metabolic engineering of *Bacillus subtilis* for terpenoid production. *Applied Microbiology and Biotechnology*, *99*(22), 9395–9406.

Guédez, G., Hipp, K., Windeisen, V., Derrer, B., Gengenbacher, M., Böttcher, B., Sinning, I., Kappes, B., & Tews, I. (2012). Assembly of the eukaryotic PLP-synthase complex from *Plasmodium* and activation of the Pdx1 enzyme. *Structure*, *20*(1), 172–184.

Guérout-Fleury, A. M., Shazand, K., Frandsen, N., & Stragier, P. (1995). Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene*, *167*(1–2), 335–336.

Gundlach, J., Herzberg, C., Kaever, V., Gunka, K., Hoffmann, T., Weiss, M., Gibhardt, J., Thurmer, A., Hertel, D., Daniel, R., Bremer, E., Commichau, F. M., & Stulke, J. (2017). Control of potassium homeostasis is an essential function of the second messenger cyclic di-AMP in *Bacillus subtilis*. *Science Signaling*, *10*(475). https://doi.org/10.1126/scisignal.aal3011

Gundlach, J., Mehne, F. M. P., Herzberg, C., Kampf, J., Valerius, O., Kaever, V., & Stülke, J. (2015). An essential poison: Synthesis and degradation of cyclic di-AMP in *Bacillus subtilis*. *Journal of Bacteriology*, *197*(20), 3265–

Gundlach, J., Rath, H., Herzberg, C., Mäder, U., & Stülke, J. (2016). Second messenger signaling in *Bacillus subtilis*: Accumulation of cyclic di-AMP inhibits biofilm formation. *Frontiers in Microbiology*, *7*, 1–8.

Gunka, K., & Commichau, F. M. (2012). Control of glutamate homeostasis in *Bacillus subtilis*: A complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. *Molecular Microbiology*, *85*(2), 213–224.

Gunka, K., Stannek, L., Care, R. A., & Commichau, F. M. (2013). Selection-driven accumulation of suppressor mutants in *Bacillus subtilis*: The apparent high mutation frequency of the cryptic *gudB* gene and the rapid clonal expansion of *gudB*+ suppressors are due to growth under selection. *PLOS ONE*, *8*(6), e66120. https://doi.org/10.1371/journal.pone.0066120

György, P. (1956). The history of vitamin B6. *The American Journal of Clinical Nutrition*, *4*(4), 313–317.

Haack, K. R., & Roth, J. R. (1995). Recombination between chromosomal IS200 elements supports frequent duplication formation in *Salmonella typhimurium*. *Genetics*, *141*(4), 1245–1252.

Haijema, B. J., Venema, G., & Kooistra, J. (1996). The C terminus of the AddA subunit of the *Bacillus subtilis* ATP-dependent DNase is required for the ATP-dependent exonuclease activity but not for the helicase activity. *Journal of Bacteriology*, *178*(17), 5086–5091.

Hammerstad, M., Gudim, I., & Hersleth, H. P. (2020). The crystal structures of bacillithiol disulfide reductase Bdr (YpdA) provide structural and functional insight into a new type of FAD-containing NADPH-dependent oxidoreductase. *Biochemistry*, *59*(51), 4793–4798.

Hamoen, L. W., Venema, G., & Kuipers, O. P. (2003). Controlling competence in *Bacillus subtilis*: shared use of regulators. *Microbiology (Reading, England)*, *149*(Pt 1), 9–17.

Han, H., Xu, B., Zeng, W., & Zhou, J. (2020). Regulating the biosynthesis of pyridoxal 5'-phosphate with riboswitch to enhance L-DOPA production by *Escherichia coli* whole-cell biotransformation. *Journal of Biotechnology*, *321*(May), 68–77.

Hanes, J. W., Burns, K. E., Hilmey, D. G., Chatterjee, A., Dorrestein, P. C., & Begley, T. P. (2008). Mechanistic studies on pyridoxal phosphate synthase: the reaction pathway leading to a chromophoric intermediate. *Journal of the American Chemical Society*, *130*(10), 3043–3052.

Hao, T., Han, B., Ma, H., Fu, J., Wang, H., Wang, Z., Tang, B., Chen, T., & Zhao, X. (2013). In *silico* metabolic engineering of *Bacillus subtilis* for improved production of riboflavin, Egl-237, (R,R)-2,3-butanediol and isobutanol. *Molecular BioSystems*, *9*(8), 2034–2044.

Harms, N., Ras, J., Reijnders, W. N. M., Van Spanning, R. J. M., & Stouthamer, A. H. (1996). S-formylglutathione hydrolase of *Paracoccus denitrificans* is homologous to human esterase D: A universal pathway for formaldehyde detoxification? *Journal of Bacteriology*, *178*(21), 6296–6299.

Harris, H. (1990). The human alkaline phosphatases: What we know and what we don't know. *Clinica Chimica Acta*, *186*(2), 133–150.

Hartl, J., Kiefer, P., Meyer, F., & Vorholt, J. A. (2017). Longevity of major coenzymes allows minimal de *novo* synthesis in microorganisms. *Nature Microbiology*, *2*(7), 17073.

Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends in Biotechnology*, *10*, 247–256.

Hauf, K., Kayumov, A., Gloge, F., & Forchhammer, K. (2016). The molecular basis of TnrA control by glutamine synthetase in *Bacillus subtilis*. *Journal of Biological Chemistry*, *291*(7), 3483–3495.

Heaton, M. P., Johnston, R. B., & Thompson, T. L. (1988). Controlled lysis of bacterial cells utilizing mutants with defective synthesis of D-alanine. *Canadian Journal of Microbiology*, *34*(3), 256–261.

Hecker, M., Pané-Farré, J., & Völker, U. (2007). SigB-dependent general stress response in *Bacillus subtilis* and related Gram-positive bacteria. *Annual Review of Microbiology*, *61*, 215–236.

Hegde, S. P., Qin, M. H., Li, X. H., Atkinson, M. A., Clark, A. J., Rajagopalan, M., & Madiraju, M. V. (1996). Interactions of RecF protein with RecO, RecR, and single-stranded DNA binding proteins reveal roles for the RecF-RecO-RecR complex in DNA repair and recombination. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(25), 14468–14473.

Heinrich A, Woyda K, Brauburger K, Meiss G, Detsch C, Stülke J, Forchhammer K. (2006). Interaction of the membrane-bound GlnK-AmtB complex with the master regulator of nitrogen metabolism TnrA in *Bacillus subtilis*. In *The Journal of biological chemistry* (Vol. 281, Issue 46), 34909-34917.

Helmann, J. D., Fang, M., Wu, W., Gaballa, A., Kobel, P. A., Morshedi, M. M., Fawcett, P., & Paddon, C. (2003). The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. *Journal of Bacteriology*, *185*(1), 243–253.

Herbig, A. F., & Helmann, J. D. (2001). Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Molecular Microbiology*, *41*(4), 849–859.

Herzberg, C., Weidinger, L. A. F., Dörrbecker, B., Hübner, S., Stülke, J., & Commichau, F. M. (2007). SPINE: A method for the rapid detection and analysis of protein-protein interactions in *vivo*. In *Proteomics* (Vol. 7, Issue 22, pp. 4032–4035).

Hiras, J., Sharma, S. V., Raman, V., Tinson, R. A. J., Arbach, M., Rodrigues, D. F., Norambuena, J., Hamilton, C. J., & Hanson, T. E. (2018). Physiological studies of *chlorobiaceae* suggest that bacillithiol derivatives are the most widespread thiols in bacteria. *MBio*, *9*(6), 1–13.

Hochgräfe, F., Mostertz, J., Albrecht, D., & Hecker, M. (2005). Fluorescence thiol modification assay: oxidatively modified proteins in *Bacillus subtilis*. *Molecular Microbiology*, *58*(2), 409–425.

Hochgräfe, F., Mostertz, J., Pöther, D. C., Becher, D., Helmann, J. D., & Hecker, M. (2007). S-cysteinylation is a general mechanism for thiol protection of *Bacillus subtilis* proteins after oxidative stress. *Journal of Biological Chemistry*, *282*(36), 25961–25985.

Hoegl, A., Nodwell, M. B., Kirsch, V. C., Bach, N. C., Pfanzelt, M., Stahl, M., Schneider, S., & Sieber, S. A. (2018). Mining the cellular inventory of pyridoxal phosphate-dependent enzymes with functionalized cofactor mimics. *Nature Chemistry*, *10*(12), 1234–1245.

Hondorp, E. R., & Matthews, R. G. (2004). Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. *PLoS Biology*, *2*(11), e336. https://doi.org/10.1371/journal.pbio.0020336

Hoshino, T., Ichikawa, K., Nagahashi, Y., & Tazoe, M. (2006). *Microorganism and Process for Preparing Vitamin B6* (Patent No. US2006/0127992 A1.). US Patent Application. https://patentimages.storage.googleapis.com/f3/1d/70/f037672daa5929/US20060127992A1.pdf

Hu, P., Leighton, T., Ishkhanova, G., & Kustu, S. (1999). Sensing of nitrogen limitation by *Bacillus subtilis* comparison to enteric bacteria. *Journal of Bacteriology*, *181*(16), 5042 – 5050.

Huang, S.-C., Wei, J. C.-C., Lin, P.-T., Wu, D. J., & Huang, Y.-C. (2012). Plasma pyridoxal 5'-phosphate is not associated with inflammatory and immune responses after adjusting for serum albumin in patients with rheumatoid arthritis: a preliminary study. *Annals of Nutrition & Metabolism*, *60*(2), 83–89.

Huq, M. D. M., Tsai, N. P., Lin, Y. P., Higgins, L. A., & Wei, L. N. (2007). Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. *Nature Chemical Biology*, *3*(3), 161–165.

Huyen, N. T. T., Eiamphungporn, W., Mäder, U., Liebeke, M., Lalk, M., Hecker, M., Helmann, J. D., & Antelmann, H. (2009). Genome-wide responses to carbonyl electrophiles in *Bacillus subtilis*: Control of the thiol-dependent formaldehyde dehydrogenase AdhA and cysteine proteinase YraA by the MerR-family regulator YraB (AdhR). *Molecular Microbiology*, *71*(4), 876–894.

Huynh, T. N., Luo, S., Pensinger, D., Sauer, J.-D., Tong, L., & Woodward, J. J. (2015). An HD-domain phosphodiesterase mediates cooperative hydrolysis of c-di-AMP to affect bacterial growth and virulence. *Proceedings of the National Academy of Sciences*, *112*(7), E747–E756.

Imber, M., Pietrzyk-Brzezinska, A. J., & Antelmann, H. (2019). Redox regulation by reversible protein S-thiolation in Gram-positive bacteria. *Redox Biology*, *20*(July 2018), 130–145.

Imlay, James A, & Linn, S. (1988). DNA damage and oxygen radical toxicity. *Science (New York, N.Y.)*, *240*(4857), 1302–1309.

Imlay, James A. (2003). Pathways of oxidative damage. *Annual Review of Microbiology*, *57*, 395–418.

Imlay, James A. (2008). Cellular defenses against superoxide and hydrogen peroxide. *Annual Review of Biochemistry*, *77*, 755–776.

Imlay, James A. (2006). Iron-sulphur clusters and the problem with oxygen. *Molecular Microbiology*, *59*(4), 1073–1082.

Institute of Medicine. (1998). Dietary Reference Intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. In *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. Washington, DC: The National Academies Press. https://doi.org/10.17226/6015

Institute of Medicine. (2000). Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. In *Washington, DC: The National Academies Press.* The National Academies Press. https://doi.org/10.17226/9810

Institute of Medicine. (2006). *Dietary Reference Intakes: The Essential Guide to Nutrient Requirements* (J. J. Otten, J. P. Hellwig, & L. D. Meyers (eds.)). The National Academies Press. https://doi.org/10.17226/11537

Itagaki, S., Haga, M., Oikawa, Y., Sakoda, A., Ohke, Y., Sawada, H., Eguchi, T., & Tamegai, H. (2013). Differences in the roles of a glutamine amidotransferase subunit of pyridoxal 5'-phosphate synthase between *Bacillus circulans* and *Bacillus subtilis*. *Bioscience, Biotechnology and Biochemistry*, *77*(7), 1481–1485.

Ito, T., & Downs, D. M. (2020). Pyridoxal reductase, PdxI, Is critical for salvage of pyridoxal in *Escherichia coli*. *Journal of Bacteriology*, *202*(12), e00056-20. https://doi.org/10.1128/JB.00056-20

Ito, T., Hori, R., Hemmi, H., Downs, D. M., & Yoshimura, T. (2020). Inhibition of glycine cleavage system by pyridoxine 5'-phosphate causes synthetic lethality in glyA yggS and serA yggS in *Escherichia coli*. *Molecular Microbiology*, *113*(1), 270–284.

Ito, T., Iimori, J., Takayama, S., Moriyama, A., Yamauchi, A., Hemmi, H., & Yoshimura, T. (2013). Conserved pyridoxal protein that regulates ile and val metabolism. *Journal of Bacteriology*, *195*(24), 5439 – 5449.

Ito, T., Yamamoto, K., Hori, R., Yamauchi, A., Downs, D. M., Hemmi, H., & Yoshimura, T. (2019). Conserved pyridoxal 5'-phosphate-binding protein YggS impacts amino acid metabolism through pyridoxine 5'-phosphate in *Escherichia coli*. *Applied and Environmental Microbiology*, *85*(11), e00430-19.

Jain, K., Wood, E. A., Romero, Z. J., & Cox, M. M. (2020). RecA-independent recombination: Dependence on the *Escherichia coli* RarA protein. *Molecular Microbiology*, 00, 1– 16

Jang, Y. M., Kim, D. W., Kang, T. C., Won, M. H., Baek, N. I., Moon, B. J., Choi, S. Y., & Kwon, O. S. (2003). Human pyridoxal phosphatase: Molecular cloning, functional expression, and tissue distribution. *Journal of Biological Chemistry*, *278*(50), 50040–50046.

Jansonius, J. N. (1998). Structure, evolution and action of vitamin B6-dependent enzymes. *Current Opinion in Structural Biology*, *8*(6), 759–769.

Jessop, A. P., & Clugston, C. (1985). Amplification of the ArgF region in strain HfrP4X of *E. coli* K-12. *Molecular & General Genetics : MGG*, *201*(2), 347–350.

Jochmann, N., Götker, S., & Tauch, A. (2011). Positive transcriptional control of the pyridoxal phosphate biosynthesis genes *pdxST* by the MocR-type regulator PdxR of *Corynebacterium glutamicum* ATCC 13032. *Microbiology*, *157*(1), 77–88.

John, R. A. (1995). Pyridoxal phosphate-dependent enzymes. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular*, *1248*(2), 81–96.

Johnson, B. C., Pinkos, J. A., & Burke, K. A. (1950). Pyridoxine deficiency in the calf. *The Journal of Nutrition*, *40*(2), 309–322.

Juhas, M., Reuß, D. R., Zhu, B., & Commichau, F. M. (2014). *Bacillus subtilis* and *Escherichia coli* essential genes and minimal cell factories after one decade of genome engineering. *Microbiology (United Kingdom)*, *160*(11), 2341–2351.

Kahan, F. M., Kahan, J. S., Cassidy, P. J., & Kropp, H. (1974). The mechanism of action of fosfomycin (phosphonomycin). *Annals of the New York Academy of Sciences*, *235*(0), 364–386.

Kannan, L., Li, H., Rubinstein, B., & Mushegian, A. (2013). Models of gene gain and gene loss for probabilistic reconstruction of gene content in the last universal common ancestor of life. *Biology Direct*, *8*, 32.

Kanter, M. M. (1994). Free radicals, exercise, and antioxidant supplementation. *International Journal of Sport Nutrition*, *4*(3), 205–220.

Karimova, G., Pidoux, J., Ullmann, A., & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(10), 5752–5756.

Katz, E., Mason, K. T., & Mauger, A. B. (1974). The presence of *α*-amino-*β*, *γ*-dihydroxybutyric acid in hydrolysates of actinomycin z1. *The Journal of Antibiotics*, *27*(12), 952–955.

Kaundinya, C. R., Savithri, H. S., Rao, K. K., & Balaji, P. V. (2018). EpsN from *Bacillus subtilis* 168 has UDP-2,6-dideoxy 2-acetamido 4-keto glucose aminotransferase activity in *vitro*. *Glycobiology*, *28*(10), 802–812.

Kay, K. L., Hamilton, C. J., & Le Brun, N. E. (2016). Mass spectrometry of: *B. subtilis* CopZ: Cu(i)-binding and interactions with bacillithiol. *Metallomics*, *8*(7), 709–719.

Kayumov, A., Heinrich, A., Fedorova, K., Ilinskaya, O., & Forchhammer, K. (2011). Interaction of the general transcription factor TnrA with the PII-like protein GlnK and glutamine synthetase in *Bacillus subtilis*. *The FEBS Journal*, *278*(10), 1779–1789.

Kearns, D. B., Chu, F., Branda, S. S., Kolter, R., & Losick, R. (2005). A master regulator for biofilm formation by *Bacillus subtilis*. *Molecular Microbiology*, *55*(3), 739–749.

Keller, A. N., Yang, X., Wiedermannová, J., Delumeau, O., Krásný, L., & Lewis, P. J. (2014). *ε*, a new subunit of RNA polymerase found in Gram-positive bacteria. *Journal of Bacteriology*, *196*(20), 3622–3632.

Khersonsky, O., & Tawfik, D. S. (2010). Enzyme promiscuity: A mechanistic and evolutionary perspective. *Annual Review of Biochemistry*, *79*(1), 471–505.

Kidane, D., & Graumann, P. L. (2005). Dynamic formation of RecA filaments at DNA double strand break repair centers in live cells. *The Journal of Cell Biology*, *170*(3), 357–366.

Kiley, P. J., & Beinert, H. (2003). The role of Fe-S proteins in sensing and regulation in bacteria. *Current Opinion in Microbiology*, *6*(2), 181–185.

Kim, J., & Copley, S. D. (2012). Inhibitory cross-talk upon introduction of a new metabolic pathway into an existing metabolic network. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(42), E2856-E2864.

Kim, J., Flood, J. J., Kristofich, M. R., Gidfar, C., Morgenthaler, A. B., & Fuhrer, T. (2019). Hidden resources in the *Escherichia coli* genome restore PLP synthesis and robust growth after deletion of the essential gene *pdxB*. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(48), 24164–24173.

Kim, J., Kershner, J. P., Novikov, Y., Shoemaker, R. K., & Copley, S. D. (2010). Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5'-phosphate synthesis. *Molecular Systems Biology*, *6*(1), 436.

Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S. C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L. C., Danchin, A., Debarbouille, M., Dervyn, E., … Ogasawara, N. (2003). Essential *Bacillus subtilis* genes. *Proceedings of the National Academy of Sciences*, *100*(8), 4678–4683.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, *130*(5), 797–810.

Konkol, M. A., Blair, K. M., & Kearns, D. B. (2013). Plasmid-encoded comi inhibits competence in the ancestral 3610 strain of *Bacillus subtilis*. *Journal of Bacteriology*, *195*(18), 4085–4093.

Koo, B.-M. M., Kritikos, G., Farelli, J. D., Todor, H., Tong, K., Kimsey, H., Wapinski, I., Galardini, M., Cabal, A., Peters, J. M., Hachmann, A.-B. B., Rudner, D. Z., Allen, K. N., Typas, A., & Gross, C. A. (2017). Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Systems*, *4*(3), 291-305.e7.

Kooistra, J., Haijema, B. J., Hesseling-Meinders, A., & Venema, G. (1997). A conserved helicase motif of the AddA subunit of the *Bacillus subtilis* ATP-dependent nuclease (AddAB) is essential for DNA repair and recombination. *Molecular Microbiology*, *23*(1), 137–149.

Korolev, S. (2017). Advances in structural studies of recombination mediator proteins. *Biophysical Chemistry*, *225*, 27–37.

Kraemer, K., Semba, R. D., Eggersdorfer, M., & Schaumberg, D. A. (2012). Introduction: The diverse and essential biological functions of vitamins. *Annals of Nutrition and Metabolism*, *61*(3), 185–191.

Kresge, N., Simoni, R. D., & Hill, R. L. (2005). Esmond E. Snell and the B vitamins. *Journal of Biological Chemistry*, *280*(13), e10–e11.

Kreuzer, K. N. (2013). DNA damage responses in prokaryotes: regulating gene expression, modulating growth patterns, and manipulating replication forks. *Cold Spring Harbor Perspectives in Biology*, *5*(11), a012674. https://doi.org/10.1101/cshperspect.a012674

Kriel, A., Brinsmade, S. R., Tse, J. L., Tehranchi, A. K., Bittner, A. N., Sonenshein, A. L., & Wang, J. D. (2014). GTP dysregulation in *Bacillus subtilis* cells lacking (p)ppGpp results in phenotypic amino acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes. *Journal of Bacteriology*, *196*(1), 189–201.

Krüger, L., Herzberg, C., Rath, H., Pedreira, T., Ischebeck, T., Poehlein, A., Gundlach, J., Daniel, R., Völker, U., Mäder, U., & Stülke, J. (2021b). Essentiality of c-di-AMP in *Bacillus subtilis*: Bypassing mutations converge in potassium and glutamate homeostasis. *PLoS Genetics*, *17*(1), e1009092. https://doi.org/10.1371/journal.pgen.1009092

Krüger, L., Herzberg, C., Wicke, D., Bähre, H., Heidemann, J. L., Dickmanns, A., Schmitt, K., Ficner, R., & Stülke, J. (2021a). A meet-up of two second messengers: the c-di-AMP receptor DarB controls (p)ppGpp synthesis in *Bacillus subtilis*. *Nature Communications*, *12*(1), 1210. https://doi.org/10.1038/s41467-021-21306-0

Kumar, A., Sarkar, S. K., Ghosh, D., & Ghosh, A. S. (2012). Deletion of penicillin-binding protein 1b impairs biofilm formation and motility in *Escherichia coli*. *Research in Microbiology*, *163*(4), 254–257.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V, Caldwell, B., … Danchin, A. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature*, *390*(6657), 249–256.

Kunst, F., & Rapoport, G. (1995). Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *Journal of Bacteriology*, *177*(9), 2403–2407.

Kuzminov, A. (1999). Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. *Microbiology and Molecular Biology Reviews : MMBR*, *63*(4), 751–813

Kuzminov, Andrei. (2001). DNA replication meets genetic exchange: Chromosomal damage and its repair by homologous recombination. *Proceedings of the National Academy of Sciences*, *98*(15), 8461 LP – 8468.

Kuznetsova, E., Proudfoot, M., Gonzalez, C. F., Brown, G., Omelchenko, M. V., Borozan, I., Carmel, L., Wolf, Y. I., Mori, H., Savchenko, A. V., Arrowsmith, C. H., Koonin, E. V., Edwards, A. M., & Yakunin, A. F. (2006). Genome-wide analysis of substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphatase family. *Journal of Biological Chemistry*, *281*(47), 36149–36161.

Kuzuyama, T., Takagi, M., Takahashi, S., & Seto, H. (2000). Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces sp*. Strain CL190, which uses both the mevalonate and nonmevalonate pathways for isopentenyl diphosphate biosynthesis. *Journal of Bacteriology*, *182*(4), 891–897.

Laber, B., Maurer, W., Scharf, S., Stepusin, K., & Schmidt, F. S. (1999). Vitamin B6 biosynthesis: Formation of pyridoxine 5'-phosphate from 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose-5-phosphate by PdxA and PdxJ protein. *FEBS Letters*, *449*(1), 45–48.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685.

Laer, K. Van, Hamilton, C., & Messens, J. (2013). *Low molecular weight thiols in thiol disulfide exchange Antiox & Red Signal*, *18*(13),1642-1653

Lagunas, R., & Solis, A. (1968). Arsenate induced activity of certain enzymes on their dephosphorylated subtrates. *FEBS Letters*, *1*(1), 32–34.

Lamers, A. P., Keithly, M. E., Kim, K., Cook, P. D., Stec, D. F., Hines, K. M., Sulikowski, G. A., & Armstrong, R. N. (2012). Synthesis of bacillithiol and the catalytic selectivity of FosB-type fosfomycin resistance proteins. *Organic Letters*, *14*(20), 5207–5209.

Lee, J. W., Soonsanga, S., & Helmann, J. D. (2007). A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(21), 8743–8748.

Lee, W. M., Elliott, J. E., & Brownsey, R. W. (2005). Inhibition of acetyl-CoA carboxylase isoforms by pyridoxal phosphate. *Journal of Biological Chemistry*, *280*(51), 41835–41843.

Leejeerajumnean, A., Ames, J. M., & Owens, J. D. (2000). Effect of ammonia on the growth of *Bacillus* species and some other bacteria. *Letters in Applied Microbiology*, *30*(5), 385–389.

Leelakriangsak, M., Kobayashi, K., & Zuber, P. (2007). Dual negative control of *spx* transcription initiation from the P 3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *Journal of Bacteriology*, *189*(5), 1736–1744.

Lehner, A. F., & Hill, C. W. (1980). Involvement of ribosomal ribonucleic acid operons in *Salmonella typhimurium* chromosomal rearrangements. *Journal of Bacteriology*, *143*(1), 492–498.

Leistner, E., & Drewke, C. (2010). Ginkgo biloba and Ginkgotoxin. *Journal of Natural Products*, *73*(1), 86–92.

Lenhart, J. S., Schroeder, J. W., Walsh, B. W., & Simmons, L. A. (2012). DNA repair and genome maintenance in *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews*, *76*(3), 530–564.

Lerma-Ortiz, C., Jeffryes, J. G., Cooper, A. J. L., Niehaus, T. D., Thamm, A. M. K., Frelin, O., Aunins, T., Fiehn, O., De Crécy-Lagard, V., Henry, C. S., & Hanson, A. D. (2016). Nothing of chemistry disappears in biology': The Top 30 damage-prone endogenous metabolites. *Biochemical Society Transactions*, *44*(3), 961–971.

Lheureux, P., Penaloza, A., & Gris, M. (2005). Pyridoxine in clinical toxicology: a review. *European Journal of Emergency Medicine*, *12*(2), 78-85.

Liao, S., Bitoun, J. P., Nguyen, A. H., Bozner, D., Yao, X., & Wen, Z. T. (2015). Deficiency of PdxR in *Streptococcus mutans* affects vitamin B6 metabolism, acid tolerance response and biofilm formation. *Molecular Oral Microbiology*, *30*(4), 255–268.

Liebeke, M., Pöther, D. C., Van Duy, N., Albrecht, D., Becher, D., Hochgräfe, F., Lalk, M., Hecker, M., & Antelmann, H. (2008). Depletion of thiol-containing proteins in response to quinones in *Bacillus subtilis*. *Molecular Microbiology*, *69*(6), 1513–1529.

Lin, R. J., Capage, M., & Hill, C. W. (1984). A repetitive DNA sequence, rhs, responsible for duplications within the *Escherichia coli* K-12 chromosome. *Journal of Molecular Biology*, *177*(1), 1–18.

Linster, C. L., Van Schaftingen, E., & Hanson, A. D. (2013). Metabolite damage and its repair or pre-emption. *Nature Chemical Biology*, *9*(2), 72–80.

Linzner, N., Van Loi, V., Fritsch, V. N., Tung, Q. N., Stenzel, S., Wirtz, M., Hell, R., Hamilton, C. J., Tedin, K., Fulde, M., & Antelmann, H. (2019). *Staphylococcus aureus* uses the bacilliredoxin (BrxAB)/bacillithiol disulfide reductase (YpdA) redox pathway to defend against oxidative stress under infections. *Frontiers in Microbiology*, *10*, 1–15.

Liu, K., Bittner, A. N., & Wang, J. D. (2015). Diversity in (p)ppGpp metabolism and effectors. *Current Opinion in Microbiology*, *24*, 72–79.

Liu, S., Bayles, D. O., Mason, T. M., & Wilkinson, B. J. (2006). A cold-sensitive *Listeria monocytogenes* mutant has a transposon insertion in a gene encoding a putative membrane protein and shows altered (p)ppGpp levels. *Applied and Environmental Microbiology*, *72*(6), 3955–3959.

López de Saro, F. J., Woody, A. Y., & Helmann, J. D. (1995). Structural analysis of the *Bacillus subtilis* delta factor: a protein polyanion which displaces RNA from RNA polymerase. *Journal of Molecular Biology*, *252*(2), 189–202.

López de Saro, F. J., Yoshikawa, N., & Helmann, J. D. (1999). Expression, abundance, and RNA polymerase binding properties of the delta factor of *Bacillus subtilis*. *The Journal of Biological Chemistry*, *274*(22), 15953–15958.

Losick, R., & Shapiro, L. (1993). Checkpoints that couple gene expression to morphogenesis. *Science (New York, N.Y.)*, *262*(5137), 1227–1228.

Lovett, S T, Drapkin, P. T., Sutera, V. A. J., & Gluckman-Peskind, T. J. (1993). A sister-strand exchange mechanism for *recA*-independent deletion of repeated DNA sequences in *Escherichia coli*. *Genetics*, *135*(3), 631–642.

Lovett, S T, & Feschenko, V. V. (1996). Stabilization of diverged tandem repeats by mismatch repair: evidence for deletion formation via a misaligned replication intermediate. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(14), 7120–7124.

Lovett, S T, Gluckman, T. J., Simon, P. J., Sutera, V. A. J., & Drapkin, P. T. (1994). Recombination between repeats in *Escherichia coli* by a *recA*-independent, proximity-sensitive mechanism. *Molecular & General Genetics : MGG*, *245*(3), 294–300.

Lovett, Susan T. (2017). Template-switching during replication fork repair in bacteria. *DNA Repair*, *56*, 118–128.

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7), 632–644.

Lumeng, L., Brashear, R. E., & Li, T. K. (1974). Pyridoxal 5'-phosphate in plasma: source, protein-binding, and cellular transport. *The Journal of Laboratory and Clinical Medicine*, *84*(3), 334–343.

Luttinger, A., Hahn, J., & Dubnau, D. (1996). Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Molecular Microbiology*, *19*(2), 343–356.

Lyngberg, L., Healy, J., Bartlett, W., Miller, S., Conway, S. J., Booth, I. R., & Rasmussen, T. (2011). KefF, the regulatory subunit of the potassium efflux system KefC, shows quinone oxidoreductase activity. *Journal of Bacteriology*, *193*(18), 4925–4932.

Ma, Z., Chandrangsu, P., Helmann, T. C., Romsang, A., Gaballa, A., & Helmann, J. D. (2014). Bacillithiol is a major buffer of the labile zinc pool in *Bacillus subtilis*. *Molecular Microbiology*, *94*(4), 756–770.

Man, T. K., Zhao, G., & Winkler, M. E. (1996). Isolation of a pdxJ point mutation that bypasses the requirement for the PdxH oxidase in pyridoxal 5'-phosphate coenzyme biosynthesis in *Escherichia coli* K-12. *Journal of Bacteriology*, *178*(8), 2445–2449.

Manfredi, C., Carrasco, B., Ayora, S., & Alonso, J. C. (2008). *Bacillus subtilis* RecO nucleates RecA onto SsbA-coated single-stranded DNA. *The Journal of Biological Chemistry*, *283*(36), 24837–24847.

Martin-Verstraete, I., Débarbouillé, M., Klier, A., & Rapoport, G. (1994). Interactions of wild-type and truncated LevR of *Bacillus subtilis* with the upstream activating sequence of the levanase operon. *Journal of Molecular Biology*, *241*(2), 178–192.

Matsuura, M., Yamada, H., Karai, N., & Tani, Y. (1983). Effect of intracellular concentration of vitamin B6 on tryptophanase activity in *Escherichia coli*. *Agricultural and Biological Chemistry*, *47*(8), 1717–1724.

Matxain, J. M., Padro, D., Ristilä, M., Strid, Å., & Eriksson, L. A. (2009). Evidence of high •oh radical quenching efficiency by vitamin B6. *The Journal of Physical Chemistry B*, *113*(29), 9629–9632.

Matxain, J. M., Ristilä, M., Strid, Å., & Eriksson, L. A. (2006). Theoretical study of the antioxidant properties of pyridoxine. *The Journal of Physical Chemistry A*, *110*(48), 13068–13072.

McGregor, N., Ayora, S., Sedelnikova, S., Carrasco, B., Alonso, J. C., Thaw, P., & Rafferty, J. (2005). The structure of *Bacillus subtilis* RecU Holliday junction resolvase and its role in substrate selection and sequence-specific cleavage. *Structure (London, England : 1993)*, *13*(9), 1341–1351.

McLoon, A. L., Guttenplan, S. B., Kearns, D. B., Kolter, R., & Losick, R. (2011). Tracing the domestication of a biofilm-forming bacterium. *Journal of Bacteriology*, *193*(8), 2027–2034.

Mehta, P. K., & Christen, P. (2000). The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. In *Advances in Enzymology and Related Areas of Molecular Biology*,74, 129–184.

Meima, R., Haijema, B. J., Venema, G., & Bron, S. (1995). Overproduction of the ATP-dependent nuclease AddAB improves the structural stability of a model plasmid system in *Bacillus subtilis*. *Molecular & General Genetics : MGG*, *248*(4), 391–398.

Merzbacher, M., Detsch, C., Hillen, W., & Stulke, J. (2004). *Mycoplasma pneumoniae* HPr kinase/phosphorylase. *European Journal of Biochemistry*, *271*(2), 367–374.

Mikheyeva, I. V, Thomas, J. M., Kolar, S. L., Corvaglia, A.-R., Gaïa, N., Leo, S., Francois, P., Liu, G. Y., Rawat, M., & Cheung, A. L. (2019). YpdA, a putative bacillithiol disulfide reductase, contributes to cellular redox homeostasis and virulence in *Staphylococcus aureus*. *Molecular Microbiology*, *111*(4), 1039–1056.

Milano, T., Contestabile, R., Lo Presti, A., Ciccozzi, M., & Pascarella, S. (2015). The aspartate aminotransferase-like domain of Firmicutes MocR transcriptional regulators. *Computational Biology and Chemistry*, *58*, 55–61.

Milano, T., Gulzar, A., Narzi, D., Guidoni, L., & Pascarella, S. (2017). Molecular dynamics simulation unveils the conformational flexibility of the interdomain linker in the bacterial transcriptional regulator GabR from *Bacillus subtilis* bound to pyridoxal 5'-phosphate. *PLoS ONE*, *12*(12), e0189270. https://doi.org/10.1371/journal.pone.0189270

Mills, P. B., Surtees, R. A. H., Champion, M. P., Beesley, C. E., Dalton, N., Scamber, P. J., Heales, S. J. R., Briddon, A., Scheimberg, I., Hoffmann, G. F., Zschocke, J., & Clayton, P. T. (2005). Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5'-phosphate oxidase. *Human Molecular Genetics*, *14*(8), 1077–1086.

Minami, J., Kishi, T., & Kondo, M. (1982). Effects of thiamin on vitamin B6 synthesis in yeasts. *Journal of General Microbiology*, *128*(12), 2909–2917.

Mirouze, N., Bidnenko, E., Noirot, P., & Auger, S. (2015). Genome-wide mapping of TnrA-binding sites provides new insights into the TnrA regulon in *Bacillus subtilis*. *MicrobiologyOpen*, *4*(3), 423–435.

Mittenhuber, G. (2001). Phylogenetic analyses and comparative genomics of vitamin B6 (pyridoxine) and pyridoxal phosphate biosynthesis pathways. *Journal of Molecular Microbiology and Biotechnology*, *3*(1), 1–20.

Miyamoto, T., Katane, M., Saitoh, Y., Sekine, M., & Homma, H. (2020). Involvement of penicillin-binding proteins in the metabolism of a bacterial peptidoglycan containing a non-canonical D-amino acid. *Amino Acids*, *52*(3), 487–497.

Mizushina, Y., Xu, X., Matsubara, K., Murakami, C., Kuriyama, I., Oshige, M., Takemura, M., Kato, N., Yoshida, H., & Sakaguchi, K. (2003). Pyridoxal 5'-phosphate is a selective inhibitor in *vivo* of DNA polymerase *α* and *ε*. *Biochemical and Biophysical Research Communications*, *312*(4), 1025–1032.

Moccand, C., Boycheva, S., Surriabre, P., Tambasco-Studart, M., Raschke, M., Kaufmann, M., & Fitzpatrick, T. B. (2014). The pseudoenzyme PDX1.2 boosts vitamin B6 biosynthesis under heat and oxidative stress in *Arabidopsis*. *Journal of Biological Chemistry*, *289*(12), 8203–8216.

Moccand, C., Kaufmann, M., & Fitzpatrick, T. B. (2011). It takes two to tango: Defining an essential second active site in pyridoxal 5'-phosphate synthase. *PLoS ONE*, *6*(1), 16042.

Molle, V., Fujita, M., Jensen, S. T., Eichenberger, P., González-Pastor, J. E., Liu, J. S., & Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Molecular Microbiology*, *50*(5), 1683–1701.

Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., & Sonenshein, A. L. (2003). Additional targets of the *Bacillus subtilis* global regulator *codY* identified by chromatin immunoprecipitation and genome-wide transcript analysis. *Journal of Bacteriology*, *185*(6), 1911–1922.

Mols, M., & Abee, T. (2011). Primary and secondary oxidative stress in *Bacillus*. *Environmental Microbiology*, *13*(6), 1387–1394.

Mols, M., Pier, I., Zwietering, M. H., & Abee, T. (2009). The impact of oxygen availability on stress survival and radical formation of *Bacillus cereus*. *International Journal of Food Microbiology*, *135*(3), 303–311.

Mooney, S., & Hellmann, H. (2010). Vitamin B6: Killing two birds with one stone. *Phytochemistry*, *71*(5–6), 495–501.

Mooney, S., Leuendorf, J. E., Hendrickson, C., & Hellmann, H. (2009). Vitamin B6: A long known compound of surprising complexity. *Molecules*, *14*(1), 329–351.

Morag, A. S., Saveson, C. J., & Lovett, S. T. (1999). Expansion of DNA repeats in *Escherichia coli*: effects of recombination and replication functions. *Journal of Molecular Biology*, *289*(1), 21–27.

Morett, E., Saab-Rincón, G., Olvera, L., Olvera, M., Flores, H., & Grande, R. (2008). Sensitive genome-wide screen for low secondary enzymatic activities: The YjbQ family shows thiamin phosphate synthase activity. *Journal of Molecular Biology*, *376*(3), 839–853.

Morimatsu, K., Wu, Y., & Kowalczykowski, S. C. (2012). RecFOR proteins target RecA protein to a DNA gap with either DNA or RNA at the 5' terminus: implication for repair of stalled replication forks. *The Journal of Biological Chemistry*, *287*(42), 35621–35630.

Mossessova, E., & Lima, C. D. (2000). Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Molecular Cell*, *5*(5), 865–876.

Msadek, T., Kunst, F., Klier, A., & Rapoport, G. (1991). DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *Journal of Bacteriology*, *173*(7), 2366–2377.

Mukherjee, T., Hanes, J., Tews, I., Ealick, S. E., & Begley, T. P. (2011). Pyridoxal phosphate: Biosynthesis and catabolism. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1814*(11), 1585–1596.

Mukherjee, T., Hilmey, D. G., & Begley, T. P. (2008). PLP catabolism: Identification of the 2-(acetamidomethylene)succinate hydrolase gene in *Mesorhizobium loti* MAFF303099. *Biochemistry*, *47*(23), 6233–6241.

Murray, D. S., Chinnam, N., Tonthat, N. K., Whitfill, T., Wray, L. V. J., Fisher, S. H., & Schumacher, M. A. (2013). Structures of the *Bacillus subtilis* glutamine synthetase dodecamer reveal large intersubunit catalytic conformational changes linked to a unique feedback inhibition mechanism. *The Journal of Biological Chemistry*, *288*(50), 35801–35811.

Nagahashi, Y., Tazoe, M., & Hoshino, T. (2008). Cloning of the pyridoxine 5'-phosphate phosphatase gene (*pdxP*) and vitamin B6 production in *pdxP* recombinant *Sinorhizobium meliloti*. *Bioscience, Biotechnology and Biochemistry*, *72*(2), 421–427.

Nakano, S., Erwin, K. N., Ralle, M., & Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Molecular Microbiology*, *55*(2), 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A. D., & Zuber, P. (2003). Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(23), 13603–13608.

Nakano, S.,. M., Zhang, Y., Leelakriangsak, M., & Zuber, P. (2003). A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 4233–4238.

Nam, H., Lewis, N. E., Lerman, J. A., Lee, D. H., Chang, R. L., Kim, D., & Palsson, B. O. (2012). Network context and selection in the evolution to enzyme specificity. *Science*, *337*(6098), 1101–1104.

Nesterenko, M. V, Tilley, M., & Upton, S. J. (1994). A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *Journal of Biochemical and Biophysical Methods*, *28*(3), 239–242.

Neuwirth, M., Strohmeier, M., Windeisen, V., Wallner, S., Deller, S., Rippe, K., Sinning, I., Macheroux, P., & Tews, I. (2009). X-ray crystal structure of *Saccharomyces cerevisiae* Pdx1 provides insights into the oligomeric nature of PLP synthases. *FEBS Letters*, *583*(13), 2179–2186.

Newberry, K. J., Nakano, S., Zuber, P., & Brennan, R. G. (2005). Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the *α* C-terminal domain of RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(44), 15839–15844.

Newton, G. L., Buchmeier, N., & Fahey, R. C. (2008). Biosynthesis and functions of mycothiol, the unique protective thiol of actinobacteria. *Microbiology and Molecular Biology Reviews*, *72*(3), 471–494.

Newton, G. L., Leung, S. S., Wakabayashi, J. I., Rawat, M., & Fahey, R. C. (2011). The DinB superfamily includes novel mycothiol, bacillithiol, and glutathione S-transferases. *Biochemistry*, *50*(49), 10751–10760.

Newton, G. L., Rawat, M., La Clair, J. J., Jothivasan, V. K., Budiarto, T., Hamilton, C. J., Claiborne, A., Helmann, J. D., Fahey, R. C., Clair, J. J. La, Jothivasan, V. K., Hamilton, C. J., Claiborne, A., Helmann, J. D., Fahey, R. C., La Clair, J. J., Jothivasan, V. K., Budiarto, T., Hamilton, C. J., … Fahey, R. C. (2012). Bacillithiol is an antioxidant thiol produced in *Bacilli*. *Nature Chemical Biology*, *5*(9), 625–627.

Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S., Becher, D., Bisicchia, P., Botella, E., Delumeau, O., Doherty, G., Denham, E. L., Fogg, M. J., Fromion, V., Goelzer, A., … Noirot, P. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, *335*(6072), 1103–1106.

Notebaart, R. A., Kintses, B., Feist, A. M., & Papp, B. (2018). Underground metabolism: network-level perspective and biotechnological potential. *Current Opinion in Biotechnology*, *49*, 108–114.

Oberhardt, M. A., Zarecki, R., Reshef, L., Xia, F., Duran-Frigola, M., Schreiber, R., Henry, C. S., Ben-Tal, N., Dwyer, D. J., Gophna, U., & Ruppin, E. (2016). Systems-wide prediction of enzyme promiscuity reveals a new underground alternative route for pyridoxal 5'-phosphate production in *E. coli*. *PLoS Computational Biology*, *12*(1), e1004705. https://doi.org/10.1371/journal.pcbi.1004705

Ohsawa, H., & Gualerzi, O. (1981). Structure-function relationship in *Escherichia coli* initiation factors. Identification of a residue in the ribosomal binding site of initiation factor by site-specific chemical modification with pyridoxal phosphate. *Journal of Biological Chemistry*, *256*(10), 4905–4912.

Oka, T., Sugitatsu, H., Nordin, H., Thakur, M. K., Aoyama, M., Sasagawa, T., Suzuki, I., & Tsuji, H. (2001). Pyridoxal 5'-phosphate inhibits DNA binding of HNF1. *Biochimica et Biophysica Acta - General Subjects*, *1568*(3), 189–196.

Okuda, K., Ito, T., Goto, M., Takenaka, T., Hemmi, H., & Yoshimura, T. (2015). Domain characterization of *Bacillus subtilis* GabR, a pyridoxal 5-phosphate-dependent transcriptional regulator. *Journal of Biochemistry*, *158*(3), 225–234.

Okuda, K., Kato, S., Ito, T., Shiraki, S., Kawase, Y., Goto, M., Kawashima, S., Hemmi, H., Fukada, H., & Yoshimura, T. (2015). Role of the aminotransferase domain in *Bacillus subtilis* GabR, a pyridoxal 5'-phosphate-dependent transcriptional regulator. *Molecular Microbiology*, *95*(2), 245–257.

Padan, E., Bibi, E., Ito, M., & Krulwich, T. A. (2005). Alkaline pH homeostasis in bacteria: new insights. *Biochimica et Biophysica Acta*, *1717*(2), 67–88.

Page, A. N., George, N. P., Marceau, A. H., Cox, M. M., & Keck, J. L. (2011). Structure and biochemical activities of *Escherichia coli* MgsA. *The Journal of Biological Chemistry*, *286*(14), 12075–12085.

Park, J.-H. H., Burns, K., Kinsland, C., & Begley, T. P. (2004). Characterization of two kinases involved in thiamine pyrophosphate and pyridoxal phosphate biosynthesis in *Bacillus subtilis*: 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase and pyridoxal kinase. *Journal of Bacteriology*, *186*(5), 1571–1573.

Park, S. A., Park, Y. S., & Lee, K. S. (2017). Crystal structure of the C-terminal domain of *Bacillus subtilis* GabR reveals a closed conformation by *γ*-aminobutyric acid binding, inducing transcriptional activation. *Biochemical and Biophysical Research Communications*, *487*(2), 287–291.

Parris, K. D., Lin, L., Tam, A., Mathew, R., Hixon, J., Stahl, M., Fritz, C. C., Seehra, J., & Somers, W. S. (2000). Crystal structures of substrate binding to *Bacillus subtilis* holo-(acyl carrier protein) synthase reveal a novel trimeric arrangement of molecules resulting in three active sites. *Structure*, *8*(8), 883–895.

Paxhia, M. D., & Downs, D. M. (2019). SNZ3 encodes a PLP synthase involved in thiamine synthesis in *Saccharomyces cerevisiae*. *G3: Genes, Genomes, Genetics*, *9*(2), 335–344.

Pease, A. J., Roa, B. R., Luo, W., & Winkler, M. E. (2002). Positive growth rate-dependent regulation of the *pdxA*, *ksgA*, and *pdxB* genes of *Escherichia coli* K-12. *Journal of Bacteriology*, *184*(5), 1359–1369.

Peng, H., Shen, J., Edmonds, K. A., Luebke, J. L., Hickey, A. K., Palmer, L. D., Chang, F.-M. J., Bruce, K. A., Kehl-Fie, T. E., Skaar, E. P., & Giedroc, D. P. (2017a). Sulfide homeostasis and nitroxyl intersect via formation of reactive sulfur species in *Staphylococcus aureus*. *MSphere*, *2*(3), 1–21.

Peng, H., Zhang, Y., Palmer, L. D., Kehl-Fie, T. E., Skaar, E. P., Trinidad, J. C., & Giedroc, D. P. (2017b). Hydrogen sulfide and reactive sulfur species impact proteome s-sulfhydration and global virulence regulation in *Staphylococcus aureus*. *ACS Infectious Diseases*, *3*(10), 744–755.

Percudani, R., & Peracchi, A. (2003). A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO Reports*, *4*(9), 850–854.

Percudani, R., & Peracchi, A. (2009). The B6 database: A tool for the description and classification of vitamin B6-dependent enzymatic activities and of the corresponding protein families. *BMC Bioinformatics*, *10*(1), 273.

Perera, V. R., Newton, G. L., Parnell, J. M., Komives, E. A., & Pogliano, K. (2014). Purification and characterization of the *Staphylococcus aureus* bacillithiol transferase BstA. *Biochimica et Biophysica Acta*, *1840*(9), 2851–2861.

Pertea, M., Shumate, A., Pertea, G., Varabyou, A., Chang, Y.-C., Madugundu, A. K., Pandey, A., & Salzberg, S. L. (2018). Thousands of large-scale RNA sequencing experiments yield a comprehensive new human gene list and reveal extensive transcriptional noise. *BioRxiv*, 332825. https://doi.org/10.1101/332825

Peterson, B. N., Young, M. K. M., Luo, S., Wang, J., Whiteley, A. T., Woodward, J. J., Tong, L., Wang, J. D., & Portnoy, D. A. (2020). (P)ppGpp and c-di-AMP homeostasis is controlled by Cbpb in *Listeria monocytogenes*. *MBio*, *11*(4), 1–16.

Petes, T. D., & Hill, C. W. (1988). Recombination between repeated genes in microorganisms. *Annual Review of Genetics*, *22*, 147–168.

Pettersson, M. E., Sun, S., Andersson, D. I., & Berg, O. G. (2008). Evolution of new gene functions: simulation and analysis of the amplification model. *Genetica*, *135*(3), 309.

Pinder, D. J., Blake, C. E., Lindsey, J. C., & Leach, D. R. (1998). Replication strand preference for deletions associated with DNA palindromes. *Molecular Microbiology*, *28*(4), 719–727.

Potter, A. J., Trappetti, C., & Paton, J. C. (2012). *Streptococcus pneumoniae* uses glutathione to defend against oxidative stress and metal ion toxicity. *Journal of Bacteriology*, *194*(22), 6248–6254.

Prunetti, L., El Yacoubi, B., Schiavon, C. R., Kirkpatrick, E., Huang, L., Bailly, M., El Badawi-Sidhu, M., Harrison, K., Gregory, J. F., Fiehn, O., Hanson, A. D., & De Crécy-Lagard, V. (2016). Evidence that COG0325 proteins are involved in PLP homeostasis. *Microbiology (United Kingdom)*, *162*(4), 694–706.

Pysz, M. A., Conners, S. B., Montero, C. I., Shockley, K. R., Johnson, M. R., Ward, D. E., & Kelly, R. M. (2004). Transcriptional analysis of biofilm formation processes in the anaerobic, hyperthermophilic bacterium *Thermotoga maritima*. *Applied and Environmental Microbiology*, *70*(10), 6098–6112.

Qaidi, S. El, Yang, J., Zhang, J. R., Metzger, D. W., & Bai, G. (2013). The vitamin B6 biosynthesis pathway in *Streptococcus pneumoniae* is controlled by pyridoxal 5'-phosphate and the transcription factor PdxR and has an impact on ear infection. *Journal of Bacteriology*, *195*(10), 2187–2196.

Rajan, S. S., Yang, X., Shuvalova, L., Collart, F., & Anderson, W. F. (2004). YfiT from *Bacillus subtilis* is a probable metal-dependent hydrolase with an unusual four-helix bundle topology. *Biochemistry*, *43*(49), 15472–15479.

Ramos, R. J., Albersen, M., Vringer, E., Bosma, M., Zwakenberg, S., Zwartkruis, F., Jans, J. J. M., & Verhoeven-Duif, N. M. (2019). Discovery of pyridoxal reductase activity as part of human vitamin B6 metabolism. *Biochimica et Biophysica Acta - General Subjects*, *1863*(6), 1088–1097.

Rao, F., See, R. Y., Zhang, D., Toh, D. C., Ji, Q., & Liang, Z. X. (2010). YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *Journal of Biological Chemistry*, *285*(1), 473–482.

Raschle, T., Amrhein, N., & Fitzpatrick, T. B. (2005). On the Two Components of Pyridoxal 5-Phosphate Synthase from *Bacillus subtilis*. *(37) Journal of Biological Chemistry*, *280*(37), 32291–32300.

Ravikumar, V., Nalpas, N. C., Anselm, V., Krug, K., Lenuzzi, M., Šestak, M. S., Domazet-Lošo, T., Mijakovic, I., & Macek, B. (2018). In-depth analysis of *Bacillus subtilis* proteome identifies new ORFs and traces the evolutionary history of modified proteins. *Scientific Reports*, *8*(1), 17246.

Reams, A. B., Kofoid, E., Duleba, N., & Roth, J. R. (2014). Recombination and annealing pathways compete for substrates in making rrn duplications in *Salmonella enterica*. *Genetics*, *196*(1), 119–135.

Reams, A. B., Kofoid, E., Savageau, M., & Roth, J. R. (2010). Duplication frequency in a population of *Salmonella enterica* rapidly approaches steady state with or without recombination. *Genetics*, *184*(4), 1077–1094.

Reams, A. B., & Neidle, E. L. (2004). Selection for gene clustering by tandem duplication. *Annual Review of Microbiology*, *58*, 119–142.

Reams, A. B., & Roth, J. R. (2015). Mechanisms of gene duplication and amplification. *Cold Spring Harbor Perspectives in Biology*, *7*(2), a016592. https://doi.org/10.1101/cshperspect.a016592

Reuß, D. R., Altenbuchner, J., Mäder, U., Rath, H., Ischebeck, T., Sappa, P. K., Thürmer, A., Guérin, C., Nicolas, P., Steil, L., Zhu, B., Feussner, I., Klumpp, S., Daniel, R., Commichau, F. M., Völker, U., & Stülke, J. (2017). Large-scale reduction of the *Bacillus subtilis* genome: consequences for the transcriptional network, resource allocation, and metabolism. *Genome Research*, *27*(2), 289–299.

Reuß, D. R., Commichau, F. M., Gundlach, J., Zhu, B., & Stülke, J. (2016). The blueprint of a minimal cell: *MiniBacillus*. *Microbiology and Molecular Biology Reviews*, *80*(4), 955–987.

Reuß, D. R., Faßhauer, P., Mroch, P. J., Ul-Haq, I., Koo, B. M., Pöhlein, A., Gross, C. A., Daniel, R., Brantl, S., & Stülke, J. (2019). Topoisomerase IV can functionally replace all type 1A topoisomerases in *Bacillus subtilis*. *Nucleic Acids Research*, *47*(10), 5231–5242.

Revuelta, J. L., Buey, R. M., Ledesma-Amaro, R., & Vandamme, E. J. (2016). Microbial biotechnology for the synthesis of (pro)vitamins, biopigments and antioxidants: challenges and opportunities. *Microbial Biotechnology*, *9*(5), 564–567.

Reyes, A. M., Pedre, B., De Armas, M. I., Tossounian, M. A., Radi, R., Messens, J., & Trujillo, M. (2018). Chemistry and redox biology of mycothiol. *Antioxidants and Redox Signaling*, *28*(6), 487–504.

Richts, B., & Commichau, F. M. (2021). Underground metabolism facilitates the evolution of novel pathways for vitamin B6 biosynthesis. *Applied Microbiology and Biotechnology*, *105*(6), 2297–2305.

Richts, B., Hertel, R., Potot, S., Poehlein, A., Daniel, R., Schyns, G., Prágai, Z., & Commichau, F. M. (2020). Complete genome sequence of the prototrophic *Bacillus subtilis* subsp. subtilis strain SP1. *Microbiology Resource Announcements*, *9*(32), e00825-20.

Richts, B., Lentes, S., Poehlein, A., Daniel, R., & Commichau, F. M. (2021). A *Bacillus subtilis* Δ*pdxT* mutant suppresses vitamin B6 limitation by acquiring mutations enhancing *pdxS* gene dosage and ammonium assimilation. *Environmental Microbiology Reports*, *13*(2), 218–233.

Richts, B., Rosenberg, J., & Commichau, F. M. (2019). A survey of pyridoxal 5'-phosphate-dependent proteins in the Gram-positive model bacterium *Bacillus subtilis*. *Frontiers in Molecular Biosciences*, *6*, 32. https://doi.org/10.3389/fmolb.2019.00032

Roberts, A. A., Sharma, S. V., Strankman, A. W., Duran, S. R., Rawat, M., & Hamilton, C. J. (2013). Mechanistic studies of FosB: a divalent-metal-dependent bacillithiol-S-transferase that mediates fosfomycin resistance in *Staphylococcus aureus*. *Biochemical Journal*, *451*(1), 69–79.

Rogers, P. A., & Ding, H. (2001). L-cysteine-mediated destabilization of dinitrosyl iron complexes in proteins. *The Journal of Biological Chemistry*, *276*(33), 30980–30986.

Romero, D, & Palacios, R. (1997). Gene amplification and genomic plasticity in prokaryotes. *Annual Review of Genetics*, *31*, 91–111.

Romero, Diego, Aguilar, C., Losick, R., & Kolter, R. (2010). Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(5), 2230–2234.

Romero, H., Serrano, E., Hernández-Tamayo, R., Carrasco, B., Cárdenas, P. P., Ayora, S., Graumann, P. L., & Alonso, J. C. (2020). *Bacillus subtilis* RarA Acts as a Positive RecA Accessory Protein. *Frontiers in Microbiology*, *11*, 92.

Romo, A. J., & Liu, H. W. (2011). Mechanisms and structures of vitamin B6-dependent enzymes involved in deoxy sugar biosynthesis. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1814*(11), 1534–1547.

Rosales-Mendoza, S., & Angulo, C. (2015). *Bacillus subtilis* comes of age as a vaccine production host and delivery vehicle. *Expert Review of Vaccines*, *14*(8), 1135–1148.

Rosario-Cruz, Z., Chahal, H. K., Mike, L. A., Skaar, E. P., & Boyd, J. M. (2015). Bacillithiol has a role in Fe-S cluster biogenesis in *Staphylococcus aureus*. *Molecular Microbiology*, *98*(2), 218–242.

Rosenberg, I. H. (2012). A history of the isolation and identification of vitamin B6. *Annals of Nutrition and Metabolism*, *61*(3), 236–238.

Rosenberg, J. (2017). http://hdl.handle.net/11858/00-1735-0000-002E-E32C-B

Rosenberg, J., & Commichau, F. M. (2018). Harnessing underground metabolism for pathway development. *Trends in Biotechnology*, *37*(1), 29–37.

Rosenberg, J., Ischebeck, T., & Commichau, F. M. (2016). Vitamin B6 metabolism in microbes and approaches for fermentative production. *Biotechnology Advances*, *35*(1), 31–40.

Rosenberg, J., Müller, P., Lentes, S., Thiele, M. J., Zeigler, D. R., Tödter, D., Paulus, H., Brantl, S., Stülke, J., & Commichau, F. M. (2016). ThrR, a DNA-binding transcription factor involved in controlling threonine biosynthesis in *Bacillus subtilis*. *Molecular Microbiology*, *101*(5), 879–893.

Rosenberg, J., Richts, B., & Commichau, F. M. (2020). Fermentative production of vitamin b6. *Pharmaceutical Biocatalysis*, 1–34.

Rosenberg, J., Yeak, K. Y. C., & Commichau, F. M. (2018). A two-step evolutionary process establishes a non-native vitamin B6 pathway in *Bacillus subtilis*. *Environmental Microbiology*, *20*(1), 156–168.

Roth, J. R., Benson, N., Galitsky, T., Haack, K., Lawrence, J. G., & Miesel, L. (1996). Rearrangement of the bacterial chromosome: Formation and Applications. In FC Neidhardt, R Curtiss III, JL Ingraham, ECC Lin, KB Low (Ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd ed.* (2nd ed., pp. 2256–76). Washington, D.C.: American Society for Microbiology.

Rudolph, J., Kim, J., & Copley, S. D. (2010). Multiple turnovers of the nicotino-enzyme PdxB require *α*-keto acids as cosubstrates. *Biochemistry*, *49*(43), 9249–9255.

Sachla, A. J., & Helmann, J. D. (2019). A bacterial checkpoint protein for ribosome assembly moonlights as an essential metabolite-proofreading enzyme. *Nature Communications*, *10*(1), 1526.

Safo, M. K., Musayev, F. N., Di Salvo, M. L., & Schirch, V. (2001). X-ray structure of *Escherichia coli* pyridoxine 5'-phosphate oxidase complexed with pyridoxal 5'-phosphate at 2.0 Å resolution. *Journal of Molecular Biology*, *310*(4), 817–826.

Safo, M. K., Musayev, F. N., & Schirch, V. (2005). Structure of *Escherichia coli* pyridoxine 5'-phosphate oxidase in a tetragonal crystal form: Insights into the mechanistic pathway of the enzyme. *Acta Crystallographica Section D: Biological Crystallography*, *61*(5), 599–604.

Saito, N., Robert, M., Kitamura, S., Baran, R., Soga, T., Mori, H., Nishioka, T., & Tomita, M. (2006). Metabolomics approach for enzyme discovery. *Journal of Proteome Research*, *5*(8), 1979–1987.

Sakai, A., Kita, M., Katsuragi, T., Ogasawara, N., & Tani, Y. (2002). *yaaD* and *yaaE* are involved in vitamin B6 biosynthesis in *Bacillus subtilis*. *Journal of Bioscience and Bioengineering*, *93*(3), 309–312.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, 3rd edn.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez, H., Carrasco, B., Ayora, S., & Alonso, J. C. (2007a). *Homologous Recombination in Low dC + dG Gram-Positive Bacteria BT - Molecular Genetics of Recombination* (A. Aguilera & R. Rothstein (eds.); 27–52). Springer Berlin Heidelberg. https://doi.org/10.1007/4735_2006_0203

Sanchez, H., Carrasco, B., Ayora, S., & Alonso, J. C. (2007b). Dynamics of DNA double-strand break repair in *Bacillus subtilis*. In P. Graumann (Ed.), *Bacillus: Cellular and Molecular Biology* (35–66). Caister Academic Press, U.K. https://doi.org/10.21775/9781910190579-02

Sanchez, H., Carrasco, B., Cozar, M. C., & Alonso, J. C. (2007). *Bacillus subtilis* RecG branch migration translocase is required for DNA repair and chromosomal segregation. *Molecular Microbiology*, *65*(4), 920–935.

Sanchez, H., Kidane, D., Castillo Cozar, M., Graumann, P. L., & Alonso, J. C. (2006). Recruitment of *Bacillus subtilis* RecN to DNA double-strand breaks in the absence of DNA end processing. *Journal of Bacteriology*, *188*(2), 353–360.

Sanger, F., Nicklen, S., & Coulson, A. R. (1992). DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology (Reading, Mass.)*, *24*, 104–108.

Sarge, S., Haase, I., Illarionov, B., Laudert, D., Hohmann, H. P., Bacher, A., & Fischer, M. (2015). Catalysis of an essential step in vitamin B2 biosynthesis by a consortium of broad spectrum hydrolases. *ChemBioChem*, *16*(17), 2466–2469.

Saveson, C. J., & Lovett, S. T. (1997). Enhanced deletion formation by aberrant DNA replication in *Escherichia coli*. *Genetics*, *146*(2), 457–470.

Schaeffer, M. C., Sampson, D. A., Skala, J. H., Gietzen, D. W., & Grier, R. E. (1989). Evaluation of vitamin B6 status and function of rats fed excess pyridoxine. *The Journal of Nutrition*, *119*(10), 1392–1398.

Schoenlein, P. V, Roa, B. B., & Winkler, M. E. (1989). Divergent transcription of *pdxB* and homology between the *pdxB* and *serA* gene products in *Escherichia coli* K-12. *Journal of Bacteriology*, *171*(11), 6084 LP – 6092.

Schumacher, M. A., Chinnam, N. B., Cuthbert, B., Tonthat, N. K., & Whitfill, T. (2015). Structures of regulatory machinery reveal novel molecular mechanisms controlling *B. subtilis* nitrogen homeostasis. *Genes and Development*, *29*(4), 451–464.

Schuster, K., Bailey, L. B., Cerda, J. J., & Gregory, J. F. 3rd. (1984). Urinary 4-pyridoxic acid excretion in 24-hour versus random urine samples as a measurement of vitamin B6 status in humans. *The American Journal of Clinical Nutrition*, *39*(3), 466–470.

Schwechheimer, S. K., Park, E. Y., Revuelta, J. L., Becker, J., & Wittmann, C. (2016). Biotechnology of riboflavin. *Applied Microbiology and Biotechnology*, *100*(5), 2107–2119.

Scott, T. A., & Hockney, R. C. (1979). Synthesis of vitamin B6 by a mutant of *Escherichia coli* K12 and the action of 4'-deoxypyridoxine. *Journal of General Microbiology*, *110*(2), 285–289.

Seack, J., Perovic, S., Gamulin, V., Schröder, H. C., Beutelmann, P., Müller, I. M., & Müller, W. E. G. (2001). Identification of highly conserved genes: SNZ and SNO in the marine sponge *Suberites domuncula*: their gene structure and promoter activity in mammalian. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, *1520*(1), 21–34.

Sewalt, V., Shanahan, D., Gregg, L., La Marta, J., & Carrillo, R. (2016). The generally recognized as safe (GRAS) process for industrial microbial enzymes. *Industrial Biotechnology*, *12*(5), 295–302.

Shames, S. L., Ash, D. E., Wedler, F. C., & Villafranca, J. J. (1984). Interaction of aspartate and aspartate-derived antimetabolites with the enzymes of the threonine biosynthetic pathway of *Escherichia coli*. *Journal of Biological Chemistry*, *259*(24), 15331–15339.

Sharma, S. V., Arbach, M., Roberts, A. A., Macdonald, C. J., Groom, M., & Hamilton, C. J. (2013). Biophysical features of bacillithiol, the glutathione surrogate of *Bacillus subtilis* and other Firmicutes. *ChemBioChem*, *14*(16), 2160–2168.

Shemesh, M., & Chaia, Y. (2013). A combination of glycerol and manganese promotes biofilm formation in *Bacillus subtilis* via histidine kinase KinD signaling. *Journal of Bacteriology*, *195*(12), 2747–2754.

Shin, B. S., Choi, S. K., Smith, I., & Park, S. H. (2000). Analysis of *tnrA* alleles which result in a glucose-resistant sporulation phenotype in *Bacillus subtilis*. *Journal of Bacteriology*, *182*(17), 5009–5012.

Shinagawa, H., & Iwasaki, H. (1995). Molecular mechanisms of Holliday junction processing in *Escherichia coli*. *Advances in Biophysics*, *31*, 49–65.

Shyamala, V., Schneider, E., & Ames, G. F. (1990). Tandem chromosomal duplications: role of REP sequences in the recombination event at the join-point. *The EMBO Journal*, *9*(3), 939–946.

Simonen, M., & Palva, I. (1993). Protein secretion in *Bacillus* species. *Microbiological Reviews*, *57*(1), 109–137.

Slotboom, D. J. (2014). Structural and mechanistic insights into prokaryotic energy-coupling factor transporters. *Nature Reviews Microbiology*, *12*(2), 79–87.

Smirnov, S. V., Sokolov, P. M., Kodera, T., Sugiyama, M., Hibi, M., Shimizu, S., Yokozeki, K., & Ogawa, J. (2012). A novel family of bacterial dioxygenases that catalyse the hydroxylation of free l-amino acids. *FEMS Microbiology Letters*, *331*(2), 97–104.

Smith, A. M., Brown, W. C., Harms, E., & Smith, J. L. (2015). Crystal structures capture three states in the catalytic cycle of a pyridoxal phosphate (PLP) Synthase. *Journal of Biological Chemistry*, *290*(9), 5226–5239.

Sonenshein, A. L., Hoch, J. A., & Losick, R. (1993). *Bacillus subtilis and other Gram-positive bacteria : biochemistry, physiology, and molecular genetics*. American Society for Microbiology. Doi:10.1128/9781555818388.

Sonti, R. V, & Roth, J. R. (1989). Role of gene duplications in the adaptation of *Salmonella typhimurium* to growth on limiting carbon sources. *Genetics*, *123*(1), 19–28.

Soonsanga, S., Fuangthong, M., & Helmann, J. D. (2007). Mutational analysis of active site residues essential for sensing of organic hydroperoxides by *Bacillus subtilis* OhrR. *Journal of Bacteriology*, *189*(19), 7069–7076. https://doi.org/10.1128/JB.00879-07

Soonsanga, S., Lee, J. W., & Helmann, J. D. (2008). Conversion of *Bacillus subtilis* OhrR from a 1-Cys to a 2-Cys peroxide sensor. *Journal of Bacteriology*, *190*(17), 5738–5745.

Spies, M., & Kowalczykowski, S. C. (2004). Homologous Recombination by the RecBCD and RecF Pathways. In *The Bacterial Chromosome*, 389–403.

Spiro, S. (2007). Regulators of bacterial responses to nitric oxide. *FEMS Microbiology Reviews*, *31*(2), 193–211.

Stannek, L., Thiele, M. J., Ischebeck, T., Gunka, K., Hammer, E., Völker, U., & Commichau, F. M. (2015). Evidence for synergistic control of glutamate biosynthesis by glutamate dehydrogenases and glutamate in *Bacillus subtilis*. *Environmental Microbiology*, *17*(9), 3379–3390.

Stolz, J., & Vielreicher, M. (2003). Tpn1p, the plasma membrane vitamin B6 transporter of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *278*(21), 18990–18996.

Stolz, J., Wöhrmann, H. J. P., & Vogl, C. (2005). Amiloride uptake and toxicity in fission yeast are caused by the pyridoxine transporter encoded by bsu1^{+} (car1^{+}). *Eukaryotic Cell*, *4*(2), 319–326.

Straus, D. S. (1975). Selection for a large genetic duplication in *Salmonella typhimurium*. *Genetics*, *80*(2), 227–237.

Straus, D. S. (1976). Large overlapping tandem genetic duplications in *Salmonella typhimurium*. *Journal of Molecular Biology*, *103*(1), 143–153.

Strohmeier, M., Raschle, T., Mazurkiewicz, J., Rippe, K., Sinning, I., Fitzpatrick, T. B., & Tews, I. (2006). Structure of a bacterial pyridoxal 5'-phosphate synthase complex. *Proceedings of the National Academy of Sciences*, *103*(51), 19284–19289.

Strohmeier, Marco, Raschle, T., Mazurkiewicz, J., Rippe, K., Sinning, I., Fitzpatrick, T. B., & Tews, I. (2006). Structure of a bacterial pyridoxal 5'-phosphate synthase complex. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(51), 19284–19289.

Studier, F. W., & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology*, *189*(1), 113–130.

Sugimoto, R., Saito, N., Shimada, T., & Tanaka, K. (2017). Identification of YbhA as the pyridoxal 5'-phosphate (PLP) phosphatase in *Escherichia coli*: Importance of PLP homeostasis on the bacterial growth. *Journal of General and Applied Microbiology*, *63*(6), 362–368.

Sun, G., Yang, M., Jiang, L., & Huang, M. (2021). Regulation of pro-*σ*(K) activation: a key checkpoint in *Bacillus subtilis* sporulation. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.15415

Sundararaj, S., Guo, A., Habibi-Nazhad, B., Rouani, M., Stothard, P., Ellison, M., & Wishart, D. S. (2004). The CyberCell Database (CCDB): A comprehensive, self-updating, relational database to coordinate and facilitate in silico modeling of *Escherichia coli*. *Nucleic Acids Research*, *32*(DATABASE ISS.), D293–D295.

Suvorova, I. A., & Rodionov, D. A. (2016). Comparative genomics of pyridoxal 5'-phosphate-dependent transcription factor regulons in Bacteria. *Microbial Genomics*, *2*(1), e000047. https://doi.org/10.1099/mgen.0.000047

Szydlowski, N., Bürkle, L., Pourcel, L., Moulin, M., Stolz, J., & Fitzpatrick, T. B. (2013). Recycling of pyridoxine (vitamin B6) by PUP1 in *Arabidopsis*. *Plant Journal*, *75*(1), 40–52.

Tanaka, T., Tateno, Y., & Gojobori, T. (2005). Evolution of vitamin B6 (pyridoxine) metabolism by gain and loss of genes. *Molecular Biology and Evolution*, *22*(2), 243–250.

Tatsuo, H., Keiko, I., & Masaaki, T. (2006). Recombinant microorganism for the production of vitamin B6 (Patent No. US2006/0228785 A1.). In *US patent 10/528,891* (US2006/0228785 A1.). US Patent Application.

Tazoe, M., Ichikawa, K., & Hoshino, T. (2005). Purification and characterization of pyridoxine 5'-phosphate phosphatase from *Sinorhizobium meliloti*. *Bioscience, Biotechnology and Biochemistry*, *69*(12), 2277–2284.

Tazoe, M., Ichikawa, K., & Hoshino, T. (2006). Flavin adenine dinucleotide-dependent 4-phospho-D-erythronate dehydrogenase is responsible for the 4-phosphohydroxy-L-threonine pathway in vitamin B 6 biosynthesis in *Sinorhizobium meliloti*. *Journal of Bacteriology*, *188*(13), 4635–4645.

Thiaville, J. J., Flood, J., Yurgel, S., Prunetti, L., Elbadawi-Sidhu, M., Hutinet, G., Forouhar, F., Zhang, X., Ganesan, V., Reddy, P., Fiehn, O., Gerlt, J. A., Hunt, J. F., Copley, S. D., & De Crécy-Lagard, V. (2016). Members of a novel kinase family (DUF1537) can recycle toxic intermediates into an essential metabolite. *ACS Chemical Biology*, *11*(8), 2304–2311.

Tramonti, A., Fiascarelli, A., Milano, T., Di Salvo, M. L., Nogués, I., Pascarella, S., & Contestabile, R. (2015). Molecular mechanism of PdxR - A transcriptional activator involved in the regulation of vitamin B6 biosynthesis in the probiotic bacterium *Bacillus clausii*. *FEBS Journal*, *282*(15), 2966–2984.

Tramonti, A., Milano, T., Nardella, C., di Salvo, M. L., Pascarella, S., & Contestabile, R. (2017). *Salmonella typhimurium* PtsJ is a novel MocR-like transcriptional repressor involved in regulating the vitamin B6 salvage pathway. *FEBS Journal*, *284*(3), 466–484.

Tramonti, A., Nardella, C., di Salvo, M. L., Barile, A., D'Alessio, F., de Crécy-Lagard, V., & Contestabile, R. (2021). Knowns and unknowns of vitamin B6 metabolism in *Escherichia coli*. *EcoSal Plus*, *9*(2), 1–22.

Tramonti, A., Nardella, C., di Salvo, M. L., Pascarella, S., & Contestabile, R. (2018). The MocR-like transcription factors: pyridoxal 5'-phosphate-dependent regulators of bacterial metabolism. *FEBS Journal*, *285*(21), 3925–3944.

Trinh, T. Q., & Sinden, R. R. (1991). Preferential DNA secondary structure mutagenesis in the lagging strand of replication in E. coli. *Nature*, *352*(6335), 544–547.

Trumbo, P., Yates, A. A., Schlicker, S., & Poos, M. (2001). Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association*, *101*(3), 294–301.

Umezu, K., Chi, N. W., & Kolodner, R. D. (1993). Biochemical interaction of the *Escherichia coli* RecF, RecO, and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(9), 3875–3879.

Umezu, K., & Kolodner, R. D. (1994). Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. *The Journal of Biological Chemistry*, *269*(47), 30005–30013.

van Dijl, J. M., & Hecker, M. (2013). *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. In *Microbial cell factories* (Vol. 12, p. 3). https://doi.org/10.1186/1475-2859-12-3

Van Loi, V., Rossius, M., & Antelmann, H. (2015). Redox regulation by reversible protein S-thiolation in bacteria. *Frontiers in Microbiology*, *6*, 1–22.

van Tilburg, A. Y., Cao, H., van der Meulen, S. B., Solopova, A., & Kuipers, O. P. (2019). Metabolic engineering and synthetic biology employing *Lactococcus lactis* and *Bacillus subtilis* cell factories. *Current Opinion in Biotechnology*, *59*, 1–7.

Van Tilburg, A. Y., Van Heel, A. J., Stülke, J., De Kok, N. A. W., Rueff, A. S., & Kuipers, O. P. (2020). *MiniBacillus* PG10 as a convenient and effective production host for lantibiotics. *ACS Synthetic Biology*, *9*(7), 1833–1842.

Vandamme, E. J., & Revuelta, J. L. (2016). Vitamins, biopigments, antioxidants and related compounds: a historical, physiological and (bio)technological perspective. In *Industrial Biotechnology of Vitamins, Biopigments, and Antioxidants*, 1–14.

Vanderschuren, H., Boycheva, S., Li, K. Te, Szydlowski, N., Gruissem, W., & Fitzpatrick, T. B. (2013). Strategies for vitamin B6 biofortification of plants: A dual role as a micronutrient and a stress protectant. In *Frontiers in Plant Science 4*, (MAY), 143.

Varghese, S., Wu, A., Park, S., Imlay, K. R. C., & Imlay, J. A. (2007). Submicromolar hydrogen peroxide disrupts the ability of Fur protein to control free-iron levels in *Escherichia coli*. *Molecular Microbiology*, *64*(3), 822–830.

Vermeersh, J. J., Christmann-Franck, S., Karabashyan, L. V., Fermandjian, S., Mirambeau, G., & Der Garabedian, P. A. (2004). Pyridoxal 5'-phosphate inactivates DNA topoisomerase IB by modifying the lysine general acid. *Nucleic Acids Research*, *32*(18), 5649–5657.

Vu, H. N., & Downs, D. M. (2020). An unexpected role for the periplasmic phosphatase PhoN in the salvage of B6 vitamers in *Salmonella enterica*. *Applied and Environmental Microbiology,* 87 (3) e02300-20; doi:

Vu, H. N., Ito, T., & Downs, D. M. (2020). The role of YggS in Vitamin B6 homeostasis in *Salmonella enterica* is informed by heterologous expression of yeast SNZ3. *Journal of Bacteriology*, *202*(22), 1–10.

Wach, A. (1996). PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast (Chichester, England)*, *12*(3), 259–265.

Wada, K., & Haga, M. (1997). *Food Poisoning by Ginkgo biloba Seeds BT - Ginkgo Biloba A Global Treasure: From Biology to Medicine* (T. Hori, R. W. Ridge, W. Tulecke, P. Del Tredici, J. Trémouillaux-Guiller, & H. Tobe (eds.); 309–321. Springer Japan. https://doi.org/10.1007/978-4-431-68416-9_24

Wang, P. Z., & Doi, R. H. (1984). Overlapping promoters transcribed by *Bacillus subtilis* sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. *The Journal of Biological Chemistry*, *259*(13), 8619–8625.

Wang, T. C., Chang, H. Y., & Hung, J. L. (1993). Cosuppression of *recF*, *recR* and *recO* mutations by mutant *recA* alleles in *Escherichia coli* cells. *Mutation Research*, *294*(2), 157–166.

Wang, T., De Jesus, A. J., Shi, Y., & Yin, H. (2015). Pyridoxamine is a substrate of the energy-coupling factor transporter HmpT. *Cell Discovery*, *1*, 1–10.

Webb, B. L., Cox, M. M., & Inman, R. B. (1997). Recombinational DNA repair: the RecF and RecR proteins limit the extension of RecA filaments beyond single-strand DNA gaps. *Cell*, *91*(3), 347–356.

Weinrauch, Y., Msadek, T., Kunst, F., & Dubnau, D. (1991). Sequence and properties of *comQ*, a new competence regulatory gene of *Bacillus subtilis*. *Journal of Bacteriology*, *173*(18), 5685–5693.

Wenzel, M., & Altenbuchner, J. (2015). Development of a markerless gene deletion system for *Bacillus subtilis* based on the mannose phosphoenolpyruvate-dependent phosphotransferase system. *Microbiology (Reading, England)*, *161*(10), 1942–1949.

West, S. C. (1996). The RuvABC proteins and Holliday junction processing in *Escherichia coli*. *Journal of Bacteriology*, *178*(5), 1237–1241.

White, R. S., & Dempsey, W. B. (1970). Purification and properties of vitamin B6 kinase from *Escherichia coli. Biochemistry*, *9*(21), 4057–4064.

Wicke, D., Schulz, L. M., Lentes, S., Scholz, P., Poehlein, A., Gibhardt, J., Daniel, R., Ischebeck, T., & Commichau, F. M. (2019). Identification of the first glyphosate transporter by genomic adaptation. *Environmental Microbiology*, *21*(4), 1287–1305.

Williamson, V. M., & Doi, R. H. (1978). Delta factor can displace sigma factor from *Bacillus subtilis* RNA polymerase holoenzyme and regulate its initiation activity. *Molecular & General Genetics : MGG*, *161*(2), 135–141.

Winkler, C., Denker, K., Wortelkamp, S., & Sickmann, A. (2007). Silver- and coomassie-staining protocols: detection limits and compatibility with ESI MS. *Electrophoresis*, *28*(12), 2095–2099.

Winterbourn, C. C., & Hampton, M. B. (2008). Thiol chemistry and specificity in redox signaling. *Free Radical Biology & Medicine*, *45*(5), 549–561.

Wolf, M., Müller, T., Dandekar, T., & Pollack, J. D. (2004). Phylogeny of Firmicutes with special reference to *Mycoplasma* (*Mollicutes*) as inferred from phosphoglycerate kinase amino acid sequence data. *International Journal of Systematic and Evolutionary Microbiology*, *54*(Pt 3), 871–875.

Woodmansee, A. N., & Imlay, J. A. (2003). A mechanism by which nitric oxide accelerates the rate of oxidative DNA damage in *Escherichia coli*. *Molecular Microbiology*, *49*(1), 11–22.

Woolley, D. W. (1963). *Antimetabolites of the water-soluble vitamins*. In R. M. Hochster & J. H. B. T.-M. I. Quastel (Eds.), *Metabolic Inhibitors* (pp. 445–480). Academic Press.

Wray Jr, Lewis V, Ferson, A. E., Rohrer, K. & Fisher, S. H- (1996). TnrA, a transcription factor required for global nitrogen regulation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(17), 8841–8845.

Wray Jr, Lewis V, & Fisher, S. H. (2007). Functional analysis of the carboxy-terminal region of *Bacillus subtilis* TnrA, a MerR family protein. *Journal of Bacteriology*, *189*(1), 20–27.

Wray Jr, Lewis V., Atkinson, M. R., & Fisher, S. H. (1994). The nitrogen-regulated *Bacillus subtilis nrgAB* operon encodes a membrane protein and a protein highly similar to the *Escherichia coli glnB*-encoded P(II) protein. *Journal of Bacteriology*, *176*(1), 108–114.

Wray Jr, Lewis V., & Fisher, S. H. (2005). A feedback-resistant mutant of *Bacillus subtilis* glutamine synthetase with pleiotropic defects in nitrogen-regulated gene expression. *Journal of Biological Chemistry*, *280*(39), 33298–33304.

Wray Jr, Lewis V., & Fisher, S. H. (2010). Functional roles of the conserved Glu304 loop of *Bacillus subtilis* glutamine synthetase. *Journal of Bacteriology*, *192*(19), 5018–5025.

Wray Jr, Lewis V., Zalieckas, J. M., & Fisher, S. H. (2001). *Bacillus subtilis* glutamine synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA. *Cell*, *107*(4), 427–435.

Wu, R., Sanishvili, R., Belitsky, B. R., Juncosa, J. I., Le, H. V., Lehrer, H. J. S., Farley, M., Silverman, R. B., Petsko, G. A., Ringe, D., & Liu, D. (2017). PLP and GABA trigger GabR-mediated transcription regulation in *Bacillus subtilis* via external aldimine formation. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(15), 3891–3896.

Xue, X., Li, J., Wang, W., Sztajer, H., & Wagner-Döbler, I. (2012). The global impact of the delta subunit RpoE of the RNA polymerase on the proteome of *Streptococcus mutans*. *Microbiology (Reading, England)*, *158*(Pt 1), 191–206.

Xue, X., Tomasch, J., Sztajer, H., & Wagner-Döbler, I. (2010). The delta subunit of RNA polymerase, RpoE, is a global modulator of *Streptococcus mutans* environmental adaptation. *Journal of Bacteriology*, *192*(19), 5081–5092.

Yamada, Ryo hei, Tsuji, T., & Nose, Y. (1977). Uptake and utilization of vitamin B6 and its phosphate esters by *Escherichia coli*. *Journal of Nutritional Science and Vitaminology*, *23*(1), 7–17.

Yamada, Ryo hei, & Furukawa, Y. (1981). Role of pyridoxal kinase in vitamin B6 Uptake by *Escherichia coli*. *Journal of Nutritional Science and Vitaminology*, *27*(3), 177–191.

Yang, E. S., & Schirch, V. (2000). Tight binding of pyridoxal 5'-phosphate to recombinant *Escherichia coli* pyridoxine 5'-phosphate oxidase. *Archives of Biochemistry and Biophysics*, *377*(1), 109–114.

Yang, Y., Tsui, H. C. T., Man, T. K., & Winkler, M. E. (1998). Identification and function of the *pdxY* gene, which encodes a novel pyridoxal kinase involved in the salvage pathway of pyridoxal 5'-phosphate biosynthesis in *Escherichia coli* K-12. *Journal of Bacteriology*, *180*(7), 1814–1821.

Yang, Y., Zhao, G., & Winkler, M. E. (1996). Identification of the *pdxK* gene that encodes pyridoxine (vitamin B6) kinase in *Escherichia coli* K-12 . *FEMS Microbiology Letters*, *141*(1), 89–95.

Yeeles, J. T. P., & Dillingham, M. S. (2010). The processing of double-stranded DNA breaks for recombinational repair by helicase-nuclease complexes. *DNA Repair*, *9*(3), 276–285.

Yocum, R. R., Williams, M. K., & Pero, J. G. (2005). *Methods and organisms for production of b6 vitamers* (Patent No. 20050164335).

Yoshida, K. I., Yamaguchi, H., Kinehara, M., Ohki, Y. H., Nakaura, Y., & Fujita, Y. (2003). Identification of additional TnrA-regulated genes of *Bacillus subtilis* associated with a TnrA box. *Molecular Microbiology*, *49*(1), 157–165.

Yoshikane, Y., Yokochi, N., Ohnishi, K., Hayashi, H., & Yagi, T. (2006). Molecular cloning, expression and characterization of pyridoxamine-pyruvate aminotransferase. *Biochemical Journal*, *396*(3), 499–507.

Zalkin, H., & Smith, J. L. (1998). Enzymes utilizing glutamine as an amide donor. *Advances in Enzymology and Related Areas of Molecular Biology*, *72*, 87–144.

Zaprasis, A., Hoffmann, T., Wünsche, G., Flórez, L. A., Stülke, J., & Bremer, E. (2014). Mutational activation of the RocR activator and of a cryptic *rocDEF* promoter bypass loss of the initial steps of proline biosynthesis in *Bacillus subtilis*. *Environmental Microbiology*, *16*(3), 701–717.

Zeigler, D. R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., & Perkins, J. B. (2008). The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *Journal of Bacteriology*, *190*(21), 6983–6995.

Zhang, J., Weng, H., Zhou, Z., Du, G., & Kang, Z. (2019). Engineering of multiple modular pathways for high-yield production of 5-aminolevulinic acid in *Escherichia coli*. *Bioresource Technology*, *274*(November 2018), 353–360.

Zhang, P. (2013). Structure and mechanism of energy-coupling factor transporters. *Trends in Microbiology*, *21*(12), 652–659.

Zhang, X., Carter, M. S., Vetting, M. W., Francisco, B. S., Zhao, S., Al-Obaidi, N. F., Solbiati, J. O., Thiaville, J. J., Crécy-Lagard, V. De, Jacobson, M. P., Almo, S. C., & Gerlt, J. A. (2016). Assignment of function to a domain of unknown function: DUF1537 is a new kinase family in catabolic pathways for acid sugars. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(29), E4161–E4169.

Zhao, G., Pease, A. J., Bharani, N., & Winkler, M. E. (1995). Biochemical characterization of *gapB*-encoded erythrose 4-phosphate dehydrogenase of *Escherichia coli* K-12 and its possible role in pyridoxal 5'- phosphate biosynthesis. *Journal of Bacteriology*, *177*(10), 2804–2812.

Zhao, G., & Winkler, M. E. (1995). Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5'-phosphate oxidase of *Escherichia coli* K-12. *Journal of Bacteriology*, *177*(4), 883–891.

Zhu, B., & Stülke, J. (2018). SubtiWiki in 2018: From genes and proteins to functional network annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Research*, *46*(D1), D743–D748.

Zuber, P. (2009). Management of oxidative stress in *Bacillus*. *Annual Review of Microbiology*, *63*, 575–597.

Zuber, P., Chauhan, S., Pilaka, P., Nakano, M. M., Gurumoorthy, S., Lin, A. A., Barendt, S. M., Chi, B. K., Antelmann, H., & Mäder, U. (2011). Phenotype enhancement screen of a regulatory *spx* mutant unveils a role for the ytpQ

gene in the control of iron homeostasis. *PLoS ONE*, *6*(9), e25066. https://doi.org/10.1371/journal.pone.0025066

Zweers, J. C., Barák, I., Becher, D., Driessen, A. J. M., Hecker, M., Kontinen, V. P., Saller, M. J., Vavrová, L., & van Dijl, J. M. (2008). Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. *Microbial Cell Factories*, *7*(1), 10.

6. Appendix

6.1 Materials

Enzymes

Enzymes	Supplier
Dig Mix	ThermoFischer
DNase I	Roche Diagnostics, Mannheim
FastAP™	ThermoFisher, Waltham
Lysozyme	Merck, Darmstadt
Fusion™ DNA Polymerase	Biozym, Hessisch-Oldendorf
Restriction endonucleases	ThermoFischer, Waltham
RNase A	Roche Diagnostics, Mannheim
T4-DNA ligase	Roche Diagnostics, Mannheim
Taq DNA polymerase	ThermoFischer, Waltham

Equipment

Device	Manufacturer
Biofuge fresco	Heraeus Christ, Osterode
Blotting device VacuGeneTMXI	Amersham, Freiburg
ChemoCam imager	Intas, Göttingen
Cuvettes (microliter, plastic)	Sarstedt,
Electronic scale Sartorius universal	Sartorius, Göttingen
French pressure cell	G. Heinemann
Fiberlite F9 / F40 rotors	ThermoFisher, Bonn
GelDoc™ XR+	Bio-Rad, München
Gel electrophoresis device	Waasetec, Göttingen
Heating block Dri Block DB3	Waasetec, Göttingen
Heraeus Pico 21	ThermoFisher, Bonn
Hydro tech vacuum pump	Bio-Rad, Munich
Incubator Innova R44	New Brunswick, Neu-Isenburg,
Incubator shaker Innova 2300	New Brunswick, Neu-Isenburg
LabCycler	LabCycler SensorQuest, Göttingen
Magnetic stirrer	JAK Werk, Staufen
Microplate reader SynergyMx Mini-Protean	BioTek, Bad Friedrichshall
Mikroprozessor pH-Meter 766 Calimatic	Knick, Berlin
Mini-Protean III System	Bio-Rad, Munich
Nanodrop ND-1000	ThermoFisher, Bonn
Nitrocellulose membrane	Bio-Rad, München
Open air shaker Innova 2300	New Brunswick, Neu-Isenburg
Polyvinylidendifluoride membrane (PVDF)	Bio-Rad, München
Refrigerated centrifuge PrimoR	Heraeus Christ, Osterode
Scale Sartorius universal	Sartorius, Göttingen
SDS-PAGE glass plates	Bio-Rad
Southern blotting device	VacuGene™ XL (GE Healthcare)
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer Ultraspec 2000	Amersham, Freiburg
Steam autoclave	Zirbus, Bad Grund
Stereo Lumar V12 stereo microscope	Carl Zeiss, Göttingen
Sterile bench Hera Safe	ThermoFisher, Bonn
Thermocycler	Biometra, Göttingen
TS Sorvall WX utraseries centrifuge / RC 6+	Beckmann Coulter, Krefeld
Ultra centrifuge, Sorvall Ultra Pro 80	ThermoFisher, Bonn
UV Transilluminator 2000	Bio-Rad Laboratories GmbH, München
Vortex	Bender and Hobein, Bruchsal
Water desalination plant	Millipore, Schwalbach

Commercial systems

System	Supplier
NucleoSpin Plasmid-Kit	Macherey-Nagel, Düren
PageRuler™ Plus Prestained Protein Ladder	ThermoFisher, Waltham
peqGOLD Bacterial DNA Kit	PEQLAB, Erlangen
PCR Purification Kit	Quiagen,

Chemicals

Chemical	Supplier
Blocking reagent	Roche
HDGreen™ Plus DNA Stain	Intas, Göttingen

Bacterial strains

Strains constructed in this work

Name	Genotype	Construction[1]
BP1100	*trp+ ΔpdxST::tet*	BV604 -> SP1
BP1101	*trp+ ΔytoQ::aphA3*	BKK29850 -> SP1
BP1102	*trp+ ΔpdxST::tet amyE::(pdxJ-aad9) aprE::(pdxH) ΔytoQ::aphA3*	BKK29850 -> BP1017
BP1103	*trp+ ΔpdxST::tet amyE::(pdxJ-aad9) aprE::(pdxH) ΔbshC::cat ΔytoQ::aphA3*	BKK29850 -> BP978
BP1104	*trp+ ΔbshC::cat ΔytoQ::aphA3*	BKK29850 -> BP977
BP1105	*trp+ ΔpdxT::cat*	LFH [(BR77 + BR83; BR82 + BR83; cat-fwd(kan) + cat-rev(kan)] -> SP1
BP1106	*trp+ ΔpdxT::cat amyE::P_{nrgA}-lacZ aphA3*	pGP168 -> BP1105
BP1107	*trp+ ΔpdxST::tet amyE::(pdxST-strep-aphA3)*	pBP773 -> BP1100
BP1108	evolved PG10 strain	6 weeks of daily passages of PG10 in LB-Glc
BP1109	evolved PG39 strain	1 week of daily passages of PG39 in LB-Glc
BP1110	evolved PG39 strain	2 weeks of passages of PG39 in LB-Glc
BP1111	*trpC2 amyE::$P_{mhqN}{}^{-T139C}$-lacZ apha3*	pBP760 -> 168
BP1112	*trpC2 amyE::$P_{mhqN}{}^{wt}$-lacZ apha3*	pBP761 -> 168
BP1113	PG10 background *amyE::$P_{mhqN}{}^{-T139C}$-lacZ apha3*	pBP760 -> PG10
BP1114	PG10 background *amyE::$P_{mhqN}{}^{WT}$-lacZ apha3*	pBP761 -> PG10
BP1115	*trp+ ΔpdxT::cat;* ~12x amplification of a 15 kb region including *pdxS,* other mutations present	BP1105; S1; cultivation of BP1105 on MSSM plates with 1.3mM Q and 10 mM NH4
BP1116	*trp+ ΔpdxT::cat*; ~3x amplification of a 15 kb region including *pdxS.*	BP1105; S4; cultivation of BP1105 on MSSM plates with 1.3mM Q and 10 mM NH4
BP1117	*trp+ ΔpdxT::cat*; truncation: *pgpH*^{C574A}	BP1105; S14; cultivation of BP1105 on MSSM plates with 1.3mM Q and 20 mM NH4
BP1118	BP1115 background *; P_{nrgA}-lacZ aphA3*	pGP168 -> BP1115
BP1119	BP1116 background *P_{nrgA}-lacZ aphA3*	pGP168 -> BP1116
BP1120	BP1117 background *P_{nrgA}-lacZ aphA3*	pGP168 -> BP1117
BP1121	*trp+ amyE::(P_{grac}aroE cat)*	pBP763 -> SP1
BP1122	*trp+ aroE::aad9 (pBQ200::aroE^{1256trunc} ermC). mutation in aroE^{1256G} to restores WT aroE length.*	BP239 cultivation on CS-Glc plate with Phe, Tyr gradient Suppressor 7
BP1123	*trp+ aroE::aad9 (pBQ200::aroE$_{1256\text{-}trunc}$ ermC). mutation in aroE^{1256G} restores WT aroE length.*	BP239 cultivation on CS-Glc plate with Phe, Tyr gradient Suppressor 12
BP1124	*trp+ P_{nrgA}-lacZ aphA3*	pGP168 -> SP1
BP1125	*trp+ΔpdxS::cat*	LFH [BR103 + BR104, BR105 + BR106, cat-fwd(kan) + cat-rev(kan)] -> SP1
BP1126	*trp+ΔpgpH::ermC*	GP2034 -> SP1
BP1127	*trp+ ΔpgpH::ermC pdxT::cat*	GP2034 -> BP1105

BP1128	*trp+ΔpgpH::ermC pdxT::cat* P_{nrgA}*-lacZ apha3*	GP2034 -> BP1106
BP1129	*trp+ ΔpdxT::cat serS-ermC-gfP*	LFH [BR121 + BR122, BR123 + BR124, mls-rev(kan) + SW41 gDNA1966] -> BP1105
BP1130	*trp+ serS-ermC-gfP*	LFH [BR121 + BR122, BR123 + BR124, mls-rev(kan) + SW41 gDNA1966] -> SP1
BP1131	*trp+ amyE::(pdxST-strep-aphA3)*	pBP773 -> SP1
BP1132	*trp+ ΔaroA::ermC*	LFH [BR126 + BR127, BR129 + BR130, mls-fwd(kan) + ml-rev(kan)]-> SP1
BP1133	*trp+ ΔaroB::ermC*	LFH [BR133 + BR134, BR135 + BR136, mls-fwd(kan) + ml-rev_o.T.(kan)]-> SP1
BP1134	*trp+ ΔaroC:ermC*	LFH [BR139 + BR140, BR141 + BR142, mls-fwd(kan) + ml-rev(kan)] -> SP1
BP1135	*trp+ ΔaroD::ermC*	LFH [BR145 + BR146, BR147 + BR148, mls-fwd(kan) + ml-rev_o.T.(kan)]-> SP1
BP1136	*trpC2 ΔaroA::ermC*	LFH [BR126 + BR127, BR129 + BR130, mls-fwd(kan) + ml-rev(kan)]-> 168
BP1137	*trpC2 ΔaroB::ermC*	LFH [BR133 + BR134, BR135 + BR136, mls-fwd(kan) + ml-rev_o.T.(kan)]-> 168
BP1138	*trpC2 ΔaroC::ermC*	LFH [BR139 + BR140, BR141 + BR142, mls-fwd(kan) + ml-rev(kan)] -> 168
BP1139	*trpC2 ΔaroD::ermC*	LFH [BR145 + BR146, BR147 + BR148, mls-fwd(kan) + ml-rev_o.T.(kan)]-> 168
BP1140	*trp+ amyE::(*P_{grac}*aroE cat) ΔaroE::aad9*	pBP762 -> BP1121
BP1141	BKE25660 suppressor 2	cultivation on SP-EL for several days
BP1142	BKE25660 suppressor 1	cultivation on SP-EL for several days
BP1143	BKE25660 suppressor 3	cultivation on SP-EL for several days
BP1144	BKE25660 suppressor 4	cultivation on SP-EL for several days
BP1145	*trp+ amyE::*P_{rpoE}^{WT}*-lacZ*	pBP777 -> SP1
BP1146	*trp+ amyE::*$P_{rpoE}^{G->A}$*-lacZ*	pBP778 -> SP1
BP1147	*trp+ ΔpdxT::cat amyE::*P_{nrgA}*-lacZ aphA3 serS-ermC-gfP ΔrecA::aad9*	gDNA GP2542 -> BP1182
BP1148	BP1147 S1	cultivation of BP1147 on MSSM 20mM NH4 1.3 mM Q
BP1149	trpC+ Δ(ybxA-ybaE-ybaf)::cat	LFH [BR227 + BR228, BR229 + 230, cat-fwd(kan) + cat-rev(kan)]-> SP1
BP1150	PG39 evolved for 6 weeks. Replicate A	6 weeks evolution in LB-Glc
BP1151	PG39 evolved for 6 weeks. Replicate B	6 weeks evolution in LB-Glc
BP1152	PG39 evolved for 6 weeks. Replicate C.	6 weeks evolution in LB-Glc
BP1153	evolved PG10	1 week evolution in LB-Glc
BP1154	evolved PG10	2 weeks evolution in LB-Glc
BP1155	BP1105 Suppressor #2	evolved BP1105; cultivation on MSSM 1.3mM Q; 10 mM NH4
BP1156	BP1105 Suppressor #3	evolved BP1105; cultivation on MSSM 1.3mM Q; 10 mM NH4
BP1157	BP1105 Suppressor #5	evolved BP1105; cultivation on MSSM 1.3mM Q; 10 mM NH4
BP1158	BP1105 Suppressor #6	evolved BP1105; cultivation on MSSM 1.3mM Q; 10 mM NH4
BP1159	BP1105 Suppressor #7	evolved BP1105; cultivation on MSSM 1.3mM Q; 10 mM NH4
BP1160	BP1105 Suppressor #8	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1161	BP1105 Suppressor #9	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1162	BP1105 Suppressor #10	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1163	BP1105 Suppressor #11	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1164	BP1105 Suppressor #12	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4

BP1165	BP1105 Suppressor #13	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1166	BP1105 Suppressor #15	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1167	BP1105 Suppressor #16	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1168	BP1105 Suppressor #17	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1169	BP1105 Suppressor #19	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1170	BP1105 Suppressor #20	evolved BP1105; cultivation on MSSM 1.3mM Q; 20 mM NH4
BP1171	BP1106 Suppressor #1 Blue. Only sequenced by PCR: *glnA*$^{G79A,\ C1016A}$	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1172	BP1106 Suppressor #2 Blue *tnrA*$^{280T\ other}$ mutations present	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1173	BP1106 Suppressor #3 Blue *glnA*^{C180A} other mutations present	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1174	BP1106 Suppressor #4 White rpoE$^{82\text{-}T,T87A}$ other mutations present	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1175	BP1106 Suppressor #5 White pgpH$^{508\text{-}C,T512G}$ other mutations present	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1176	BP1106 Suppressor #6 White	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1177	BP1106 Suppressor #7 White	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1178	BP1106 Suppressor #8 White	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1179	evolved PG10	3 weeks of passages -> LB-Glc
BP1180	evolved PG10	4 weeks of passages -> LB-Glc
BP1181	evolved PG10	5 weeks of passages -> LB-Glc
BP1182	*trp+ ΔpdxT::cat amyE::*P_{nrgA}*-lacZ aphA3 serS-ermC-gfP*	LFH [BR121 + BR122, BR123 + BR124, mls-rev(kan) + SW41 gDNA1966] -> -> BP1106
BP1183	*trp+ ΔpdxST::tet amyE::*P_{dacA}*-pdxS aphA3*	pBP776 -> BP1100
BP1184	BP1106 suppressor blue *glnA*^{G79A}	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1185	BP1106 suppressor fladder phenotype	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1186	BP1106 suppressor fladder phenotype	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1187	BP1106 suppressor volcano phenotype	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1188	BP1106 suppressor volcano phenotype	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1189	BP1106 suppressor brown	evolved BP1106; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1190	BP1182 suppressor with strong gfp signal	evolved BP1182; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1191	BP1182 suppressor with strong gfp signal	evolved BP1182; cultivation on MSSM 1.3mM Q 20mM NH_4
BP1192	evolved BP1191	BP1191 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1193	evolved BP1191	BP1191 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1194	evolved BP1191	BP1191 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1195	BP1182 blue Suppressor #1	evolved BP1182; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1196	*trpC+ pdxS::cat amyE::*P_{dacA}*-PdxS aphA3*	pBP776 -> BP1125
BP1197	BP1182 blue Suppressor #11	evolved BP1182; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1198	*trpC+ ΔpdxST::tet Δ(ybxA-ybaE-ybaf)::cat*	LFH [BR227 + BR228, BR229 + 230, cat-fwd(kan) + cat-rev(kan)]-> BP1100

BP1199	BP1182 blue Suppressor #17	evolved BP1182; cultivation on MSSM 1.3mM Q 20mM NH_4 X-Gal
BP1200	evolved BP1191	BP1192 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1201	evolved BP1192	BP1193 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1202	evolved BP1192	BP1194 evolved for 1 week -> MSSM 1.3 Q 20 NH4
BP1203	BP1191 cultivated for 3 week in MSSM 1.3 Q 20 NH4. diluted 1:80 twice a day -> 24 dilutions. Replicate 1	BP1200 evolved for 1 week -> MSSM 1.3 Q 20 NH5
BP1204	BP1191 cultivated for 3 week in MSSM 1.3 Q 20 NH4. diluted 1:80 twice a day -> 24 dilutions. Replicate 2	BP1201 evolved for 1 week -> MSSM 1.3 Q 20 NH5
BP1205	BP1191 cultivated for 3 week in MSSM 1.3 Q 20 NH4. diluted 1:80 twice a day -> 24 dilutions. Replicate 3	BP1202 evolved for 1 week -> MSSM 1.3 Q 20 NH6
BP1206	*trpC+ amyE::(PdxR-P_{pdxS})^{lmo}-lacZ aphA3*	pBP781 -> SP1
BP1207	*trpC+ ΔpdxST::tet amyE::(PdxR-P_{pdxS})^{lmo}-lacZ aphA3*	pBP781 -> BP1100
BP1208	*trpC+ ΔpdxT::cat amyE::(PdxR-P_{pdxS})^{lmo}.-lacZ aphA3*	pBP781 -> BP1105
BP1209	*trpC+ ΔpdxT::cat amyE::P_{nrgA}-lacZ aphA3 glnA^{C79A,C1016A} spec*	LFH [BR189 + BR189 gDNA BP1171, BR191 + BR192 gDNA BP1171, spec-fwd(kan) +spec-rev(kan)] -> BP1106
BP1210	*trpC+ ΔpdxT::cat amyE::P_{nrgA}-lacZ aphA3 glnA^{C180A} spec*	LFH [BR189 + BR189 gDNA BP1173, BR191 + BR192 gDNA BP1173, spec-fwd(kan) +spec-rev(kan)] -> BP1106
BP1211	*trpC+ ΔpdxT::cat amyE::P_{nrgA}-lacZ aphA3 glnA^{C79A} spec*	LFH [BR189 + BR189 gDNA BP1184, BR191 + BR192 gDNA BP1184, spec-fwd(kan) +spec-rev(kan)] -> BP1106
BP1212	*trpC+ ΔpdxT::cat amyE::P_{nrgA}-lacZ aphA3 tnrA^{280T} spec*	LFH [AL22 + BR197 gDNA BP1172, BR198 + BR199 gDNA BP1172, spec-fwd(kan) +spec-rev(kan)] -> BP1106
BP1213	*trpC+ ΔpdxST::tet amyE::(PdxR-P_{pdxS})^{lmo}-lacZ aphA3 xkdE::(pdxS-ermC)*	pBP782 -> BP1207
BP1214	*BP1191 cultivated for 1 week in MSSM 1.3 Q 20 NH4. Replikat 1*	
BP1215	*BP1191 cultivated for 1 week in MSSM 1.3 Q 20 NH4. Replikat 2*	
BP1216	*trpC+ xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	LFH [BR256 + BR257, BR262 + BR263, gDNA *L. monocytogenes*, BR264 + BR265, pHT01, CZ126 + CZ127 pDG148, BR258 + BR259]-> SP1
BP1217	*trpC+ ΔpdxST::tet xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	gDNA BP1216 -> BP1100
BP1218	*trpC+ ΔpdxT::cat xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	gDNA BP1216 -> BP1105
BP1219	*trpC+ ΔpdxS::cat xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	gDNA BP1216 -> BP1125
BP1220	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 rpoE::aad9*	LFH [BR213 + BR214, BR215 + BR216, spec-fwd(kan) + spec-rev.(kan)] -> BP1106
BP1221	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 pgpH::tet*	PCR [JN188 + JN187, gDNA GP2033] -> BP1106
BP1222	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 gdpP::aad9*	LFH [BR207 + BR208, BR209 + BR210, spec-fwd(kan) + JG67_o.T.(kan)] -> BP1106
BP1223	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 pgpH::aad9*	LFH [JN185 + JN187, JN188 + JN186, spec-fwd(kan) + JG67_o.T.(kan)] -> BP1106
BP1224	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 xkdE::(P_{xyl}rpoE) ermC*	pBP793 -> BP1106
BP1225	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 xkdE::(P_{xyl}pgpH) ermC*	pBP794 -> BP1106
BP1226	*trp+ ΔpdxT::cat amyE::PnrgA-lacZ aphA3 xkdE:(P_{xyl}gdpP) ermC*	pBP795 -> BP1106
BP1227	*trpC+ amyE::((P_{grac})^{Eco}-lacZ aphA3) xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	pBP769 -> BP1216
BP1228	*trpC+ ΔpdxST::tet amyE::((P_{grac})^{Eco}-lacZ aphA3) xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	pBP769 -> BP1217
BP1229	*trpC+ ΔpdxT::cat amyE::((P_{grac})^{Eco}-lacZ aphA3) xkde::(pdxR-P_{pdxS})^{Lmo}-lacI^{Eco} phleo)*	pBP769 -> BP1218
BP1230	*trp+ ytoQ-gfP ermC*	LFH [BR300 + BR301, BR302 + BR303, mls-fwd(kan) + mls-rev_o.T.(kan), BR306 + SW41] -> SP1
BP1231	*trp+ PahpA-lacZ kan*	pBP820 -> SP1
BP1232	*trp+ PahpA*-lacZ kan*	pBP821 -> SP1

BP1233	*trp+ PykuT-lacZ kan*	pBP822 -> SP1
BP1234	*trp+ PykuT*-lacZ kan*	pBP823 -> SP1
BP1235	*trp+ pdxT::cat PahpA-lacZ kan*	pBP820 -> BP1105
BP1236	*trp+ pdxT::cat PahpA*-lacZ kan*	pBP821 -> BP1105
BP1237	*trp+ pdxT::cat PykuT-lacZ kan*	pBP822 -> BP1105
BP1238	*trp+ pdxT::cat PykuT*-lacZ kan*	pBP823 -> BP1105
BP1239	*trp+ΔpdxS::cat amyE::(PdxR-PpdxS)lmo-lacZ aphA3*	pBP781 -> BP1125
BP1240	*trp+ΔytoP::phleo*	LFH [BR282 + BR283, BR284 + BR285, CZ126 + CZ127] ->SP1
BP1241	*trp+ΔytoQ::kan ΔytoP::phleo*	LFH [BR282 + BR283, BR284 + BR285, CZ126 + CZ127] -> BP1101
BP1242	*trp+ΔytoQ::kan amyE::(P_{ytoP}-lacZ cat)*	pBP799 in BP1101
BP1243	*trp+ amyE::(P_{ytoP}-lacZ cat)*	pBP799 in SP1
BP1244	*trp+ ΔpdxST::tet amyE::(pdxJ-aad9) aprE::(pdxH) ΔbshC::cat ΔytoP::phleo*	LFH in BP978
BP1245	*trp+ ΔpdxST::tet amyE::(pdxJ-aad9) aprE::(pdxH) ΔbshC::cat ΔytoQ::aphA3 ΔytoP::phleo*	LFH in BP1103
BP1246	*trp+ ΔpdxST::tet Δbsh::cat ΔytoQ::aphA3*	gDNA BP1100 -> BP1104
BP1250	*trpC+ amyE::($(P_{grac})^{Eco}$-lacZ aphA3)*	pBP769 -> SP1
BP1251	*BP1207 Suppressor raised on C-Glc 4 mM PL #1(K1)*	
BP1252	*BP1207 Suppressor raised on C-Glc 4 mM PL #2(K7)*	
BP1253	*BP1207 Suppressor raised on C-Glc 4 mM PL #3(K10)*	
BP1254	*BP1207 Suppressor raised on C-Glc 4 mM PL #4(K11)*	
BP1255	*BP1207 Suppressor raised on C-Glc 4 mM PL #4(K17)*	
BP1256	*trpC+ pbuX::cat*	LFH [BR309 + BR310, BR311 + BR312, cat-fwd(kan) + cat-rev(kan)] -> SP1
BP1257	*trpC+ pucJK::cat*	LFH [BR315 + BR316, BR317 + BR318, cat-fwd(kan) + cat-rev_o.T.(kan)]-> SP1
BP1258	*trpC+ pyrP::cat*	LFH [BR321 + BR322, BR323 + BR324, cat-fwd(kan) + cat-rev_o.T.(kan)] -> SP1
BP1259	*trpC+ ywdJ:cat*	LFH [BR327 + BR377, BR 378 + BR330 cat-fwd(kan) + cat-rev_o.T.(kan)]-> SP1
BP1260	*trpC+ yxlA:cat*	LFH [BR335 + BR336, BR337 + BR338, cat-fwd(kan) + cat-rev(kan)]-> SP1
BP1261	*trpC+ pucI:cat*	LFH [BR340 + BR341, BR342 + BR343, cat-fwd(kan) + cat-rev_o.T.(kan)]-[> SP1
BP1262	*trpC+ yvcA-PmtlA-comKS-cat-hisI*	gDNA 2620 -> Sp1
BP1263	*trpC+ ΔpdxST::tet amyE::(PdxR-P_{pdxS})^{lmo}-lacZ aphA3 yvcA-PmtlA-comKS-cat-hisI*	gDNA 2620 -> BP1207
BP1264	*yitU::kan*	
BP1265	*pdxT::tet*	
BP1266	*trpC+ΔpdxST::tet pbuX::cat*	gDNA BP1256 -> BP1100
BP1267	*trpC+ ΔpdxST::tet pucJK::cat*	gDNA BP1257 -> BP1100
BP1268	*trpC+ ΔpdxST::tet pyrP::cat*	gDNA BP1258 -> BP1100
BP1269	*trpC+ ΔpdxST::tet ywdJ:cat*	gDNA BP1259 -> BP1100
BP1270	*trpC+ ΔpdxST::tet yxlA:cat*	gDNA BP1260 -> BP1100
BP1271	*trpC+ ΔpdxST::tet pucI:cat*	gDNA BP1261 -> BP1100
BP1272	*trpC+ cpgA::cat*	LFH [BR352 + BR353, BR354 + BR355, cat-fwd(kan) + cat-rev_o.T.(kan)] -> SP1
BP1273	*trpC+ serA::apha3*	LFH [AK235 + AK236, AK237 + AK238, kan-fwd + kan-rev -> SP1
BP1274	*trpC+ serC::ermC*	LFH [BR358 + BR359, BR360 + BR361, mls-fwd(kan) + CZ86] -> SP1
BP1275	*trpC+ thrB::phleo*	LFH [BR364 + BR365, BR366 + BR367, CZ126 + CZ127] -> SP1
BP1276	*trpC+ ΔpdxST:tet amyE::pdxJ aad9 aprE::pdxH ΔbshC(ΔA464)P*ytoQ cpgA::cat*	LFH [BR352 + BR353, BR354 + BR355, cat-fwd(kan) + cat-rev_o.T.(kan)] ->BP1036
BP1277	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC(ΔA464)P*ytoQ serA::apha3*	LFH [AK235 + AK236, AK237 + AK238, kan-fwd + kan-rev -> ->BP1036
BP1278	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC(ΔA464)P*ytoQ serC::ermC*	LFH [BR358 + BR359, BR360 + BR361, mls-fwd(kan) + CZ86] BP1036

BP1279	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC(ΔA464)P*ytoQ ThrB::phleo*	LFH [BR364 + BR365, BR366 + BR367, CZ126 + CZ127] ->BP1036
BP1280	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH xkde::(pdxB^{Eco} phleo)*	LFH [BR256 + BR370, BR371 + BR372 gDNA W3110, CZ126 + CZ127, BR258 + BR259] -> BP965
BP1281	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC xkde::(pdxB^{Eco} phleo)*	LFH [BR256 + BR370, BR371 + BR372 gDNA W3110, CZ126 + CZ127, BR258 + BR259 -> BP978
BP1282	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ganA::(pdxA^{Eco} kan)*	LFH [CZ228 + BR374, BR375 + BR376 gDNA W3110, kan-fwd + kan-rev, CZ230 + CZ231] -> BP965
BP1283	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC ganA::(pdxA^{Eco} kan)*	LFH [CZ228 + BR374, BR375 + BR376 gDNA W3110, kan-fwd + kan-rev, CZ230 + CZ231] -> BP978
BP1284	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH xkde::(pdxB^{Eco} phleo) ganA::(pdxA^{Eco} kan)*	BP1283 -> BP1281
BP1285	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC xkde::(pdxB^{Eco} phleo) ganA::(pdxA^{Eco} kan)*	BR1282 -> BP1280

[1]if not stated otherwise, gDNA of SP1 served as template for the PCR. Templates for the resistance genes can be seen in plasmid list below

Other strains used for this study

Name	Genotype	Reference
B. subtilis		
168	*trpC2*	Laboratory collection
SP1	*trp+* Prototrophic derivative of strain 168	(*Richts et al.*, 2020)
BKE29850	*trpC2 ytoQ::ermC*	(*Koo et al.*, 2017)
BP965	*trp+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH*	(*J. Rosenberg et al.*, 2018)
BP977	*trp+ ΔbshC::cat*	(Rosenberg PhD Thesis, 2019)
BP978	*trp+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC::cat*	(*J. Rosenberg et al.*, 2018)
BP1036	*trpC+ ΔpdxST::tet amyE::pdxJ aad9 aprE::pdxH ΔbshC^{-A464}* P*$_{ytoQ}$	(*J. Rosenberg et al.*, 2018)
BV604	*ΔpdxST::tet*	(*Commichau et al.*, 2014)
DK1042	Genetically competent derivative of strain NCIB3610	(*Konkol et al.*, 2013)
GP922	*ΔymdB::cat*	(*Diethmaier et al.*, 2011)
GP1966	*trpC2 ΔynfC::(P$_{alf4}$-gfp ermC)*	(*Reuß et al.*, 2019)
GP2542	*trpC2 recA::aad9*	(*Reuß et al.*, 2019)
GP2033	*trpC2 ΔpgpH::tet*	(*Gundlach*, PhD thesis)
GP2034	*trpC2 ΔpgpH::ermC w/o terminator*	(*Gundlach et al.*, 2015)
GP3153	*trpC2 ΔohrA::tet ΔohrB::aad9*	(*Blötz*, PhD thesis)
PG10	Genome reduced *B. subtilis* strain with about 36% genome reduction	(*Reuß et al.*, 2017)
PG39	Genome reduced *B. subtilis* strain with about 40% genome reduction	(Klewing PhD Thesis, 2019)
E. coli		
DH5α	*recA1 endA1 gyrA96 thi hsdR17rK- mK+relA1 supE44 Φ80ΔlacZΔM15 Δ(lacZYA-argF)U169*	Sambrook, Fritsch and Maniatis, (1989)
XL1-Red	F- *endA1gyrA96*(*nal^R*) *thi-1 relA1 lac glnV44hsdR17*(r_K^- m_K^+) *mutS mutT mutD5 Tn10*	Stratagene
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F´ [*proAB lacI^{q}ZΔM15* Tn*10*(*tet^R amy cm^R*)]	Stratagene
BTH101	F$^-$*cya-99´araD139, galE15 galK16, rpsL1 (str^R)hsdR2 mcrA1 mcrB1*	EUROMEDEX
BL21 (DE3)	F$^-$*ompT gal dcm lon hsdS$_B$*(r_B^- m_B^-) λ(DE3 [*lacI lacUV5*-T7 *gene 1 ind1 sam7 nin5*])	(*Studier & Moffatt*, 1986)
BL21 Rosetta	F$^-$*ompT hsdS$_B$*(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^R)	NOVAGEN

Oligonucleotides constructed in this study

Name	Sequence (5'->3')	Reference/ Purpose
AK239	GGAGTCAGAAGTTGATGATTCTATTGCC	Deletion of *serA* (laboratory collection)

AK235	CATGTTAAACGGCAGGATACCTGCG	Deletion of *serA* (laboratory collection)
AK236	CCTATCACCTCAAATGGTTCGCTGGTCTGAGACCAAT ACTCGAAACATCG	Deletion of *serA* (laboratory collection)
AK237	CGAGCGCCTACGAGGAATTTGTATCGGTGTCTGTGAA GCTCATTGATCTGCC	Deletion of *serA* (laboratory collection)
AK238	GTGCCAGCTGCTCCAAATCCG	Deletion of *serA* (laboratory collection)
BR26	AAAGGATCCCAAAATGCGTAATTCCGATTCTGG	Fwd primer for amplification of a 700 bp long left border to *fadE* for LFH PCR. Provides a *Bam*HI restriction site for cloning into pGP1022 (pBP760)
BR27	CGCAAGCTGACACGCG	Rev primer for amplification of a 700 bp long left border of *fadE* for LFH PCR (pBP760)
BR28	TAGGCGTTACGCGTGTCAGCTTGCGTTAGCTTGAACT GCTTTCTGTCTG	Fwd primer for amplification of a 700 bp long right border of *mrgA* for LFH PCR, provides an overhang to the left border (pBP760)
BR29	TTTGAATTCGGAAATCAGCGGCAAGAATGT	Rev primer for amplification of a 700 bp long right border of *mrgA* for LFH PCR, provides an *Eco*RI restriction site for cloning into pGP1022
BR30	GTTTTAACGGATTCAACACTGCC	Fwd check primer for pBP760
BR31	TTGAAGGCGCGAACAAGC	Rev check primer for pBP760
BR32	AAAGGATCCTCTGATTCCGCAACTGCCA	Fwd Primer for amplification of a 700 bp long left border to *yflD* for LFH PCR. provides *a Bam*HI restriction site for cloning into pGP1022 (pBP761)
BR33	CCTTTATTTCTAGAAAGATACCTTGTTTTACAT	Rev Prime for amplification of a 700 bp long left border to *yflD* for LFH PCR (pBP761)
BR34	CAAGGTATCTTTCTAGAAATAAAGG GGGACGGGGAGCCAGTG	Fwd Primer for amplification of a 700 bp long right border to *yfhE* for LFH PCR. provides an overhang to the left flank. (pBP761)
BR35	TTTCTCGAGGGCCAGCATCTGACAGGC	Rev Primer for amplification of a 700 bp long right border to *yfhE* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP761)
BR36	CGCTGGAATCGGCAAGC	Fwd check primer for pBP761 deletion
BR37	CGCGTTTTCTTTTATTACAATGAGGT	Rev check primer for pBP761 deletion
BR38	AAAGGATCCAATCCCCCCAGAAACCGC	Fwd for amplification of a 700 bp long left border to *ywnJ* for LFH PCR. provides a *Bam*HI restriction site for cloning into pGP1022 (pBP762)
BR39	TTAGAAATTTTGATCGGTTGGTTTTTTCG	Rev primer for amplification of a 700 bp long left border to *ywnJ* for LFH PCR (pBP762)
BR40	AAAACCAACCGATCAAAATTTCTAAGACATACTGATT TTCAAGTAAGTTTATGCA	Fwd primer for amplification of a 700 bp long right border to *spoIID* for LFH PCR. provides an overhang to the left flank. (pBP762)
BR41	TTTCTCGAGCACCGGCACCATCAAAATTG	Rev primer for amplification of a 700 bp long right border to *spoIID* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP762)
BR42	ATTTGTCTATGAAATCAGCAATACGATCA	Fwd check primer for pBP762 deletion
BR43	CTCTAGCTGAAGGAACAACAACG	Rev check primer for pBP762 deletion
BR44	CATTTATTACTCCACAGTAACACTCTTCGC	Rev primer for amplification of a 700 bp long left border to *ycbL* for LFH PCR (pBP763)
BR45	GAGTGTTACTGTGGAGTAATAAATGGAGAGCAGGGT CTATCTTCTGGA	Fwd primer for amplification of a 700 bp long right border to *ycdG* for LFH PCR. provides an overhang to the left flank. (pBP763)
BR46	AAACTCGAGGCTTCACGATTTGAGCCGTAA	Rev primer for amplification of a 700 bp long right border to *ycdG* for LFH PCR. provides an *Xho*I restriction site for cloning into pGP1022 (pBP763)
BR47	GCTTTTTTTGCGGTTGTACGAT	Rev check primer for pBP763 deletion
BR48	AAAGGATCCAGTATTCTGCGTTCGACCTGG	Fwd primer for amplification of a 700 bp long left border to *sda* for LFH PCR. provides a *Bam*HI restriction site for cloning into pGP1022 (pBP764)
BR49	CAATCGTTCTGTTATCCACACCAA	Rev primer for amplification of a 700 bp long left border to *sda* for LFH PCR (pBP764)

BR50	TTTGGTGTGGATAACAGAACGATTGTTATGAAATTTTCACAATTTTCACGAGCA	Fwd primer for amplification of a 700 bp long right border to *pbpI* for LFH PCR. provides an overhang to the left flank. (pBP764)
BR51	TTTCTCGAGGCAGGAAGGGAATGTGCGA	Rev primer for amplification of a 700 bp long right border to *pbpI* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP764)
BR52	CCTGTTTTGTCCTCGGTTTGG	Fwd check Primer for pBP764 deletion
BR53	AACGCGATTTTGTAATACCATAGCA	Rev check primer for pBP764 deletion
BR54	AAAGGATCCCAAAAAACCCTTTCCCCATGAGAA	Fwd primer for amplification of a 700 bp long left border to *spoIIM* for LFH PCR. provides a *Bam*HI restriction site for cloning into pGP1022 (pBP765)
BR55	TGCTGGTCATTCTGTTTTTAGCG	Rev primer for amplification of a 700 bp long left border to *spoIIM* for LFH PCR (pBP765)
BR56	AGCGCTAAAAACAGAATGACCAGCATCAGCCTCGCGGGAC	Fwd primer for amplification of a 700 bp long right border to *yqjT* for LFH PCR. provides an overhang to the left flank. (pBP765)
BR57	TTTCTCGAGGCTTCTGGGGAGCCGTTC	Rev primer for amplification of a 700 bp long right border to *yqjT* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP765)
BR58	ATCTTCCCATGTCCTAATCGATCC	Fwd check primer for pBP765 deletion
BR59	GGGGAAGCCACACAGCAT	Rev check primer for pBP765 deletion
BR60	AAAGGATCCCCATACAAACGCGATGGCTAAAG	Fwd primer for amplification of a 700 bp long left border to *slp* for LFH PCR. provides a *Bam*HI restriction site for cloning into pGP1022 (pBP766)
BR61	TTATTTTACGATGTGAATCGGACTTCC	Rev primer for amplification of a 700 bp long left border to *slp* for LFH PCR (pBP766)
BR62	AAGTCCGATTCACATCGTAAAATAATCGGGATCGGCATATTCGG	Fwd primer for amplification of a 700 bp long right border to *ylaM* for LFH PCR. provides an overhang to the left flank. (pBP766)
BR63	TTTCTCGAGAACATAACATCCTCTTCCCTGCTT	Rev primer for amplification of a 700 bp long right border to *ylaM* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP766)
BR64	CGTATGCTAACTTCGGTACTGAAC	Fwd check primer for pBP766 deletion
BR65	CGGGAGACAGCCGTAATCAT	Rev check primer for pBP766 deletion
BR66	AAAGGATCCCGAACCGTTTTCCGCTCTG	Fwd primer for amplification of a 700 bp long left border to *yqjF* for LFH PCR. provides a *Bam*HI restriction site for cloning into pGP1022 (pBP767)
BR67	GCTTTTTTATTTCACCGACTCAGTAAGA	Rev primer for amplification of a 700 bp long left border to *yqjF* for LFH PCR (pBP767)
BR68	TACTGAGTCGGTGAAATAAAAAAGCTTACCCAGTTGTTTTGACTTGATCC	Fwd primer for amplification of a 700 bp long right border to *spo0A* for LFH PCR. provides an overhang to the left flank. (pBP767)
BR69	TTTCTCGAGGACAGCCACCTTCAAAGCTTG	Rev primer for amplification of a 700 bp long right border to *spo0A* for LFH PCR. provides a *Xho*I restriction site for cloning into pGP1022 (pBP767)
BR70	TCTTTTTGATCGTATTATGCTCGGG	Fwd check primer for pBP767 deletion
BR71	CGCGACTTAATGAAATCAAACAGC	Rev check primer for pBP767 deletion
BR72	AAAGGATCCATGGAGTTTATCGTCTATTTAGCAGGA	Fwd primer for amplification of *ytoQ*. Provides a *Bam*HI restriction site for cloning into pGP380 (pBP770) / pGP382 (pBP771)
BR73	TTTAAGCTTTTATTCTGTTTCAAATAGATAGGAAAGGGC	Rev primer for amplification of *ytoQ*. Provides a *Hind*III restriction site for cloning into pGP380 (pBP770)
BR74	TTTAAGCTTTTCTGTTTCAAATAGATAGGAAAGGGC	Rev primer for amplification of *ytoQ*. Provides a *Hind*III restriction site for cloning into pGP382 (pBP771). PCR product without terminator for correct Strep-Tag fusion
BR75	GGGCTCTTTCGCGTAATATACTGA	Fwd check primer for *pdxST* deletion
BR76	CACCAACCATCGGCTCTCTTAG	Rev check primer for *pdxST* deletion

BR77	TTTGTCGACATTAGGGGGACCAAGAAATGGCTCAAAC	Fwd primer for *pdxS(T)* amplification. Includes the native RBS and *Sal*I restriction site for cloning into pGP382.
BR78	AAACTGCAGTACAAGTGCCTTTTGCTTATATTCCTC	Rev primer for *pdx(S)T* amplification (w/o terminator). Includes a restriction site for *Pst*I
BR79	TTTGGATCCCTTGTCAAGTGAAGGCGCGCTATGCTACAATACAGCTTGGTTTAAAGGAGGAAACAATCATGGCTCAAACAGGTACTGAACG	Fwd primer for *pdxS(T)* amplification. Incudes constitutive Palf4 promoter + *gapA* SD. Contains a restriction site for cloning (Pac7)
BR80	AAAGGATCCATGATTGTTTCCTCCTTTATTTTTCGAACTGCGGGTGGCTCCATACAAGTGCCTTTTGCTTATATTCCTC	Rev primer for *pdx(S)T* amplification with Strep-Tag, stop, SD and start codon for *lacZ* expression to find positive clones. Contains *Bam*HI restriction site for PAC7
BR81	CCTATCACCTCAAATGGTTCGCTGTTACCAGCCGCGTTCTTGC	Rev primer for amplification of a 1000 bp up fragment (*pdxT*) for LFH PCR (with BR77). Has an kan-flag
BR82	CCGAGCGCCTACGAGGAATTTGTATCGAACAGTTGAAAGCTGTGGAAACT	Fwd primer for amplification of a 1000 bp down fragment (*pdxT*) for LFH PCR. Has an kan-flag
BR83	ATTTAGGAAGCTGCCCCGTT	Rev primer for amplification of a 1000 bp down fragment (*pdxT*) for LFH PCR. (with BR82)
BR84	CCTGCTGTAATAATGGGTAGAAGGT	Fwd check primer for pAC7 utilization where Promoter + goi are fused to a Strep tag by fwd and Rev primer and an additional RBS is added after STOP to amplify *lacZ*. Primer binds ~80 bp before the MCS.
BR85	GGGGGATGTGCTGCAAGG	Rev check primer for pAC7 utilization where promoter + goi are fused to a Strep Tag by fwd and rev primers and an additional RBS is added after STOP to amplify *lacZ*. Primer binds ~80 bp after start of *lacZ* gene.
BR86	TTTGGATCCATGGCTCAAACAGGTACTGAACG	Fwd primer for *pdxS(T)* amplification. Includes *Bam*HI restriction site for cloning
BR87	AAAGAGCTCTTATACAAGTGCCTTTTGCTTATATTCCTC	Rev primer for *pdx(S)T* amplification. Includes *Sac*I restriction site for cloning
BR88	AAAGAGCTCATGGCTCAAACAGGTACTGAACG	Fwd primer for *pdxS(T*) amplification. Includes *SacI* restriction site for cloning into pGP574
BR89	TTTGGATCCTACAAGTGCCTTTTGCTTATATTCCTC	Rev primer for *pdx(S)T* amplification. Includes *Bam*HI restriction site for cloning into pGP574
BR90	TTTCCCGGGCATACCAATTCAATTTCGAGTGGGTC	Fwd primer for amplification of *mhqN* UTR for cloning into pAC7. Harbors a *Xma*I restriction site for cloning into pAC7
BR91	AAAGGATCCCCCATTACGCTACACTCCCTTTTATCTC	Rev Primer for amplification of *mhqN* UTR+ATG for cloning into pAC7. Has a *Bam*HI restriction site with "CC"-Spacer to keep the frame.
BR92	AAAGGATCCATGAAACGAGATAAGGTGCAGAC	Fwd primer for amplification of *aroE* in *B. subtilis* for cloning into pGP3273. Harbors a *Bam*HI restriction site
BR93	TTTGAATTCTCAGGATTTTTTCGAAAGCTTATTTAAATG	Rev primer for amplification of *aroE* in *B. subtilis* for cloning into pGP3273. Harbors a *Eco*RI restriction site
BR94	GCGGTAGCGGCATTAAAATTAGG	Fwd primer for amplification of a 1000bp upstream region of *aroE* for LFH PCR (kan-cassette)
BR95	CCTATCACCTCAAATGGTTCGCTGTTTTTCCACCTCAATCAGCATAAAAAG	Rev primer for amplification of a 1000bp upstream region of *aroE* for LFH PCR. Has an overhang to the kan resistance cassette.
BR96	CCGAGCGCCTACGAGGAATTTGTATCGAGTTTTACTTCAGGATTTTTTATGCAGATC	Fwd primer for amplification of a 1000bp downstream region of *aroE* for LFH PCR (kan-cassette). Has an overhang to the kan resistance cassette.
BR97	TTTCACAAGCCCCGCCTG	Rev primer for amplification of a 1000bp downstream region of *aroE* for LFH PCR.

BR98	AAACCCGGGAAAGACCTTTCCTGCCATTAAGATC	Fwd primer for amplification of *mhqN* UTR for cloning into pAC7. Harbors a *Xma*I restriction site for cloning into pAC7. (Longer Fragment than BR90)
BR99	AAAGGATCCATTACGCTACACTCCCTTTTATCTC	Rev primer for amplification of *mhqN* UTR+ATG (from restriction site) for cloning into pAC7. Has a *Bam*HI restriction site
BR100	AAAGGATCCCCCATCTCATTGAGATCTTCTTTTGTGATTG	Rev primer for amplification of *mhqN* UTR+100bp from gene for cloning into pAC7. Has a *Bam*HI restriction site
BR101	AAACTGCAGTTACCAGCCGCGTTCTTGC	Rev primer for cloning of *pdxS* into pBQ200. Harbors a *Pst*I restriction site
BR102	GCGTGCGCTAAGAGAAGAAG	Fwd primer for amplification of Promoter region of PdxST. Can be used together with BP120 amplify promoter region of *pdxS* to check for mutations
BR103	AACAAAGATCTTCACGGGCATC	Fwd primer for amplification of 1000 bp upstream of *pdxS* for LFH PCR.
BR104	CCTATCACCTCAAATGGTTCGCTGTTCTTGGTCCCCCTAATCAAGG	Rev primer for amplification of 1000 bp upstream of *pdxS* for LFH PCR. Harbors a kan-flag.
BR105	CCGAGCGCCTACGAGGAATTTGTATCGAACATAGGAGCGCTGCTGA	Fwd primer for amplification of 1000 bp downstream of *pdxS* for LFH PCR. Has a kan flag for the resistance cassette
BR106	CGCCTTTGTGTACAAGCTTTGC	Rev primer for amplification of 1000 bp downstream of *pdxS* for LFH PCR.
BR107	AGAAATCGTTGCCGGTTCTGA	Fwd check primer for LFH knockout of *pdxS*
BR108	GCAACCTGCTGAGAAACTTCATTT	Rev check primer for LFH knockout of *pdxS*
BR109	GTAAGCGTAAGTCAGTAGTGTACATT	Fwd primer for check of *aroA* gene deletion
BR110	ACATTGCCCTCACATCATGTAGG	Rev primer for check of *aroA* gene deletion
BR111	GTAGCCGACAGAAGCATTTGAAC	Fwd primer for check of *aroB* gene deletion
BR112	TTTCTGCCAGCATTGAACGTTC	Rev primer for check of *aroB* gene deletion
BR113	TCATGATGCTTTCCTTTGACAGAC	Fwd primer for check of *aroC* gene deletion with AK238
BR114	CGCAATTCTGAAGCACGCTG	Fwd primer for check of *aroD* gene deletion
BR115	GGTCGCCCATACGTTTACGA	Rev primer for check of *aroD* gene deletion
BR116	AAACCCGGGCCGCATGACGTACAGATTACAAAAG	Fwd primer for amplification of *pdxST* Promoter region for LFH PCR. Harbors a *Xma*I restriction site for cloning in pAC7
BR117	CACGTTCAGTACCTGTTTGAGCCATTCGTACGACCTCCGTATTTCATTT	Rev primer for amplification of *pdxST* promoter region. Has an overhang to *pdxS* for LFH.
BR118	ATGGCTCAAACAGGTACTGAACG	Fwd primer for amplification of *pdxS*
BR119	AAAGGATCCATTTGTTAGTATCCTCCTTTACCAGCCGCGTTCTTGC	Rev primer for *pdxS* amplification with SD and start codon for *lacZ* expression to find positive clones. Contains *Bam*HI restriction site for pAC7
BR120	GCCAGACACGTGACAGCTAG	Rev primer for amplification of *pdxST* promoter region. Has an overhang to *pdxS* for LFH.
BR121	GGACAGTGGAAGCTGAGCTTG	Fwd primer for amplification of 1000 bp upstream fragment in *serS* for LFH PCR)
BR122	CCGAGCGCCTACGAGGAATTTGTATCGTTACGGTTTCATTACTTCTCTATTCCC	Rev primer for amplification of a 1000 bp upstream fragment of *serS*. Harbors an overhang to the *ermC gfp* construct for LFH PCR
BR123	AGCATAGCGCGCCTTCACTTGACAAATTATGGAAAGGCGTGCCTGA	Fwd primer for amplification of a 1000 bp downstream fragment of *serS* for LFH PCR with *gfp* cassette. Harbors an overhang to *gfp* cassette
BR124	GGAGCTTATCGTATGAAGGAACATCA	Rev primer for amplification of a 1000 bp downstream fragment of *serS* for LFH PCR.
BR125	AAAGGATCCTTTAAAGGAGGAAACAATCATGGCTCAAACAGGTACTGAACG	Forward primer for amplification of *pdxS* and cloning into pBQ200. Harbors a *Bam*HI restriction site and SD sequence.
BR126	AAAGTCGACTCAGGATTTTTTCGAAAGCTTATTTAAATG	Reverse primer for amplification of *aroE* from BP1122 to check influence of base exchange. Has a *Sal*I restriction site for cloning into pBQ200

BR127	GCAAAGAACTTCGCGACGA	Fwd primer for amplification of 1000 bp upstream region for deletion of *aroA*.
BR128	ACCTATCACCTCAAATGGTTCGCTGTTTTTTCATCCTTTCCCTGCACATTC	Rev primer for amplification of 1000 bp upstream region for deletion of *aroA*. Has a kan-flag for LFH PCR
BR129	GCGCCTACGAGGAATTTGTATCGATTTGAACAATCCAAAAGGCCGC	Fwd primer for amplification of 1000 bp downstream fragment of *aroA*. Has a kan-flag for LFH PCR.
BR130	AGTCCGGAATCATATGTGTAATCCC	Rev primer for amplification of 1000 bp downstream fragment of *aroA*.
BR131	GGAATAATGATGAGCAAAGATGGAATTAA	Fwd check primer for *aroA* deletion
BR132	GCCCTGATCCTGAGCGG	Rev check primer for *aroA* deletion
BR133	TATGAGCGGGGTGCGC	Fwd primer for amplification of 1000 bp upstream region for deletion of *aroB*.
BR134	ACCTATCACCTCAAATGGTTCGCTGTAAAATTCCCTCGACAGTTTTCTCATATT	Rev primer for amplification of 1000 bp upstream region for deletion of *aroB*. Has a kan-flag for LFH PCR
BR135	TTTTACTGGATGAATTGTTTTAGTAATGGCGATTGGAGGAGACATC	Fwdp for amplification of 1000 bp downstream fragment of *aroB*. Has a kan-flag for LFH PCR and the RBS of *aroH*. Deletion cassette may not have terminator!
BR136	ACCCGACAGCTCCGCC	Rev primer for amplification of 1000 bp downstream fragment of *aroB*.
BR137	CGGGCTTTACATAACGGAGGAA	Fwd check primer for *aroB* deletion
BR138	TCTTTGGTATGCGAAGGAACAGA	Rev check primer for *aroB* deletion
BR139	GCACAGCCTCAACAAAAAGAAGC	Fwd primer for amplification of 1000 bp upstream region for deletion of *aroC*.
BR140	CCTATCACCTCAAATGGTTCGCTGCGTCTCAAATCCTTTCAATCTTTACCT	Rev primer for amplification of 1000 bp upstream region for deletion of *aroC*. Has a kan-flag for LFH PCR
BR141	GAGCGCCTACGAGGAATTTGTATCGGGGGATAGAACAATAAAAAAACTCAAGCT	Fwd primer for amplification of 1000 bp downstream fragment of *aroC*. Has a kan-flag for LFH PCR.
BR142	GCTTGAAAGTGCGGATATCATTACC	Rev primer for amplification of 1000 bp downstream fragment of *aroC*.
BR143	GCGTAAAAATAAGCCCGCTGT	Fwd check primer for *aroC* deletion
BR144	CGAATCGGAAGTGAAATTGCA	Rev check primer for *aroC* deletion
BR145	TCAGACTGAAAAACTATAATGAAATACAAGATG	Fwd primer for amplification of 1000 bp upstream region for deletion of *aroD*.
BR146	ACCTATCACCTCAAATGGTTCGCTGACACCTCTCCCCTTTTCTTAAATTAATG	Rev primer for amplification of 1000 bp upstream region for deletion of *aroD*. Has a kan-flag for LFH PCR
BR147	TTTTACTGGATGAATTGTTTTAGTATATTGTAATAGGAAAATTAGGAGGAACAGAAT	Fwd primer for amplification of 1000 bp downstream fragment of *aroD*. Has a kan-flag for LFH PCR and RBS of *yqeI*. Deletion cassette may not have terminator!
BR148	ACGCTCCGCAAGTTCGATC	Rev primer for amplification of 1000 bp downstream fragment of *aroC*.
BR149	GGAGTTACGATCCAAACCGAGG	Fwd check primer for *aroD* deletion
BR150	CGCAGGCATTTTTTCACGC	Rev check primer for *aroD* deletion
BR151	ACCCGATCGCGCGTTATC	Primer for *pgpH* sequencing
BR152	TTCACGCTCAGCGGCA	Primer for *pgpH* sequencing
BR153	TCAGACCGATGATCAATACAGATGAA	Primer for *pgpH* sequencing
BR154	TCATCTGTATTGATCATCGGTCTGAT	Primer for *pgpH* sequencing
BR155	TGTTTCACCAGCCTGTCAATCA	Fwd check primer for *yfhK* mutation
BR156	GGCCATCTTCCCGTCCTG	Rev check primer for *yfhK* mutation
BR157	GGGAAACATGAATGAAGCTGCAG	Fwd check primer for *yhfW* mutation
BR158	CAATGCCTGCTTCCTATGTATGAA	Rev check primer for *yhfW* mutation
BR159	GCTCCGGGTGAAATCTGTTTCA	Fwd primer for *yrrS* check
BR160	GGATACACCGGGAGATAAGG	Rev primer for *yrrS* check
BR161	CCGTCAGCACGCTTTGAAG	Fwd check primer for *yfhJK* mutation
BR162	GGCTCAGTCCGGACATTCAG	Rev check primer for *yfhJK* mutation

BR163	AAAGAATTCCACGCAAATCTATGAAGGCACTC	Fwd primer for amplification of *rpoE* promoter area. For cloning into pAC7 and LacZ measurements. Has a *Eco*Ri restriction site
BR164	TTTGGATCCCCTTCCTGTGAATATTGTTTGATACCCAA	Rev primer for amplification of *rpoE* promoter area. For cloning into pAC7 and LacZ measurements. Has a *Bam*Hi restriction site and 2 bp Spacer (CC)
BR165	GCAGAAATGCAAAAAGGCG	Fwd Primer for *pdxS* probe.
BR166	CTAATACGACTCACTATAGGGAGATCCTCACTCATCG CAACTA	Rev primer for *pdxS* probe. Has T7 Binding Site
BR167	AAAGAATTCGGAAGAAAGAGTTATTGTGATGAGTTA TATTTT	Fwd primer for amplification of *pdxR* (Incl terminator) + promoter *pdxS* from *Listeria monocytogenes.* Adds an *Eco*RI restriction site. For cloning into pAC7
BR168	TTTGGATCCCCCTCCATATTAATAACCTCCATCAAAGT TA	Rev primer for amplification of *pdxR* + Promoter *pdxS* from *Listeria monocytogenes*. Adds an *Bam*HI restriction site. For cloning into pAC7
BR169	TTTCACGTCTCAATTGAAAGAGCTCTGATGGATGT	Fwd primer for amplification of *pdxS* with its native promoter. Adds a *Bmg*BI restriction site for cloning into pGP886
BR170	TTTGTCGACTTACCAGCCGCGTTCTTGC	Revp for amplification ofp. Adds a *Sal*I restriction Site for cloning
BR171	AAAAGGCCTTCAATTGAAAGAGCTCTGATGGATGT	Fwd primer for amplification of *pdxS* with its native promoter. Adds a *Stu*I restriction site for cloning into pGP888
BR172	AAAGAATTCTCAATTGAAAGAGCTCTGATGGATGT	Fwd primer for amplification of the *pdxS* promoter. Adds an *Eco*RI restriction site for cloning into pAC5 / pAC7
BR173	TTTGGATCCAGCCATTTCTTGGTCCCCCTA	Rev primer for amplification of the *pdxS* promoter. Adds a *Bam*HI restriction site for cloning into pAC5/ pAC7
BR174	AAAGGGCCCCTCCATATTAATAACCTCCATCAAAGTT A	Fwd primer for amplification of *Listeria monocytogenes pdxR* with its native promoter. Adds an *Apa*I restricton site for cloning.
BR175	AAAAGGCCTCTCCATATTAATAACCTCCATCAAAGTTA	Fwd primer for amplification of *Listeria monocytogenes pdxR* with its native promoter. Adds a *Stu*I restriction site for cloning.
BR176	TTTGTCGACGGAAGAAAGAGTTATTGTGATGAGTTAT ATTTT	Rev primer for amplification of *Listeria monocytogenes pdxR*. Adds a *SalI* Restriction site for cloning.
BR177	TTTCCGGTCTCATGGTATGGCTCAAACAGGTACTGAA CG	Fwd primer for amplification of *pdxS*. Adds a *BsaI* restriction site with spacer for cloning into pETSUMOadapt.
BR178	TTTCTCGAGTTACCAGCCGCGTTCTTGC	Rev primer for amplification of *pdxS*. Adds a *Xho*I Restriction site with spacer for cloning into pETSUMOadapt.
BR179	TTTCCGGTCTCATGGTATGTTAACAATAGGTGTACTA GGACTTCA	Fwd primer for amplification of *pdxT*. Adds a *BsaI* restriction site with spacer for cloning into pETSUMOadapt.
BR180	AAACTCGAGTTATACAAGTGCCTTTTGCTTATATTCCT C	Rev primer for amplification of *pdxT*. Adds an *Xho*I restriction site with spacer for cloning into pETSUMOadapt.
BR181	CCATTTTCAGAACTGGGTGCG	Rev sequencing primer for *pdxR* plasmids
BR182	GCCAGCTTCGTCCTCCTG	Fwd sequencing primer for *pdxR* plasmids
BR183	GCAGAGAAGTATCAAGCAAATGAAG	Fwd sequencing primer for pBP786
BR184	CCGGTGCATTCTGTCCTAAGG	Fwd sequencing primer for pBP787
BR185	AAACCGGTCTCATGGTATGGTATGCCAGCATAATGAT GAG	Fwd primer for amplification of *ylaM*. Has a *Bsa*I restriction Site
BR186	TTTCTCGAGTTAAAAAATACTCAGCCTGTACATCTCAG	Rev primer for amplification of *ylaM*. Has an *Xho*I restriction Site.
BR187	GCCAAACACGCCGATGAAAA	Fwd primer for amplification of *recA*::*spc* LFH product

BR188	GGCTGACGTTTGATTCCCTGA	Rev primer for amplification of *recA::spc* LFH Product
BR189	GGAGCAAGCATCGAATTATCAGC	Fwd primer for upstream fragment (*glnA* mutation insertion)
BR190	TCCTATCACCTCAAATGGTTCGCTGTTAATACTGAGAC ATATACTGTTCGCGT	Rev primer for upstream fragment (*glnA* mutation insertion). Has a kan flank
BR191	CCGAGCGCCTACGAGGAATTTGTATCGTATCTCAATC CCTTGGCACTAAAAGT	Fwd primer for downstream fragment (*glnA* mutation insertion). Has a kan flank
BR192	CGTGCCTCTATGGAAATGGTCA	Rev primer for upstream fragment (*glnA* mutation insertion)
BR193	TGCCAGGCTATTCAATTTGCATC	Fwd check primer for *glnA* mutation insertion (Left flank)
BR194	GGAATCTGTATATAATCTTCATCGCTTG	Rev check primer for *glnA* mutation insertion (Right Flank)
BR195	CCGCCTTTGTCGTTTAGTTCAAG	Rev check primer for *glna* mutation insertion
BR196	CAGTCCGCTCTTGTGATGACA	Fwd check primer for *glna* mutation insertion
BR197	CCTATCACCTCAAATGGTTCGCTGTTAACGGTTTTTGT ACCGAAAGTGAG	Rev primer for upstream fragment (*tnrA* mutation insertion) used with AL22. Has a kan flank
BR198	CCGAGCGCCTACGAGGAATTTGTATCGATAATAAAAG TCCGGCTCGCAGT	Fwd primer for down fragment (*tnrA* mutation insertion). Has a kan flank
BR199	CCCGCGTCATATCAGCAGAT	Rev primer for down fragment (*tnrA* mutation insertion).
BR200	TTCAGTCCCTTACTATACGTATTCGC	Fwd check primer for *tnrA* LFH
BR201	GGATCCCTCAGCATTACACGG	Rev check primer for *tnrA* LFH
BR202	AAAGGATCCATTGGGTATCAAACAATATTCACAGGAA G	Fwd primer for amplification of *rpoE*. Has a *Xba*I restriction site for cloning and adds an additional base to keep the frame in pGP888
BR203	TTTGAATTCCTATTTAATTTCCTCTTCTTCATCATCATA GTCT	Rev primer for amplification of *rpoE*. Has a *Bam*HI restriction Site for cloning.
BR204	TTTTCTAGAAGTGTTGAAAAAGAAGGCTAAGACCAAA	Fwd primer for amplification of *pgpH*. Has a *Xba*I restriction site for cloning and adds an additional base to keep the frame in pGP888. Can be used together with JN209
BR205	TTTTCTAGAAATGCCAAGCTTTTATGAAAAACCG	Fwd primer for amplification of *gdpP*. Has a *Xba*I restriction site for cloning and adds an additional base to keep the frame in pGP888.
BR206	TTTGGATCCTCATCTCTGTACGCCTCCCTC	Rev primer for amplification of *gdpP*. Has a *Bam*HI restriction Site for cloning.
BR207	GCACTGCCGCTGCCA	Fwd primer for amplification of up fragment of *gdpP*.
BR208	CCTATCACCTCAAATGGTTCGCTGTTCTATCACTCCCC ACCATGTG	Rev primer for amplification of up fragment of *gdpP*. Has a kan-flank
BR209	CCGAGCGCCTACGAGGAATTTGTATCGGGAGGCGTA CAGAGATGAAGG	Fwd primer for amplification of down fragment of *gdpP*. Has a kan-flank
BR210	GGCTCCTGTCCTATCTGAGAAAAC	Rev primer for amplification of down fragment of *gdpP*.
BR211	CAGGATGATACGAGGTGACTAAGTG	Fwd check primer for *gdpP* LFH
BR212	TGGTCAGCAACGCCGC	Rev check primer for *gdpP* LFH
BR213	GTTTATCACAAACGGCGGCG	Fwd primer for amplification of up fragment of *rpoE*
BR214	CCTATCACCTCAAATGGTTCGCTGGGTCGGACACTCC CTTTCTATTAC	Rev primer for amplification of up fragment of *rpoE*. Has a kan-flank
BR215	CCGAGCGCCTACGAGGAATTTGTATCGTATCAGATTT CATCTTCAAAACGATCTTGAC	Fwd primer for amplification of down fragment of *rpoE*. Has a kan-flank
BR216	CCAAGCTGCGCAATTCTTTTAC	Rev primer for amplification of down fragment of *rpoE*.
BR217	ACACAGCTGGCTCTCGGA	Fwd check primer for *rpoE* LFH
BR218	GGTTGTCCGCATCTTCACATTC	Rev check primer for *rpoE* LFH
BR219	CGGTTCTTGACCAGGACGTAA	Fwd check primer for presence of *mhq*
BR220	TTCCAATCGTAATCATCATGACAACC	Rev check primer for presence *mhqN*
BR221	CCGAGCGCCTACGAGGAATTTGTATCGGGAACCTTTG CCGAACTGTT	Rev primer for *recA* up fragment. Has a kan flank (with BR187)
BR222	CCTATCACCTCAAATGGTTCGCTGAGCAAGCTGAAGA GACACAAGA	Fwd primer for downstream fragment of *recA*. Has a kan flank (with BR188)

BR223	TTTGGCGGCTGATGGAGAG	Fwd check primer for *recA* LFH
BR224	CCTGCATGCGTCATTCCCG	Rev check primer for *recA* LFH
BR225	TGTTTTGACAATGCGCAGTATCG	Fwd check primer *pdxS* promoter
BR226	CCCGTCAACTTCGTTCAGCT	Rev check primer *pdxS* promoter
BR227	ATGCTGAAATCATGGTTGAAAAAGAAGA	Fwd primer for upstream fragment for *ybxA-ybaF* deletion LFH
BR228	CCTATCACCTCAAATGGTTCGCTGGGAAAACCGGCCTCCTCTC	Rev primer for upstream fragment for *ybxA-ybaF* deletion LFH. Has a kan-flag
BR229	CCGAGCGCCTACGAGGAATTTGTATCGGCTTAATTGTATTAGCTGCTTTGTTATTTTT	Fwd primer for downstream fragment for *ybxA-ybaF* deletion LFH. Has a kan-flag
BR230	GGTTTAGTTGTCATAGTAAACGTTCCATA	Rev primer for downstream fragment for *ybxA-ybaF* deletion LFH
BR231	TGGACTGATGGAAGCACTGG	Fwd check primer for *ybxA-ybaF* deletion.
BR232	TAAGTTCCGGGGCTTATCGTG	Rev check primer for *ybxA-ybaF* deletion.
BR233	AAATCTAGAGATGGCAGACACATTAGAGCGT	Fwd primer for *acpA* amplification. Has a *Xba*I restriction site and a spacer to keep the frame in BACTH plasmids
BR234	AAAGGTACCCCTTGCTGGTTTTGTATGTAGTTCACAG	Rev primer for *acpA* amplification. Has a *Kpn*I restriction site and a spacer to keep the frame in BACTH plasmids
BR235	AAATCTAGAGATGGCAGAACGTATGGTAGGTAAA	Fwd primer for *aphA* amplification. Has a *Xba*I restriction site and a spacer to keep the frame in BACTH plasmids
BR236	AAAGGTACCCCAAGTGTTTTTTGGCCTGGTTTCC	Rev primer for *acpA* amplification. Has a *Kpn*I restriction site and a spacer to keep the frame in BACTH plasmids
BR237	AAATCTAGAGATGGCTCAAACAGGTACTGAACG	Fwd primer for *pdxS* amplification. Has a *Xba*I restriction site and a spacer to keep the frame in BACTH plasmids
BR238	AAAGGTACCCCCCAGCCGCGTTCTTGC	Rev primer for *pdxS* amplification. Has a *Kpn*I restriction site and a spacer to keep the frame in BACTH plasmids
BR239	AAATCTAGAGATGTTAACAATAGGTGTACTAGGACTTCA	Fwd primer for *pdxT* amplification. Has a *Xba*I restriction site and a spacer to keep the frame in BACTH plasmids
BR240	AAAGGTACCCCTACAAGTGCCTTTTGCTTATATTCCTC	Rev primer for *pdxT* amplification. Has a *Kpn*I restriction site and a spacer to keep the frame in BACTCH plasmids
BR241	AAAGAATTCGTTGAGCTCCTTTTCTAATTGATTGCT	Fwd primer for amplification of *aphA* promoter. Has a *Eco*RI restriction site for cloning into pAC7
BR242	AAAGGATCCCCATGTATCCCTCCAATTTATTGTTTGGC	Rev primer for amplification of *aphA* Promoter. Has a *Bam*Hi restriction site for cloning into pAC7
BR243	AAAGAATTCATGTATCCCTCCAATTTATTGTTTGGC	Fwd primer for amplification of ykuT Promoter. Has a *Eco*RI restriction Site for cloning into pAC7
BR244	AAAGGATCCCCGTTGAGCTCCTTTTCTAATTGATTGCT	Rev primer for amplification of *ykuT* Promoter. Has a *Bam*HI restriction Site for cloning into pAC7
BR245	AGAAAGCAGTCGAAGAAGATCTTGAT	Fwd primer for amplification of upstream fragment for resistance change of BP1125 (*rpoE*)
BR246	CCGAGCGCCTACGAGGAATTTGTATCGGAGGGTTGCCAGATTTAAAGGATC	Rev primer for amplification of upstream fragment for resistance change of pGP886 integration. Has a kan-flank
BR247	CCTATCACCTCAAATGGTTCGCTGCCGGGGTCAGGCTGACA	Fwd primer for amplification of downstream fragment for resistance change of pGP886 integration. Has a kan-flank
BR248	GCATTTGCTTGAATACACTAAAGAACC	Rev primer for amplification of downstream fragment for resistance change of pGP886 integration
BR249	CTTCGCAGCTGGTATCCTTATGA	Fwd check primer for BP1125 resistance change (*rpoE*)
BR250	CTGCTCCGGCTTTTCCTCC	Rev check primer for resistance change of pGP886 integration.

BR251	GGAAGCCGCAATTATATCAGTGG	Fwd primer for amplification of upstream fragment for resistance change of BP1126 (*pgpH*)
BR252	GCTTGTCGATATTGCTGAGCA	Fwd check primer for BP1126 resistance change (*pgpH*)
BR253	TGCTATCGATGAGTGAGGTTGAG	Fwd primer for amplification of upstream fragment for resistance change BP1127(*pgpH*)
BR254	TGATTCCTATATTAAACGGGCGAAG	Fwd check primer for BP1127 resistance change (*gdpP*)
BR255	-	-
BR256	GAAACGGTTTGTGCTGGATGAC	Fwd primer for LFH upstream fragment (integration into xkdE)
BR257	GATTTATGACCTCCTCCTTTCTCGG	Rev primer for LFH upstream fragment v(integration into xkdE)
BR258	CGAGCGCCTACGAGGAATTTGTATCGAGGGAGGTGAATCAAGCAGG	Fwd primer for LFH downstream fragment (integration into *xkdE*) has a kan flag
BR259	GTATTGCCGCCCGTATCGT	Rev primer for LFH downstream fragment (integration into *xkdE*)
BR260	TCGCCATCCTGAGCTAAAGC	Fwd check primer for *xkdE* LFH
BR261	CAATTGATTTCCTCCTCCTTGACTG	Rev check primer for *xkdE* LFH
BR262	CCGAGAAAGGAGGAGGTCATAAATCGGAAGAAAGAGTTATTGTGATGAGTTATATTTT	Fwd primer for amplification of *pdxR* (Incl terminator) + promoter *pdxS* from *Listeria monocytogenes*. Has an overlapping sequence to *xkdE* for LFH PCR
BR263	ATTAATAACCTCCATCAAAGTTAATTGAAATTACC	Rev primer for amplification of *pdxR* (Incl terminator) + promoter *pdxS* from *Listeria monocytogenes*.
BR264	ATTAACTTTGATGGAGGTTATTAATATGAAACCAGTAACGTTATACGATGTC	Fwd primer for amplification of *lacI* from pHT01. Has an overhang to *pdxR-*P_{pdxS} construct
BR265	CCTATCACCTCAAATGGTTCGCTGTCACTGCCCGCTTTCCAG	Rev primer for amplification of *lacI* from pHT01. Has a kan-flag
BR266	CACGATTGACCCGCATACG	Fwd check primer for Screening-System Fragment #1
BR267	AAGAAGGAAGCGAGACGTGTAG	Rev check primer for Screening-System Fragment #1
BR268	CCAACTGTCTTTCTGGAGGCA	Fwd check primer for Screening-System Fragment #2
BR269	CCGCCATCGCCGCTT	Rev check primer for Screening-System Fragment #2
BR270	GAAAAACCACCCTGGCGC	Fwd check primer for Screening-System Fragment #3
BR271	AAAGAATTCAGCTAACGGAAAAGGGAGCG	Fwd primer for amplification of IPTG inducible P*grac* Promoter from pTH01. Has an *Eco*RI restriction site
BR272	AAAGGATCCCCCATATTTATTTCCTCCTTTAATTGGGAATTGTTATCC	Rev primer for amplification of IPTG inducible P*grac* promoter from pTH01. Has an *Bam*HI restriction site. Adds the start codon + spacer to the construct
BR273	AAAGAATTCCTTGTCAAGTGAAGGCGCGCTATGCTACAATACAGCTTGGTTTAAAGGAGGAAACAATCATGGCTCAAACAGGTACTGAACG	Fwd primer for amplification of *pdxS*. Adds an *Eco*RI restriction dite and the P*alf4* promoter.
BR274	AAAGAATTCCAACATAGTACATAGCGAATCTTCCC	Fwd primer for amplification of P*xyl* from pGP888 Adds an *Eco*RI restriction dite
BR275	GATTGTTTCCTCCTTTCAGATGCAT	Rev primer for amplification of P*xyl* from pGP888
BR276	ATGCATCTGAAAGGAGGAAACAATCATGGCTCAAACAGGTACTGAACG	Fwd primer for amplification of *pdxS*. Adds an overhang to P*xyl*.
BR277	AAACTGCAGAAAGGAGGAAACAATCATGGCTGAATTACTGTTAGGCGT	Fwd primer *pdxJ* amplification from *E. coli*. Adds SD + spacer and a restriction site *Pst*I
BR278	TTTAAGCTTTTAGCCACGCGCTTCCAG	Rev primer for *pdxJ* amplification from *E. coli*. Has a *Hind*III restriction site
BR279	CACGTTGTGATAGGTCAGACTGTGTCCG	5'phosphorylated CCR primer for *pdxJ* amplification
BR280	AAAGAATTCCAAAATAAAGCACCTCCTGGTTGAT	Fwd primer for amplification of *ytoP* promoter. Adds a *Eco*RI restriction site
BR281	TTTGGATCCCCATTCATGTTTTCCCCTCATTTCTATGTAG	Rev primer for amplification of *ytoP* promoter. Adds a *Bam*HI restriction site and a framekeeper
BR282	CCTGGTCTCGGTCTTGGAAC	Fwd LFH primer for amplification of upstream fragment for *ytoP* deletion
BR283	CCTATCACCTCAAATGGTTCGCTGGTTTTCCCCTCATTTCTATGTAGTCC	Rev LFH primer for amplification of upstream fragment for *ytoP* deletion. Adds a kan overhang

BR284	CCGAGCGCCTACGAGGAATTTGTATCGGGTTTCATAA AAGCTTGTTGAACGAG	Fwd LFH primer for amplification of downstream fragment for *ytoP* deletion. Adds a kan overhang
BR285	AGACCAATCATCCTTTACCGCTT	Rev LFH Primer for amplification of downstream fragment for *ytoP* deletion
BR286	CGGAGGATTTAATCGAATGGACAGA	Fwd check primer for *ytoP* knockout
BR287	CGTAGTAGTAGGTGAATTCAGGGAAA	Rev check primer for *ytoP* knockout
BR288	AAAGGATCCAAAGGAGGAAACAATCATGAATCAAGA AACGAAAGCGCT	Fwd primer for amplification of *ytoP*. Adds SD +Spacer and *Bam*HI Restriction Site
BR289	TTTCTGCAGCCTACATATTTTCTTTAATCGCATCCACT	Rev primer for amplification of *ytoP*. Adds *Pst*I testriction Site
BR290	GCTTGAACGAATCGGGAAGAC	Fwd primer for amplification of *bshC::cat* knockout gragment
BR291	CAGAATGAGCTGTCCTTTATAATCGG	Rev primer for amplification of *bshC::cat* knockout fragment
BR292	AGTCTGAAGGAATCCGGCTTTATAA	Fwd check primer fo *bshC* knockout
BR293	AATCAAATAAAATGCCGTCTACTTCTGT	Rev check primer for *bshC* knockout
BR294	TTTTCTGTGAAATTGCGTTTTGGG	Fwd check primer for *MiniBacillus* check PCR
BR295	GTTATCAAAAGCAAGTTCAATGATAGCC	Rev check primer for *MiniBacillus* check PCR
BR296	CCATCGAAGCTTGAGGTCCG	Alternative rev check primer for *MiniBacillus* check PCR
BR297	AAAGGATCCCCTTCCATTTTTGTTGCCTCCTTTAATTG GGAATTGTTATCCGCTCACAATTCCAGTGTGTAACCA CACAGTATATACTATCC	Rev primer for amplification of P*cspC*. Adds Lac operator, SD and start codon. Adds *Bam*HI restriction site
BR298	AAAGGATCCCCTTCCATTTTTGTTTCCTCCTCAATTAA GGAATTGTGAGCGGATAACAATTCCGGTGAACGAGT CCTAGGTATTTGAT	Rev primer for amplification of P*Deg36* from pBQ200. Adds Lac Operator, SD and start codon Adds *Bam*HI restriction site
BR299	GTAAAACGACGGCCAGTGAATT	Fwd primer for amplification of P*Deg36*. PCR Product contains *Eco*RI Site.
BR300	AGTTCAGGCAGGAAAGGCTC	Fwd primer for 800 bp *ytoQ* amplification for *gfp* fusion
BR301	CAGTGAAAAGTTCTTCTCCTTTACTCATTTCTGTTTCAA ATAGATAGGAAAGGGC	Rev primer for *ytoQ* amplification. STOP codon skipped. Has an overhang to *gfp*
BR302	CCGAGCGCCTACGAGGAATTTGTATCGGAAAAAAGC TTGTCGATCCGGG	Fwd primer for 800 bp *ytoQ* downstream amplification for *gfp* fusion. Has a kan overhang to *ermC* cassette (w/o terminator)
BR303	TGAAATTCAAACAGATAACGGGCC	Rev primer for 800 bp *ytoQ* downstream fragment
BR304	GCTTGGGATTCCGTAAATTTCCC	Fwd check primer *ytoQ gfp* fusion
BR305	GCCGGGCACATGGACGA	Rev check primer *ytoQ* gfp fusion
BR306	ATGAGTAAAGGAGAAGAACTTTTCACTG	Amplification of *gfP ermC* cassette.
BR307	CGGGACATTTCGAAACAGCAG	Fwd *bioW* promoter check primer
BR308	TCCGCCGGATATATGCTTTCC	Rev *bioW* promoter check primer
BR309	GTTCGGGAATTTTTATTTTCAGCCTATG	Fwd primer for amplification of *pbuX* upstream fragment for LFH. Has a kan_flag
BR310	CCTATCACCTCAAATGGTTCGCTGTCATGAATGAACCT CCTGTACGAAG	Rev primer for amplification of *pbuX* upstream fragment for LFH. Has a kan_flag
BR311	CCGAGCGCCTACGAGGAATTTGTATCGGCAGTCTAAC TCCGCCGC	Fwd primer for amplification of *pbuX* downstream fragment for LFH. Has a kan_flag
BR312	TCGGTAAAGTCCCAGCCCA	Rev primer for amplification of *pbuX* downstream fragment for LFH. Has a kan_flag
BR313	AATCTCTATCTGTTATAATCAAAAGCCTGG	Fwd check primer for *pubX* deletion
BR314	GCCAAAAAAACACTGATATCATGGAAT	Rev check primer for *pubX* deletion
BR315	GGCAAACTCGATCGCAATGAC	Rev primer for amplification of *pucJK* upstream fragment for LFH. Has a kan_flag
BR316	CCTATCACCTCAAATGGTTCGCTGTATAGTCCCTCCCT GTATGTGAACT	Fwd primer for amplification of *pucJK* upstream fragment for LFH. Has a kan_flag
BR317	CCGAGCGCCTACGAGGAATTTGTATCGAGGAAGATG CCAATGTTCACAATG	Rev primer for amplification of *pucJK* downstream fragment for LFH. Has a kan_flag
BR318	ATAGTGTAGGAACCCCTCGGT	Fwd primer for amplification of *pucJK* downstream Fragment for LFH. Has a kan_flag
BR319	GGCCCGCAATGAGTTTTTCAA	Rev primer for *pucJK* deletion
BR320	AGGCGGCTTGTGCTGAG	Fwd check Primer for *pucJK* deletion

BR321	AGGCGCGCACCTTGTC	Rev primer for amplification of *pyrP* upstream fragment for LFH.
BR322	CCTATCACCTCAAATGGTTCGCTGGATGATTTCCCCCT GATTGTTGAC	FwdPrimer for amplification of *pyrP* upstream fragment for LFH. Has a kan_flag
BR323	CCGAGCGCCTACGAGGAATTTGTATCGAACCTTTTAA TGAAAGTCCAGAGAGG	Rev primer for amplification of *pyrP* Downstream fragment for LFH. Has a kan_flag
BR324	GCGGACTGATGTCGTTCATTTT	Fwd primer for amplification of *pyrP* Downstream fragment for LFH.
BR325	GAAAAAACGGTTGACAGAGGGTTT	Rev check primer for *pyrP* deletion
BR326	CCTCTGTTTACCGGAGCAGG	Fwd check primer for *pyrP* deletion
BR327	CAGGGATGATGATGCTTTTGGAG	Fwd primer for amplification of *ywdJ* upstream fragment for LFH.
BR328	CCGAGCGCCTACGAGGAATTTGTATCGGTAAGCTCAA CCTCTCTCTTTAAAAATCA	Rev primer for amplification of *ywdJ* upstream fragment for LFH. Has a kan_flag
BR329	CCTATCACCTCAAATGGTTCGCTGATTTTGGTGCAGG TGCGATAAG	Fwd primer for amplification of *ywdJ* downstream fragment for LFH. Has a kan_flag
BR330	GGGCGGCGGTTTTCAAATT	Rev primer for amplification of *ywdJ* downstream fragment for LFH.
BR331	CGACCGTGTTCCCCGC	Fwd check primer for *ywdJ* deletion
BR332	CACAAATGCAGACAATGGGGATG	Rev check primer for *ywdJ* deletion
BR333	GGTATAGAGGGATTGGCTGTTTGA	Rev primer for amplification of a *yxlA* upstream fragment
BR334	CCGAGCGCCTACGAGGAATTTGTATCGTGCATCGTCC TCCATATTGGATG	Fwd primer for amplification of a *yxlA* u*Pst*ream fragment. Has a kan flag.
BR335	GGGCCACGCTTCCTGAAA	Fwd primer for amplification of a *yxlA* downstream fragment.
BR336	CCTATCACCTCAAATGGTTCGCTGAGACAGCTTTTTTA TCATTCAAATCGTTTC	Rev primer for amplification of *yxlA* downstream Fragment. Has a kan Flag
BR337	CAACCATCAGCGCGCAG	Rev check primer for *yxlA* deletion
BR338	CCCTAAAAGGCTGTCTTTTTTGTTTG	Fwd check primer for *yxlA* deletion
BR339	AAGGATTTCATCGCGATGCATG	Fwd seq Primer for *pdxT* knockout
BR340	CGCGAGCTGAAAACGGATAAG	Fwd primer for amplification of *pucI* upstream fragment for LFH.
BR341	CCTATCACCTCAAATGGTTCGCTGGCCGGCTCCCCTTT CC	Rev primer for amplification of *pucI* upstream Fragment for LFH.
BR342	CCGAGCGCCTACGAGGAATTTGTATCGCCGCCAGGCT GAATAAGATCTA	Fwd primer for amplification of *pucI* Downstream Fragment for LFH.
BR343	TGCGGACCACATTGCCTG	Rev primer for amplification of *pucI* Downstream Fragment for LFH.
BR344	GGGGGAATCATTTTCCTTCTATCCT	Fwd check primer *pucI* deletion
BR345	GCAGGATTGCCGTAAGTCGA	Rev check primer *pucI* deletion
BR346	GCGCCGTGCCAAAATAAAAG	Fwd primer for amplification of *yitU* upstream fragment for LFH.
BR347	ACCTATCACCTCAAATGGTTCGCTGGAAAAGCTCCTTT AACCTTAGATTTTGC	Rev Primer for amplification of *yitU* upstream fragment for LFH.
BR348	CCGAGCGCCTACGAGGAATTTGTATCGAGAAGGTCC GTATTAATTTTTCCCACT	Fwd Primer for amplification of *yitU* downstream fragment for LFH.
BR349	TTGACGCAATTCAGGAGCCT	Rev Primer for amplification of *yitU* downstream frragment for LFH.
BR350	GGTCAGCGTTTTCATCTCCGA	Fwd check primer *yitU* deletion
BR351	CTGCTTGTGATGCTCCTCCC	Rev check primer *yitU* deletion
BR352	GGCGCCACGGTCACC	Fwd primer for *cpgA* LFH upstream fragment
BR353	CCTATCACCTCAAATGGTTCGCTGGTTATTCATCTTTC GGATACTCAATGGTT	Rev primer for *cpgA* LFH upstream fragment. Has a kan flag
BR354	CCGAGCGCCTACGAGGAATTTGTATCGATGATAAAG GTTGCACCATCTATTCTTTC	Fwd primer for *cpgA* LFH downstream fragment has a kan Flag.
BR355	AGAGCTTAGGCTATTGTATGCCC	Rev primer for *cpgA* LFH downstream fragment
BR356	CAGGGCTCTTGGAAAAAGAAGG	Fwd check primer for *cpgA* LFH
BR357	CCGATCCAAAGCGTGTGTTC	Rev primer for *cpgA* LFH
BR358	CTTCATATAAGCTCTTCCTCTGCATTC	Fwd primer for *serC* LFH upstream fragment

BR359	CCTATCACCTCAAATGGTTCGCTGGATCTCTCCCTGTTTTCCACGT	Rev primer for *serC* LFH upstream fragment. Has a kan flag
BR360	CCGAGCGCCTACGAGGAATTTGTATCGAAAGTCTGGCTATGCAAACCGT	Fwd primer for *serC* LFH downstream fragment. Has a kan Flag.
BR361	AAACATGCTGACATGCTGCG	Rev primer for *serC* LFH downstream fragment
BR362	GTCAAATGAGGTGTTTATCGCAGG	Fwd check primer for *serC* LFH
BR363	AGCCAGGCTGCTTCTTATCC	Rev check primer for *serC* LFH
BR364	TCGCTCAAGCTGTCATGTACG	Fwd primer for *thrB* LFH upstream fragment
BR365	CCTATCACCTCAAATGGTTCGCTGTCATACACGGGCCGCTC	Rev primer for *thrB* LFH upstream fragment. Has a kan flag
BR366	CCGAGCGCCTACGAGGAATTTGTATCGATCATGCCAAGCGTTCCTCAG	Fwd primer for thrB LFH downstream fragment. Has a kan Flag.
BR367	GCCATTTGCCGTCAGGAGA	Rev primer for thrB LFH downstream fragment
BR368	GTGCGCGTCAACAGGTAAC	Fwd check primer for thrB LFH
BR369	AATGAGCCGCGATGAGGTG	Rev check primer for thrB LFH
BR370	*AGCATAGCGCGCCTTCACTTGACAA*GATTTATGACCTCCTCCTTTCTCGG	Rev primer for amplification of *xkdE* upstream fragment for LFH. Has an overhang to P*alf4* promoter
BR371	*TTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATGGATCTCTGGATCCTCTAGAAGGAGGAAACAATCATG*AAAATCCTTGTTGATGAAAATATGCCTTATG	Fwd primer for amplification of *E. coli pdxB*. Adds P*alf* promoter + SD + ATG.
BR372	*CCTATCACCTCAAATGGTTCGCTG*TTAACGTGCCGGATGATGAACG	Rev primer for amplification of *E. coli pdxB*. Has a kan-overhang
BR373	AGCACACAAACGAATGCGG	Seq primer of *pdxB* LFH
BR374	*AGCATAGCGCGCCTTCACTTGACAA*CCGCTCGGCGTTGCTA	Rev primer for *ganA* upstream fragment for LFH. Has an overhang to P*alf4* promoter
BR375	*TTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATGGATCTCTGGATCCTCTAGAAGGAGGAAACAATC*ATGGTTAAAACCCAACGTGTTGTG	Fwd primer for amplification of *E.coli pdxA*. Adds P*alf4* promoter.
BR376	*CCTATCACCTCAAATGGTTCGCTG*TCATTGGGTGTTAACAATCATTTTGATG	Rev primer for amplification of *pdxA*. Has a kan overhang
BR377	*CCTATCACCTCAAATGGTTCGCTG*GTAAGCTCAACCTCTCTCTTTAAAAATCA	Rev primer for amplification of *ywdJ* upstream fragment for LFH. Has a kan_flag
BR378	*CCGAGCGCCTACGAGGAATTTGTATCG*ATTTTGGTGCAGGTGCGATAAG	Fwd primer for amplification of *ywdJ* downstream fragment for LFH. Has a kan_flag
CD03	AAAGGATCCTGATAGAACAAGGTGTAAACATGGC	Amplification/ sequencing of *glnA* (laboratory collection)
CD04	AAAGAATTCTTCTTTTACTAATTTTTCGATATC	Amplification/ sequencing of *glnA* (laboratory collection)
CZ126	CAGCGAACCATTTGAGGTGATAGGGAACGATGACCTCTAATAATTG	Amplification of phleomycin resistance cassette (laboratory collection)
CZ68	CGATACAAATTCCTCGTAGGCGCTCGGTTACTTATTAAATAATTTATAGCTATTG	Amplification of erythromcycin resistance cassette without terminator (laboratory collection)
CZ228	GAGGAAATACCGCTTTAACGCTC	LFH in *ganA* (laboratory collection)
CZ229	GCTTCTCCAAGCTGCTTAAATCCCGTATCGTTCTGCTAATAAGC	LFH in *ganA* (laboratory collection)
CZ230	CCGAGCGCCTACGAGGAATTTGTATCGCTCTCCAGTTTTTCCGGTTCG	LFH in *ganA* (laboratory collection)
CZ231	CTTCCAGCCGTTTCAGGATTG	LFH in *ganA* (laboratory collection)
CZ127	CGATACAAATTCCTCGTAGGCGCTCGGGTAGTATTTTTTGAGAAGATCAC	Amplification of phleomycin resistance cassette (laboratory collection)
CZ128	CCAAAGTGAAACCTAGTTTATC	Check primer for phleomycin resistance cassette (laboratory collection)
CZ129	CGAGACTTTGCAGTAATTGATC	Check primer for phleomycin resistance cassette (laboratory collection)

JN185	**CCTATCACCTCAAATGGTTCGCTG**GAGCTGCTTTTGGTCTTAGCC	Deletion of *pgpH* (laboratory collection)
JN186	**CCGAGCGCCTACGAGGAATTTGTATCG**GAATTTTCCACTCCCGGATTGAATATC	Deletion of *pgpH* (laboratory collection)
JN187	CCAAGATTCTTTTTTGAAATTGATGGAGAA	Deletion of *pgpH* (laboratory collection)
JN188	GTTCATGTGTACATTCCTCTCTTACCTATAA	Deletion of *pgpH* (laboratory collection)
JG67	GATGCATGCACATGAAATTGGTGAGCAG	Amplification of *spc* resistance casstette without terminator (laboratory collection)
ML84	CTAATGGGTGCTTTAGTTGAAGA	*Cat*_cassette_check_Rev (laboratory collection)
ML85	CTCTATTCAGGAATTGTCAGATAG	*Cat*_cassette_check_Fwd (laboratory collection)
ML103	CTCTTGCCAGTCACGTTAC	*ermC*_cassette_check_Rev (laboratory collection)
ML104	TCTTGGAGAGAATATTGAATGGAC	*ermC*_cassette_check_Rev (laboratory collection)
ML107	GCTTCATAGAGTAATTCTGTAAAGG	Kan_cassette check primer (laboratory collection)
ML108	GACATCTAATCTTTTCTGAAGTACATCC	Kan_cassette check primer (laboratory collection)
mls-fwd(kan)	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC	Amplification of erythromycin resistance cassette (laboratory collection)
mls-Rev(kan)	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG	Amplification of erythromycin resistance cassette (laboratory collection)
spec-Fwd	CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG	*aad9*_cassette_Fwd (laboratory collection)
spec-rev	CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG	*aad9*_cassette_rev (laboratory collection)
SW41	TTGTCAAGTGAAGGCGCGCTAT	Construction of BP1182 (laboratory collection)
cat-fwd	CAGCGAACCATTTGAGGTGATAGGCGGCAATAGTTACCCTTATTATCAAG	Amplification of *cat* cassette (laboratory collection)
cat-rev	CGATACAAATTCCTCGTAGGCGCTCGGCCAGCGTGGACCGGCGAGGCTAGTTACCC	Amplification of *cat* cassette (laboratory collection)
cat-rev_o.T.	CGATACAAATTCCTCGTAGGCGCTCGGTTATAAAAGCCAGTCATTAGGCCTATC	Amplification of *cat* cassette (laboratory collection)
Kan-check-rev	CTGCCTCCTCATCCTCTTCATCC	check primer deletion (laboratory collection)
Kan-Fwd	CAGCGAACCATTTGAGGTGATAGG	Amplification of the *kan*-resistance cassette (laboratory collection)
Kan-rev	CGATACAAATTCCTCGTAGGCGCTCGG	Amplification of the *kan*-resistance cassette (laboratory collection)
Kan-rev o.T.	TTACTAAAACAATTCATCCAGTAAAATAT	Amplification of the *kan*-resistance cassette without terminator (laboratory collection)
ML108	GACATCTAATCTTTTCTGAAGTACATCC	check primer deletion by gene replacement with a kan-cassette (laboratory collection)
Spec-fwd(kan)		Amplification of the *spc*-resistance cassette (laboratory collection)
Spec-rev(kan)		Amplification of the *spc*-resistance cassette (laboratory collection)
Spec-rev(o.T.)		Amplification of the *spc*-resistance cassette without terminator (laboratory collection)

T7-prom	CACGGGAGAACAGGGCTTAAA	check primer for plasmids containing a T7 promoter (laboratory collection)
T7-term	GCTAGTTATTGCTCAGCGG	check primer for plasmids containing a T7 terminator (laboratory collection)

Plasmids constructed in this work

Name	Vector / restriction nucleases	Insert: Primer, template
pBP760	pAC7 / *Bam*HI + *Eco*RI	P_{mhqN*}: BR90 + BR91, gDNA BP1108
pBP761	pAC7 / *Bam*HI + *Eco*RI	P_{mhqN}: BR90 + BR91, gDNA 168
pBP762	pGP1022 / *Xho*I + *Bam*HI	LFH BR38 - BR42, gDNA PG39
pBP763	pGP3273 / *BamH*I + *EcoR*I	aroE: BR92 + BR93, gDNA 168
pBP764	pBQ200 / *Bam*HI + *Sal*I	PCR iGEM2018_27 + BR126, extracted plasmid from BP1123
pBP765	pBQ200 / *Bam*HI + *Sal*I	PCR iGEM2018_27 + BR126, gDNA 168
pBP766	pETSUMOadapt / XhoI + *Bsa*I	*pdxS:* BR177 + BR178, gDNA 168
pBP767	pETSUMOadapt / XhoI + *Bsa*I	*pdxT:* BR179 & BR180, gDNA 168
pBP768	pETSUMOadapt / XhoI + *Bsa*I	*ylaM:* BR185 + BR186, gDNA 168
pBP769	pAC7 / *Bam*HI + *Eco*RI	P_{grac}: BR271 + BR272, pTH01
pBP770	pGP380 / *Bam*HI + *Hind*III	*ytoQ:* BR72 + BR73, gDNA SP1
pBP771	pGP382 / *Bam*HI + *Hind*III	*ytoQ:* BR72 - BR74, gDNA SP1
pBP772	pGP382 / *Sal*I + *Pst*I	*pdxST:* BR77 + BR78, gDNA SP1
pBP773	pAC7 / *Xma*I + *Bam*HI	*pdxST:* BR79 + BR80, gDNA SP1
pBP774	pGP574 /*Bam*HI + *Sac*I	*pdxST:* BR86 + BR87, gDNA SP1
pBP775	pBQ200 / *Bam*HI + *Pst*I	*pdxS:* BR125 + BR101, gDNA SP1
pBP777	pAC7 / *Eco*RI + *Bam*HI	P_{rpoE}: BR163 + BR164, gDNA SP1
pBP778	pAC7 / *Eco*RI + *Bam*HI	P_{rpoE}*: BR163 + BR164, gDNA BP1172
pBP779	pGP382 / *Bam*HI + *Hind*III	*ytoQ:* JR266 + BR74, gDNA SP1
pBP780	pAC5 / *Eco*RI + *Bam*HI	*pdxR*-P_{pdxS}: BR167 + BR168, gDNA *Listeria monocytogenes*
pBP781	pAC7 / *Eco*RI + *Bam*HI	*pdxR*-P_{pdxS}: BR167 + BR168, gDNA *Listeria monocytogenes*
pBP782	pGP886 / *Bmg*BI (AjiI) + *Sal*I	*pdxS:* BR169 + BR170, gDNA SP1
pBP783	pGP888 / *Stu*I (*Eco1*471) + *Sal*I	*pdxS:* BR169 + BR171, gDNA SP1
pBP784	pAC5 / *Eco*RI + *Bam*HI	P_{pdxS}: BR172 + BR173, gDNA SP1
pBP785	pAC7/ *Eco*RI + *Bam*HI	P_{pdxS}: BR172 + BR173, gDNA SP1
pBP786	pGP886 / *Apa*I + *Sal*I	*pdxR:* BR174 + BR176, gDNA *Listeria monocytogenes*
pBP787	pGP888 / *Stu*I (*Eco*1471) + *Sal*I	*pdxR:* BR175 + BR176, gDNA *Listeria monocytogenes*
pBP788	pBQ200 / *Eco*RI + *Pst*I	P_{alf4}-*pdxS*: BR273 + BR101, gDNA SP1
pBP789	pBQ200 / *Eco*RI + *Pst*I	P_{pdxS}-*pdxS*: BR172 + BR101, gDNA SP1
pBP790	pGP888 /*Xba*I + *Bam*HI	*rpoE*: BR201 + BR202, gDNA SP1
pBP791	pGP888 /*Xba*I + *Bam*HI	*pgpH*: BR203 + JN209, gDNA SP1
pBP792	pGP888 /*Xba*I + *Bam*HI	*gdpP*: BR204 + BR205, gDNA SP1
pBP793	pGP886 / *Xba*I + *Bam*HI	*rpoE*: BR201 + BR202, gDNA SP1
pBP794	pGP886 / *Xba*I + *Bam*HI	*pgpH*: BR203 + JN209, gDNA SP1
pBP795	pGP886 / *Xba*I + *Bam*HI	*gdpP*: BR204 + BR205, gDNA SP1
pBP797	pBQ200 / *Pst*I + *Hind*III	*pdxJ**: CCR BR277/BR278/BR279, gDNA W3110
pBP798	pBQ200 / *Pst*I + *Hind*III	*pdxJ*: BR277/BR278, gDNA W3110
pBP799	pAC5 / *Eco*RI + *Bam*HI	*ytoP*: BR280/BR281, gDNA SP1
pBP800	p25-N / *Kpn*I + *Xba*I	*acpA*: BR233 + BR234, gDNA SP1
pBP801	pKT25 / *Kpn*I + *Xba*I	*acpA*: BR233 + BR234, gDNA SP1
pBP802	pUT18 / *Kpn*I + *Xba*I	*acpA*: BR233 + BR234, gDNA SP1
pBP803	pUT18C / *Kpn*I + *Xba*I	*acpA*: BR233 + BR234, gDNA SP1
pBP804	p25-N / *Kpn*I + *Xba*I	*aphA*: BR235 + BR236, gDNA SP1
pBP805	pKT25 / *Kpn*I + *Xba*I	*aphA*: BR235 + BR236, gDNA SP1
pBP806	pUT18 / *Kpn*I + *Xba*I	*aphA*: BR235 + BR236, gDNA SP1
pBP807	pUT18C / *Kpn*I + *Xba*I	*aphA*: BR235 + BR236, gDNA SP1
pBP808	p25-N / *Kpn*I + *Xba*I	*pdxS*: BR237 + BR238, gDNA SP1
pBP809	pKT25 / *Kpn*I + *Xba*I	*pdxS*: BR237 + BR238, gDNA SP1
pBP810	pUT18 / *Kpn*I + *Xba*I	*pdxS*: BR237 + BR238, gDNA SP1
pBP811	pUT18C / *Kpn*I + *Xba*I	*pdxS*: BR237 + BR238, gDNA SP1
pBP812	p25-N / *Kpn*I + *Xba*I	*pdxT*: BR239 + BR240, gDNA SP1
pBP813	pKT25 / *Kpn*I + *Xba*I	*pdxT*: BR239 + BR240, gDNA SP1

pBP814	pUT18 / *Kpn*I + *Xba*I	*pdxT*: BR239 + BR240, gDNA SP1
pBP815	pUT18C / *Kpn*I + *Xba*I	*pdxT*: BR239 + BR240, gDNA SP1
pBP816	p25-N / *Kpn*I + *Xba*I	*pdxST*: BR237 + BR240, gDNA SP1
pBP817	pKT25 / *Kpn*I + *Xba*I	*pdxST*: BR237 + BR240, gDNA SP1
pBP818	pUT18 / *Kpn*I + *Xba*I	*pdxST*: BR237 + BR240, gDNA SP1
pBP819	pUT18C / *Kpn*I + *Xba*I	*pdxST*: BR237 + BR240, gDNA SP1
pBP820	pAC7 / *Eco*RI + *Bam*HI	P_{ahpA}: BR241 + BR242, gDNA SP1
pBP821	pAC7 / *Eco*RI + *Bam*HI	P_{ahpA}: BR241 + BR242, gDNA BP1174
pBP822	pAC7 / *Eco*RI + *Bam*HI	P_{ykuT}: BR243 + BR244, gDNA SP1
pBP823	pAC7 / *Eco*RI + *Bam*HI	P_{ykuT}: BR243 + BR244, gDNA BP1174
pBP824	pBQ200 / *Eco*RI + *Pst*I	*ytoP*: BR288 + BR289, gDNA SP1
pBP825	pAC7 / *Eco*RI + *Bam*HI	P_{DeqQ}* + lacOperator + SD + Start-Codon: BR298 + BR299, pBQ200
pBP826	pAC5 / *Eco*RI + *Bam*HI	P_{DeqQ}* + lacOperator + SD + Start: BR298 + BR299, pBQ200

Other plasmids used in this work

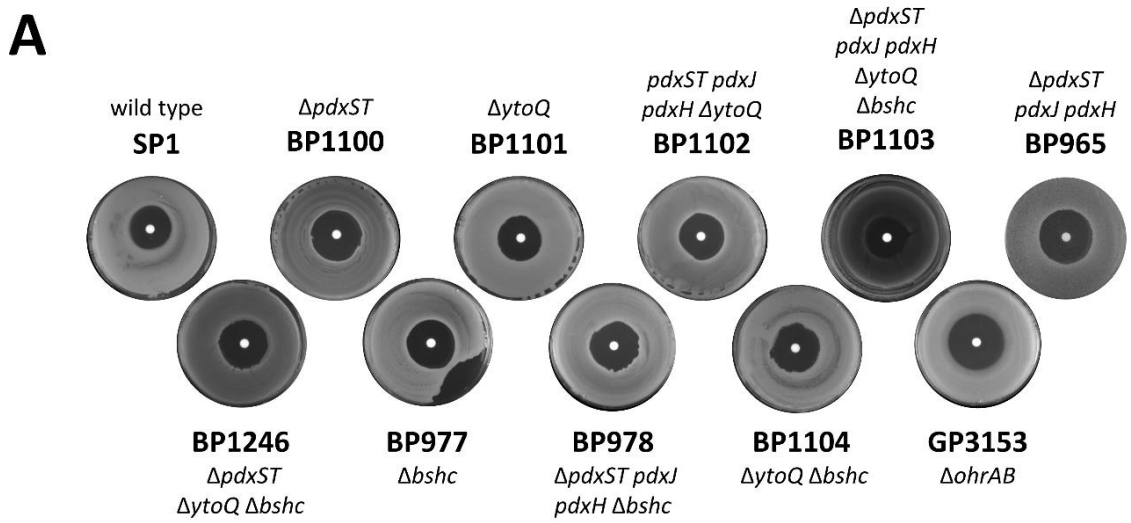
Name	Description	Reference/ Construction
p25N	MCS-T25. Two-hybrid plasmid	(*Karimova et al.*, 1998)
pAC7	Plasmid for translational promoter *lacZ* fusions	(*Weinrauch et al.*, 1991)
pBP639	Overexpression of YtoQ in *B. subtilis*	(*J. Rosenberg et al.*, 2018)
pBP640	Expression of N-terminally Strep-tagged YtoQ in *E. coli*	(*J. Rosenberg*, 2017)
pBP641	Expression of C-terminally Strep-tagged YtoQ in *E. coli*	(*J. Rosenberg*, 2017)
pBQ200	Constitutive overexpression of proteins in *B. subtilis*	(*Martin-Verstraete et al.*, 1994)
pDG148	Template for phleomycine resistance gene	(*Guérout-Fleury et al.*, 1995)
pDP646	Template for erythromycin resistance gene	(*Guérout-Fleury et al.*, 1995)
pDG780	Template for kanamycin resistance gene	(*Guérout-Fleury et al.*, 1995)
pDG1513	Template for tetracycline resistance gene	(*Guérout-Fleury et al.*, 1995)
pDG1726	Template for spectinomycin resistance gene	(*Guérout-Fleury et al.*, 1995)
pGEM-cat	Template for chloramphenicol resistance gene	(*Guérout-Fleury et al.*, 1995)
pGP172	Overexpression in *E. coli* BL21 (DE3) with N-terminal Strep-tag	(*Merzbacher et al.*, 2004)
pBP168	Translational P_{nrgAB}-*lacZ* fusion	(*Detsch & Stülke*, 2003)
pGP380	Constitutive overexpression of N-terminally Strep-tagged proteins in *B. subtilis*	(*Herzberg et al.*, 2007)
pGP382	Constitutive overexpression of C-terminally Strep-tagged proteins in *B. subtilis*	(*Herzberg et al.*, 2007)
pETSUMOadapt	Inducible overexpression of N-terminally His-tagged proteins in *B. subtilis* with cleavable SUMO-tag	(*Mossessova & Lima*, 2000)
pKT25	T25-MCS Two-hybrid plasmid	(*Karimova et al.*, 1998)
pUT18	MCS-T18 Two-hybrid plasmid	(*Karimova et al.*, 1998)
pUT18C	T18-MCS Two-hybrid plasmid	(*Karimova et al.*, 1998)

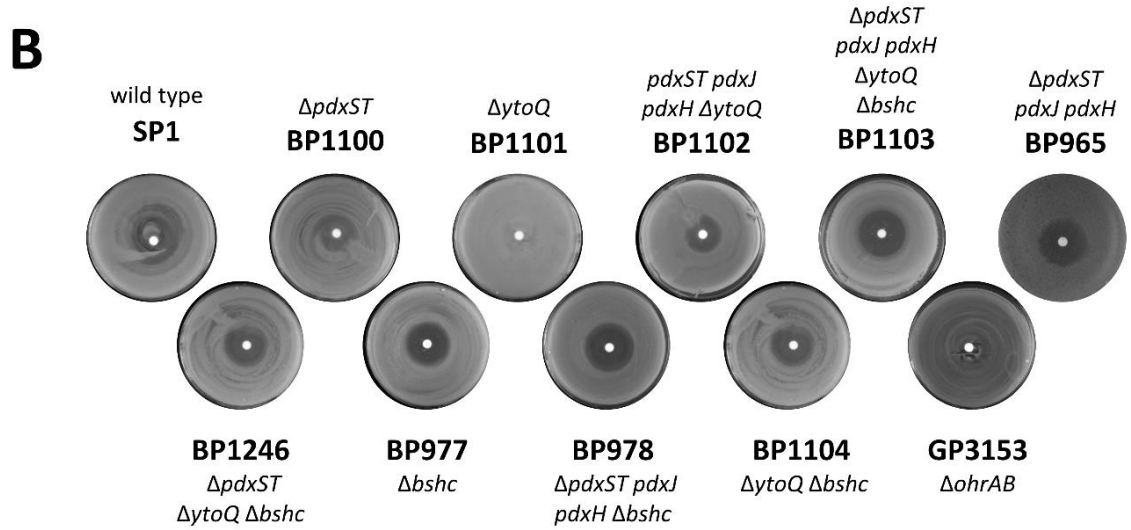
Softwares and web pages

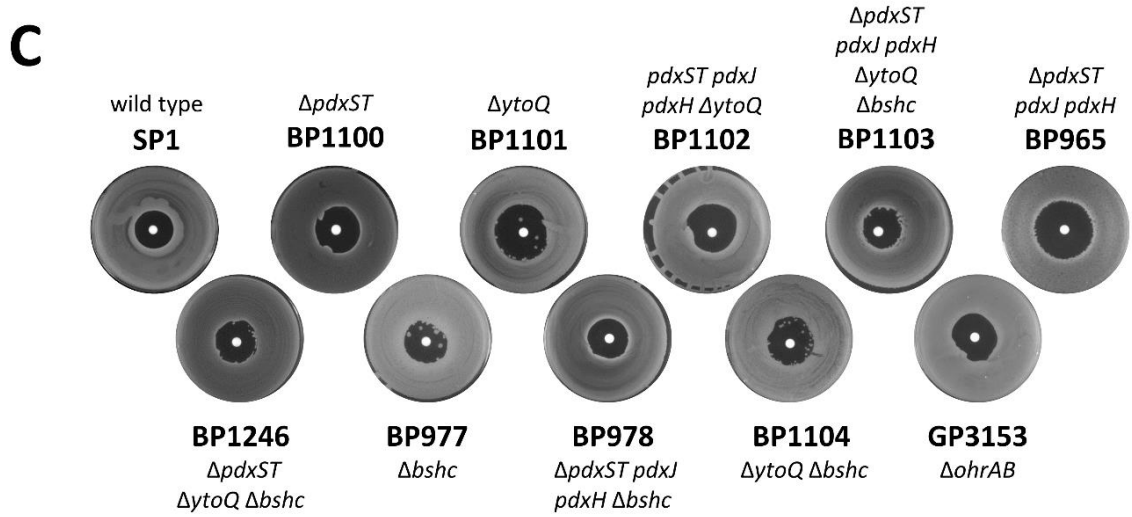
Program/ web page	Provider	Application
Basic Local Alignment Search Tool	NCBI	Gene and protein comparison between different organisms
Gen5™Data analysis software	BioTek®	Analysis of plate reader Data and management of the plate reader
Geneious Prime 2021.03.1	Biomatters	In silico DNA data management
ImageLab™ Software	BioRad	Imaging of blot pictures
Mendeley Desktop	PDFTron™ Systems Inc.	Citation manager
Microbes online		www.microbesonline.org
Microsoft Office	Microsoft Inc.	Writing, Presentation
Rare tRNA calculator	Bioline	Evaluation of efficiency for recombinant Protein expression in *E. coli*

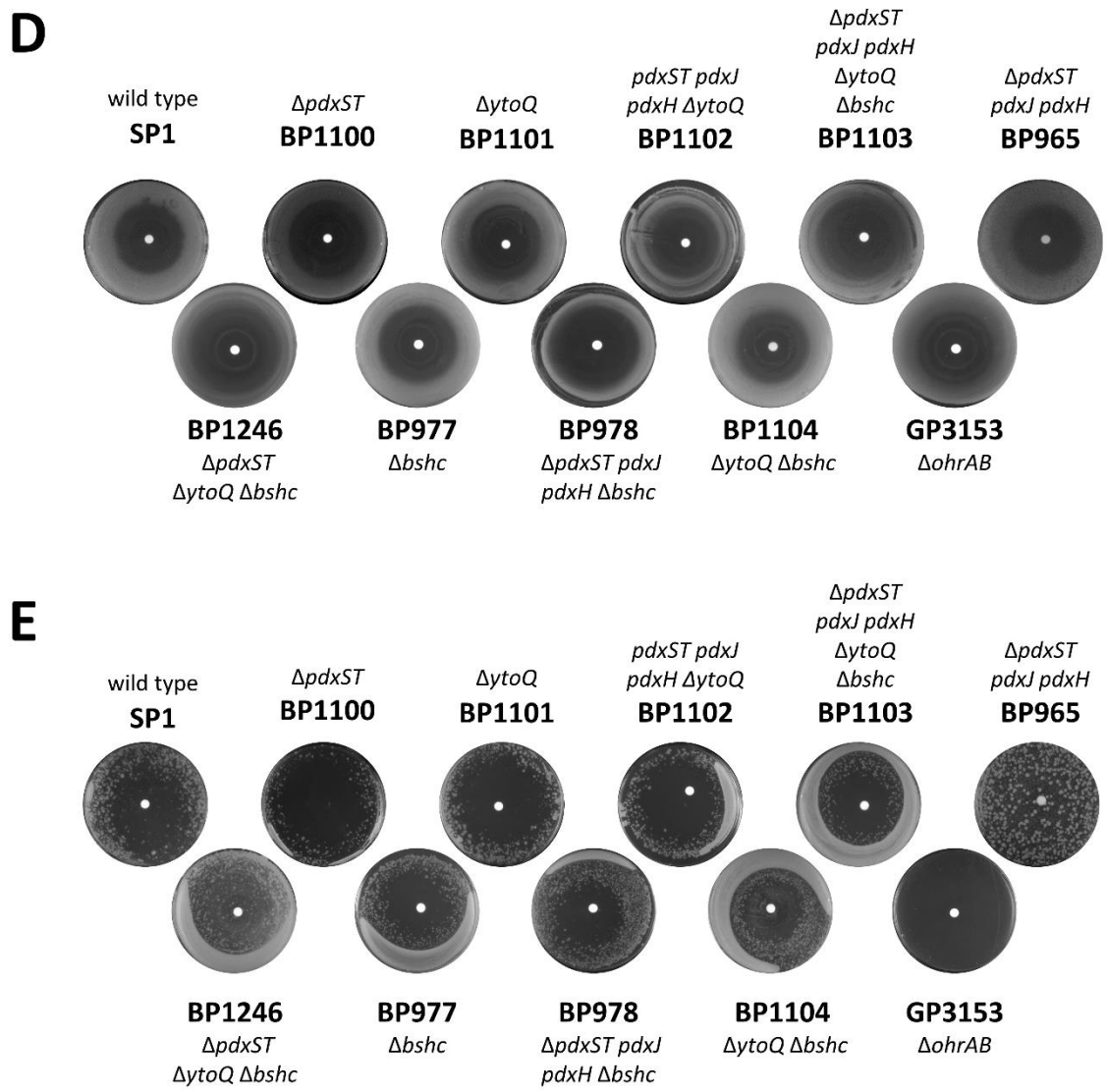
SubtiWiki	(*Zhu & Stülke*, 2018)	*Bacillus subtilis* Database: http://subtiwiki.uni-goettingen.de/v4/
Pymol	Version 2.0 Schrödinger, LLC.	The PyMOL Molecular Graphics System,
Vitamin B6 Database	Universita di Parma	http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/bib.pl

6.2 **Supporting material**

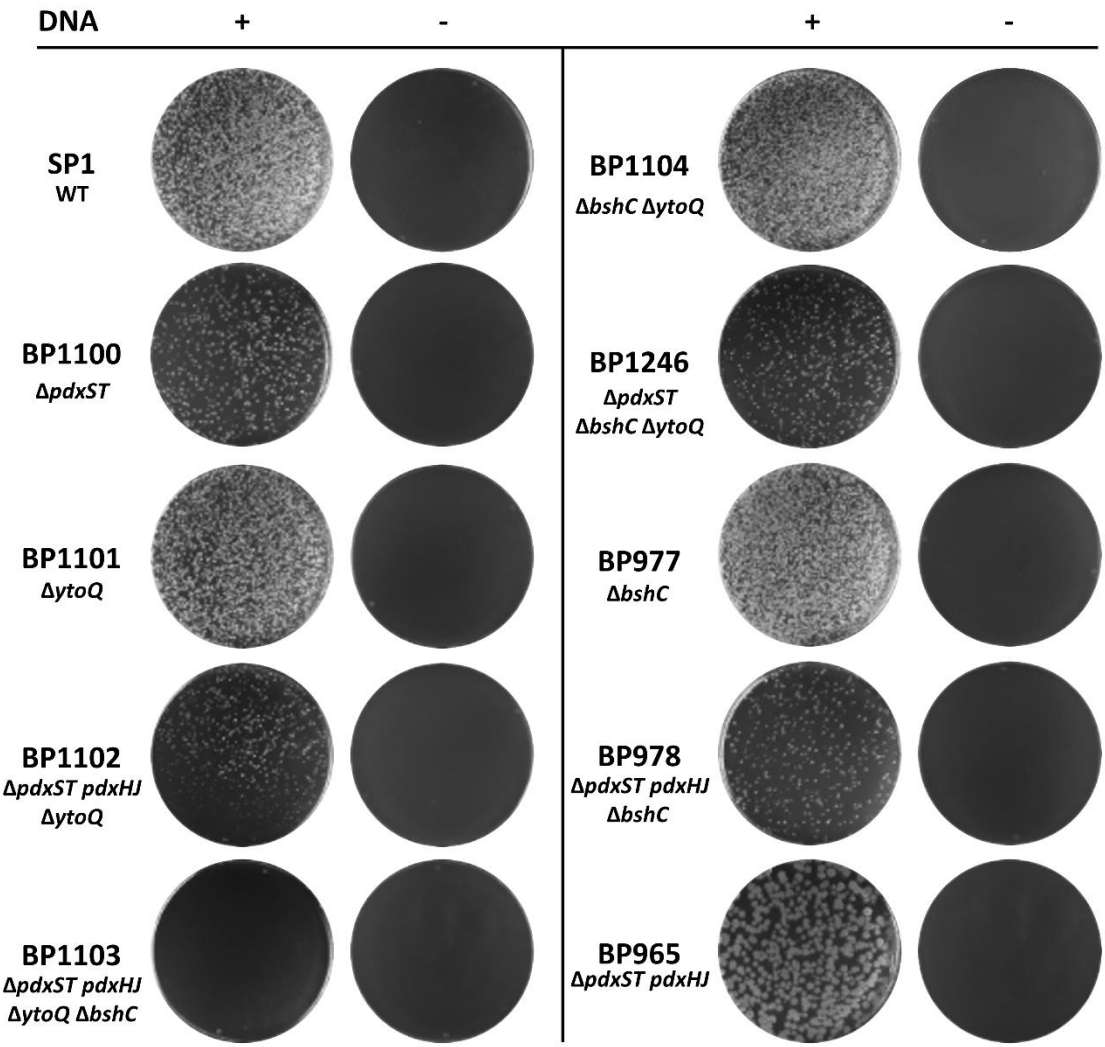




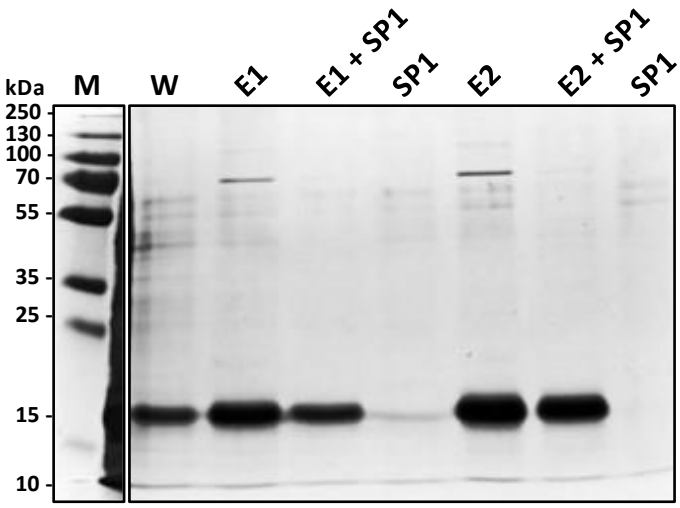




Suppl. Figure 1 Stress test plates.
Bacteria were streaked out on LB agar plates supplemented with PL and respective antibiotics and incubated over night at 37°C. Cell material was taken from the plates and resuspended in 1 ml sterile saline solution, washed two times by centrifugation at 10,000 × g and subsequent resuspension in saline solution. The OD_{600} was set to 1 and 100 µl of the cell suspension was plated on LB-PL agar plates. A filter paper was placed in the middle of the plate and 10 µl of the following stressors were added: **A**: Cumene hydroperoxide (0.5 M), **B**: Fosfomycin: 5 µg/µl, **C**: H_2O_2 (0.8 M), **D**: Paraquat (0.5 M), **E**: t*ert*-Butyl hydroperoxide (0.5 M).



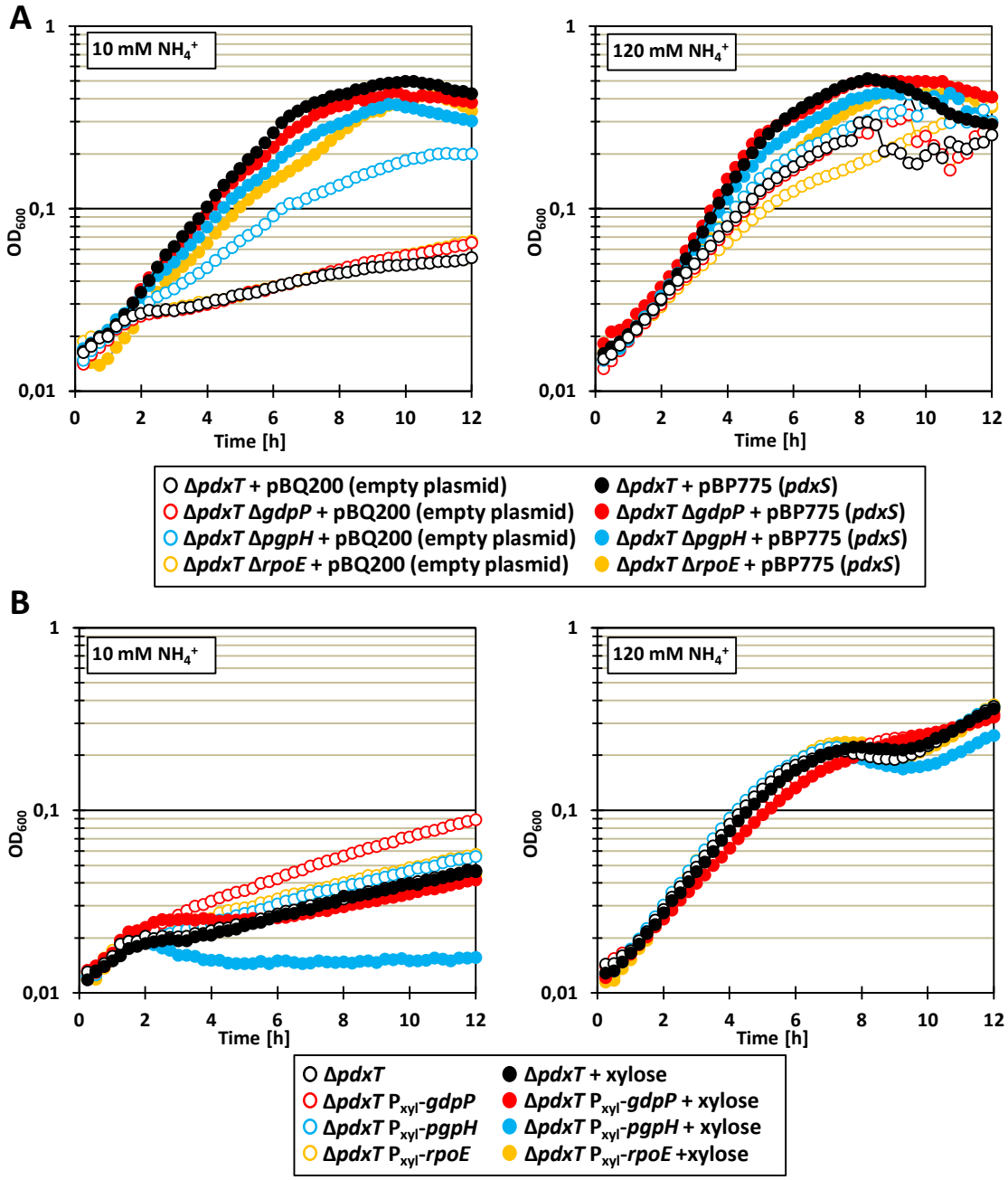
Suppl. Figure 2 Competence test of different heterologous pathway mutants
Bacterial strains were inoculated from LB culture to MNGE+CAA minimal medium and incubated at 37°C until OD_{600} was 1. The cells were diluted with fresh MNGE medium an incubated for 1 h. The cells were centrifuged at 10,000 × g and the supernatant was used to set the OD_{600} to 1. 1 µg of pBQ200 plasmid DNA was used to transform the competent cells. 4/5 of the cells were applied to the LB-PL plates and images were taken one day after incubation at 37°C.



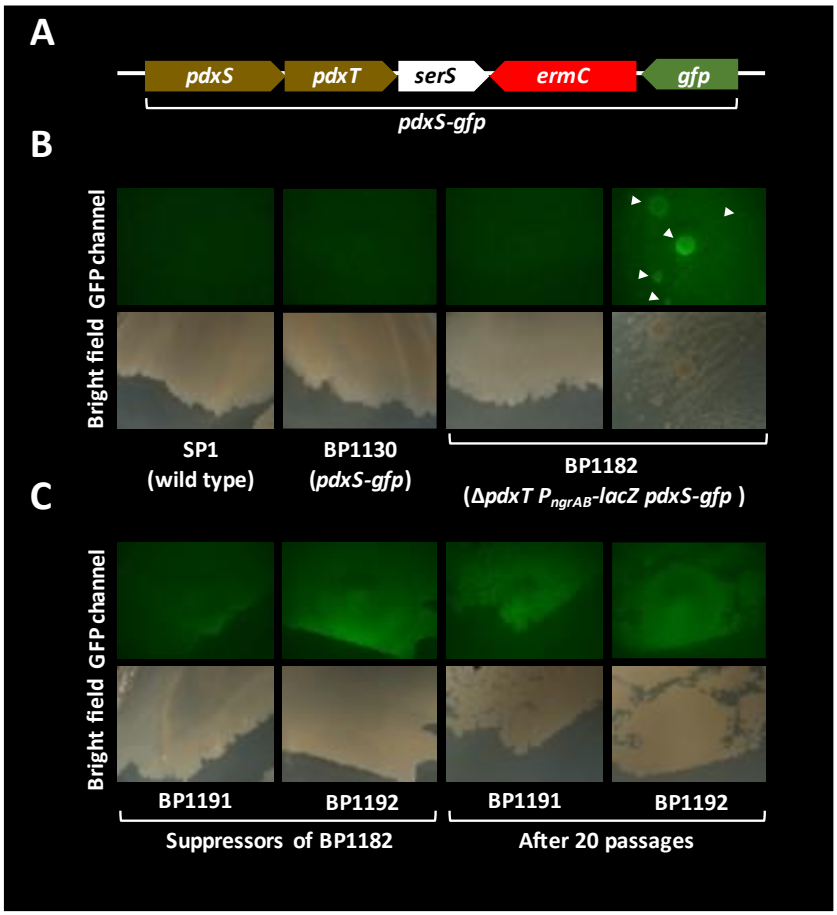
Suppl. Figure 3 Pulldown with Strep-YtoQ
YtoQ-STREP (pBP642), expressed by Rosetta (DE3) was coupled to a Strep-Tactin matrix. The matrix was washed with 15 ml buffer and bound proteins were either directly eluted or crude extract of *B. subtilis* WT SP1 was added beforehand. Elution fractions were analyzed by SDS –PAGE and silver staining. M: protein size marker, W: fraction after washing step, E_x: elution fraction 1 - x of bound STREP-YtoQ, E+SP1: elution fraction of bound STREP-YtoQ saturated with *B. subtilis* crude extract, SP1: crude extract of SP1 added to the Strep-Tactin matrix.

Suppl. Table 1: Genes included in the amplified region

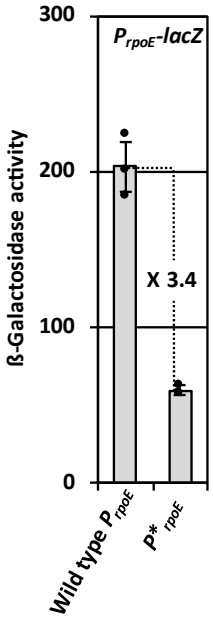
Gen	Function	Possible effect on B6/NH_4 metabolism
rrnO-16S	Translation	-
rrnO-23S	Translation	-
yaaC	Unknown	-
guaB	biosynthesis of GMP	-
dacA	carboxypeptidase	Peptide degradation could lead to increased NH_4 levels
pdxS	pyridoxal-5-phosphate biosynthesis	Increased synthesis of PLP
cat	Chloramphenicol resistance	-
serS	translation	-
dck	purine salvage and interconversion	-
dgk	purine salvage and interconversion	-
yaaH	survival of ethanol stress, protection of the spore	-
yaaI	survival of ethanol stress	-
yaaJ	tRNA modification	-
scr	presecretory protein translocation	causes RNA-driven elongation arrest
dnaX	part of the clamp-loader complex and the replisome	-
yaak	Unknown (similar to DNA-binding protein)	Possible regulatory protein
recR	required for the formation of RecA DNA repair centers	-
yaaL	unknown	-
bofA	control of Sigma-K activation	-
rrnA-16S	translation	-
rrnA-23S	translation	-



Suppl. Figure 4 Effect of *pgpH* and *rpoE* deletion on growth of a Δ*pdxT* mutant.
Precultures of the strains were cultivated in MSSM medium supplemented with PL and 30 mM ammonium until the OD_{600} was 0.5 – 0.8. The cells were washed in saline solution and 100 µl MSSM medium containing different concentrations of ammonium were inoculated and monitored in the platereader. A: Ammonium dependent growth of BP1106 (Δ*pdxT*) BP1220 (Δ*pdxT* Δ*rpoE*), BP1221 (Δ*pdxT* Δ*gdpP*) and BP1223 (Δ*pdxT* Δ*pgpH*) in presence or absence of overexpression of *pdxS*. Strains were transformed with the *pdxS* overexpression plasmid pBP775 or the empty plasmid pBQ200. B: Ammonium dependent growth of BP1106 (Δ*pdxT*), BP1224 (Δ*pdxT* P_{xyl}-*rpoE*), BP1225 (Δ*pdxT* P_{xyl}-*pgpH*) and BP1226 (Δ*pdxT* P_{xyl}-*gdpP*) in presence of absence of the inducer xylose.



Suppl. Figure 5 A system to monitor the amplification of the *pdxS* locus.
A: Integration of the *ermC-gfp* construct downstream of the *pdxST* and *pdxS* genes in the genomes of the strains BP1130 (Δ*serS*::(P_{alf4}-*gfp ermC*)) and BP1182 (Δ*pdxT* P_{nrgAB}-*lacZ* Δ*serS*::(P_{alf4}-*gfp ermC*)), respectively. **B**: Visualization of the amplification of the genomic region in the strain BP1182 containing the *pdxS* gene using a stereo fluorescence microscope Lumar.V12 (see Experimental procedures). The bacteria were propagated on MSSM plates and incubated for 2 days at 37°C. The strains SP1 and BP1130 served as controls. White arrows indicate suppressor mutants. Exposure time, 5 s (GFP); 1 s bright field. **C**: The fluorescence signals of two suppressors of the strain BP1182 (designated as BP1191 and BP1192) were compared with that of their derivatives that were passaged 20 times under selective growth conditions (MSSM 20 mM NH_4 medium without PL supplementation). The bacteria were propagated on MSSM plates and incubated for 2 days at 37°C. Exposure time, 5 s (GFP); 1 s bright field; magnification, 12x.



Suppl. Figure 6 *β*-Galactosidase assays to monitor the activities of the wild type PrpoE and the mutated P*rpoE promoters in the strains BP1145 and BP1146, respectively. Strains MSSM minimal medium (30mM NH4) and the cells were harvested at an OD600 between 0.5 and 0.8. -Galactosidase activities are given as units per mg of protein. Bars indicate mean values.