MiniBacillus – the construction of a minimal organism

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Statement of Authorship

I hereby declare that this doctoral thesis entitled "*MiniBacillus*- the construction of a minimal organism" has been written independently and with no other sources and aids than quoted.

Anika Klewing

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List of abbreviations

General

ABC	ATP-binding cassette
AP	alkaline phosphatase
APC	amino acid-polyamine-organocation
APS	alkaline phosphatase
ATP	adenosine triphosphate
B.	Bacillus
CAA	casamino acids
cat	chloramphenicol
CE	crude extract
CoA	coenzyme A
dH2O	deionized water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosidtriphosphate
E.	*Escherichia*
e.g.	lt. for example
ECF	Energy coupling factor
erm	Erythromycine
et al.	*et alia*
EV	Empty vector
FT	flow through
fwd	forward
gDNA	genomic DNA
glc	glucose
H_2O	water
IPTG	isopropyl β-D-1-thiogalactopyranoside
kan	kanamycin
kb	kilo base pair
LB	lysogeny broth
LFH	long flanking homology
M.	*Mycoplasma*
Mb	mega base pairs
NAD/NADH	nicotinamide adenine dinucleotide
NADP/NADPH	nicotinamide-adeninedinucleotide phosphate
OD	optical density
P	promoter
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
pH	power of hydrogen
PTS	phosphotransferase system
rev	reverse
RNA	ribonucleic acid
RNase	ribonulease
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SP	sporulation medium
SSS	solute:sodium symporter
TAE	tris-acetate-EDTA
TCA	tricaboxylic acid
WGS	whole genome sequencing
wt	wild type

Amino acids

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Pro	proline
Phe	phenylalanine
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine

Units

°C	degree Celsius
A	ampere
bp	base pair
Da	Dalton
g	gram
h	hour
l	liter
m	meter
min	minute
mol	mol
M	molar
s	second
V	volt

Prefixes

M	Mega
k	kilo
m	milli
µ	micro
n	nano
p	pico

Summary

The *MiniBacillus* project is a minimal genome project. It attempts to reduce the genome of the model organism *Bacillus subtilis* in a top-down approach by deleting not necessary parts of the genome step by step. In this work, a *MiniBacillus* strain was created with a genome reduction of 40.51%, which is the most extensive genome reduction, achieved in a top-down approach. The data obtained from previous multi-omics experiments were used to adapt the deletion process and maintain a stable strain. Furthermore, new proteome data were analysed. A glycolytic cassette was inserted as a first attempts of a defragmentation approach to counteract the deceleration of the deletion process. Another important goal of the *MiniBacillus* project is to gain more knowledge about the mechanisms in the cell. The final minimal cell will be able to utilize glucose as the single carbon source. Therefore, glycolysis and the pentose-phosphate pathway will remain in the cell and the TCA cycle will be deleted. To analyse the effect of a deletion of this central pathway, a TCA cycle mutant in the wild type strain was created. This strain is viable, but shows severe problems in sporulation, competence and cell morphology. Especially the reduced competence is a disadvantage for the *MiniBacillus* project. The final minimal cell will import all amino acids from the complex medium and all biosynthesis pathways will be deleted. However, not for every amino acid the particular importers are characterized. In this work, the three new serine/ threonine transporters YbeC, BcaP and YbxG could be identified. The YbeC transporter seems to be the low-affintiy serine transporter, which transports the major part of serine into the cell at high serine concentrations. BcaP and YbxG have just a minor function. Furthermore, the major import of threonine is mediated by BcaP, which also transports isoleucine and valine into the cell. In contrast, YbeC and YbxG have a minor threonine import function. This information can be used for the *MiniBacillus* project and the biosynthesis pathways of serine and threonine can be deleted in the final strain. Furthermore, BcaP will remain in the *MiniBacillus* to ensure the necessary import of serine and threonine.To analyse the serine import, the toxicity of high serine concentrations on wild type cells was utilized. However, the mechanism and physiological role of serine inhibition was unknown. The results of this work indicate a function in the inhibition of the threonine pathway. Serine might bind to the homoserine dehydrogenase protein and inhibit its activity. This causes a reduced level of threonine in the cell. The information of this work was incorporated into the *Minibacillus* project and a blueprint 2.0 was created.

1. Introduction

1.1. The model organism *Bacillus subtilis*

Bacillus subtilis is a rod-shaped, gram-positive bacterium. It belongs to the phylum *Firmicutes*, together with, amongst others, *Staphylococcus* and *Clostridium* (Wolf *et al.*, 2004). The genome of 4.2 Mbp was completely sequenced in 1997 and harbours around 4100 genes (Kunst *et al.*, 1997). Since the genome of *B. subtilis* is well-studied and many methods are established for the genetic manipulation, it has a high importance as a model organism. Furthermore, *B. subtilis* is also frequently used for biotechnological applications, *e.g.* in the production of different products, like riboflavin and vaccines (Hao *et al.*, 2013; Rosales-Mendoza and Angulo, 2015). The wild type strain 168 is a commonly used laboratory strain and harbours a tryptophan auxotrophy (Zeigler *et al.*, 2008). A lot of information gathered about *B. subtilis* are collected in the *Subti*Wiki database. It contains *e.g.* expression and interaction data about all genes and proteins and is therefore an important tool for the work with *B. subtilis* (Michna *et al.*, 2016). *B. subtilis* is perfectly adapted to the life in the soil. Its abilities to form spores or biofilms allow the adaption to changing environmental conditions (Piggot and Hilbert, 2004).

1.2. Minimal genomes and the *MiniBacillus* project

1.2.1. Naturally occurring minimal genomes

The lifestyle and environmental niches play an important role in the evolution of the genome. Changing environmental conditions or the lack of nutrients lead to a more complex metabolic machinery and therefore to more genes. However, organisms that have adapted to a specific niche with almost constant conditions, show often reduced genomes. These minimal genomes can often be found in pathogenic bacteria. The group of bacteria, called mycoplasma, are organisms with small genomes. *Mycoplasma pneumoniae* is a human pathogen, causing respiratory infections and it has a genome of around 816 kbp (Himmelreich *et al.*, 1996; Hammerschlag, 2001). Due to the pathogenic lifestyle, many nutrients are provided by the environment and the corresponding biosynthetic pathway are lacking in these bacteria. Furthermore, the bacteria do not form a cell wall (Trachtenberg, 1998). An even smaller genome can be found in the bacterium *Mycoplasma genitalium* with 580 kbp (Fraser *et al.*, 1995). It was for a long time the smallest known genome, but in 2013 the even smaller genome of *Nasuia deltocephalinicola* was discovered. This obligate symbiont has a genome of only 112 kbp and is capable to survive without its symbiosis partner the leafhopper (Bennett and Moran, 2013; Ishii *et al.*, 2013).

1.2.2. Synthetic minimal genomes

Minimal genomes are often studied to understand the essence of life. Also synthetic minimal genomes are created and two approaches, top-down and bottom-up, are used for this purpose and the basis is the knowledge about what is essential to sustain life.

In the top-down approach, a naturally occurring genome is reduced stepwise by the deletion of unnecessary regions of the genome. An advantage of this method is that if a problem or an error occurs, it is always possible to get back to the ancestor strain. The error can be easily analysed, since the deletion of one of the genes in this step, must be the reason for this phenotype. An example for a top-down approach is the genome reduced *E. coli* strain created by Hirokawa *et al.* (2013). This strain showed an improved growth caused by the genome reduction, which could also be beneficial for industrial application.

In a bottom-up approach, in contrast, genomes are synthesised *de novo* and transplanted into the cell. This approach needs an exact plan of what is needed to sustain life, to assemble the final genome. However, a disadvantage is that errors are not easily identified, since the error occurs only in the final step, the transplantation of the genome. If the cell is not viable, the missing elements need to be identified and the genome needs to be newly synthesized. But if no errors occur, this approach is faster than the top-down approach, since it requires less steps. The creation of a synthetic *Mycoplasma* cell by the group of Craig Venter is an example for a bottom-up approach. The first milestone in this project was the chemical synthesis of a *M. genitalium* genome (Gibson *et al.*, 2008). This was followed by the first transplantation of a *M. mycoides* genome into a *M. capricolum* cell, which shows that the general principle of genome transplantation is possible (Lartigue *et al.*, 2007). These techniques were then applied to create the strain JCVI-syn3.0, a *M. mycoides* offspring with a minimized and chemically synthesized genome of 531 kbp and 473 genes. The genome is smaller than that of any autonomously replicating cell. However, it still contains 149 genes of unknown function (Hutchison *et al.*, 2016).

A synthetic minimal genome might also be useful for biotechnological applications. No unknown factors or self-defense mechanisms will lead to complications in the *e.g.* overproduction of proteins. By the deletion of unnecessary pathways, more energy can be used for the desired product. However, in a real minimal genome, the addition of some functions might be necessary to create a useful strain for biotechnology, since important functions for the production might be deleted. It could be shown, that genome reduction can lead the improved production of recombinant proteins (Morimoto *et al.*, 2008). However, in other cases, disadvantageous changes could be observed, like morphology changes (Hashimoto *et al.*, 2005).

The model organism *B. subtilis* was previously used for minimal genome projects. A genome reduction of 25% was achieved in the MGIM strain, which shows a slightly reduced growth in comparison to the wild type (Ara *et al.*, 2007).

1.2.3. The *MiniBacillus* project

This work is part of the *MiniBacillus* project. The goal of this project is to construct a *B. subtilis* strain with a minimal gene set, that is completely understood. Every gene should have an assigned function in the cell. A top-down approach is used, and not necessary parts of the genome are deleted step-by-step. If a growth defect occurs, the strains should be evolved so that arising suppressor mutations can be isolated. These can lead to the restoration of growth and the resulting strain can be used again for new deletions. If a final mini cell is achieved, a transposon mutagenesis can be performed to analyse which genes are still or newly dispensable (Juhas *et al.*, 2014).

The fundament of each minimal genome project is the basic concept, which genes are incorporated into the minimal cell. Therefore, the essential genes need to be first considered. Essential genes cannot be deleted under standard growth conditions. For *B. subtilis* these growth conditions are LB-glc medium at 37°C. To identify the essential gene set, a computational or an experimental method can be used (Burgard *et al.*, 2001). In the experimental approach, every single gene is disrupted using different methods, *e.g.* the integration of a plasmid via single crossover recombination (Kobayashi *et al.*, 2003). The set of essential genes of *B. subtilis* was extensively studied (Juhas *et al.*, 2011; Commichau *et al.*, 2013). An important study in this context was the recently published work of Koo *et al.* (2017). Two single genes deletion libraries of *B. subtilis* were created, which comprise the single deletion mutants of each almost 4000 genes. This led to the identification of 257 essential genes. Furthermore, new competence genes were identified, by testing the single deletion strains.

The list of essential genes is not sufficient for the building of a minimal genome. Some functions are essential for *B. subtilis*, but if two genes encode for this function, the single deletion of one is possible and they are therefore not listed as essential genes. In such cases, one of the genes needs to be chosen for the minimal gene set. Furthermore, the competence genes and the genes for genome stability are important to ensure the stability of the strain during the deletion process (Reuß *et al.*, 2016). In some cases, essential genes can be deleted if another gene is already deleted. The antitoxin gene *yxxD* can only be deleted, if the toxin gene *yxiD* is already deleted (Holberger *et al.*, 2012).

Starting with this data, a blueprint for a minimal *B. subtilis* cell was created (Reuß *et al.*, 2016). The conditions for the *MiniBacillus* are growth at 37°C in LB-glc medium. This medium provides many

nutrients and amino acids. The uptake is favoured instead of the synthesis, since the import needs less genes. This is the case for the amino acids. Mainly the importers remain in the *MiniBacillus* strain, but some biosynthesis pathways are also listed, since not all amino acid importers are known. The main carbon source of the cell will be glucose. Therefore, the carbon metabolism is reduced to glycolysis and the pentose-phosphate pathway. The citric acid cycle should be completely deleted. Since the LB-glc medium contains not enough nucleotides, that the import would be sufficient to sustain stable growth, nucleotides can still be synthesized in the final *MiniBacillus* strain. This leads in total to a minimal gene set of 523 protein coding genes, of which 243 are essential and 119 RNA coding genes of which two are essential (Reuß *et al.*, 2016). A scheme of the *MiniBacillus* cell is shown in Figure 1.

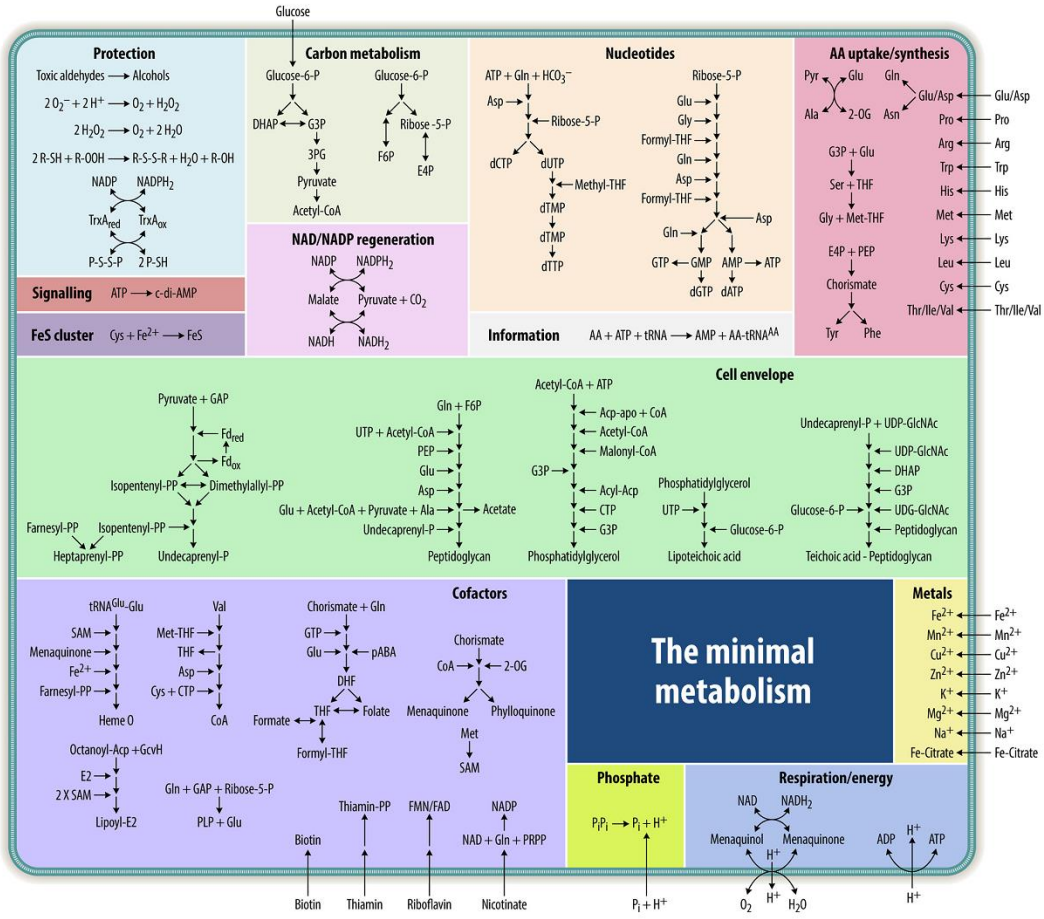


Figure 1: The Blueprint of a *MiniBacillus* cell. The pathways and importer systems that should retain in the genome minimized *B. subtilis* cell. *E.g.* the carbon metabolism is reduced to the gylcolysis and the pentose-phosphate pathway (Reuß *et al.*, 2016).

For the creation of the minimal *B. subtilis* genome, a marker-free deletion system is used. This has the advantage, that no selection marker or scar remains in the genome, which could interfere with the deletion process. The method is based on a selection-counter selection system. A constructed plasmid with the upstream and downstream regions of the desired deletion region

and a spectinomycin resistance is introduced into the strain. The strain lacks the native phosphotransferase system for the import of mannose, encoded by the genes *manA* and *manP*. The introduced plasmid integrates into the chromosome by a single crossover. In a second internal recombination, the cells could, in some cases, lose the deletion region and therefore also the *manP* gene, that was also encoded on the plasmid (Figure 2). Therefore, the cells will be grown in the presence of mannose. If *manP* is still expressed in the cells, mannose-phosphate accumulates to a toxic level and the strain cannot grow. However, the cells that lost the plasmid and the gene are able to grow in the presence of mannose (Wenzel and Altenbuchner, 2015) .

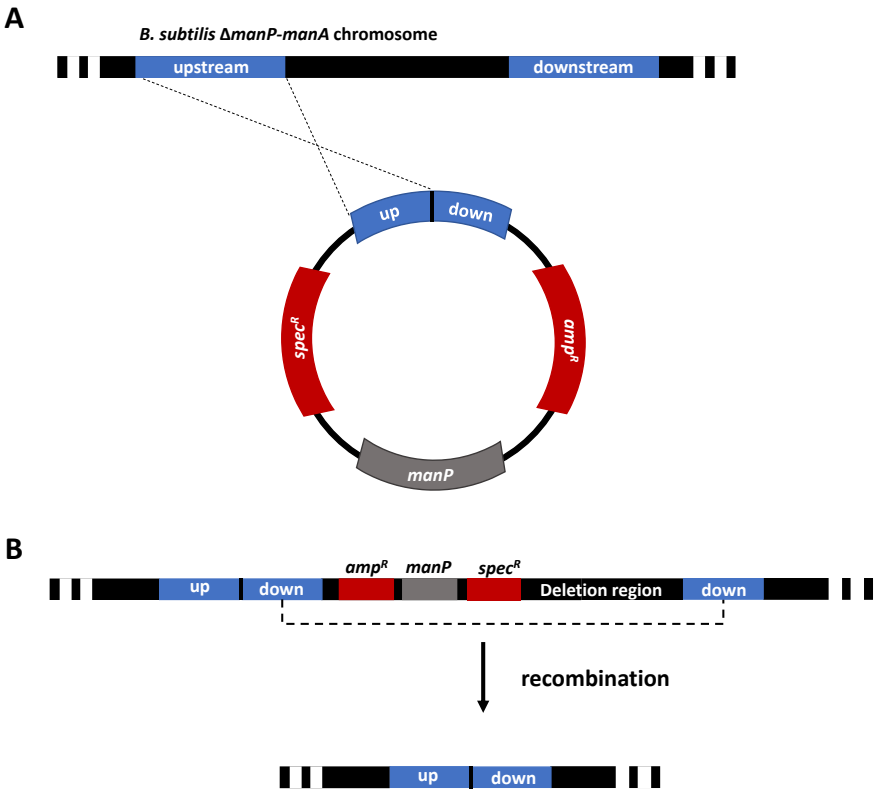


Figure 2: The marker-free deletion system of the *MiniBacillus* project. A The introduction of a constructed plasmid is mediated via a single crossover and the cells are grown in the presence of spectinomycin. **B** In a second recombination step, the plasmid is removed from the chromosome, together with the desired deletion region. This deletion needs to be verified via PCR.

The deletion strains also utilize a special competence system, developed by Rahmer *et al.* (2015), the *comKS* system. A *comKS* cassette is introduced into the chromosome with the mannitol inducible promoter of *mtlA*. ComK is the master regulator of competence and if a certain threshold of ComK is reached in the cell, the competence is induced (Maamar and Dubnau, 2005; Smits *et al.*, 2005). With this method, the transformation efficiency is about 7-fold higher in comparison to the competence induction with the amino acid starvation method (Rahmer *et al.*, 2015). Furthermore, the cells can be grown in complex media like LB and not in minimal media that is normally used to induce competence. This is an advantage, especially for the *MiniBacillus* project, since the

MiniBacillus strains needs to uptake many compounds, like amino acids, from the complex medium, instead of synthesizing them.

The project was started by the group of Josef Altenbuchner in Stuttgart and the first deletion were performed in the strain Δ6, in which the main prophages are missing (Wenzel and Altenbuchner, 2015). The strains constructed during the *MiniBacillus* project are shown in Figure 3. At the point of the deletion strain IIG-Bs27-47-24, with a genome size of 2.83 Mbp, a second parallel line was started in Göttingen. Further deletions were performed and the next milestone was achieved with the creation of the *MiniBacillus* strain PG10. This strain has a genome reduction of 34.54% in comparison to the wild type strain 168. Interestingly, a multi-omics analysis was performed to compare the strain PG10 with the strain Δ6. This led to a good overview about the metabolism and expression in PG10. From the transcriptome data, it could be seen that the expression levels of some genes had changed. This information can now be used to adapt the deletion process and counteract occurred problems (Reuß *et al.*, 2017).

Figure 3: The constructed strains of the *MiniBacillus* project and the corresponding genome sizes. Some dead end occurred in the deletion process with the strains PG21 and the PG28.

More deletions were performed and the strain PG18 was created, with a genome reduction of 36.61%. This strain shows a good growth under the selected conditions and no problems with competence or genome stability. Several genes were deleted to create the strain PG19, which led to a decreased competence in the following strains PG20 and PG21. This effect was caused by the deletion of *nrnA*, encoding for the nanoRNase NrnA (Reuß, 2017). The deletion of other RNases, *e.g.* PnpA can also cause a competence reduction (Luttinger *et al.*, 1996). The reduced competence was a dead end for the project and new deletions were again performed based on the stable strain PG18. PG22 was the next constructed strain and its genome was further reduced up to the strain PG28. However, a problem with the genome integrity was recognized. Some deletions could be verified by PCR, but not by whole genome sequencing (WGS). This could already be observed in the

strain PG22. However, it was discussed if this might be due to a problem with the DNA replication, but the reason for this phenotype is not clear (Reuß, 2017). Therefore, the strain PG18 will be used to create a new line in this work.

It was already mentioned, that a minimal genome strain can be useful for industrial applications. A recent study shows that PG10 is able to produce and secrete proteins that cannot be produced by the wild type strain 168. In this case, four antigens from *Staphylococcus aureus* were expressed in PG10 (Aguilar Suárez *et al.*, 2019). The reason for the improved production and secretion was seen to be the lack of some proteases in the strain PG10, which could previously been shown to influence the heterologous protein production (Stephenson and Harwood, 1998; Pohl *et al.*, 2013). This shows that genome minimized strains can be useful for the production of proteins in the industry.

1.3. Central carbon metabolism and the citric acid cycle

B. subtilis is able to utilize different carbohydrates, like glucose and arabinose, as the sole carbon and energy source (Stülke and Hillen, 2000). Several proteins, like the amylase AmyE, are secreted into the surrounding medium to degrade polysaccharides (Yang *et al.*, 1983). The different sugars are then each imported into the cell and phosphorylated by a specialized phosphotransferase system (PTS) (Saier and Reizer, 1992; Postma *et al.*, 1993). Glucose is the preferred carbon source of *B. subtilis* and the uptake is facilitated by the glucose PTS encoded by *ptsGHI* (Gonzy-Tréboul *et al.*, 1989; Stülke and Hillen, 2000). However, the metabolism of different carbon and energy sources is highly regulated by carbon catabolite control (Fujita, 2009). Glucose is further metabolized in glycolysis and the pentose-phosphate pathway (Sauer *et al.*, 1997). In glycolysis, glucose-6-phosphate is converted in several steps to pyruvate. Furthermore, this pathway provides precursors for the synthesis of amino acids and cell wall components (Ludwig *et al.*, 2001). The genes *pdhABCD* encode the pyruvate dehydrogenase, which catalyses the reaction of pyruvate to acetyl-coenzyme A (CoA) (Lowe *et al.*, 1983). Acetyl-CoA can further be introduced into the tricarboxylic acid (TCA) cycle or into overflow metabolism by the production of acetate, which is secreted to dispose excess amounts of carbon (Speck and Freese, 1973). The TCA cycle is a central point in the metabolism of many organisms and it has two main functions in the cell. First, the degradation of intermediates to produce energy, and second, the production of important building blocks, *e.g.* for amino acids (Hanson and Cox, 1967). The TCA cycle of *B. subtilis* is illustrated in Figure 4.

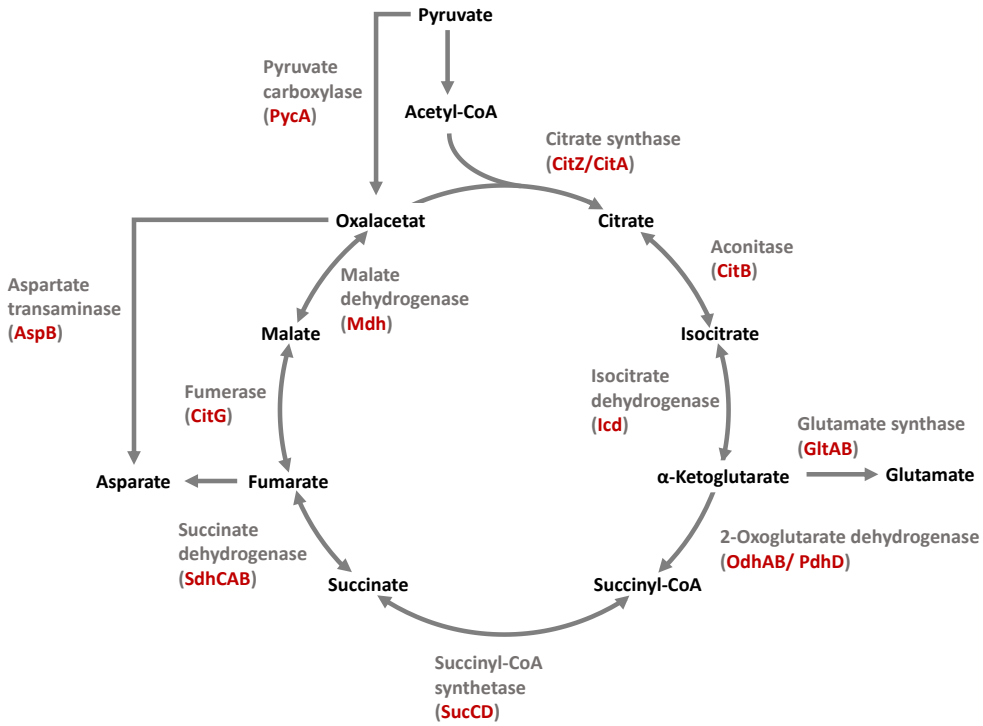


Figure 4: Overview about the TCA cycle in *B. subtilis*. The connection of the TCA cycle to the nitrogen metabolism is mediated via the synthesis of glutamate from α-ketoglutarate. Additionally, oxaloacetate can be converted to aspartate, by the aspartate transaminase AspB.

The first three enzymes of the TCA cycle, the citrate synthase, the aconitase and the isocitrate dehydrogenase have an important role in *B. subtilis*. Deletion mutants of each of the enzymes are glutamate auxotroph and show defects in sporulation. Acetyl-CoA is introduced into the TCA cycle by the reaction with oxaloacetate to citrate. This reaction is catalysed by the citrate synthase. Interestingly, there are two citrate synthases CitZ and CitA annotated in the *B. subtilis* genome. CitZ is responsible for the major citrate synthase activity (Jin and Sonenshein, 1994a; Jin and Sonenshein, 1994b). The citrate synthase CitA can compensate the loss of CitZ only partially and it is therefore annotated as a minor citrate synthase (Zschiedrich, 2014). The expression of both enzymes is also differently regulated. CitZ is transcriptionally regulated by CcpA and CcpC and its expression is therefore repressed by glucose and glutamate (Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2002). In contrast, the expression of CitA is repressed by glucose, but the combination of glucose with glutamate activates the expression again (Jin and Sonenshein, 1994a). A known but poorly characterized, transcriptional repressor of the *citA* gene is the LysR-type protein CitR (Jin and Sonenshein, 1994b). However, it is not known, why *B. subtilis* harbours two citrate synthases. The next step in the TCA cycle is the conversion of citrate to isocitrate, catalysed by the aconitase CitB. Citrate accumulates is a *citB* deletion mutant and leads to a block in sporulation. This is caused by the formation of chelating complexes of citrate and divalent cations, like Mn^{2+} and Fe^{2+}, that are necessary to initiate sporulation (Craig *et al.*, 1997). The accumulation of citrate is not only a

consequence of the missing citrate degradation pathway, the aconitase is also able to bind the *citZ* RNA and destabilize it (Alén and Sonenshein, 1999; Pechter *et al.*, 2013). This enzyme is therefore a trigger enzyme, which can act as an RNA-binding protein in response to the availability of iron, beside its enzymatic activity as an aconitase (Alén and Sonenshein, 1999; Commichau and Stülke, 2008). These two mechanisms ensure the exact regulation of the rate-limiting initial steps of the TCA cycle. Furthermore, the *citB* deletion mutant or a mutant without enzymatic activity is able to form suppressor mutants, which harbour a mutation in the citrate synthase gene *citZ*. (Pechter *et al.*, 2013). A similar problem of an increasing citrate level and the resulting problem in sporulation can be observed in a deletion mutant of the isocitrate dehydrogenase Icd. This enzyme catalyses the reaction from isocitrate to α-ketoglutarate and it is the only enzyme of the TCA cycle, which utilizes NADP as a cofactor (Ramaley and Hudock, 1973). The block in sporulation in an *icd* deletion mutant, can be reversed by an additional deletion of the citrate synthase CitZ, which leads to the assumption that the level of citrate is here also responsible for the phenotype (Matsuno *et al.*, 1999). CcpC is the repressor of the citrate synthase CitZ, the aconitase CitB and the isocitrate synthase Icd. Since *citZ*, *icd* and *mdh*, the malate dehydrogenase, are encoded in one operon, CcpC binds in the respective promoter region. CcpC exclusively regulates the expression of TCA cycle genes and responds to the citrate level in the cell. Low citrate levels lead to the repression of *citZ* and *citB*, which is again reversed if the citrate concentration increases. In contrast, very high levels of citrate activate the expression of the aconitase by CcpC (Kim *et al.*, 2003b; Kim *et al.*, 2003a).

The TCA cycle intermediate α-ketoglutarate is the link to nitrogen metabolism. It can be converted to glutamate by the glutamate synthase GltAB (Wacker *et al.*, 2003). Furthermore, the reaction from glutamate to α-ketoglutarate is catalysed by the glutamate dehydrogenases RocG and GudB (Belitsky and Sonenshein, 1998). However, α-ketoglutarate can also be converted to succinyl-CoA in the TCA cycle. This reaction is catalysed by the α-ketoglutarate dehydrogenase complex encoded by the genes *odhAB* and *pdhD* (Carlsson and Hederstedt, 1989). The enzyme PdhD was already mentioned as a part of the pyruvate dehydrogenase complex (Gao *et al.*, 2002). The succinyl-CoA synthetase, encoded by *sucCD*, catalyses the conversion of succinyl-CoA to succinate (Condon *et al.*, 2002). Succinate is further metabolised to fumarate by the succinate dehydrogenase complex SdhCAB (Melin *et al.*, 1987). SdhC is the membrane bound part of the enzyme and additionally part of the cytochrome b558. The SdhCAB complex is involved in the electron transfer to menaquinone and thereby in the respiratory chain (Hederstedt and Rutberg, 1981; Baureder and Hederstedt, 2011). The next step is catalysed by the fumarase CitG, which is the conversion of fumarate to malate (Miles and Guest, 1985). The fumarase is additionally involved in the DNA damage response, since DNA damage induces the expression of *citG* and the protein seems to co-

localize with the DNA in the cell (Singer *et al.*, 2017). The malate dehydrogenase Mdh catalyses the reaction of malate to oxaloacetate (Jin *et al.*, 1996).

A second, TCA-independent pathway for the production of oxaloacetate exists in *B. subtilis*. It can be produced from pyruvate by the pyruvate carboxylase PycA. This enzyme is bound to biotin as a cofactor (Henke and Cronan, 2014). This oxaloacetate can be further converted to aspartate. The reaction is catalysed by the aspartate transaminase AspB. Since this is a transaminase reaction, glutamate is needed for this reaction and α-ketoglutarate is produced in addition. Aspartate is the precursor of several other amino acids like threonine and isoleucine and a deletion mutant of *aspB* is auxotrophic for aspartate and asparagine (Dajnowicz *et al.*, 2017; Zhao *et al.*, 2018). Furthermore, aspartate can also be again introduced into the TCA cycle, via the conversion to fumarate. The reaction is catalysed by the L-aspartase AnsB (Sun and Setlow, 1991).

Since the TCA cycle is a major point of the metabolism in *B. subtilis*, the expression of the TCA cycle genes is tightly regulated. Most of the TCA cycle genes are under control of carbon catabolite repression. The regulator CcpA represses the expression of *citZ-icd-mdh*, *citB*, *odhAB* and *sucCD,* if enough glucose is available in the cell. Furthermore, the expression of the TCA cycle specific repressor CcpC is also regulated by CcpA (Jin and Sonenshein, 1996; Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2002). This complex interplay of different regulatory mechanisms emphasizes the important role of the TCA cycle in the cell.

The formation of a metabolome was observed for some enzymes of the TCA cycle. The citrate synthase CitZ, the isocitrate dehydrogenase Icd and the malate dehydrogenase Mdh form the central core of this complex. These proteins are additionally encoded in the same operon. It could be shown that the fumarase CitG and the aconitase CitB can also interact with the malate dehydrogenase (Meyer *et al.*, 2011; Jung and Mack, 2018). This metabolome shows again the important interplay of all TCA cycle enzymes and the important role of the complete TCA cycle as a central metabolic pathway. Furthermore, the TCA cycle seems to be involved in other cellular processes. As mentioned above, sporulation is affected if single TCA cycle genes, especially *citZ, citB* or *icd,* are individually deleted.

Although the TCA cycle is a central point in the metabolism of *B. subtilis*, other bacteria like *M. pneumoniae* have a metabolism without a TCA cycle. The specialized lifestyle is the reason for the reduced metabolism, since all nutrients are provided by the host (Manolukas *et al.*, 1988; Halbedel *et al.*, 2007). Furthermore, also incomplete TCA cycles can be found in *Listeria monocytogenes*, since the 2-oxoglutarate dehydrogenase, the succinyl-CoA synthetase and the succinate dehydrogenase are absent (Glaser *et al.*, 2001; Kim *et al.*, 2006; Eisenreich *et al.*, 2006).

1.4. The amino acid biosynthesis and import of *B. subtilis*

1.4.1. The amino acid metabolism of *B. subtilis*

Amino acids are essential building blocks in the cell. They are important for the synthesis of proteins and they can also be utilized as a source for carbon, energy or nitrogen. Amino acids can be either imported from the medium or synthesised by the cell itself. The central carbon metabolism provides many precursors for amino acid biosynthesis. The different pathways for amino acid synthesis in B. subtilis are shown in Figure 5.

Ribose-5-phosphate can be synthesized in the pentose-phosphate pathway and it can be further converted to the amino acid histidine. Furthermore, glutamate can be produced in the histidine utilization pathway (Wray and Fisher, 1994). Phosphoenolpyruvate (PEP), a glycolytic intermediate, is a compound for the synthesis of aromatic amino acids. In this aromatic acid biosynthesis pathway, chorismate is produced, which is an important intermediate in the cell, since it serves furthermore as a precursor for folate and menaquinone (Driscoll and Taber, 1992; Qin and Taber, 1996; de Saizieu *et al.*, 1997). The aromatic amino acids tryptophan, tyrosine and phenylalanine can be metabolized from chorismate (Nester *et al.*, 1969; Hoch and Nester, 1973).

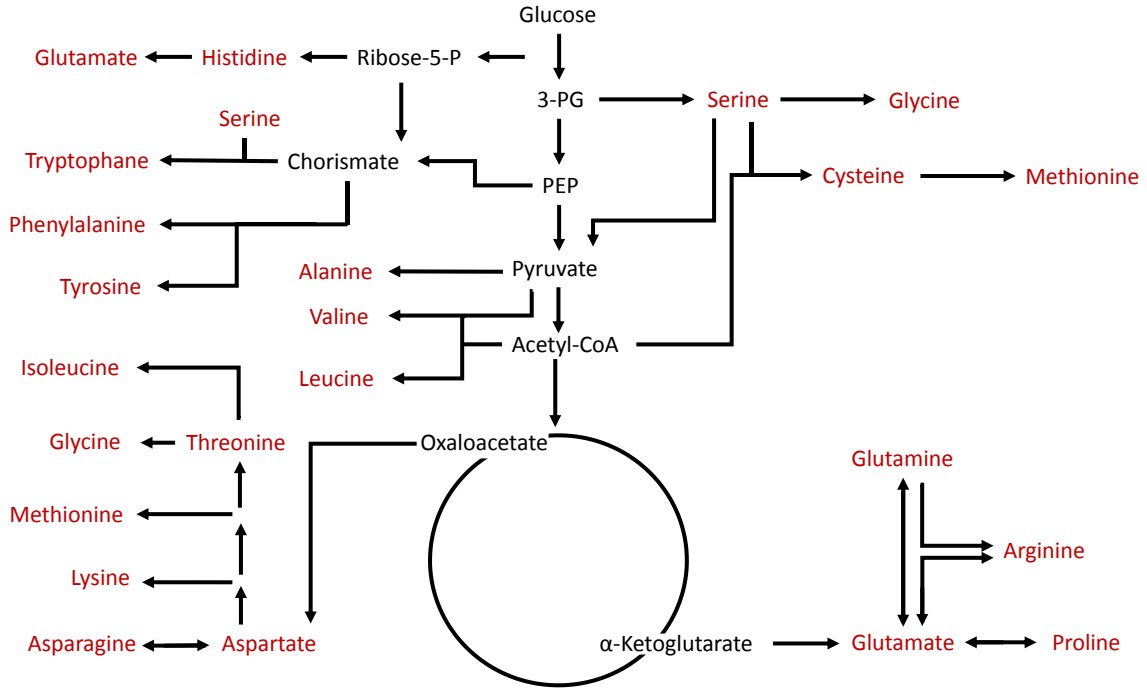


Figure 5: The general pathways of amino acid biosynthesis. Glycolysis provides intermediates like 3-phosphoglycerate (3-PG) and phosphoenolpyruvate (PEP) for the biosynthesis of different amino acids. Glutamate and aspartate can be synthesized from intermediates of the TCA cycle and can be used further to synthesize different other amino acids.

The glycolytic product pyruvate also serves as a precursor of for the synthesis of alanine, valine and leucine (Mäder *et al.*, 2004). 3-phosphoglycerate (3-PG) is produced from glucose-6-phosphate and

can be used to synthesize serine. Since the metabolism of serine is a major research topic of this work, the biosynthesis and utilization pathways are shown in detail in Figure 6.

The phosphoglycerate dehydrogenase SerA catalyses the initial step of the biosynthesis from 3-phosphoglycerate to 3-P-hydroxy-pyruvate. This enzyme is feedback inhibited by serine (Ponce-de-Leon and Pizer, 1972). The intermediate 3-P-hydroxy-pyruvate is further converted to phophoserine by the 3-phosphoserine aminotransferase SerC. Interestingly, the final step in the serine biosynthesis was unknown until YsaA was discovered to be the missing phosphoserine phosphatase. YsaA was therefore named SerB and it catalyses the conversion of phosphoserine to serine (Koo *et al.,* 2017). Serine can be degraded to pyruvate by the L-serine deaminase composed of SdaAA and SdaAB (Chen *et al.*, 2012). Furthermore, glycine can be produced form serine by the serine hydroxymethyltransferase GlyA (Saxild *et al.*, 2001). Acetyl-CoA and serine are precursors to synthesize cysteine, which can furthermore be used to synthesis methionine (Hullo *et al.*, 2007). Interestingly, high serine concentrations can inhibit the growth of *B. subtilis* and become toxic for the cell in minimal medium. The mechanism that is responsible for this effect is unknown, but the addition of some amino acids, like threonine and aspartate, is able to compensate this toxic effect and the cells can grow. Some other amino acids, like arginine and proline, are only able to compensate partially. In contrast to *B. subtilis*, several strains of *B. thuringiensis* are resistant to the growth inhibitory effect of serine (Lachowicz *et al.*, 1996).

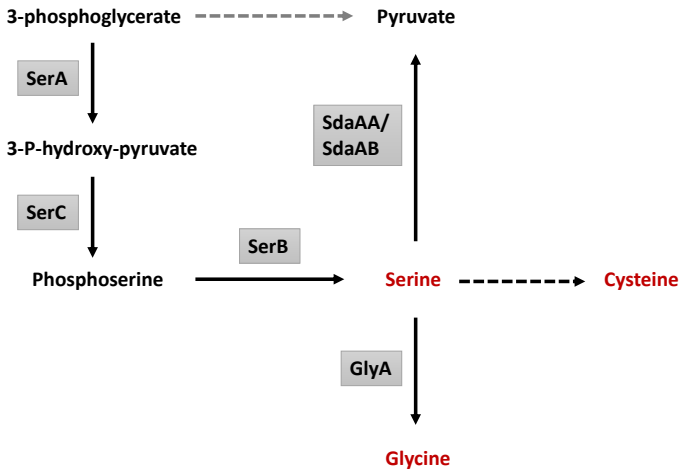


Figure 6: Serine metabolism of *B. subtilis*. Abbreviations used in this figure: SerA, phosphoglycerate dehydrogenase; SerC: 3-phosphoserine aminotransferase; SerB: phosphoserine phosphatase; SdaAA; L-serine deaminase (alpha chain); SdaAB, L-serine deaminase (beta chain); GlyA, serine hydroxymethyltransferase.

The TCA cycle provides precursors of the amino acid synthesis as well. α-ketoglutarate can be converted to glutamate, which serves as an important link between carbon and nitrogen metabolism. Glutamate has an important role in the cell and it was shown to be the most abundant

metabolite in the *E. coli* cell (Bennett *et al.*, 2009). In *B. subtilis*, the amino acid is needed in high concentrations for at least 37 transamination reactions, since the transaminases have a very low affinity for glutamate (Bennett *et al.*, 2009). Interestingly, many of these transamination reactions can be found in the biosynthesis pathways of several amino acids (Oh *et al.*, 2007). As mentioned above, the 3-phosphoserine aminotransferase SerC of the serine biosynthesis pathway catalyses also a transamination reaction. Glutamate serves as the major donor of amino groups and it is a precursor for other amino acids, like glutamine, proline and arginine.

Oxaloacetate, another intermediate of the TCA cycle, can be converted to aspartate. Figure 7 shows the amino acids that can be produced from aspartate. The *B. subtilis* genome encodes three asparagine synthases AsnO, AsnB and AsnH which catalyse the reaction of aspartate to asparagine (Yoshida *et al.*, 1999). Furthermore, aspartate can be converted to L-aspartatyl-4-phosphate. This reaction is catalysed by three aspartokinases (DapG, LysC, ThrD), that are each differently regulated by several products of the following pathways, the threonine biosynthesis, the diaminopimelate synthesis and the lysine biosynthesis. DapG, the essential aspartokinase I is feedback inhibited by diaminopimelate, a precursor of the peptidoglycan biosynthesis. Lysine repressed the expression of the aspartokinase II LysC, by a lysine dependent riboswitch. ThrD, the Aspartokinase III, is inhibited by the presence of both threonine and lysine (Graves and Switzer, 1990; Kobashi *et al.*, 2001; Rosenberg *et al.*, 2016).

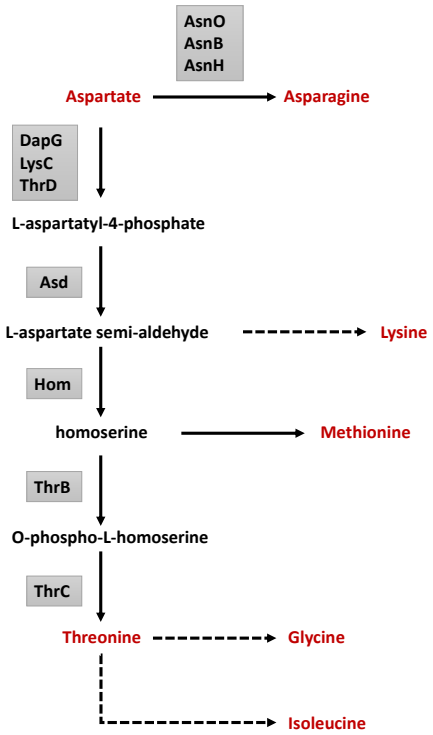


Figure 7: The amino acid biosynthesis pathways derived from aspartate. Abbreviations used in this figure: AsnO, AsnB and AsnH, Asparagine synthases; DapG, aspartokinase I; LysC, aspartokinase II; ThrD, aspartokinase III; Asd, aspartate-semialdehyde dehydrogenase; Hom, homoserine dehydrogenase; ThrB, homoserine kinase; ThrC, threonine synthase.

L-aspartate semi-aldehyde is produced from L-aspartatyl-4-phosphate by the enzyme aspartate-semialdehyde dehydrogenase Asd. This compound can be further metabolised in the diaminopimelate (DAP) pathway to meso-2,6-diaminopimelate, which is used for either the synthesis of peptidoglycan or for the synthesis of lysine (Rodionov *et al.*, 2003). However, L-aspartate semi-aldehyde can also be converted to homoserine by the homoserine dehydrogenase Hom (Parsot and Cohen, 1988). Homoserine can again be used, either for the biosynthesis of methionine or for the conversion to O-phospho-L-homoserine. The last reaction is catalysed by the homoserine kinase. The intermediate O-phospho-L-homoserine is in the next step metabolised to the amino acid threonine (Parsot, 1986). The genes *hom, thrC* and *thrB* form an operon and are regulated by several transcription factors. The DNA-binding protein ThrR represses the expression of the *hom* operon and additionally the expression of the aspartokinase III gene *thrD* (Rosenberg *et al.*, 2016). Furthermore, the protein CodY is involved in the regulation of the operon. This transcription factor regulates the expression of several nitrogen metabolism genes, competence genes and genes involved in acetate metabolism. The DNA-binding of this global regulator CodY is activated by branched-chain amino acids (BCAA) and GTP. CodY represses the genes required for nutrient-limiting conditions in rich media, since enough BCAAs and GTP stabilize the DNA-binding of CodY. This repression is released, if the BCAA and GTP levels in the cell decrease by a lower availability of nutrients. The hom-thrCB promoter is also repressed by CodY (Fisher, 1999). The expression of the *hom* operon is also regulated by TnrA (Mirouze *et al.*, 2015). TnrA is a transcription factor, which regulates the expression of several genes under nitrogen limiting conditions (Wray *et al.*, 1996). The amino acid threonine can be further converted to glycine and isoleucine (Mäder *et al.*, 2004). Interestingly, high amounts of threonine in minimal medium inhibit the growth and the formation of spores in *B. subtilis*. This effect can be compensated by the addition of valine to the medium, which suggests a inhibition of valine biosynthesis by threonine (Lamb and Bott, 1979a). Similarly, inhibitory effects of high isoleucine or valine concentrations could be observed (Teas, 1950; Lachowicz *et al.*, 1996). Therefore, the level of some amino acids could have a regulatory function in the cell.

1.4.2. Amino acid transporters

B. subtilis transports different kinds of substrates. This task is fulfilled by different transporter proteins. In general, the proteins can be divided according to the energy source, into channels, primary transporters and secondary transporters. Channels transport substrates by an energy independent concentration gradient. Primary transporters use the energy from the hydrolysis of ATP for the active transport. Furthermore, secondary transporters are dependent on the ion

gradient as a transport energy source (Saier, 2000). Transporters import and export different compounds, like vitamins, sugars and amino acids. A very important class of primary transporters are the ATP-binding cassette (ABC) transporters. These can be divided into the importers, the exporters and the proteins that are not involved in transport, but in the regulation of processes like DNA repair (Higgins *et al.*, 1986; Davidson *et al.*, 2008). The ABC transporter consist of three domains: the nucleotide-binding domain, the membrane spanning domain and the solute-binding domain (Quentin *et al.*, 1999). The arginine importer encoded by *artPQR* is an example for an ABC transporter in *B. subtilis* (Yu *et al.*, 2015).

The secondary transporters are the largest superfamily of amino acid transporters (Saier, Jr, 2000). An important group within the family is the amino acid-polyamine-organocation (APC) superfamily, which includes solute:cation symporters and solute:solute antiporters (Jack *et al.*, 2000). Many amino acid transporters of *B. subtilis* can be classified in this group. The proline and gamma-amino butyric acid permease GabP and the branched-chain amino acid and threonine transporter BcaP are examples for transporters of the APC family (Zaprasis *et al.*, 2014; Belitsky, 2015). Symporters of the secondary transporters are divided by the ions that are similarly taken up with the solute. Members of the solute:sodium symporter (SSS) family, like the proline importer PutP from *B. subtilis*, import sodium ions together with the desired compound (Moses *et al.*, 2012). Although, transporters can be divided into different classes, some are not limited to certain substrates and they are rather unspecific. Similar amino acids can often be imported by the same transporter. The importer BcaP from *B. subtilis* can import valine and isoleucine, since both are branched-chain amino acids (BCAA). Additionally, the threonine transport of BcaP was observed (Belitsky, 2015). Since the addition of serine, alanine and asparagine can decrease the uptake of isoleucine, BcaP might also be involved in the transport of these amino acids (Belitsky, 2015). The uptake of an amino acid is in most cases not facilitated by only one transporter. Although, BcaP seems to facilitate the major uptake of valine and isoleucine, two additional importers are known, BraB and BrnQ. The reason for the different uptake systems is that each system is active under different conditions. All three genes *bcaP*, *brnQ* and *braB* are repressed via CodY under high BCAA concentrations. However, BraB is active at intermediate CodY levels, since it is additionally repressed by ScoC, a transition state regulator. ScoC represses the expression of BraB only if CodY is present in low concentrations, since CodY inhibits the expression of ScoC (Belitsky *et al.*, 2015). This ensures a certain level of BCAA import, although the expression of the other transporters are still repressed. BrnQ in contrast is overall very low expressed, since the AzlB protein negatively regulates the BrnQ repression, but the reason for this is not completely understood. The three

different uptake systems for valine and isoleucine are therefore tightly regulated to ensure the adapted uptake of these amino acids to the current cell status (Belitsky, 2015).

Furthermore, some transporters are only expressed and active during low substrate conditions. These are high-affinity transporters and they exhibit a high affinity to the substrate (Bosdriesz *et al.*, 2018). All three valine and isoleucine transporters BcaP, BraB, BrnQ are high-affinity transporters and are active during low BCAA concentrations, since the amount of the repressor CodY is also low. A triple deletion mutant of the transporters, combined with a BCAA auxotrophic strain, is still able to grow on high BCAA concentrations. Therefore, at least one low-affinity system must be encoded in the *B. subtilis* genome. Low-affinity transporters are expressed at high substrate conditions (Belitsky, 2015; Bosdriesz *et al.*, 2018).

Although, many amino acid transporters of *B. subtilis* are known and they can be divided into different group of transporters, the import of some amino acids is poorly characterized. For the amino acids phenylalanine and tyrosine, no importers are annotated at all. For the serine import only the serine/threonine exchanger SteT is described. SteT is similar to known amino acid transporters from humans and it exhibit a serine transport function in proteoliposomes (Reig *et al.*, 2007). Since, high serine concentrations can inhibit the cell growth, the uptake of serine is most likely higher regulated and different uptake systems could be involved. Furthermore, there are some membrane proteins annotated, which show similarities to known amino acid transporters, but are of unknown function.

1.5. Aim of this work

This work focuses on the *MiniBacillus* project. The first goal is to reduce the genome of the *MiniBacillus* strain PG18 further and to analyse the impact of the performed deletion on the strain. Data from multi-omics experiments, obtained in previous works, will be utilized to adapt the deletion process and improve the strain. Furthermore, the strain will be phenotypically analysed and compared on a genome and proteome level. Since the *MiniBacillus* project is mainly about understanding life, the second objective of this work is to analyse the function of the TCA cycle in the cell. In the final minimal cell, the TCA cycle will be not necessary. Therefore, it needs to be analysed, if a deletion of the TCA cycle strain is possible in a wild type strain and if the deletion is a disadvantage for the cell. Not all amino acid importers are known for *B. subtilis*. However, this knowledge would be an advantage for the *MiniBacillus* project, since the mini cell could import all necessary amino acids from the complex medium. Therefore, the transporters of unknown function should be characterized further, especially for the amino acid serine. Furthermore, the mechanism

and physiological role of the serine toxicity should be further analysed. These results will all contribute to the progress of the *MiniBacillus* project.

2. Materials and Methods

2.1. Materials

Materials, chemicals, equipment, commercial systems, enzymes and oligonucleotides are listed in the appendix.

2.1.1. Bacterial strains and plasmids

Bacterial strains and plasmids are listed in the appendix.

2.1.2. Growth media

Buffers, solutions and media were prepared with deionized water and autoclaved for 20 min at 121°C and 2 bar, unless otherwise stated. Thermolabile substances were dissolved and sterilized by filtration.

Bacterial growth media

E. coli was grown in LB and M9 medium, whereas *B. subtilis* was grown in LB, SP and C-minimal media, supplemented with additives as indicated. For solidification, basic media were supplemented with 1.5% (w/v) agar (Blötz *et al.*, 2017).

Complex media

LB medium	10 g	Tryptone
(1 l)	5 g	Yeast extract
	10 g	NaCl
SP medium	8 g	Nutrient Broth
(1 l)	0.25 g	$MgSO_4$ x 7 H_2O
	1 g	KCl
	Solve and autoclave, after cooling down addition of:	
	1 ml	$CaCl_2$ (0,5 M)
	1 ml	$MnCl_2$ (10 mM)
	2 ml	Ammonium iron citrate (2.2 mg/ml)
Starch medium	7.5 g	Nutrient broth
(1 l)	5 g	Starch

Minimal media

10x MN medium		136 g	K_2HPO_4 x 3 H_20
(1 l)		60 g	KH_2PO_4
		10 g	Sodium citrate x 2 H_2O
MNGE medium	1 ml	10x MN medium	
(10 ml)		1 ml	Glucose (20%)
		50µl	Potassium glutamate (40%)
		50 µl	Ammonium iron citrate (2.2 mg/ml)
		100 µl	Tryptophan (5 mg/ml)
		30 µl	MgSO4 x 7H2O (1M)
		+/- 100 µl	CAA (10%)
C minimal medium		20 ml	5x C-salts
(100 ml)		1 ml	Tryptophan (5 mg/ml)
		1 ml	Ammonium iron citrate (2.2 mg/ml)
		1 ml	III' salts
CE medium		20 ml	5x C-salts
(100 ml)		1 ml	Tryptophan (5 mg/ml)
		1 ml	Ammonium iron citrate (2.2 mg/ml)
		1 ml	III' salts
		2 ml	Potassium glutamate (40%)
CSE medium		20 ml	5x C-salts
(100 ml)		1 ml	Tryptophan (5 mg/ml)
		1 ml	Ammonium iron citrate (2.2 mg/ml)
		1 ml	III' salts
		2 ml	Potassium glutamate (40%)
		2 ml	Sodium Succinate (30%)

M9 (-CAA) medium	50 ml	20x M9 base medium
(100 ml)	1 ml	1 M $MgSO_4$
	1 ml	0.1 M $CaCl_2$
	0.5 ml	1 mM $FeCl_3$
	1 ml	Glucose (50%)
	1 ml	Thiamine-Cl_2 (1 mg/ml)

Solutions and additives

5x C-salts	20 g	KH_2PO_4
(1 l)	80 g	K_2HPO_4 x 3 H_2O
	16.5 g	$(NH_4)_2SO_4$
III' salts	0.232 g	$MnSO_4$ x 3 H_2O
(1 l)	12.3 g	$MgSO_4$ x 7 H_2O
20x M9 base	140 g	Na_2HPO_4 x 2 H_2O
(1 l)	60 g	KH_2PO_4
	20 g	NH_4Cl
	Ad to 1000 ml with deionized water	

Antibiotics

Antibiotics were prepared as 1000-fold concentrated stock solutions. Kanamycin, spectinomycin, lincomycin, zeocin and ampicillin were dissolved in deionized water, chloramphenicol, erythromycin and tetracycline in 70% ethanol. All solutions were filtrated and stored at -20°C. For the selection of *ermC*, erythromycin and lincomycin were used in combination.

Selection concentration for *E. coli*

Ampicillin	100 µg/ml
Kanamycin	50 µg/ml

Selection concentration for *B. subtilis*

Kanamycin	10 µg/ml
Spectinomycin	150 µg/ml
Lincomycin	25 µg/ml
Zeocin	35 µg/ml
Chloramphenicol	5 µg/ml
Erythromycin	2 µg/ml
Tetracycline	12.5 µg/ml

2.2. Methods

2.2.1. General methods

Some general methods used in this work are listed in Table 1 with the corresponding literature.

Table 1: General methods

Method	Reference
Absorption measurement	Sambrook *et al.*, 1989
Precipitation of nucleic acids	Sambrook *et al.*, 1989
Gel electrophoresis of DNA	Sambrook *et al.*, 1989
Plasmid preparation from *E. coli*	Sambrook *et al.*, 1989
Ligation of DNA fragments	Sambrook *et al.*, 1989
Determination of protein amounts	Bradford, 1976
Gel electrophoresis of proteins (denaturing)	Laemmli, 1970
Sequencing according to the chain termination method	Sanger *et al.*, 1992

2.2.2. Cultivation of bacteria

Unless otherwise stated, *E. coli* was grown in LB or M9 -CAA medium at 37°C and 200 rpm in tubes and flasks. *B. subtilis* was grown in LB, SP, C-Glc, CE-Glc, CSE-Glc or MNGE medium at 37°C or 28°C in tubes and flasks. Fresh colonies from plates or -80°C cryo cultures supplemented with 10% DMSO were used for inoculation. The growth was measured as the optical density at 600 nm.

2.2.3. Storage of bacteria

E. coli was kept on LB medium agar plates up to 4 weeks at 4°C. DMSO cultures were used for long-term storage. *B. subtilis* was cultured on SP or LB plates and stored in DMSO stocks. For a DMSO stock, 900 µl of a fresh overnight culture was mixed with 100 µl of DMSO. Stocks were snap frozen and stored at -80°C. SP agar tubes were used for the long-term storage of *B. subtilis* (Blötz et al., 2017).

2.2.4. Preparation of competent *E. coli* and transformation

Preparation of competent cells in SOB medium

A culture of *E. coli* DH5α or XL1blue cells were used to inoculate 250 ml SOB-medium over night at 18°C. After reaching an OD_{600} of 0.5-0.9 the culture was cooled down by incubation for 10 min on ice. The cells were harvested by centrifugation (10 min; 4000 rpm; 4°C) and resuspended in 80 ml of ice-cold TB-Buffer. A final concentration of 7% DMSO was added and aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C (Blötz *et al*., 2017).

SOB-Medium	20 g	Tryptone
(1 l)	5 g	yeast extract
	0.584 g	NaCl
	0.188 g	KCl
	Ad. 1 l	deion. H_2O
TB-Buffer	1.51 g	PIPES
(500 ml, pH 6.7)	1.1 g	$CaCl_2 \times H_2O$
	9.32 g	KCl
	Ad 472.5 ml	deion. H_2O
	27.5 ml	$MnCl_2$ (1 M)

Preparation of competent cells in $CaCl_2$ medium

An overnight culture of *E. coli* BL21 or JM109 was used to inoculate 100 ml of LB medium. This culture was grown to an OD_{600} of 0.3 and 10 ml of cells were harvested for 6 min at 5000 rpm and 4°C. The pellet was resuspended in 5 ml of a 50 mM $CaCl_2$ solution. The cells were incubated on ice for 30 min and again centrifuged as described before. 1 ml of a 50 mM $CaCl_2$ solution was used to resuspend the cells, which were then used for the transformation method (Blötz *et al*., 2017).

Transformation of competent *E. coli* cells

200 µl competent cells were defrosted on ice and mixed with 10-100 ng DNA. After inoculation for 30 min on ice, the heat shock was performed at 42°C for 90 seconds. 500 µl of LB medium was added and the samples were incubated for 60 min at 37°C at 200 rpm. 150 µl and the rest of the solution were plated on LB selection plates (Blötz *et al*., 2017).

2.2.5. Preparation of competent *B. subtilis* cells and transformation

Preparation of competent cells in MNGE medium and transformation

An overnight culture of *B. subtilis* was used to inoculate 10 ml of MNGE medium containing 1% CAA to an optical density of 0.1. The culture was grown at 37°C at 200 rpm until an OD_{600} of 1.3 was reached. Then the culture was diluted with 10 ml MNGE medium without CAA and incubated again for one hour. After this incubation step, 400 µl of competent cells were incubated with 0.1-1 µg DNA for 30 min at 37°C with at 200 rpm. Afterwards, 100 µl of expression mix were added and the samples were incubated at 37°C for one hour. The cell suspension was spread onto SP or LB selection plate (Blötz *et al*., 2017).

Expression mix (1.05 ml)	500 µl	yeast extract
	250 µl	CAA (10%)
	250 µl	deion. H_2O
	50 µl	Tryptophan (5 mg/ml)

Preparation of competent cells with the mannitol-inducible *comKS* system

500 µl of an overnight culture is used to inoculate 5 ml of LB medium in a baffled flask, that is incubated for 90 min at 37°C and 200 rpm. To induce the competence, 5 ml LB with 0.5% mannitol and 5 mM $MgCl_2$ are added to the culture. After another incubation step of 90 min at 37°C and 200 rpm, the cells are harvested by centrifugation for 10 min at 4500 rpm. The cells are re-suspended in 10 ml LB medium and 1 ml of the suspension is mixed with 0.1-1 µg DNA. The samples are incubated for 60 min at 37°C and 200 rpm. 150 µl and the rest of the solution were plated on LB selection plates (Rahmer *et al.*, 2015; Blötz *et al.*, 2017).

2.2.6. Preparation and detection of DNA

Isolation of genomic DNA of *B. subtilis*

For the isolation of *B. subtilis* gDNA, the cells of a 4 ml LB overnight culture were harvested. The gDNA was extracted, using the peqGOLD Bacterial DNA Kit from PEQLAB. Deionized water was used for elution of the DNA from the columns (Blötz *et al*., 2017).

Isolation of plasmid DNA from *E. coli*

Plasmid DNA was extracted from *E. coli* cultures, using the NucleoSpin® Plasmid Kit from Machery-Nagel. Deionized water was used for the elution of the plasmids from the columns (Blötz *et al*., 2017).

Purification of DNA

The QIAquick PCR purification Kit was used for the purification of DNA fragments. For the elution of the DNA from the column, deionized water was used (Blötz *et al*., 2017).

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments by size and thereby analyse the respective size. 1% agarose gels (w/v) were prepared in TAE Buffer together with HDGreen™ DNA-Dye from Intas. The DNA samples were supplemented with 5x DNA loading dye and loaded onto the gel, together with an *Eco*RI/ *Hind*III digested λ-DNA marker to estimate the size of the DNA fragments. A voltage of 140 V was applied until the colour marker reached the last third of the gel. For the detection of the DNA the GelDoc™ from Biorad was used by excitation with UV light (254 nm) (Blötz *et al*., 2017).

50x TAE Buffer	242 g	Tris-base
	57.1 ml	Acetic acid (100%)
	100 ml	EDTA (0.5M, pH 8.0)
	Ad to 1000 ml with deionized water	
5x DNA loading dye	5 ml	Glycerol (100%)
	200 µl	50x TAE
	10 mg	Bromphenol blue
	10 mg	Xylene cyanol
	4.8 ml	dH_2O

Sequencing of DNA

Sequencing of plasmids and DNA fragments was performed by Microsynth AG with the chain termination method. Whole genome sequencing was carried out by the Göttingen Genomic Laboratory.

Cloning procedure

DNA restriction enzymes from ThermoFisher were used to cleave the PCR fragment and the respective plasmid. The manufacturer's instructions for enzyme concentration, incubation conditions and heat inactivation after the reaction were followed. The vector was dephosphorylated by the addition of 1 µl alkaline phosphatase (ThermoFisher) and incubation at 37°C for 15 min. Afterwards the vector and the insert were ligated using T4-DNA ligase (ThermoFisher). The ligation reaction contained 150 ng of vector DNA and a 5-fold excess of insert DNA and was incubated 2 h at RT or overnight at 16°C (Blötz *et al*., 2017).

Polymerase chain reaction (PCR)

The PCR reaction was performed with genomic or plasmid DNA as template in a total volume of 50 µl with Phusion™ polymerase or DreamTaq polymerase.

Reaction mix for Phusion™ polymerase (50 µl):

10 µl	5x HF reaction buffer
2 µl	dNTPs (12.5 µmol/ml)
2 µl	forward primer (20 pmol)
2 µl	reverse primer (20 pmol)
1 µl	template DNA (1 ng/µl)
0.2 µl	Phusion™ polymerase (2 U/µl)
32.8 µl	dH_2O

Reaction mix for DreamTaq (50 µl):

5 µl	10x DreamTaq reaction buffer
2 µl	dNTPs (12.5 µmol/ml)
2 µl	forward primer (20 pmol)
2 µl	reverse primer (20 pmol)
1 µl	template DNA (1 ng/µl)
0.25 µl	DreamTaq polymerase (2 U/µl)
37.75 µl	dH_2O

The samples were briefly vortexed, centrifuged and the reaction was placed into the Themocycler with the following programs:

Standard program for the Phusion™ polymerase

Initial denaturation	98°C	5 min	
Denaturation	98°C	30 s	30 x
Annealing	48-56°C	35 s	
Elongation	72°C	30 s per 1 kb	
Final elongation	72°C	10 min	
Break	4°C	∞	

Standard program for the DreamTaq polymerase

Initial denaturation	95°C	5 min	
Denaturation	95°C	30 s	30 x
Annealing	48-56°C	35 s	
Elongation	72°C	1 min per 1 kb	
Final elongation	72°C	10 min	
Break	4°C	∞	

Long flanking homology PCR (LFH-PCR)

The long flanking homology PCR (LFH PCR) was used to generate a DNA fragment for the deletion of genes in the genome of *B. subtilis*. Therefore, the upstream and downstream regions of the target genes are amplified by PCR (1000 bp). The genes encoding for the resistance against chloramphenicol, kanamycin, erythromycin, spectinomycin, tetracyclin and zeocin are amplified from the plasmids pGEM-cat, pDG780, pDG646, pDG1726, pDG1513 and pDG148 respectively. The flanking regions and the resistance cassette were fused together in the LFH PCR, in which the first step was the joining of the three fragments without the oligonucleotides. In a second step the oligonucleotides are added to the reaction and the complete fragment was amplified. Complementary sequences allow the joining of the fragments. Afterwards, *B. subtilis* competent cells were transformed with the LFH product and plated onto the respective selection plates.

Reaction mix for LFH PCR with Phusion™ polymerase (50µl):

10 µl	5x HF reaction buffer
2 µl	dNTPs (12.5 µmol/ml)
4 µl	forward primer (20 pmol)
4 µl	reverse primer (20 pmol)
100 ng	upstream flanking region
100 ng	downstream flanking region
150 ng	resistance gene
1 µl	Phusion™ polymerase (2 U/µl)
Ad to 50 µl with dH_2O	

Standard program for the LFH PCR with Phusion™ polymerase

Initial denaturation	98°C	3 min	
Denaturation	98°C	30 s	10 x
Annealing	52°C	35 s	
Elongation	72°C	2 min 15 s	
Hold	15°C	∞	
Addition of oligonucleotides			
Denaturation	98°C	30 s	30 x
Annealing	52°C	35 s	
Elongation	72°C	3 min 30 s + 5 s/ cycle	
Final elongation	72°C	10 min	
Break	4°C	∞	

Marker-free deletion system

The marker-free deletion system is based on the mannose phosphotransferase system. In *B. subtilis* mannose uptake and phosphorylation is performed by the permease ManP. The resulting product mannose-6-phosphate is then further metabolized by the mannose-6-phosphate isomerase ManA. Without the corresponding genes *manP* and *manA*, the uptake of mannose is not possible. However, if only *manP* is present, mannose-6-phosphate accumulates in the cell to a toxic level. The plasmids used in this method are pJOE6743 and pGP1022, which carry the *manP* gene as a counterselection marker and a spectinomycin resistance as a selection marker.

The upstream and downstream regions of the deletion target were amplified, fused together and cloned into the deletion plasmid. A strain without the *manP* and *manA* genes (Δ*manP-manA*) was transformed with the plasmid and plated on LB agar with spectinomycin. A 4 ml LB culture was inoculated with a colony from the plates and after incubation at 37°C over the day, the cells were diluted $1:10^{-4}$ In LB medium supplemented with 0.5% mannose. The cultures were incubated overnight, then diluted $2x10^{-5}$ and plated onto LB agar plate with 0.5% mannose. The cells that could grow in the presence of mannose have lost the introduced *manP* gene and the uptake of mannose was not possible anymore. These colonies were again tested for their growth on LB medium with spectinomycin. Only the colonies which did not grow in the presence of spectinomycin were used for colony PCR to check for the correct deletion. This marker-free deletion method is illustrated in Figure 8 (Wenzel and Altenbuchner, 2015; Blötz *et al.*, 2017).

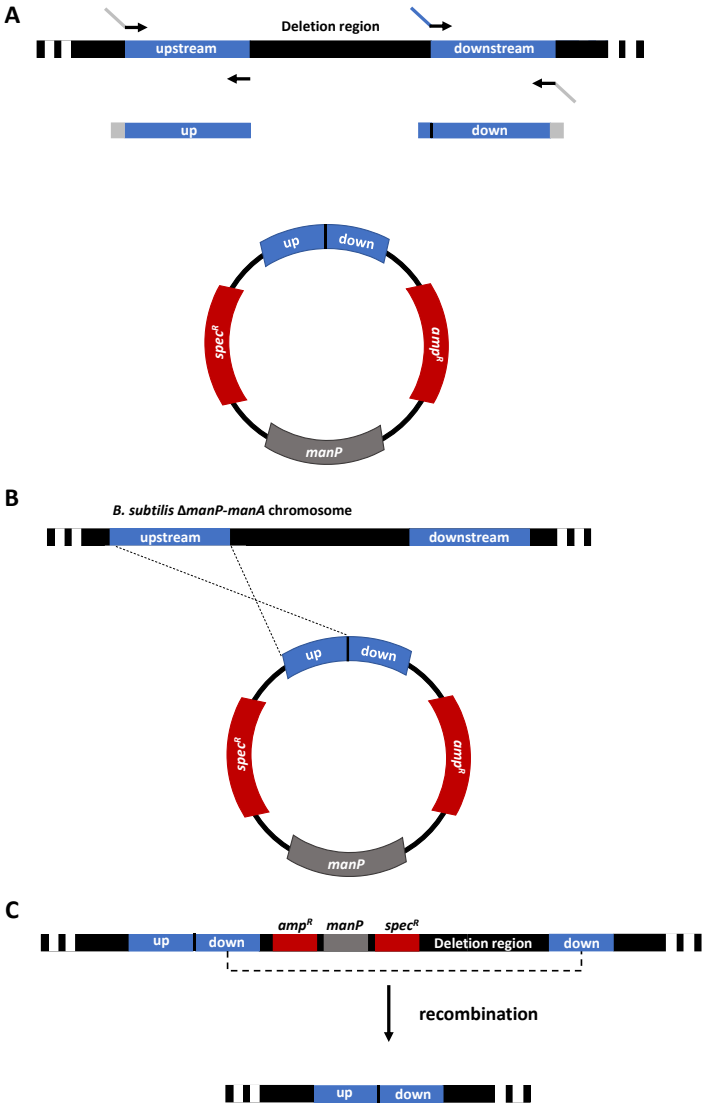


Figure 8: Marker-free deletion system. A The upstream and downstream regions of the deletion region were amplified, fused in an LFH reaction and cloned into the vector pJOE6743 or pGP1022. **B** The strain was transformed with the plasmid, which was inserted into the genome via one of the homolog flanks. **C** Since the flanking region, which was not used for the integration is now present in two copies, a recombination occurs, and the deletion region is removed.

Cre-lox deletion system

The Cre-lox system is used to generate clean deletions. In the first step, the gene of interest is exchanged with a resistance cassette from an LFH product. The LFH product contains the up- and downstream regions from the gene of interest, a resistance cassette and additionally lox sites between the flanking regions and the resistance cassette. These 34 bp lox sites, lox71and lox66, can be recognized by the Cre recombinase from the P1 bacteriophage, which cuts and recombines these specific sequences. The gene encoding this enzyme was previously introduced into the *sacA* locus of the *B. subtilis* chromosome and the expression can be induced by the addition of xylose. If the expression of the cre recombinase is induced, the resistance cassette is cut out and the lox72 site remains in the genome (Figure 9). (Yan *et al.*, 2008; Kumpfmüller *et al.*, 2013).

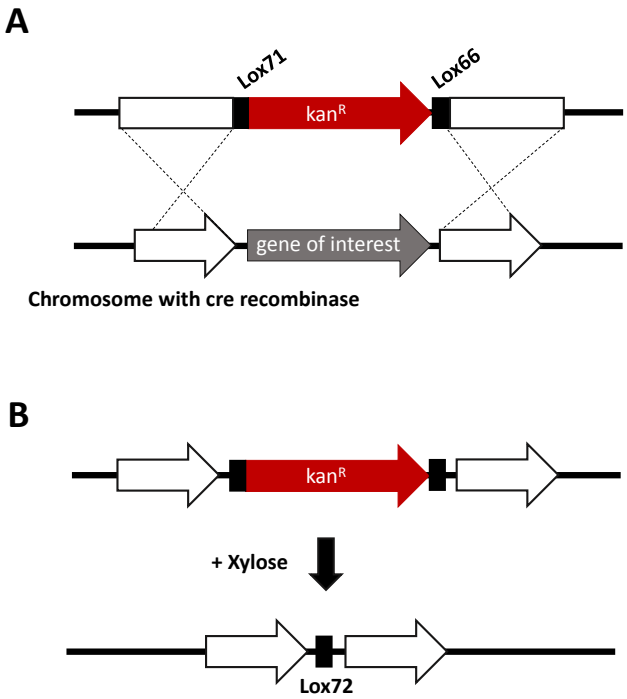


Figure 9: Cre-lox deletion. A The gene of interest was exchanged with an antibiotic resistance cassette via LFH. Additionally, lox sites were introduced. **B** The cre recombinase was induced by the addition of xylose and it cuts and recombines the lox71 and lox66 sites. Finally, the resistance cassette was removed and a lox72 site is left.

2.2.7. Preparation and detection of proteins

Overexpression of recombinant proteins in *E. coli*

The *E. coli* BL21 strain with the relevant plasmid was used to inoculate an overnight culture. The main culture of 1 l LB was inoculated to an OD_{600} of 0.1 and grown at 37°C and 200 rpm until an optical density of 0.6 to 0.8 was reached. At this point, the inducer isopropyl-β-D-thio-galactopyranoside (IPTG) was added in a final concentration of 1 mM.

Cell disruption with French pressure cell press

A cell pellet was resuspended in buffer W or ZAP buffer and filled into the precooled bomb. The remaining air was removed before the bomb was locked and placed in the French press. The disruption was performed three times with a pressure of 18.000 PSI.

Preparation of crude extracts for β-galactosidase activity assay)

The cell pellet, from a cell culture grown to an OD_{600} of 0.5-0.8, was resuspended in 400 µl Z-buffer with β-mercaptoethanol and Lysozyme/DNase I mix (60 µl on 12 ml Buffer). The samples were incubated 10 min at 37°C and 600 rpm and afterwards centrifuged 3 min at 4°C and 14800 rpm.

LD mix	100 mg	Lysozyme
	10 mg	DNase I
	Ad to 10 ml with dH_2O	

Z-Buffer	0.534 g	Na_2HPO_4 x 2 H_2O
	0.276 g	NaH_2PO_4
	0.037 g	KCl
	50 µl	1 M $MgSO_4$
	175 µl	β-mercaptoethanol
	Ad to 50 ml with dH_2O	

Purification of Strep-tagged proteins

A cell pellet of the respective *E. coli* strain was resuspended in cold buffer W and the cells were disrupted with the french pressure cell press as described above. The solution was centrifuged at 35000 rpm and 4°C for 30 min to remove cell debris. This crude extract was then loaded onto pre-equilibrated 500 µl Strep-Tactin Sepharose (IBA) in a Poly-Prep chromatography column (Biorad). Buffer W was used to wash the mixture five times and the bound proteins were then eluted with buffer E in four fractions. The fractions were analysed by SDS page (Blötz *et al*., 2017).

Buffer W (pH 8.0)	121.14 g	Tris-base
	87.7 g	NaCl
	3.72 g	Na_2EDTA
	Adjust the pH with HCl to 8.0	
	Ad to 1000 ml with dH_2O	

Buffer E	0.027 g	D-Desthiobiotin
	Add 50 ml 1x buffer W	

Purification of His-tagged proteins

An *E. coli* cell pellet was resuspended in ZAP Buffer and the cells were disrupted as described above. To remove cell debris, the cell solution was centrifuged at 17.500 rpm and 4°C for 30 min. 1.25 ml of Ni-NTA® sepharose was loaded onto a column and equilibrated with 12.5 ml ZAP Buffer. The crude extract was loaded onto the column and the flow through was collected. Five washing steps were performed with each 10 ml ZAP buffer and the elution was done with ZAP buffer with increasing concentrations of imidazole (Blötz *et al*., 2017).

10x ZAP Buffer	60.57 g	Tris-base
	116.88 g	NaCL
	Adjust the pH with HCl to 7.5	
	Ad to 1000 ml with dH_2O	

Dialysis

The dialysis was used to remove the desthiobiotin from the protein solution or to change to a desired buffer. Therefore, the elution fractions with the highest protein amount were pipetted into a dialysis tube and dialyzed against the desired buffer in an excess of 1000 fold overnight.

Denaturing gel electrophoresis of proteins (SDS-PAGE)

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method described by Laemmli (1970) was used to analyse the protein sizes. First the protein samples were mixed with 5x SDS loading dye and denatured for 10 min at 95°C. The SDS gels consist of a stacking gel with 5% polyacrylamide and a running gel with 12% polyacrylamide underneath. The samples and a protein size marker PageRuler™ Plus prestained were loaded onto the gel and the electrophoresis was performed at 100-160 (Blötz *et al*., 2017).

5x SDS loading dye	1.4 ml	Tris-HCl (1 M, pH 7.0)
	3 ml	Glycerol (100%)
	2 ml	SDS (20%)
	1.6 ml	β-Mercaptoethanol (100%)
	0.01 g	Bromphenol blue
	2 ml	dH_2O

10x Page buffer (PLP)	144 g	L-glycine
	30 g	Tris-base
	10 g	SDS
12% running gel	4.8 ml	dH_2O
	3.9 ml	Tris-HCl (1.5 M, pH 8.8)
	6 ml	Acryl-bisacrylamide (30%)
	150 µl	SDS (10%)
	150 µl	Ammonium persulfate (10%)
	15 µl	TEMED
5% stacking gel	10.25 ml	dH_2O
	1.305 ml	Tris-HCl (1.5 M, pH 6.8)
	1.95 ml	Acryl-bisacrylamide (30%)
	150 µl	SDS (10%)
	150 µl	Ammonium persulfate (10%)
	30 µl	TEMED

Coomassie staining

After the SDS-Page, the protein gels were stained with Coomassie Brilliant Blue. Therefore, the gels were first treated with a fixation solution for 30 min at RT. A staining solution was used to stain the proteins. Afterwards, the gels were destained with water (Blötz *et al*., 2017).

Fixation solution	10%	Acetic acid
	50%	Methanol
	Ad to the final volume with dH_2O	
Staining solution	0.5%	Coomassie brilliant blue
	10%	Acetic acid
	45%	Methanol

Enzyme activity assays

β-galactosidase activity assay

For the measurement of the β-galactosidase activity, 100 µl of the crude extract was mixed with 700 µl Z-Buffer with β-mercaptoethanol and incubated for 5 min at 28°C. The reaction was initiated with the addition of 200 µl o-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction is stopped with 500 ml 1M Na_2CO_3 when the solution turns yellow. To detect the amount of produced o-nitrophenyl, the OD_{420} of the samples was measured. 10 µl of the crude extract was used for the Bradford assay to determine the protein concentration. The β-galactosidase activity was calculated with the following formula:

$$\frac{OD\ 420nm}{\Delta t\ \times OD\ 595nm} \times 2005.3475$$

ONPG	4 mg	*o*-Nitrophenyl-β-D-Galactopyranoside
	1 ml	Z-Buffer without β-mercaptoethanol
1 M Na_2CO_3	26.5 g	Na_2CO_3
	Ad to 250 ml with dH_2O	

Citrate synthase activity assay

For the determination of citrate synthase activity, a colorimetric assay was performed. The citrate synthase converts acetyl-CoA and oxaloacetate to citrate and coenzyme A. This coenzyme A reacts in the assay with the Ellman's reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and the resulting compound TNB can be measured spectrophotometrically at 412 nm. The reaction mixture contains 100 mM Tris-HCl (pH 8.0), 1 mM DTNB and 50 nM of the purified citrate synthases CitZ or CitA. The reaction was initiated by the addition of 0.3 mM oxaloacetate and 0.3 mM acetyl-CoA and the absorbance at 412 nm was measured at 25°C. For the determination of Km and Vmax values, one substrate was added in a constant concentration and the other substrate was added in varying concentrations (0.03-0.45 mM) (Ellman, 1959; Johansson and Pettersson, 1974).

From the resulting data, the initial reaction rate v_o was determined as the change of absorption (ΔA) per minute. The next step was to plot the values $1/v_o$ against 1/substrate concentration [S] in a Linewaever-Burk diagram (Lineweaver and Burk, 1934). From this plot, the Km and Vmax values can be determined by the following equation:

$$\frac{1}{v_0} = \frac{K_M}{V_{max}\ [S]} + \frac{1}{V_{max}}$$

Hom assay

For the measurement of Hom enzyme activity, the reverse reaction from L-homoserine to L-aspartate 4-semialdehyde was used. The simultaneous conversion of $NADP^+$ to NADPH and the accompanying change in absorption at 340 nm was measured with a photometer. The reaction mixture contains 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM $NADP^+$ and 0.02 mM DTT. To initiate the reaction, 10 mM homoserine was added to the reaction. The change in absorption at 340 nm was measured at 25 or 37°C (Hama *et al.*, 1990; Hama *et al.*, 1991).

Preparation of samples for proteome analysis

A 4 ml overnight culture was used to inoculate a 50 ml LB preculture to an OD_{600} of 0.05 and incubated at 37°C and 200 rpm until an OD_{600} of 0.5 is reached. From this preculture, a main culture of 150 ml in a 1 l flask was inoculated to an OD_{600} of 0.05. After incubation to an OD_{600} of 0.5, 30 ml of the cells were harvested by centrifugation at 4°C for 15 min and 8500 rpm. The supernatant was removed and the cells were washed in 10 ml TE Buffer. The solution was again centrifuged as described and the supernatant was removed. The samples were frozen in liquid nitrogen and analysed by the Department of Functional Genomics in Greifswald. The analysis was performed as described in Reuß *et al*. (2017).

2.2.8. Drop dilution assay

Overnight cultures of the strains are used to inoculate a 4 ml cultures in either LB or C-glc medium, that were inoculated at 37°C and 200 pm until an OD_{600} of around 1 is reached. The cells were washed and resuspended in C-glc medium to a final OD_{600} of 1. These samples were used to prepare serial dilutions of 10^{-1} to 10^{-6} and 5µl of each dilution was dropped on the respective plates. The plates were incubated at 37°C for two days in case of the C-glc minimal plates with serine.

2.2.9. Sporulation assay

From a 4 ml LB overnight culture of the *B. subtilis* strain, a 4 ml SP culture was inoculated 1:100 and incubated at 37°C and 200 rpm until it reached an OD_{600} of 0.2-0.4. This culture was further used to inoculate a new 4 ml SP culture 1:100, which was inoculated for at least 20 h. The OD_{600} of the culture was measured and a cell solution of OD 1 was prepared in 0.9% NaCl solution. Two samples of each 1 ml were prepared. To remove all living cells from the culture, a heating step at 85°C for 30 min was applied to one sample. The heated and the not heated samples were diluted step-by-step 10^{-1} in a drop dilution assay in 0.9% NaCl and plated on LB plates. The plates were incubated at 37°C over night.

3. Results

3.1. The *MiniBacillus* project

The aim of the *MiniBacillus* project is to understand what is needed for life, by reducing the well-known genome of *B. subtilis.* A minimal set of genes should remain in the genome, that were defined in a blueprint (Reuß *et al.*, 2016). For the genome reduction, chromosomal regions with unnecessary genes are deleted step by step with a marker-free deletion system (Wenzel and Altenbuchner, 2015). The *MiniBacillu*s strain PG10, with a genome reduction of 34.54% was analysed with a multi-omics approach (Reuß *et al.,* 2017). Therefore, this strain is very well characterized. This work is based on an offspring of this strain, the strain PG18. PG18 has a genome reduction of 36.61% (Reuß, 2017).

3.1.1. The deletion progress

Previous transcriptome data showed, that some genes are highly expressed in the *MiniBacillus* strain (Table 2). Part of these is the *mhqNOP* operon, which was over 4700-fold upregulated. This was caused by the deletion of the repressor MhqR, which normally regulates the resistance to quinones and diamide (Töwe *et al.*, 2007; Reuß *et al.*, 2017). However, the upregulated expression wastes a lot of energy and leads to an imbalance in the cell. Furthermore, the function of the *mhqNOP* genes is the protection against methyl-hydroquinone, which is unnecessary for the final *MiniBacillus* strain. Therefore, the plasmid pGP2093 was used to delete the *mhqNOP* operon in the strain PG18 with the marker-free deletion system as described in chapter 2.2.6., resulting in the strain PG29.

Table 2: Operons that are higher expressed in PG10, compared to Δ6

Operon	Function	Regulators	Factor
mhqNOP	Protection against hydroquinone	MhqR (deleted)	4781
paiAB	Control of intracellular polyamine concentrations		420

The next step was to delete the *paiAB* operon with the plasmid pGP2094. This operon encodes for a spermine/ spermidine-N-acetyltransferase, which is also upregulated 420-fold. The resulting strain PG30 was further used to restore a point mutation in the *pit* gene, a low-affinity phosphate transporter. This point mutation was noticed in PG10 and leads probably to a reduction of phosphate uptake. However, this could be detrimental for the strain, since the reduced level of phosphate in the cell might activate a regulator system for phosphate metabolism, the PhoPR

system. This leads to the activation of genes for the acquisition of phosphate and similar to the repression of the tagAB and tagDEFGH operons for the biosynthesis of teichoic acids (Prágai *et al.*, 2004). Therefore, the *pit* point mutation was restored in strain PG31.

Since the essential or for the *MiniBacillus* necessary genes are scattered around the genome and the deletion regions become smaller, an approach to accelerate the deletion process might be the defragmentation. Functionally related genes, which should remain in the *MiniBacillus*, are clustered together in one locus. The native locus is then dispensable and can be deleted in one bigger deletion, instead of two smaller ones. This clustering can be done by introducing a second copy of the gene. In this case a glycolytic cassette should be introduced, containing the genes *pgi*, *fbaA*, *ptsGHI*. Pgi is the glucose-6-phosphate isomerase and FbaA the fructose-1,6-bisphophate aldolase and both enzymes are part of glycolysis, which plays a central role in the *MiniBacillus* blueprint. The operon *ptsGHI* encodes for the glucose phosphotransferase system which is responsible for the uptake of glucose in the cell. The introduction of this glycolytic cassette was shown to be functional in Zschiedrich (2014), however, the transfer of this construct would lead to the deletion of several genes including the gene *nrnA*. The deletion of *nrn*A, encoding for a nanoRNase, leads to the reduction of the competence in the *MiniBacillus* strain (Reuß, 2017). Therefore, the glycolytic cassette was newly assembled and introduced next to *nrnA*, to sustain competence. This was done in two steps as shown in Figure 10. First the genes *pgi* and *fbaA,* together with a chloramphenicol resistance were introduced with a PCR product next to *dnaE*, leading to the deletion of the unknown gene *ytrH* (PG32). In the second step, the operon *ptsGHI* with a kanamycin resistance cassette was exchanged with the chloramphenicol resistance and the genes *ytrI* and *ytzJ*. The resulting strain PG33 was selected on plates with kanamycin and also tested for the loss of the chloramphenicol resistance. The genes *pgi*, *fbaA* and *ptsGHI* can now be deleted at their native locus to fasten the deletion process.

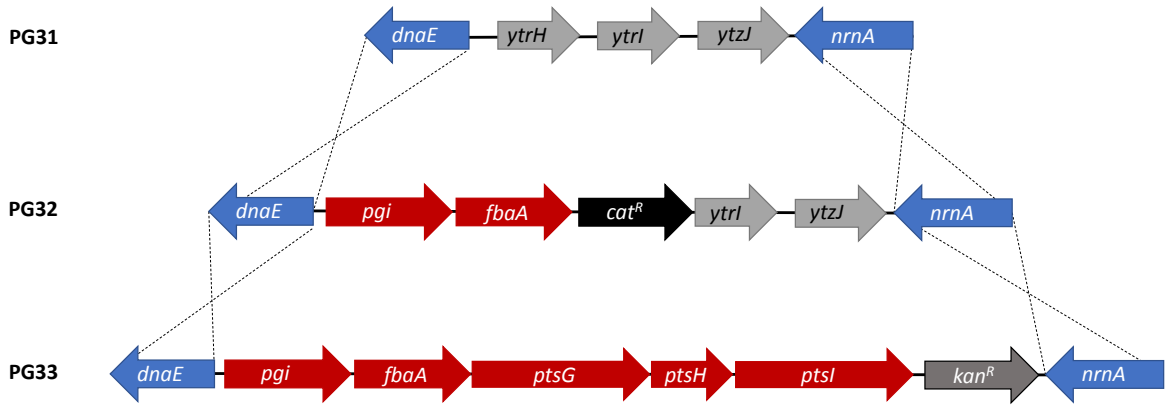


Figure 10: The two steps of the glycolytic cassette introduction into the *MiniBacillus* strain. First, the genes *pgi* and *fbaA* were integrated with a chloramphenicol cassette and in second step the genes *ptsGHI* were introduced with a kanamycin resistance.

The following deletions from PG34 to 39 are deletions of genomic regions that are not necessary for the final *MiniBacillus* strain. The deletions are listed in Table 3, together with the plasmids that were used and the resulting genome size. The consequences of these deletions and the deletions that were already done before are discussed in chapter 3.1.3. The strains PG34, PG37 and PG39 were additionally analysed by WGS.

Table 3: The *MiniBacillus* deletion strains constructed in this work

strain	Genome size	Deletion plasmid	Deletion	% reduction in comparison to wild type	WGS analysis
PG10	2759359		Reuß *et al*. (2017)	34.54	yes
PG18	2672270		Reuß (2017)	36.61	yes
PG29	2670199	pGP2093	Δ*mhqNOP*	36.66	no
PG30	2666896	pGP2094	*yuzG-sufA*	36.74	no
PG31	2666876	pJOE3256	*pit* point mutation	36.74	yes
PG32	2670874	PCR product	insertion *pgi, fbaA, cat*; Deletion *ytrH*	36.64	no
PG33	2674507	PCR product	insertion *ptsGHI, kan;* Deletion *ytrI, ytzJ, cat*	36.56	no
PG34	2652827	pGP2098	*ycgQ-yckE*	37.07	yes
PG35	2635284	pGP2088	*yvaM-yvbK*	37.49	no
PG36	2622356	pGP2073	*nhaX-yhaX*	37.79	no
PG37	2587747	pGP2270	*glpQ-ycbK*	38.62	yes
PG38	2554562	pGP2282	*yqjF-yqjG*	39.40	no
PG39	2507732	pGP2283	*yddN-ydfM*	40.51	yes

The final *MiniBacillu*s strain of this work PG39 has a genome size of about 2.5 Mbp, which corresponds to a genome reduction of 40.51% in comparison to the wild type strain 168. This is the greatest reduction of the *B. subtilis* genome published so far.

3.1.2. Phenotypical analysis

The final *MiniBacillus* strain should sustain a robust growth at 37°C in LB-glc medium. To verify that the constructed strains are still able to grow under these conditions, the growth was monitored in 15 ml LB-glc medium at 37°C (Figure 11 A).

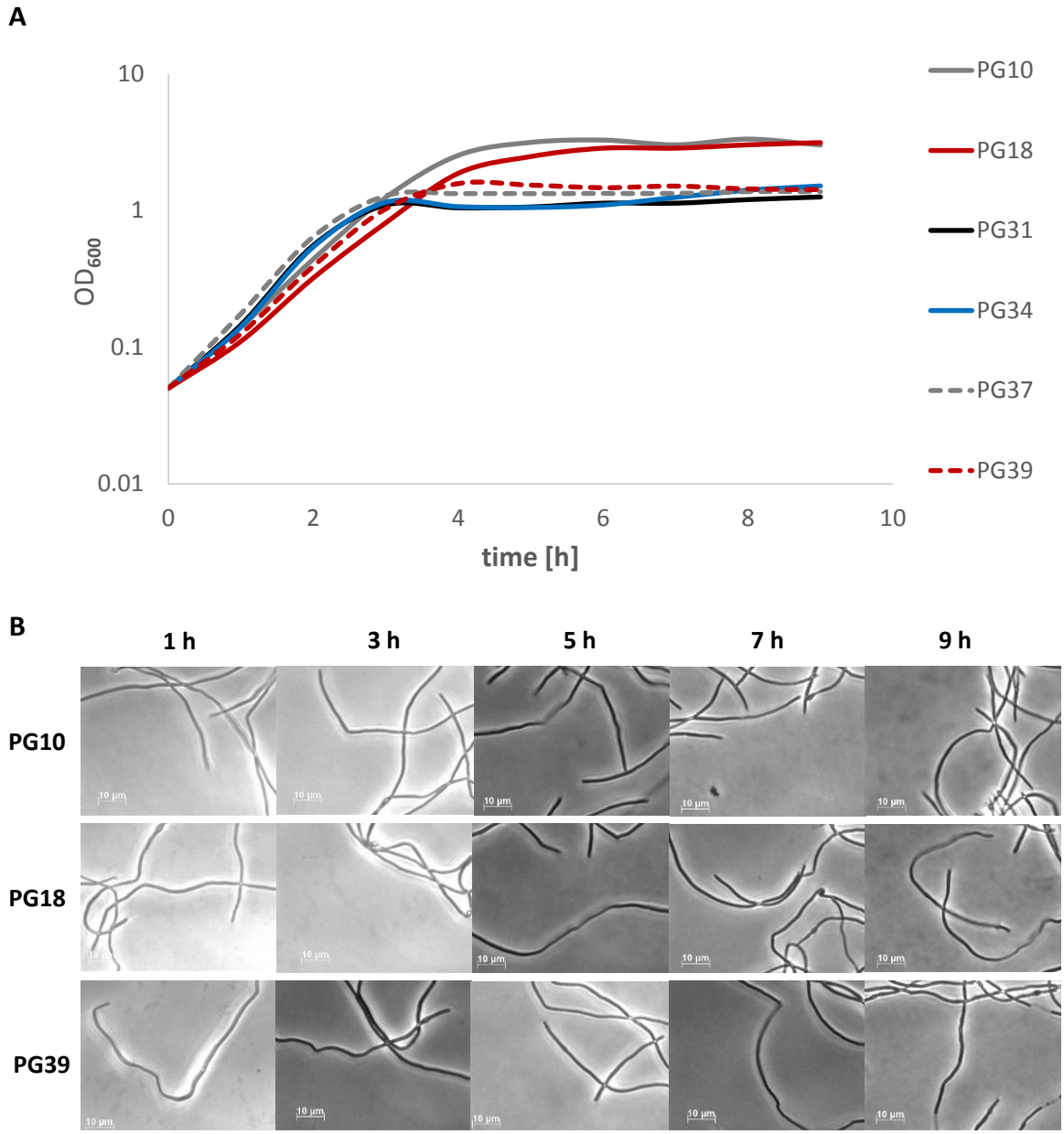


Figure 11: The growth of the *MiniBacillus* deletion strains in LB-glc. A The strains were grown in 15 ml LB-glc medium in baffled flask at 37°C. **B** Samples from the LB-glc cultures were analysed under the microscope every two hours.

The tested strains PG10, PG18, PG31, PG34, PG37 and PG39 show the same growth rate in LB-glc medium. However, the ancestor strains PG10 and PG18 are able to reach a higher OD in comparison to the other strains. These strains seem to produce a higher biomass than the others. Since the growth rate is more important for the project and the difference in the final OD is not severe.

Furthermore, the cell morphology was also observed at several time points from the growth curve (Figure 11 B). The microscopy pictures show the same cell morphology for all strains. Normally, cells of the *B. subtilis* wild type strain 168 are rod-shaped single cells. Additionally, they are able to form spores. For the *MiniBacillus* strains, long cell chains without spores can be observed. If the cells are treated with a membrane dye, it can be observed that these are indeed long chains of single cells and not several elongated cells (Figure 12). However, this formation of long cell chains is a phenotype that was already observed very early in the deletion process and the reason for this is not known (Reuß, 2017). But since the cell morphology of PG39 shows no differences to that of PG18 or PG10, the additional deletions seem to have no influence on it.

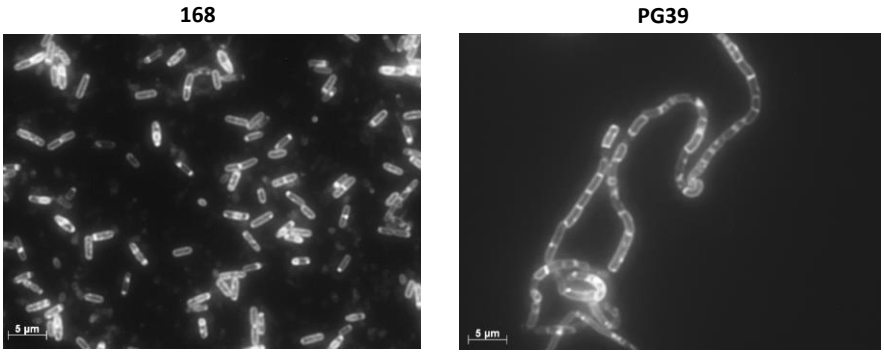


Figure 12: The comparison of the cell morphology of the wild type strain and the strain PG39. The cells were treated with the membrane dye FM 4-64 to visualize the cell membrane.

In a previously constructed *MiniBacillus* strain, the problem of genomic instability occurred and the reason for that was unknown (Reuß, 2017). However, to sustain a stable DNA replication process, the basic structure of the chromosome should be maintained. The origin and the termination of replication are located on opposite sides of the genome. In Figure 13 it can be seen, that the deletion regions are scattered around the genome and that the balance between the origin and the termination of replication is sustained.

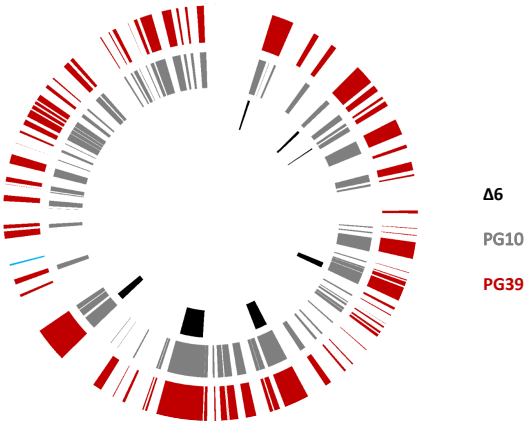


Figure 13: The chromosomes of the strains Δ6, PG10 and PG39. The deleted regions are shown, which are scattered around the genome.

Previously, multi-omics experiments were performed for the strain PG10 in comparison to the ancestor strain Δ6. A lot of information can be obtained from these experiments. Therefore, proteome analysis was performed for the strains Δ6, PG10 and PG38. Furthermore, the genes that remained in the genome were divided into the five categories: cellular processes, metabolism, information processing, lifestyles and others. The results of the gene categorization and the proteome analyse are shown in Figure 14.

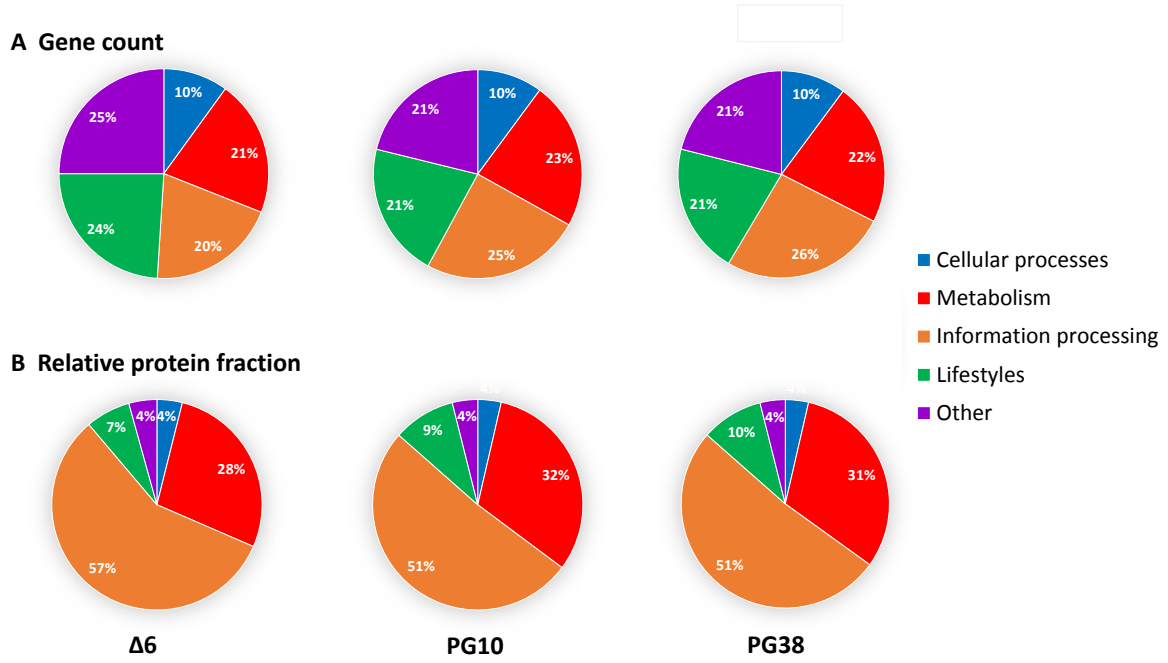


Figure 14: The relative results of the proteome analysis and the categorization of the genes. A The remaining genes in the genome are divided into the five different categories. **B** The relative protein fractions that were determined are shown for the five functional categories. (Data processing: Bingyao Zhu)

Around 2% of the genes in the chromosome of strain Δ6 are encoding for lifestyle genes. However, this number is reduced in PG10 and PG38 to 21%, since most of the genes responsible for the sporulation and other not necessary processes are deleted. The final *MiniBacillus* strain will not need several pathways for different lifestyles. In contrast, many of the genes from the information process are needed to sustain life. This is reason why the percentage of these genes even increase in the relative gene count. The relative protein fractions for each category was determined from the proteome data. Interestingly, the amount of proteins in the cell involved in the information processing is the biggest fraction with 57% in the strain Δ6. Although the genes in this fraction make up only 20%. This was already observed by Reuß *et al.*, (2017). Therefore, the relative protein fractions for cellular processes, lifestyle and other are in comparison smaller. However, the percentages for each fraction are not changing significantly in PG10 and PG39. This means that there are no major changes in the proteome balance. However, some proteins show a different

protein count in PG39 in comparison to PG10, there seems to be no significant up- or downregulation of a specific pathway (data not shown).

3.1.3. Deletion impact

Although the PG39 *MiniBacillus* strain shows almost no phenotypic difference in comparison to the PG18 ancestor strain in LB-glc medium, the deletions could have an impact under other conditions. First of all, the complex metabolism of *B. subtilis* was reduced. Since the known amino acid importers should remain in the cell, the biosynthesis pathways become dispensable. Therefore, some biosynthesis genes are already deleted, which means that the strain is already auxotrophic for the amino acids tryptophan, valine, isoleucine, leucine, proline and arginine. Furthermore, in PG38, the biosynthesis genes for biotin (*bioWAFDBI*) were deleted, which means the biotin importer YhfU is now essential for the cell. The strain is biotin auxotroph. *B. subtilis* wild type cells are able to use different carbon sources. In contrast, the *MiniBacillus* strain has some deletions in the uptake systems for several carbon sources (Table 4).

Table 4: The deletion impact on strain PG39

Auxotrophic for	**No longer utilizable carbon sources**	**Deleted genes coding for sigma factors**
Tryptophan	Maltose	*sigE* - sporulation
Valine	N-Acetyl-glucosamine	*sigG* - sporulation
Isoleucine	Oligo-ß-glucoside	*sigF* - sporulation
Leucine	Oligo-ß-mannoside	*sigZ* - ECF-sigma factor
Proline	Fructose	*sigY*- prophage
Arginine	Inositol	
	Arabinose	
Biotin	Ribose	
	Xylose	

For the final *MiniBacillus* strain, the alternative sigma factors of no interest. Therefore, PG39 has already deleted some of the responsible genes that are listed in Table 4. However, this has also an effect on the lifestyle category, since less stress responses are available. Furthermore, a lot of genes related to sporulation were deleted so that PG39 forms no spores anymore. However, the main part of the deleted genes are of unknown function.

The WGS data of the strains PG10, PG18, PG31, PG34, PG37 and PG39, were analysed for potential mutations that occured during the deletion process. All single nucleotide polymorphisms

(SNPs) and variations were compared to the strain PG10, which was already analysed (Reuß *et al.,* 2017). Potential mutations that are often found during WGS of *B. subtilis* strains are rejected. Some mutations were found for the strains PG18, PG31 and PG39 which are listed in Table 5.

Table 5: The mutations found in the *MiniBacillus* WGS data in comparison to PG10

Strain	Mutation	function
PG18	Promoter *tagAB*	Biosynthesis of teichoic acid
	Promoter *ywaG*	Putative transcriptional regulator
	YqgS L220LTEM (Insertion)	Minor lipoteichoic acid synthase
PG31	MtlA M11R	Mannitol-specific permease
	YgaE F31S	Unknown
	YhbD G152S	Unknown
PG39	AmtB A76V	Ammonium transporter
	mfd (bp 475 deleted → frameshift)	Transcription-repair coupling factor
	YqgN T9A	Unknown

Some mutations were found in genes with unknown function. In the strain PG18, the promoter of the *tagAB* operon is mutated. These genes are essential and encode for proteins involved in the biosynthesis of teichoic acid, which is an important component of the cell wall. The mutation is a substitution of G to A 69 bp in front of the *tagA* gene, but it is not located in the -35 or -10 region of the promoter. It was previously described, that the *tagAB* operon is repressed by PhoPR system (Liu *et al.*, 1998). This system is activated by phosphate starvation (Prágai *et al.*, 2004). It was already described that the *MiniBacillus* strains from PG10 to PG31 harbour a point mutation in the low-affinity transporter Pit. This mutation might have reduced the phosphate level in the cell, by which the PhoPR system was activated. Therefore, the *tagAB* operon expression was repressed. But the cell needs to synthesize the essential cell wall component lipoteichoic acid. Although, the mutation in the promoter region of *tagAB* is not in the known PhoP binding sides, it could have led to a de-repression of the operon. Interestingly, the mutated gene *yqgS* is also involved in the synthesis of lipoteichoic acid. But the resulting enzyme is only a minor lipoteichoic acid synthase and it is only active during sporulation (Wörmann *et al.*, 2011).

The mutation in *mtlA,* encoding for the mannitol-specific permease, is interesting for the *MiniBacillus* project, since the *comKS* system is induced by mannitol. Although, the effect of several mutations on the mannitol-specific phosphotransferase system (PTS) was already studied, it is not known which effect this mutation might cause (Bouraoui *et al.*, 2013). Another transporter was also

found to be mutated in PG39, the ammonium transporter AmtB. But the gene *amtB* is not part of the final *MiniBacillus* blueprint.

To conclude, the current *MiniBacillus* strain PG39 has a genome reduction of 40.51% and shows almost no differences in growth and cell morphology. However, the strain is auxotrophic for several amino acids and the import systems for several carbon sources are deleted. The deletions also led to the accumulation of some mutations.

3.2. The role of the TCA cycle in the *MiniBacillus* project

The TCA cycle is a central point in the metabolism of *B. subtilis* and several other organisms. However, some organisms, like *M. pneumoniae*, live without a TCA cycle (Manolukas *et al.*, 1988; Halbedel *et al.*, 2007). Furthermore, the final *MiniBacillus* strain will only use glycolysis and the pentose-phosphate pathway to produce energy. Therefore, the main carbon source will be glucose (Reuß *et al.*, 2016). To estimate the impact of the TCA cycle deletion and its possible effect on the *MiniBacillus* strain, a TCA cycle mutant in the wild type background should be created.

3.2.1. The two citrate synthases of *Bacillus subtilis*

In glycolysis, glucose is converted to pyruvate, which is used by the pyruvate dehydrogenase to produce acetyl-CoA. The initial step of acetyl-CoA into the TCA cycle is catalysed by the citrate synthase. Two citrate synthase genes are annotated in the genome of *B. subtilis, citZ* and *citA*. The genes and the transcription levels of two enzymes were already studied and compared (Jin and Sonenshein, 1994a). The enzyme CitZ was characterized as the major citrate synthase, since the deletion of *citZ* has a greater impact on growth than the deletion of *citA* (Zschiedrich, 2014). CitA seems to be a minor citrate synthase, but it is poorly characterized and has an unknown function in the cell. The co-transcribed gene *citR* seems to encode for a transcriptional repressor of *citA.* The effect of the deletion of the genes and the expression of *citA* under different conditions was already analysed (Zschiedrich, 2014). However, the role of CitA and also CitZ in the TCA cycle needs to be clarified to estimate which of the genes has to be deleted to construct a complete TCA cycle deletion strain.

The growth of the single and double deletion mutants of *citZ*, *citA* and *citR* in LB and C-malate medium was analysed (Figure 15). In C-malate medium, the strains need a functional citrate synthase to produce citrate and glutamate, which is essential for the cell.All the single and double mutants grow similarly in the complex medium LB, which provides a lot of nutrients and amino acids. A deletion of these genes in the *MiniBacillus* strain would lead to no growth defect in the selected medium LB-glc. However, the strains with a deletion in *citZ* show a growth disadvantage in C-malate medium.

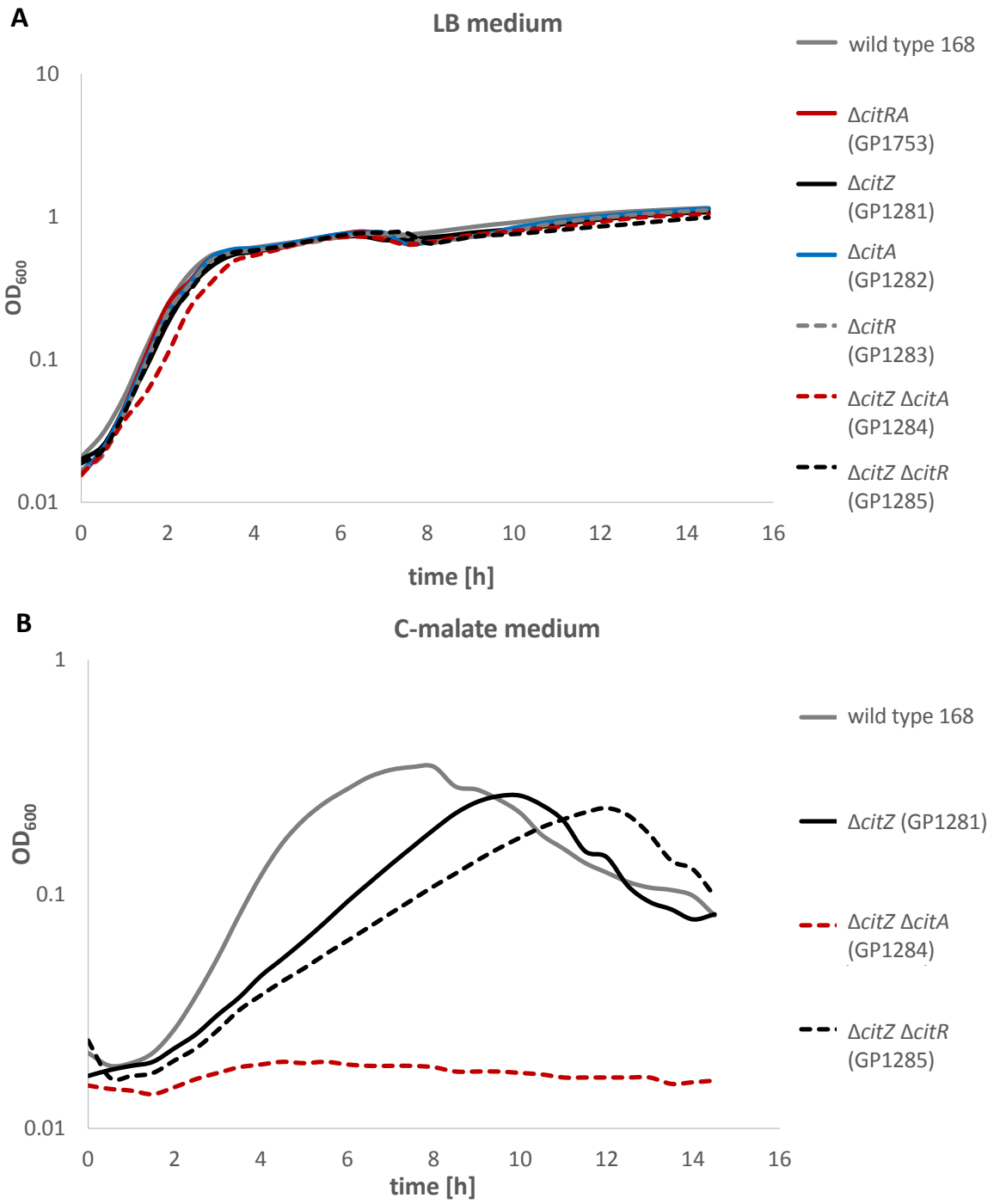


Figure 15: The growth of the *citZ, citA* and *citR* deletion mutants in LB medium (A) and C-malate medium (B). The precultures were prepared in LB medium and the growth was monitored at 37°C.

The Δ*citZ* mutant grows slower than the wild type or the Δ*citA* mutant, which supports the theory, that CitZ is the major citrate synthase. The strains Δ*citA*, Δ*citR* and Δ*citRA* grow like the wild type (data not shown). The double mutant Δ*citZ* Δ*citA* cannot grow at all in this medium. This indicates that CitA also functions as a citrate synthase and that CitZ and CitA are the only enzymes that can catalyse the reaction to citrate under these conditions. The growth defect is probably caused by the reduced glutamate production, which was already described before (Jin and Sonenshein, 1996).

Interestingly, if *citR* is deleted in addition to *citZ*, the growth is even worse than the growth of the Δ*citZ* mutant. In theory, in this strain the transcription of *citA* should be no longer repressed by CitR and so this strain should be able to produce more citrate. However, the mechanism of repression is poorly understood and it could be more complex which might lead to this observed growth disadvantage.

The activity of the major citrate synthase CitZ was already described before (Jin and Sonenshein, 1996) and a first attempt to compare the activities of the two citrate synthases *in vitro* was performed by Zschiedrich (2014). However, the verification of these results and the determination of the Km and Vmax values will further characterize the activities of both enzymes. Therefore, the N-terminal Strep-tagged proteins were each overexpressed in *E. coli* from the plasmids pGP2515 (Strep-CitZ) and pGP2516 (Strep-CitA). The cells were disrupted with the French press and the proteins were purified via Strep-Tactin as described (chapter 2.2.7). The SDS page shows the expected sizes for the purified CitZ and CitA proteins (Figure 16).

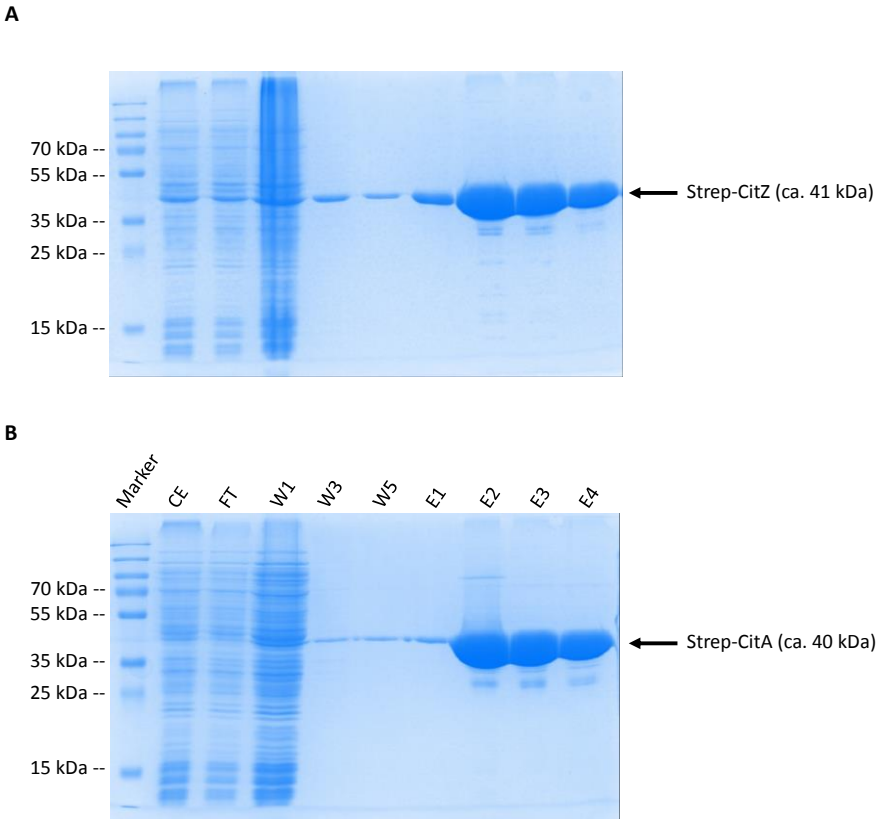


Figure 16: The SDS gel of the citrate synthase protein purifications. A The major citrate synthase CitZ was eluted with the size of about 41 kDa. **B** The strep-tagged CitA protein was purified with a size of about 40 kDa. (CE= crude extract, FT= flow through, W= wash fraction, E= elution fraction)

The amount of purified protein was measured and the enzymes were used in a citrate synthase activity assay. The citrate synthase converts oxaloacetate and acetyl-CoA to citrate and coenzyme

A. In this assay the released coenzyme A can react with the added ellmanns reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman, 1959). The resulting product 2-nitro-5-chlorobenzaldehyde (TNB) can be measured spectrophotometrically at a wavelength of 412 nm. The assay was performed with each enzyme as described in chapter 2.2.7. The change in absorption over time was measured with a plate reader at 25°C and a blank measurement without enzyme was subtracted (Figure 17).

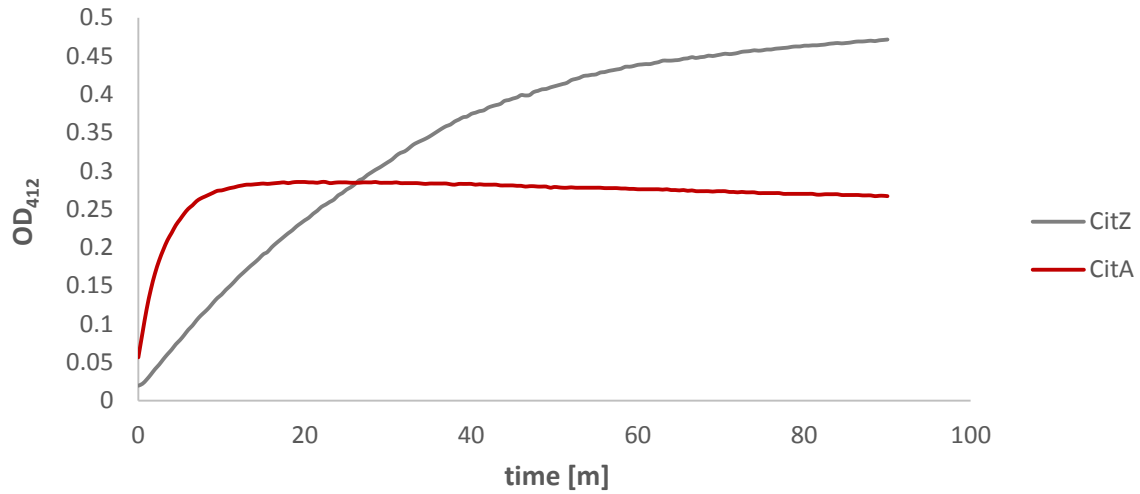


Figure 17: The activity assay of the two citrate synthases. The reaction was measured spectophotometrically at 412 nm and at 25°C.

The activity assay of the two enzymes shows that both harbour citrate synthase activity, since the produced coenzyme A could react with the DTNB. Interestingly, the previously described minor citrate synthase CitA seems to catalyse the reaction faster. However, the major citrate synthase CitZ seems to produce almost the double amount of citrate in comparison to CitA.

Table 6: The K_m and V_{max} values for the two citrate synthases CitZ and CitA for the substrates oxaloacetate and acetyl-CoA.

	Substrate	CitZ	CitA
K_m	Acetyl-CoA	164.41 µM	14.24 µM
	Oxaloacetate	64.14 µM	53.95 µM
V_{max}	Acetyl-CoA	26.7 mM/min	3.46 mM/min
	Oxaloacetate	9.71 mM/min	10.28 mM/min

The Km and Vmax values were determined for each of the substrates, oxaloacetate and acetyl-CoA. Several measurements were performed with one substrate in a constant concentration and the

substrate of interest in varying concentrations (0.03 – 0.45 mM). The Km and Vmax values for the two citrate synthases and both substrates were calculated as described in chapter 2.2.7. and they are listed in Table 6.

The higher Km values of CitZ for both substrates indicate a weaker binding of the enzyme to the substrates in comparison to CitA. So CitZ needs a higher concentration of substrate to reach Vmax. These results suggest that both proteins can be active as citrate synthases and should therefore be deleted to obtain a clean TCA cycle deletion mutant. Nevertheless, CitZ seems to be the major citrate synthase. Additionally, the deletion of the repressor CitR will also be included, since its physiological role is still unknown, but it could be linked to the TCA cycle.

3.2.2. The deletion of the TCA cycle

For the deletion of the complete TCA cycle, the single genes for the involved enzymes have to be deleted (Table 7).

Table 7: The genes and operons encoding for enzymes that are involved in the TCA cycle.

Genes	**Protein function**
citZ	Major citrate synthase
icd	Isocitrate dehydrogenase
mdh	Malate dehydrogenase
citA	Minor citrate synthase
citR	Transcriptional repressor of *citA*
citB	Aconitase
odhA	2-oxoglutarate dehydrogenase (E1 subunit)
odhB	2-oxoglutarate dehydrogenase (E2 subunit)
sucC	Succinyl-CoA synthetase (beta subunit)
sucD	Succinyl-CoA synthetase (alpha subunit)
sdhC	Succinate dehydrogenase (cytochrome b558 subunit)
sdhA	Succinate dehydrogenase (flavoprotein subunit)
sdhB	Succinate dehydrogenase
citG	Fumarase

The first deletion attempts were performed in the wild type strain 168 and every single gene or operon should be exchanged step by step with a resistance cassette. However, the strain could not

be finished, since just a few deletions lead to the loss of genetic competence. In the *MiniBacillus* strain, the mannitol inducible *comKS* system is used to increase the transformation efficiency. An additional copy of the competence genes *comK* and *comS* is introduced into the chromosome and the addition of mannitol to the medium induces the *mtlA* promoter in front of the genes and thereby genetic competence. It could be shown, that the competence increases around 7-fold in comparison to the wild type (Rahmer *et al.*, 2015). Therefore, the next deletion attempt was performed in the background of a strain with the inducible *comKS* system (GP2973). This strain also harbours the xylose-inducible Cre recombinase from P1 bacteriophage, integrated into *sacA*. This enzyme is used in the Cre-lox system to remove the resistance cassette from a deletion, by recombining the additionally introduced lox sites. Only a small scar, the lox72 site is left behind. This method is necessary, since the available resistance cassettes are not sufficient to enable the deletion of all genes. The stepwise created strains of the TCA cycle deletion are listed in Table 8.

Table 8: The strains of the stepwise TCA cycle deletion

Strain	Genotype
GP2973	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre)*
GP2974	GP2973 *ΔcitZ-icd-mdh::lox72*
GP2975	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet*
GP3024	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72*
GP3025	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec*
GP3026	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat*
GP3027	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat ΔcitB::lox72*
GP3028	GP2973 *ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat ΔcitB::lox72 ΔcitRA::lox79*

The first step was to delete the genes *citZ*, *icd* and *mdh* that are encoded in one operon. The operon was exchanged with a kanamycin resistance cassette, flanked by the two lox sites lox71 and lox66. This cassette was then removed by the Cre recombinase, which was expressed upon the addition of xylose, leaving the lox72 site behind. The next step was to delete the succinyl-CoA synthetase encoded by *sucCD* operon by the introduction of a tetracycline resistance cassette. For the deletion

of the succinate dehydrogenase encoded by the *sdhCAB* operon, the Cre-lox system was used and the gene *citG* (fumarase) was deleted with a spectinomycin resistance cassette. The 2-oxoglutarate dehydrogenase consists of three subunits OdhAB and PdhD. However, the deletion of *odhAB* is sufficient to delete the pathway. The protein PdhD is additionally involved in the conversion of pyruvate to acetyl-CoA as a part of the pyruvate dehydrogenase complex. Furthermore, a Δ*pdhD* strain is unable to grow on glucose as the single carbon source which makes this gene essential for the *MiniBacillus* strain (Gao *et al.*, 2002). The operon *odhAB* was deleted by the replacement with a chloramphenicol resistance cassette. Afterwards, *citB* and *citRA* were one after another deleted with the Cre-lox system. The final strain (GP3028) has a deletion in all genes encoding for enzymes involved in the TCA cycle.

3.2.3. The phenotype of the TCA cycle deletion strain

Although the competence needed to be improved, a *B. subtilis* strain without TCA cycle could be constructed, which means that *B. subtilis* can live without a TCA cycle. Growth experiments were performed to investigate the impact of the TCA cycle deletion on the growth (Figure 18). The growth of the TCA cycle deletion mutant was compared to the wild type and the parental strain GP2973. All three strains show a good growth in LB-glc medium. The TCA cycle mutant does not seem to have a growth disadvantage, which leads to the assumption that the deletion of the TCA cycle will not lead to a growth disadvantage in the *MiniBacillus* strain. However, the growth test in C minimal medium with only glucose shows that the lack of the TCA cycle leads to a growth defect under these conditions. The reason for this might be that the cells cannot produce glutamate anymore, which is essential for the organism. The addition of glutamate to the medium (CE-glc) leads then again to a better growth of the TCA cycle deletion strain. This supports also the assumption that the strain is auxotrophic for glutamate.

Furthermore, the strain lacking both citrate synthases was also unable to grow on minimal medium without glutamate, as described above. However, all other amino acids need to be synthesized in the CE-glc minimal medium, since they cannot be imported from the medium. Some are derived from glycolysis or the added glutamate, but aspartate is produced from oxaloacetate, an intermediate of the TCA cycle. But since the strain can grow without additional aspartate in the medium, aspartate must be synthesized. The pyruvate carboxylase PycA catalyses the reaction from pyruvate to oxaloacetate, which can then be converted to aspartate by the aspartate transaminase AspB (Figure 19).

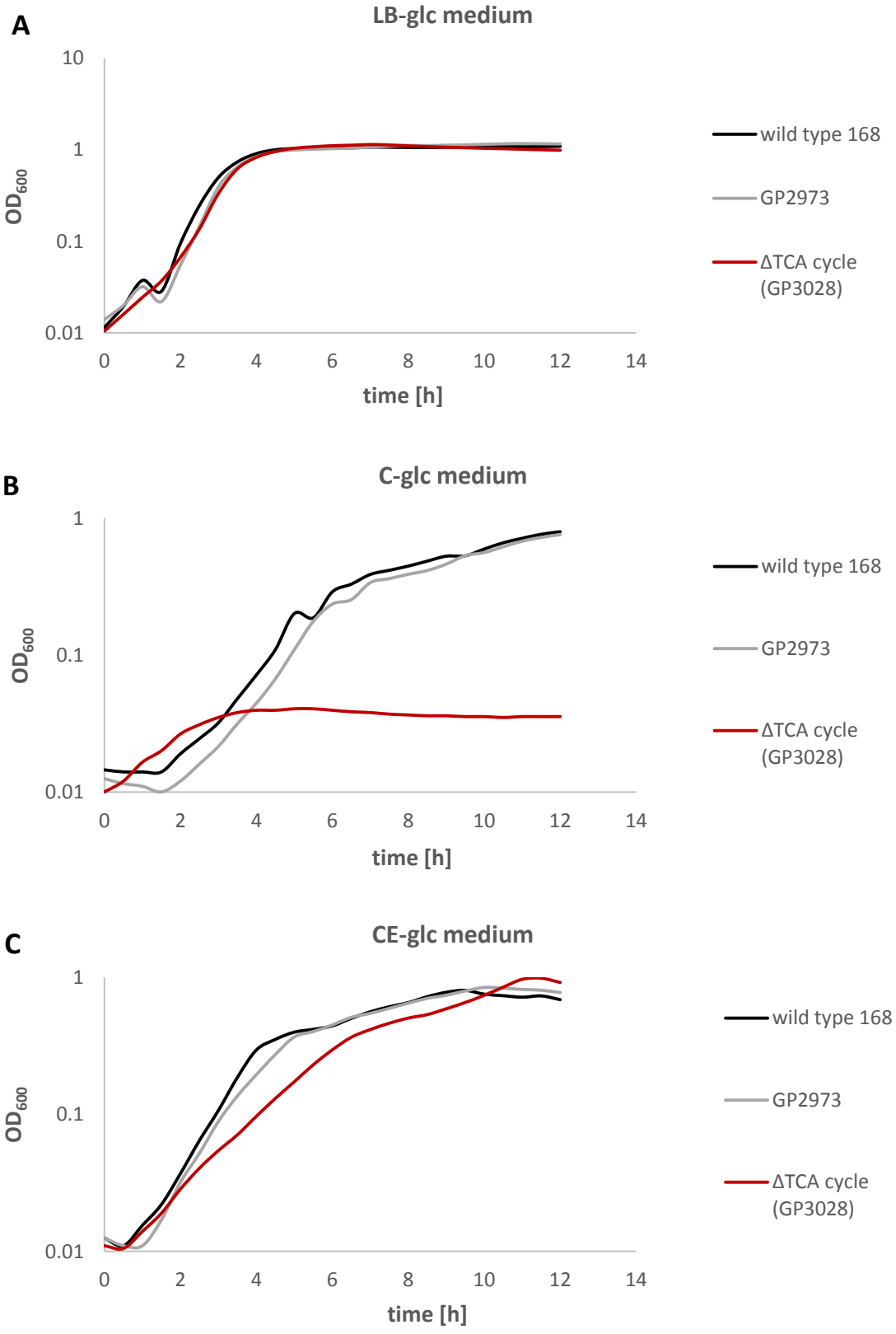


Figure 18: The growth curves for the TCA cycle deletion strain (GP3028) in comparison to the wild type strain 168 and the parental strain GP2973. Precultures were prepared in LB medium**.** The strains were grown at 37 °C in LB-glc medium (**A**), C-glc medium (**B**) and CE-glc medium (**C**).

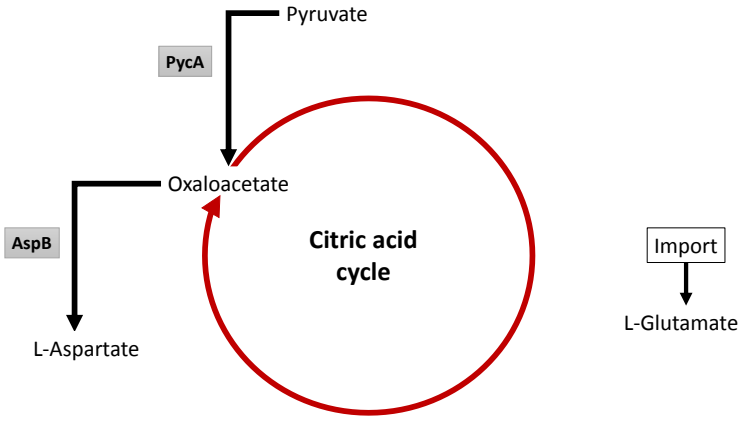


Figure 19: The impact of the TCA cycle deletion on the amino acid metabolism. The deletion of the TCA cycle causes an auxotrophy for glutamate. However, pyruvate can still be converted to oxaloacetate by PycA which is then converted to aspartate by AspB.

Since the competence was reduced in the first attempt to delete the TCA cycle, the competence of the final strain and the intermediate steps were tested. The protocol for the preparation of competente cells with the *comKS* system was used as described (chapter 2.2.5.), but the competent cells were each diluted to an OD_{600} of 0.5 and 1 ml of them were transformed with 50 ng pAC7 plasmid DNA (Figure 20).

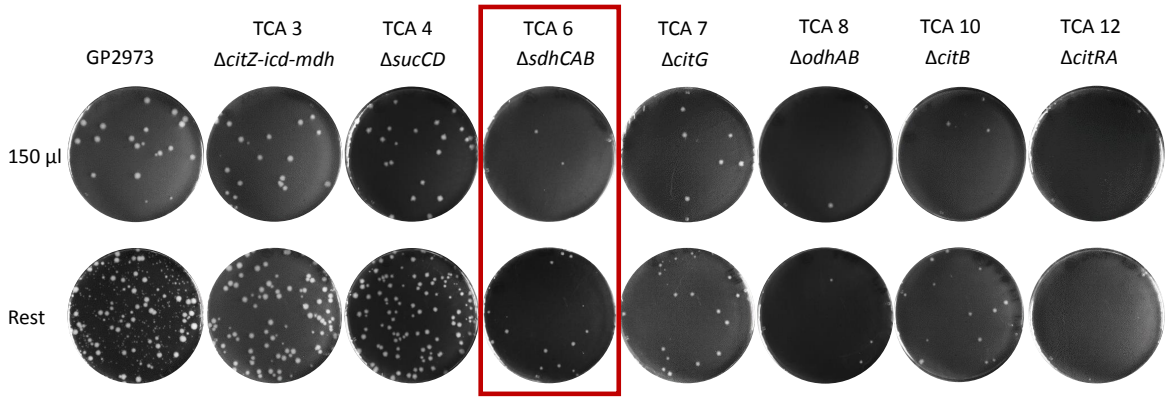


Figure 20: The competence test of the final and the intermediate strains of the TCA cycle deletion mutant. The strains were diluted to the same OD_{600} of 0.5 and transformed with 50 ng pAC7 plasmid. The cells were finally plated on LB-glc plates with kanamycin.

The competence test shows that the TCA cycle deletion strain has a highly reduced competence in comparison to the parental strain GP2973. However, some transformation colonies are still visible. Interestingly, the deletion of *sdhCAB* seems to have the greatest impact on the competence. To investigate, if the deletion of *sdhCAB* or the combination of the different deletions is causing this phenotype, a strain was created in the GP2973 background with a deletion of only *sdhCAB* and tested for the competence (Figure 21).

The competence of the strain with the single *sdhCAB* deletion is highly reduced in comparison to the parental strain GP2973. 50 ng of the plasmid pAC7 was added to 1 ml of cells

(OD_{600} of 0.3). This leads to a calculated transformation efficienty of 1,720 transformants per µg DNA of GP2973 and 280 transformants per µg DNA of GP2973 with the deletion of *sdhCAB*. This indicates that this operon is necessary to maintain the competence.

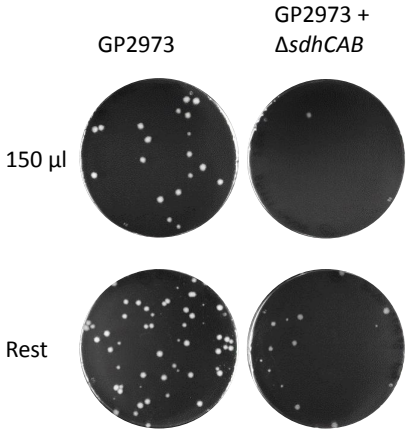


Figure 21: The competence test of the *sdhCAB* single deletion strain. The strains were diluted to the same OD_{600} of 0.3 and mixed with 50 ng of the plasmid pAC7. After another inoculation step for 1 hour, the cells were plated onto LB-glc plates with kanamycin.

The protein complex SdhCAB is not only involved in the TCA cycle, but it is also involved in the respiratory chain. SdhC is the membrane anchor of the complex and is involved in the electron transfer to menaquinone, since it is a part of cytochrome b558 (Hederstedt and Rutberg, 1983; Baureder and Hederstedt, 2011). The deletion of genes involved in the respiratory chain was already observed to lead to a reduction or loss of competence, *e.g* for the NADH dehydrogenase Ndh (Koo *et al.,* 2017). However, the loss of competence in the final TCA cycle deletion strain could also result from the combination of all mutations, since the *sdhCAB* single deletion strain seems to be still more competent than the final TCA cycle deletion strain. Since the *comKS* system is also used in the *MiniBacillus* strain, the deletion of the TCA cycle would also lead to a reduced competence. This would be detrimental for the project, since the cells need to be transformed with a plasmid in the deletion system. The reduction of genetic competence would complicate the deletion process.

Some deletion mutants of TCA cycle genes are described to have a defect in sporulation. The i*cd* deletion mutant *e.g.* exhibit a reduced ability to sporulate. The reason fo this was the accumulation of citrate and isocitrate. However, the additional deletion of the citrate synthase *citZ* seems to be able to counteract this effect (Matsuno *et al.*, 1999). In a deletion of the complete TCA cycle, no intermediates can accumulate. Furthermore, the effect of the *comKS* system on the sporulation was never investigated before. This might be interesting since the amount of the phosphorylated sporulation master regulator Spo0A influences the amount of ComK in the cell and thereby also competence (Fujita *et al.*, 2005). The sporulation of the TCA cycle deletion strain was tested as described in chapter 2.2.9. (Figure 22).

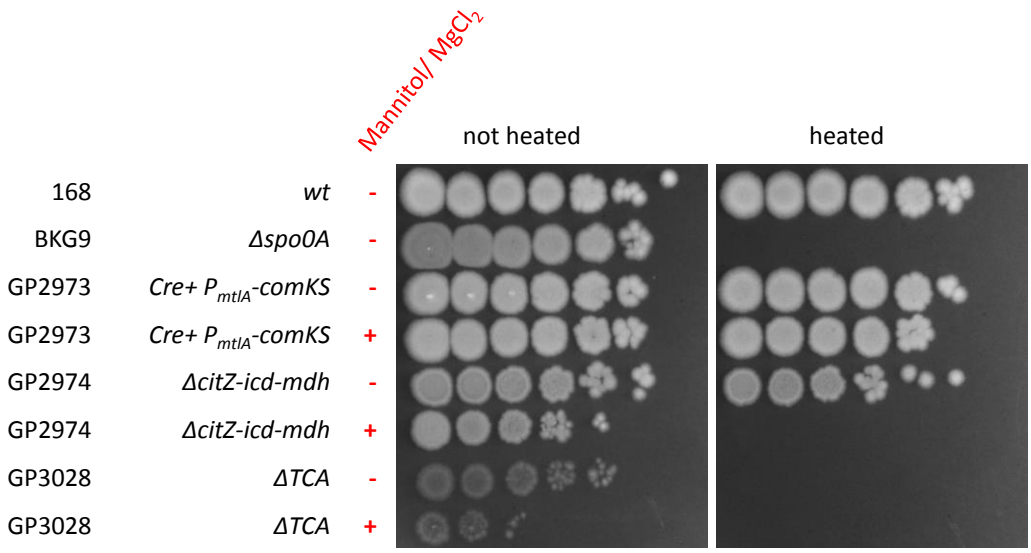


Figure 22: The sporulation test of the TCA cycle mutant in SP medium. The wild type strain 168 serves as a positive control and the BKG9 (Δ*spo0A*) as a negative control for sporulation. The strains with the inducible *comKS* system were tested with and without the inducer mannitol. The cells were plated on LB plates.

In the sporulation assay, the number of spores formed in a SP culture from different strains are compared. One sample of each strain was heated at to remove all living cells, so that only the spores survive. The wild type strain 168 formed many spores which were able to grow again on the LB plates after the heating step. In contrast, the Δ*spo0A* mutant is not able to form spores at all, since *spo0A* is the regulator of a phosphorelay to initiate sporulation (Burbulys *et al.*, 1991). The strain GP2973, with the *comKS* system and the integrated cre recombinase, forms the same number of spores as the wild type. In this case, the addition of mannitol and $MgCl_2$ for the induction of the *comKS* system, has almost no effect on the spore formation. However, the Δ*citZ-icd-mdh* operon mutant has a reduced number of spores in SP medium without mannitol and $MgCl_2$. This was already reported previously for the single deletion mutants Δ*citZ* and Δ*icd* (Jin and Sonenshein, 1994b; Jin *et al.*, 1997). If the *comKS* system is induced by the two components, there is no sporulation detectable anymore. This indicates an influence of the ComK level on sporulation. Additionally, the growth of the not heated samples is also reduced in comparison to the sample with only SP medium. The TCA cycle deletion strain GP3028 shows very weak growth in both media, especially with the addition of mannitol and $MgCl_2$. Additionally, the strain cannot form spores anymore. The reason for the loss of sporulation was often explained by the accumulation of intermediates of the TCA cycle. Especially the accumulation of citrate seemed to have a great impact, since citrate builds a complex with divalent cations which are required for the Spo0A phosphorelay (Craig *et al.*, 1997; Matsuno *et al.*, 1999). However, in a TCA cycle deletion mutant, no intermediates accumulate, since the complete pathway is deleted. But as previously described, there are still pathways that produce intermediates that are normally used by the TCA cycle, like *e.g.* oxaloacetate by the pyruvate carboxylase PycA.

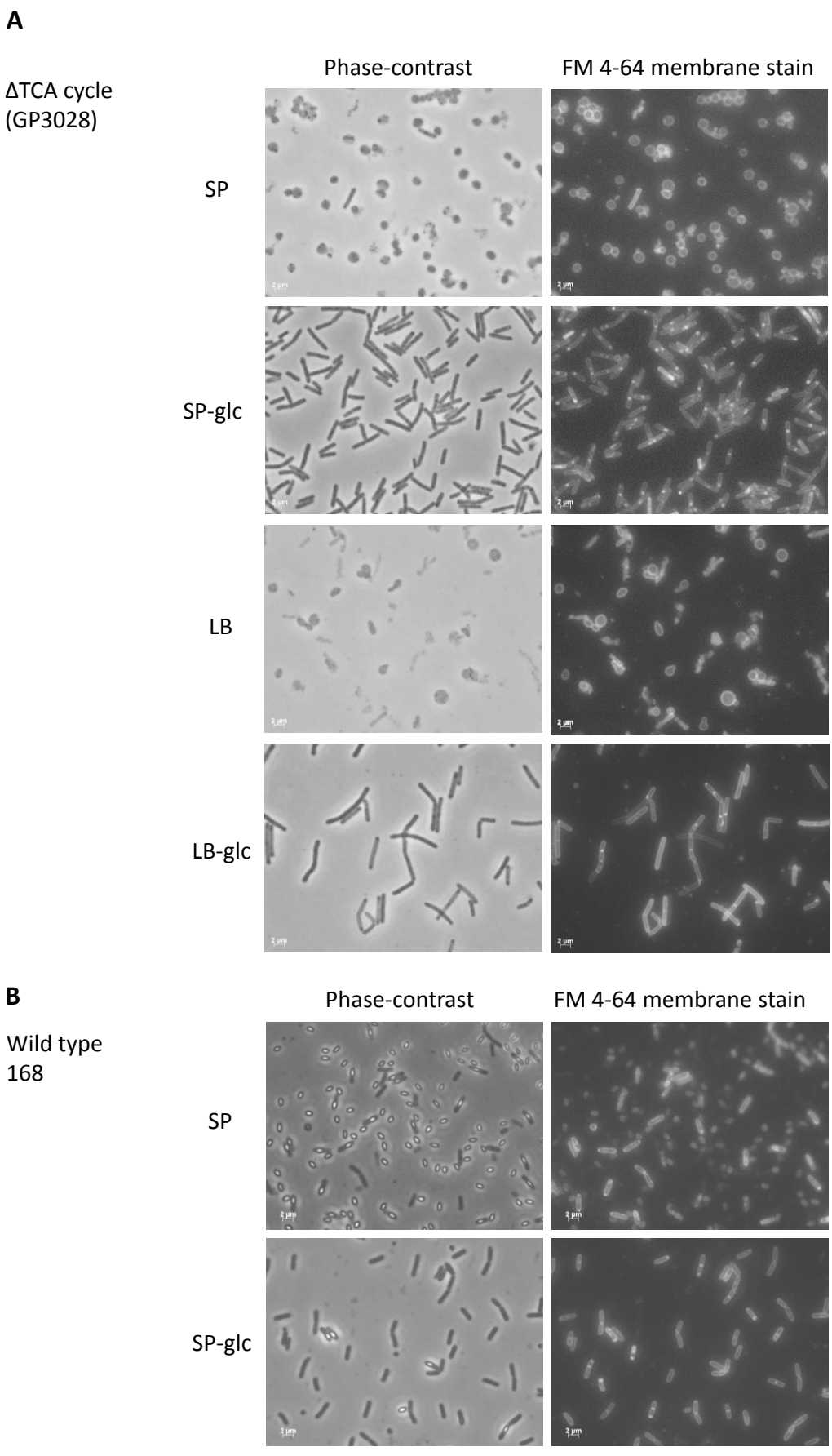


Figure 23: The microscopy of the TCA cycle deletion strain (GP3028) grown in LB and SP medium with and without 0.5% glucose (A). The wild type 168 was grown in SP and SP-glc medium as a control (**B**).

Finally, the TCA cycle deletion strain was also analysed at the single cell level under the microscope. The strain was grown in LB and SP medium with the addition of 0.5% glucose or without. The cells were additionally stained with the FM4-64 membrane stain (Figure 23). The TCA cycle deletion cells from the SP-glc cultures are rod-shaped, like the wild type cells of the control. However, they are not able to form spores what confirms the results from the sporulation assay. The wild type 168 forms spores in SP medium. In contrast, almost all cells of the TCA cycle deletion strain from the SP medium without additional carbon source are forming round structures. In the FM 4-64 stain, the lipids of the membrane are stained and can therefore be observed surrounding the round structures. This effect can also be seen in LB cultures without additional glucose.

These round structures can always be observed, if the peptidoglycan cell wall, is missing, in either protoplasts or L-form cells. L-form cells are lacking the peptidoglycan completely and are still able to grow.

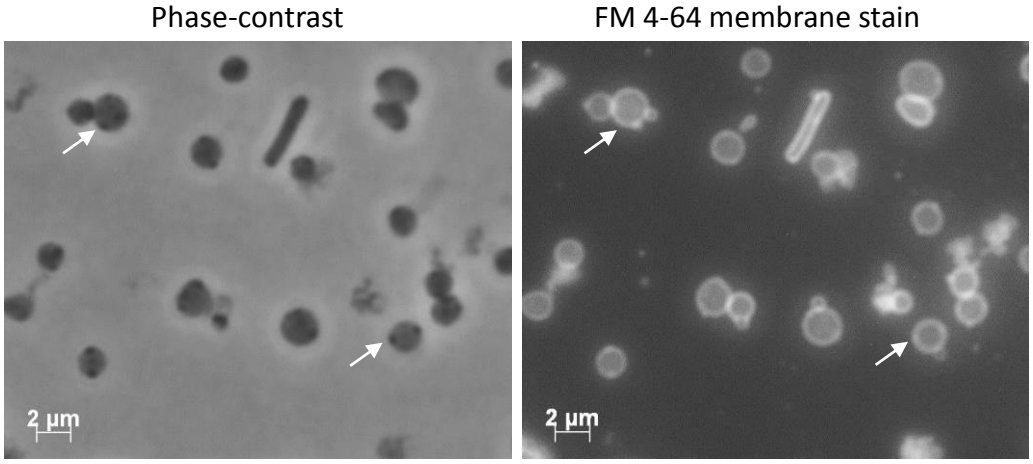


Figure 24: The spherical cells grown in SP medium without additional carbon source. The arrows indicate small darker dots on the surface of the cells.

On the magnified cells in Figure 24, small black dots can be observed on the surface. They cannot be found in the image with the membrane stain, which means those are no clusters of lipids. However, since L-form cells have often a block in peptidoglycan synthesis, theses dots could be leftover peptidoglycan, since the spherical cells are not as dark as the single rod-shaped cell in the Figure. Since the missing additional carbon source seems to be the reason for the appearance of the spherical cells, this could mean that the TCA cycle deletion strain is in this case not able to produce enough peptidoglycan to sustain the rod-shaped cell morphology. For the production of peptidoglycan, fructose-phosphate and phosphoenolpyruvate from glycolysis are used, as well as acetyl-CoA, which is produced from pyruvate (Daniel and Errington, 1993). If additional glucose is added, the cells can produce enough intermediates from glycolysis to form the peptidoglycan cell wall and the rod-shaped form. However, it still needs to be elucidated if these cells are protoplasts or L-form cells. L-form cells are able to divide and therefore also to grow in a culture. This was tested

with a preculture of the TCA cycle deletion strain in SP medium. From this culture, different cultures were inoculated, and growth was monitored (Figure 25).

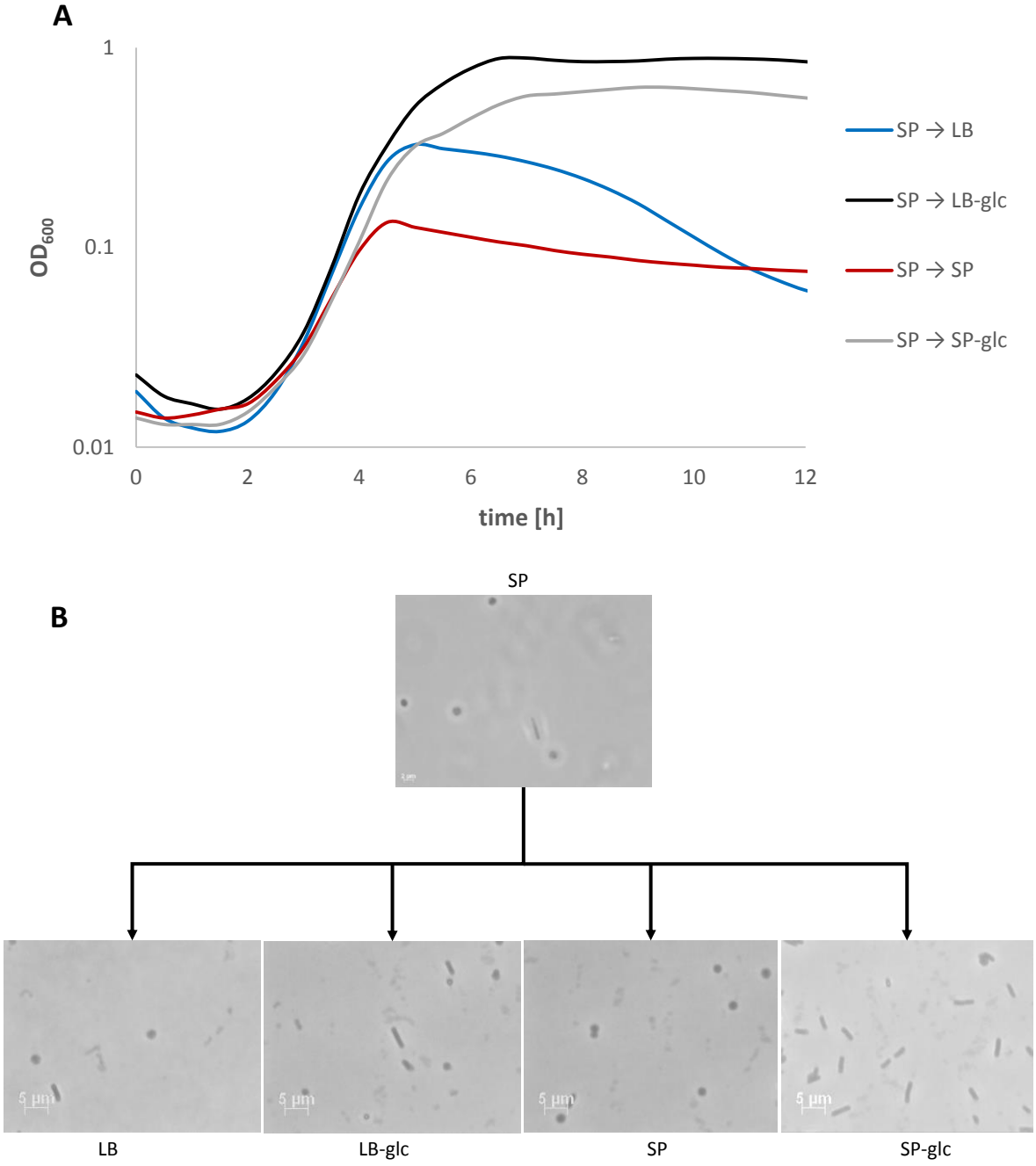


Figure 25: The growth of the TCA cycle deletion strain from a SP preculture in different media. A The growth of the strain in LB, LB-glc, SP and SP-glc medium, inoculated from the same SP preculture. **B** The cell morphology of the different cultures after 12 hours.

Almost all cells from the preculture showed the spherical phenotype, that was observed before. Only a few cells are still rod-shaped (Figure 25 B). The main cultures that were inoculated from this preculture showed first the same growth rate. However, the final OD_{600} of the cultures differ. The LB medium seemed to be better for the growth than the SP medium, since the cells from the LB

medium reached a higher OD_{600}. However, the addition of glucose to either LB or SP medium caused an even higher final OD_{600}. For the decision, if the spherical cells are still able to divide and grow, the growth curve in SP is the most interesting. It seemed that the cells are still able to grow, since the OD_{600} rises. However, there were still rod-shaped cells in the SP preculture which could have caused the raised OD_{600}. But since this is not a perfectly osmotically balanced medium, it could be possible that the spherical cells are able to grow, but some are burst because of the osmotic imbalance. Deletion mutants with reduced peptidoglycan synthesis are capable to change into the L-form growth. If *e.g.* the peptidoglycan precursor pathway is inhibited by different antibiotics, the cells can switch into the L-form state (Mercier *et al.*, 2014). Furthermore, the mutations or repression of genes involved in the respiratory chain are also capable of inducing L-form cells. This was observed for the, already previously mentioned, NADH dehydrogenase Ndh (Kawai *et al.*, 2015). Since the succinate dehydrogenase complex SdhCAB is also involved in the respiratory chain, the L-form state could be similarly triggered in the TCA cycle deletion strain. These facts all may lead to the assumption that the TCA cycle deletion strain forms L-form cells instead of protoplasts.

To conclude, the TCA cycle deletion would probably lead to no effect on the growth of the *MiniBacillus* strain in LB-glc medium. However, since the *MiniBacillus* strain uses the same *comKS* system to induce the competence, the TCA cycle deletion will lead to strong decrease of the competence. This would be a drawback for the project. Furthermore, the deletion of the TCA cycle might drastically change the cell morphology. Although, the *MiniBacillus* strain is already not able to sporulate, the peptidoglycan synthesis will be hindered, and this might lead to the formation of L-form cells, if no additional carbon source is added. However, the effect on the *MiniBacillus* strain could be different in comparison to the effect on the wild type strain 168, since over 40% of the genome is already deleted.

3.3. The identification of serine transporters in *Bacillus subtilis*

The amino acid transport is interesting for the *MiniBacillus* project, since the uptake of amino acids requires less genes than the biosynthesis pathways. However, in *B. subtilis* some amino acids have no characterized importer, *e.g.* tyrosine. This means the biosynthesis pathways remains in the blueprint of a minimal cell.

3.3.1. The serine importer YbeC

For the identification of potential amino acid transporters, the database *Subti*Wiki was searched for candidates that show similarities to known transporters (Michna *et al.*, 2016). Eight different candidates were chosen for a screening approach (Table 9).

Table 9: The putative amino acid importers, that were chosen from *Subti*Wiki database.

Gene	Product	Protein length	Deletion strain
aapA	Amino acid permease	459 aa	GP2377
steT	Serine/threonine exchanger transporter	438 aa	GP2378
yfnA (mtrA)	Methylthioribose transporter	461 aa	GP2379
ytnA	Unknown (similar to proline permease)	463 aa	GP1885
ybeC	Unknown	539 aa	GP1886
alsT	Putative glutamine transporter	465 aa	GP1888
yveA	Similar to aspartate/ glutamate transporter	520 aa	GP2385
yodF	Unknown	496 aa	GP1887

The protein SteT shows similarity to amino acid transporters from humans and it was shown to have a function in the exchange of serine and threonine in proteoliposomes (Reig *et al.*, 2007). To elucidate its function directly in *B. subtilis*, this gene was also chosen for this screening approach. Furthermore, the gene *yfnA* (now: *mtrA*) was also annotated as being similar to *steT* and therefore also chosen in this experiment (Reig *et al.*, 2007). However, a recent publication of Borriss *et al.* (2018) stated, that MtrA is involved in the uptake of methylthioribose.

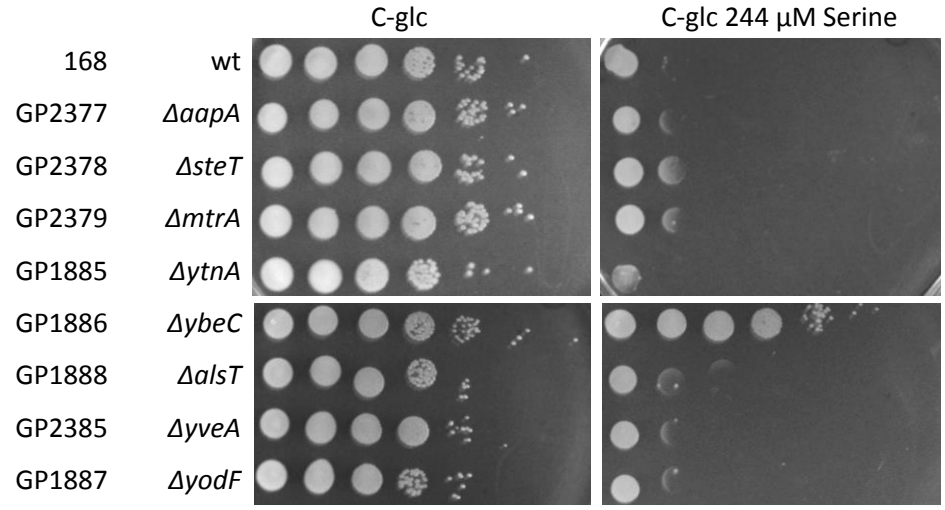


Figure 26: The drop dilution assay of the chosen potential transporters. A sample of OD_{600} of 1 was prepared for each strain. The cells were diluted 10^{-1} in several steps and plated on C-glc-minimal plates with and without 244 µM of one additional amino acid. The plates were grown for two days at 37°C.

In the screening approach, performed in this work, the deletion strains were tested for their growth on C-glc minimal media with each one amino acid added. The aim was to observe differences in

growth of mutant strains in comparison to the wild type. Interestingly, it could be observed, that the wild type strain 168 and all other mutants except for the *ybeC* deletion mutant showed a growth defect on C-glc plates with 244 µM serine (Figure 26).

Serine alone in minimal plates was shown to be toxic for the cells, although the reason for the toxicity is still unknown (Lachowicz *et al.*, 1996). However, the *ybeC* deletion mutant of seems to be resistant to serine. In theory, a deletion of a serine importer would lead to a decrease of serine import and therefore a minimization of the toxic effect. The next step was to test if the Δ*ybeC* mutant is also resistant on higher serine concentrations (Figure 27).

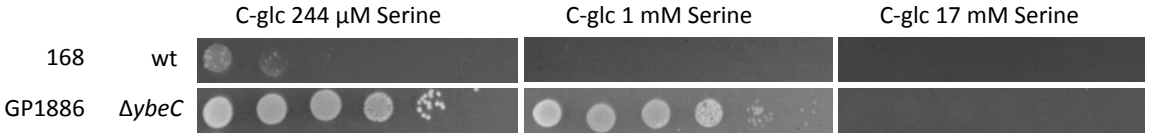


Figure 27: The growth of the Δ*ybeC* mutant in comparison to the wild type. The cells of the strains GP1886 and the wild type were diluted in several steps and plates on C-glc medium with 244 µM, 1 mM and 17 M serine. The plates were grown for two days at 37°C.

The Δ*ybeC* mutant is able to grow on an even higher serine concentration of 1 mM. However, if the concentration is increased further to even 17 mM, the strain is not able to grow anymore. In comparison, the wild type strain could not grow on 1 mM serine and only weakly on 244 µM.

Furthermore, the emergence of suppressor mutants can be observed for the wild type strain 168 and the strain GP2392 (Δ*serA)* and many of these were isolated and characterized. The suppressor wt 1 (GP2324) was isolated on C-glc plates with 1 mM serine and analysed by WGS. The strain harbours a single basepair deletion in the gene *ybeC* (bp 340), which leads to a frameshift and a shorter protein. This supports the theory, that the YbeC protein is responsible for the serine import. Several other isolated suppressor mutants were tested via PCR and sequencing for a mutation in *ybeC*. Furthermore, another suppressor of the wild type strain, isolated on 1 mM serine (GP2325), and two suppressors of the strain Δ*serA* (GP2392), isolated on C-glc plates with 244 µM (GP3049 and GP3050), showed different mutations in the *ybeC* gene (Table 10). All four *ybeC* suppressor mutants show a better growth on C-glc plates with serine in comparison to their parental strains (Figure 28).

The Δ*serA* strain needs to take up serine from the medium, since it cannot synthesis it. However, the growth is very weak and comparable with the growth of the wild type strain. The suppressors that were isolated show a better growth on the minimal medium. Although the suppressor Δ*serA* 1 grows better on 244 µM serine, the formation of new suppressors on 1 mM serine can be observed.

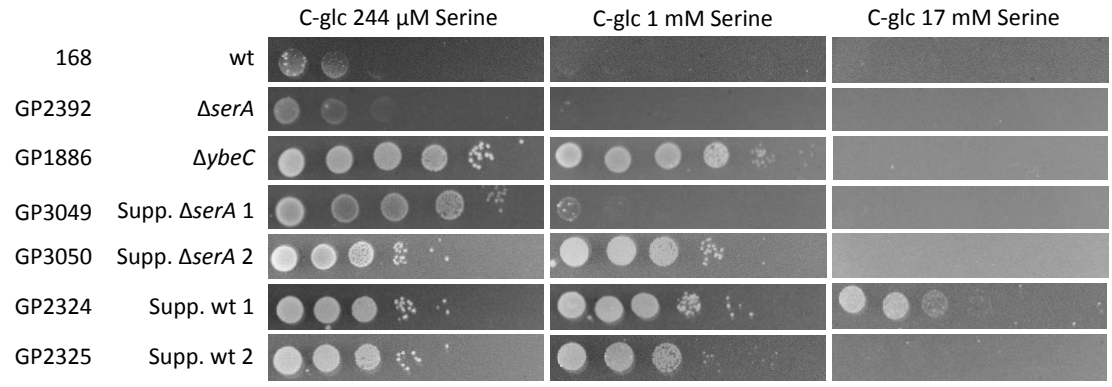


Figure 28: The drop dilution experiment of isolated suppressors in comparison to their parental strains and the Δ*ybeC* deletion sstrain. The suppressors of the wild type strain 168, GP2324 and GP2325 were isolated on C-glc plates with 1 mM serine and the Δ*serA* suppressors GP3049 and GP3050 were isolated on C-glc paltes with 244 µM serine. Precultures were prepared in C-glc medium and the plates were grown for two days at 37°C.

The acquired mutation in this suppressor Δ*serA* 1 seems to be not as beneficial as the mutation in suppressor Δ*serA* 2. This strain is able to grow on 1 mM serine comparable to the Δ*ybeC* mutant. Interestingly, the suppressor wt 1 is even able to grow on 17 mM serine.

Table 10: The suppressor mutations found in *ybeC,* isolated on C-glc serine plates.

Strain	**Suppressor mutant**	**Mutation in YbeC**
GP3049	Suppr. Δ*serA* 1	E522*
GP3050	Suppr. Δ*serA* 2	In frame deletion of 708 bp after bp 306 (236 aa missing after aa 102)
GP2324	Suppr. wt 1	bp 340 is deleted, leading to a frameshift and a shorter protein
GP2325	Suppr. wt 2	bp 974 is deleted, leading to a frameshift and a shorter protein

Different mutations can be observed in the suppressor mutants. The mutation in the strain GP3049 is interesting, since it leads to a stop codon n the C-terminus of the YbeC protein (compare Figure 29). This missing 18 amino acids seemed to lead to an impaired function of the protein, which indicates a special function of the C-terminal end of the protein. However, this suppressor mutant grows not as good as the deletion mutant of *ybeC*, which means, that the mutation of the C-terminus probably does not lead to a completely non-functional protein. The suppressor Δ*serA* 2 in contrast, has a mutation of 708 bp, after bp 306, which leads to the precise deletion of 236 amino acids in the middle of the YbeC protein. The suppressor wt 2 (GP2325) harbours, similar to the strain GP2324, a single basepair deletion which leads to a frameshift and a shorter protein. Since the

suppressor mutants GP3049, GP3050 and GP2325 were analysed by PCR and sequencing, further unidentified mutations might lead to the observed growth advantage in comparison to the parental strains.

Conclusively, the toxic effect of serine alone in the minimal plates can be compensated by the mutation of *ybeC*. This leads also to the assumption that YbeC is a serine importer and the suppressors are protected by the toxic effect of serine since the strains import less serine into the cell. Some of the isolated and analysed suppressor mutants showed no mutation in *ybeC*.

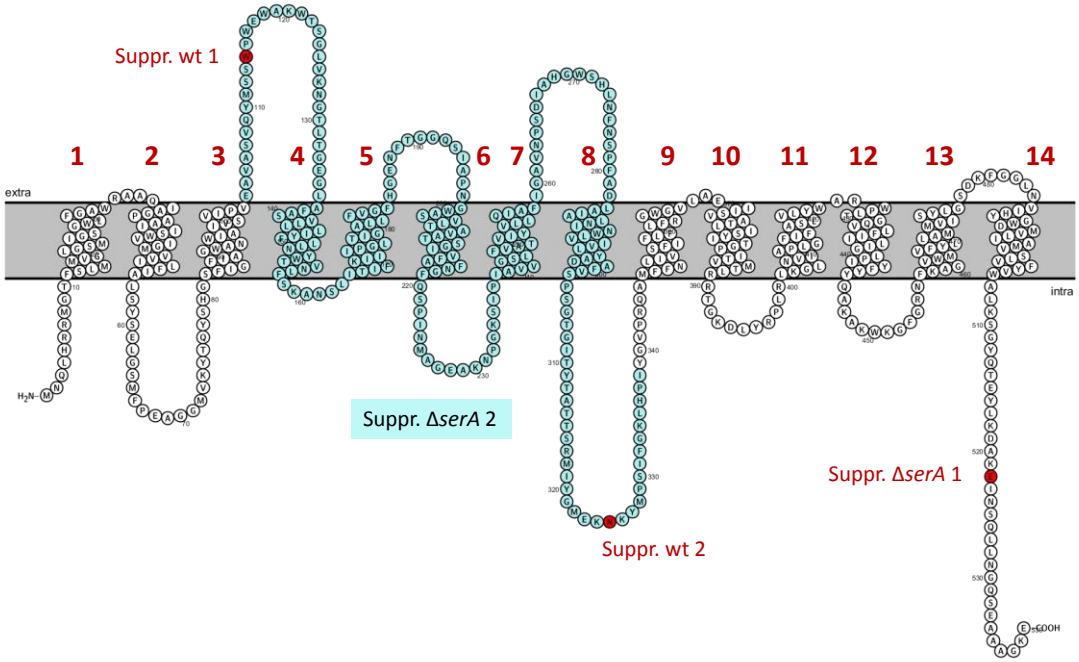


Figure 29: The observed suppressor mutations of the membrane protein YbeC. The amino acids marked in red are mutated in the corresponding suppressor mutants. The amino acids marked in blue are deleted in the suppressor Δ*serA* 2. The YbeC protein is shown according to UniProt and Protter (*http://wlab.ethz.ch/protter*) (Omasits *et al.*, 2014).

Since the Δ*ybeC* mutation seems to decrease the uptake of serine, the emerging of suppressor mutants in the strain Δ*serA* (GP2392) is interesting. The gene *serA* codes for the phosphoglycerate dehydrogenase and the enzyme catalyses the first step in the biosynthesis of serine (Chi *et al.*, 2013). The deletion mutant Δ*serA* is auxotrophic for serine and it is dependent on the import of serine. The strain GP2392 is therefore not able to grow on C-glc plates without serine. The *ybeC* suppressor mutation in the strain Δ*serA* indicates, that YbeC is not the only serine importer. To test this hypothesis the deletion mutation of *ybeC* was introduced into the strain Δ*serA* and the resulting strain GP2941 was tested for the growth on C-glc plates with serine (Figure 30). Similar to the *serA* deletion strain, the strain GP2941 is not able to grow on C-glc medium without serine.

The growth of the double mutant Δ*ybeC* Δ*serA* on C-glc medium with serine is comparable to the one of the Δ*ybeC* mutant. This leads to the assumption, that YbeC cannot be the only serine importer in *B. subtilis*, since the serine auxotrophic mutant with the *ybeC* deletion is still viable.

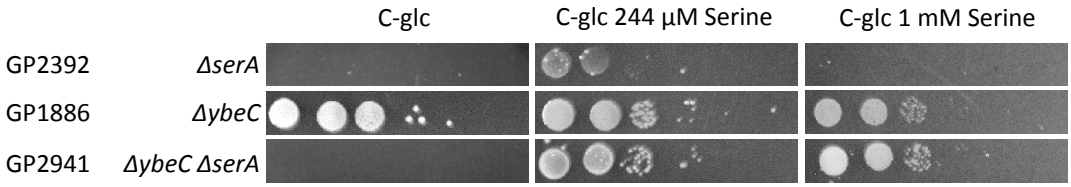


Figure 30: The combination of the *ybeC* deletion with the deletion of *serA*. The double deletion strain was compared to the single deletion strains of *serA* and *ybeC*. A serial dilution assay was performed for each strain and the cells were plated on C- glc medium with and without serine. The plates were grown for two days at 37°C.

Furthermore, the Δ*ybeC* mutant shows better growth in the presence of a toxic derivative of serine, DL-serine hydroxamate, in comparison to the wild type (chapter 6.5. supplementary information). This also indicates, that the deletion strain of *ybeC* takes up less serine or in this case toxic derivative, than the wild type.

The next step was to test if the expression of *ybeC* is influenced by the presence of serine. Therefore, the *ybeC* promoter fuzed to a *lacZ* gene by cloning the promoter into the plasmid pAC5. The wild type strain 168 was transformed with the resulting plasmid pGP2287, which integrates into the *amyE* locus of the chromosome (Martin-Verstraete *et al.*, 1992). The activity of this translational fusion of the *ybeC* promoter to the *lacZ* gene was measured with a β-galactosidase activity. The strain was grown in different media to an OD_{600} of 0.5 and the β-galactosidase activity was measured as described in chapter 2.2.7. (Table 11).

Table 11: The β-galactosidase activity assay of the *ybeC* promoter fused to *lacZ*.

Medium	**C-glc**	**C-glc 244 µM Serine**	**LB-glc**	**C-glc +244 µM serine +500 µM glutamate**
β-galactosidase activity [Miller units]	144.8 ±2.11	132.4 ±8.21	135.2 ±9.67	142.2 ±2.85

The activity of the promoter is very similar in all media. The addition of serine or serine with glutamate does not seem to influence the expression of *ybeC* in comparison to the measurements in C-glc minimal medium.

Interestingly, serine is also toxic to *E. coli* in high amounts (Hama *et al.*, 1990). To study if YbeC can also mediate the serine import in *E.coli,* a copy of the *ybeC* gene from *B. subtilis* was cloned into the vector pWH844 and transformed into the *E. coli* strain JM109 (Krüger, unpublished data). The growth of this strain was compared with a strain harbouring the empty vector (EV) on plates with increasing serine concentrations (Figure 31).

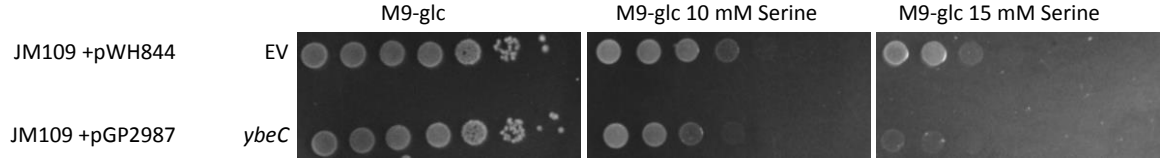


Figure 31: The growth of the *E. coli* strain JM109 with the *ybeC* plasmid pGP2987. Precultures were prepared in M9-glc medium and a serial dilution assay was performed and the cells were plated on M9-glc plates with or without serine. The plates were incubated for two days at 37°C.

It can be observed, that the strain with the *ybeC* plasmid (pGP2987) shows a growth disadvantage on M9 minimal plates with serine. The M9-glc medium was modified by removing all other amino acids to ensure that the toxic effect of serine can be seen (chapter 2.1.2.). It has to be mentioned, that the plasmid pWH844 is used to induce the expression of proteins by the addition of IPTG (Schirmer *et al.*, 1997). However, there is no IPTG added to the M9-glc plates, but the basal expression from the plasmid seems to be sufficient to increase the sensitivity against serine. This experiment proves, that the YbeC protein can also act as a serine importer in *E. coli*. However, the suppressor analyses that was performed before, showed that an YbeC protein without the C-terminus is not completely functional. The plasmid pGP2988 harbours the modified *ybeC* gene in the vector pWH844 (Krüger, unpublished data). The strain JM109 was transformed with the plasmid and also tested on M9-glc minimal plates with different concentrations of serine (Figure 32).

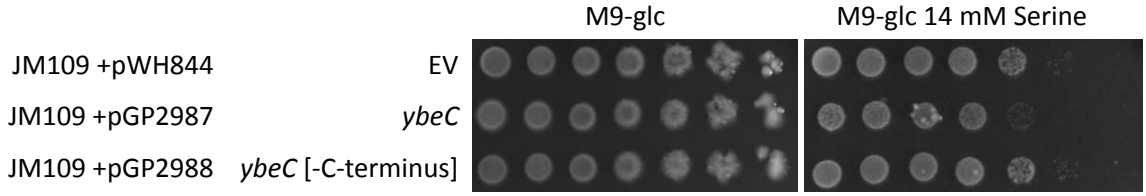


Figure 32: The growth of *E. coli* strains with different plasmids on M9-glc medium. The empty vector control was compared with a strain harbouring the *ybeC* gene from *B. subtilis* and a strain with the *ybeC* gene, lacking the C-terminus of the encoding protein. Precultures were prepared in M9-glc medium and the plates were incubated at 37°C for two days.

The *E. coli* strain with the *ybeC* gene growths again worse than the EV control. However, the strain with the deletion of the C-terminus grows again as the EV control. These results indicate, that the YbeC protein is a serine importer and that it needs the C-terminus to form a functional protein. The

deletion of the C-terminus might lead to a structural change of the protein, which leads to a non-functional protein. Furthermore, the C-terminal part of the YbeC protein could be important to sense a signal from the cell, which leads then to the activation of the import.

To ensure that the phenotype of the Δ*ybeC* deletion in *B. subtilis* is caused by the absence of the protein, a complementation assay was performed in which a new copy of *ybeC* on the plasmid pGP2980, was integrated into the *ykdE* locus of the Δ*ybeC* strain (GP2948). The plasmid pGP2980 is based on the plasmid pGP886 (Gerwig *et al.*, 2014; Krüger, unpublished data). The growth of the strain GP2948 on C-glc plates with xylose were compared to the strain GP2947 with the integrated empty vector (Figure 33).

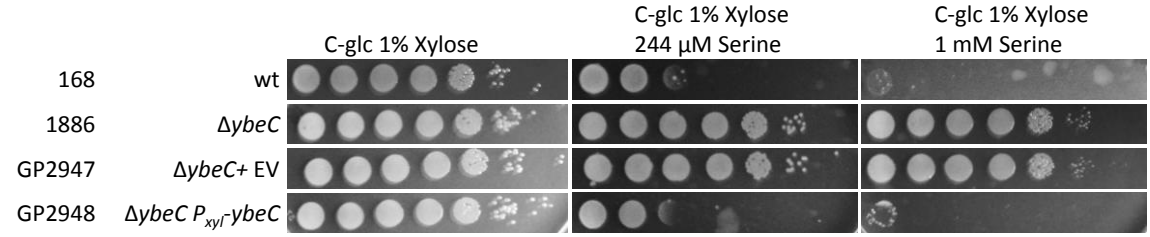


Figure 33: The complementation of the Δ*ybeC* mutant with a xylose inducible copy of *ybeC*. The strains were grown in C-glc medium and the serial drop dilution assay was performed on C-glc plates without and 244 µM or 1 mM serine. The C-glc plates are incubated at 37°C for two days.

The expression of the new copy of the *ybeC* gene can be induced by the addition of xylose. An empty vector integration into the Δ*ybeC* strain shows no difference on C-glc plates with serine, even in the presence of 1% xylose. However, if *ybeC* is expressed in the Δ*ybeC* strain by the addition of xylose, the growth is similar to the growth of the wild type strain. This confirms the previous results that *ybeC* is the reason for the resistance against serine and the effect is independent from the localisation on the chromosome.

To conclude, YbeC is a serine importer from *B. subtilis*. The deletion mutant is more resistant to serine in minimal medium than the wild type. Suppressor mutants of the wild type strain 168 and the Δ*serA* mutant emerge on C-glc plates with serine. Some of these mutations affect the ybeC gene. Furthermore, it is possible to introduce *ybeC* into *E. coli*, which makes the strain more sensitive towards serine. However, YbeC is not the only serine importer in *B. subtilis*, since the combination with a serine auxotrophic mutant Δ*serA* is possible.

3.3.2. Additional serine importers BcaP and YbxG

The experiments in chapter 3.3.1. strongly suggest, that there are more serine importer than only YbeC. The combination of another serine importer deletion with Δ*ybeC* would lead to a strain that shows either an even better growth on C-glc serine medium when there are still more serine transporters. The combined strain could also show a worse growth than the Δ*ybeC* mutant, if all

serine importers are deleted and the synthesis of serine is not enough to sustain the growth. The protein SteT was already mentioned as a potential serine / threonine exchanger and the deletion mutant Δ*steT* was combined with the Δ*ybeC* mutant. The growth of the single and double mutant was tested on C-glc serine plates (Figure 34).

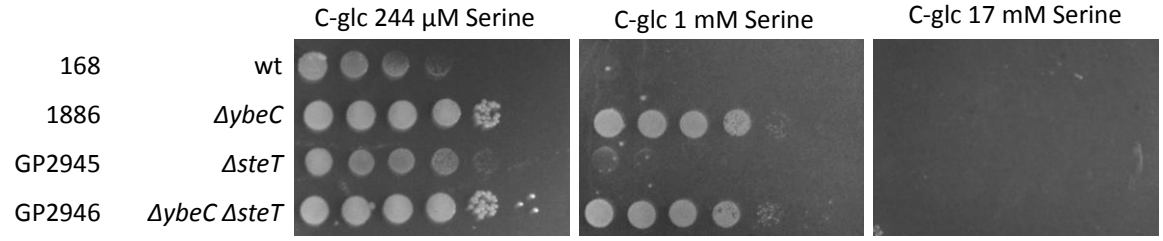


Figure 34: The gene *steT* was tested for its potential role in serine import on C-glc serine medium. The growth of the double deletion Δ*ybeC* Δ*steT* was compared to the growth of the single deletion mutants at 37°C. The plates were incubated for two days.

The additional deletion of *steT* in the Δ*ybeC* mutant shows no difference to the growth of the single deletion Δ*ybeC.* Furthermore, the growth of the Δ*steT* single deletion has also no growth advantage on C-glc minimal medium with serine. These data indicate that the protein SteT has no function in the uptake of serine under the tested conditions.

Serine is often imported by the same protein that also imports threonine, *e.g.* in *E. coli* (Hama *et al.*, 1987). Therefore, the known threonine transporters of *B. subtilis* were analysed for their potential role in the serine import. The transporter BcaP was shown to import isoleucine, valine and threonine. The import of isoleucine was shown to decrease if threonine, serine, valine, leucine, cysteine and asparagine was present in the medium (Belitsky, 2015). This leads to the assumption, that BcaP might also import serine into the cell. Furthermore, the protein YbxG was described as a potential threonine transporter since the gene was mutated in a suppressor that was found on the toxic threonine derivative 4-hydroxy-L-threonine (Commichau *et al.*, 2015). The genes *bcaP* and *ybxG* were analysed as single deletion mutants and in combination with the *ybeC* deletion for their growth on C-glc serine (Figure 35).

The single deletion mutants of *bcaP* and *ybxG* grow slightly better than the wild type on C-glc medium with 244 µM serine. If the strains are combined with the Δ*ybeC* deletion, the growth seems to be better in comparison to the Δ*ybeC* single deletion. Interestingly, the strain Δ*ybeC* Δ*bcaP* Δ*ybxG* grows even on 17 mM serine, which means the strain is highly resistant to the toxic effect of serine. This strongly indicates, that the Δ*ybeC* Δ*bcaP* Δ*ybxG* strain imports less serine than the Δ*ybeC* single deletion. Therefore, YbxG and BcaP are most likely playing a role in serine uptake.

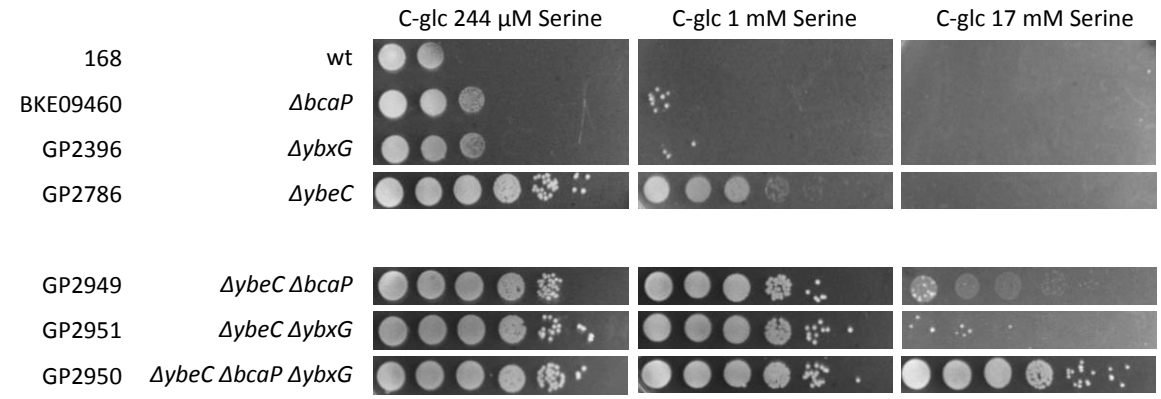


Figure 35: The drop dilution assay to test the resistance of the Δ*bcaP* and Δ*ybxG* to serine. The single mutants were combined with the deletion of the known transporter *ybeC*. Precultures were grown in C-glc medium. A serial drop dilution was performed and the plates were incubated for two days at 37°C.

Since only three serine concentrations were tested, the strains could possibly show more growth differences. Therefore, a wide range of serine concentrations were tested, and the highest serine concentration was noted on which the strains could still grow. This was analysed one and two days after inoculation on C-glc plates (Figure 36).

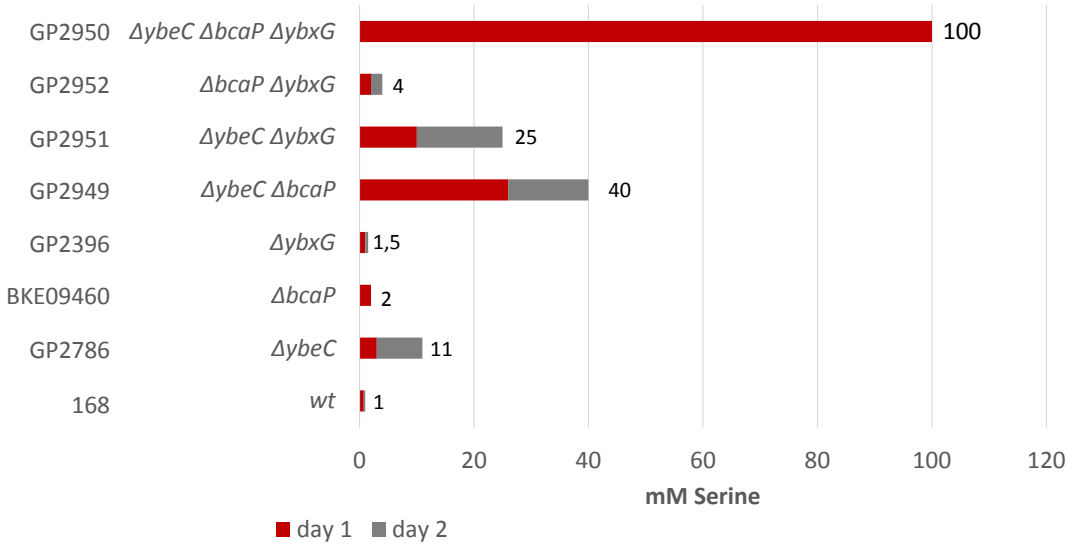


Figure 36: The different deletion strains of potential serine importers are tested for their growth of serine. The growth was observed one and two days after inoculation and the numbers indicate the highest concentration of serine that the mutant was able to grow on, on the second day.

The wild type was able to grow weakly on 1 mM serine after day two. In contrast, the Δ*ybeC* strain grows on up to 11 mM serine. The deletion mutants of the threonine and potential serine transporters *bcaP* and *ybxG*, showed a higher resistance to serine than the wild type, but not as good as the Δ*ybeC* strain. So, the serine import by BcaP and YbxG seems to be less than the import of YbeC under these conditions. The double deletion mutant Δ*bcaP* Δ*ybxG* can tolerate up to 4 mM serine in the medium, which also fits to the assumption that the two proteins are involved in serine

uptake. The combination of the Δ*ybeC* strain with either the Δ*bcaP* or the Δ*ybxG* deletion leads to strains, that are capable to grow on higher serine concentrations than the Δ*ybeC* single deletion. However, the deletion of *bcaP* has a greater impact than the deletion of *ybxG*, which could also hint to differences in the uptake efficiency of the two proteins. Therefore, BcaP seems to import more serine into the cell, than YbxG. Interestingly, the combined deletion of all three genes Δ*ybeC* Δ*bcaP* Δ*ybxG* leads to a strain, that is highly resistant to serine. It can tolerate up to 100 mM serine in the C-glc plates. These growth differences of the single and combined strains could also be shown in growth curves in liquid C-glc medium (Figure 37).

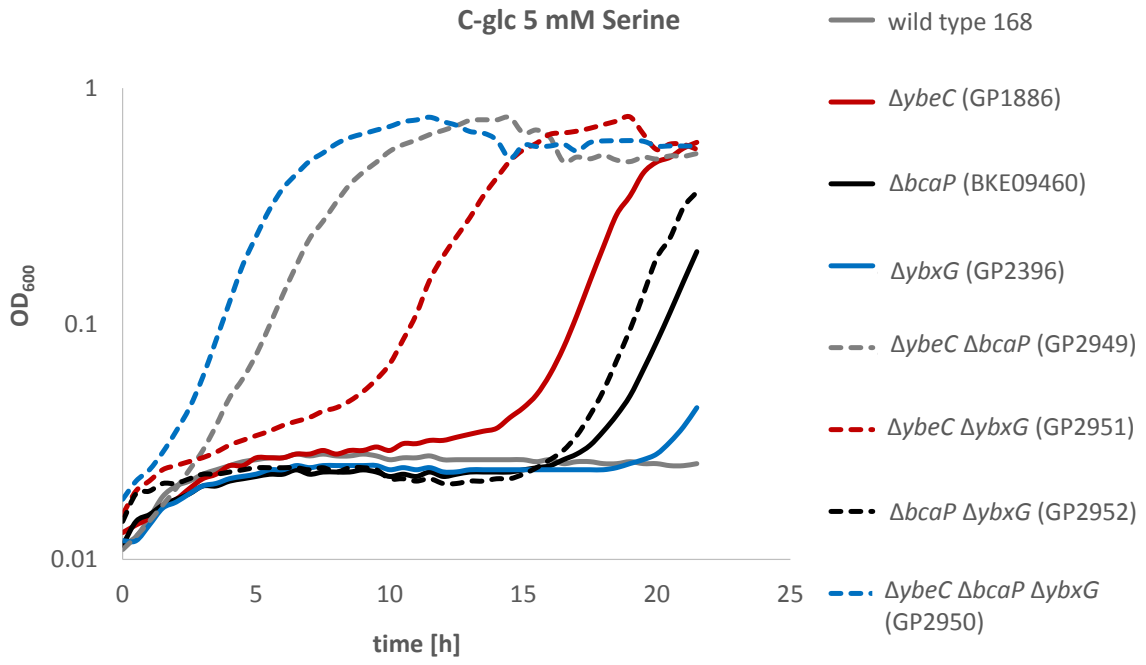


Figure 37: The growth curves of the single and combined deletion mutants of the serine importers in C-glc medium with 5 mM serine. Precultures of all strains in C-glc were used to inoculate the C-glc medium with 5 mM serine. The growth was analysed at 37°C.

The 168 wild type cannot grow with 5 mM serine in C-glc liquid medium at all. The single deletion strains show the same order of resistance against serine, like it was observed before. The Δ*ybeC* strain grows the best, followed by the Δ*bcaP* strain and finally the Δ*ybxG* strain. Combining the *bcaP* and *ybxG* deletion leads to a strain that grows better than the single mutants, but not as good as the Δ*ybeC* strain. The combination of Δ*ybeC* with the deletion of *bcaP* is cleary more beneficial for the growth in these serine concentrations than the combination with the *ybxG* deletion. However, it can be observed that the triple deletion strain Δ*ybeC* Δ*bcaP* Δ*ybxG,* grows without a growth disadvantage in these media. These results confirm the role of all three proteins in the import of serine.

To analyse if the transporters could also import serine into *E. coli* cells, the *B. subtilis* genes *bcaP* and *ybxG* were also cloned into the pWH844 vector and transferred into the *E. coli* strain JM109. The resulting strains were compared with the EV and the strains with the complete *ybeC* gene and the *ybeC* gene without the C-terminal part of the protein (Figure 38).

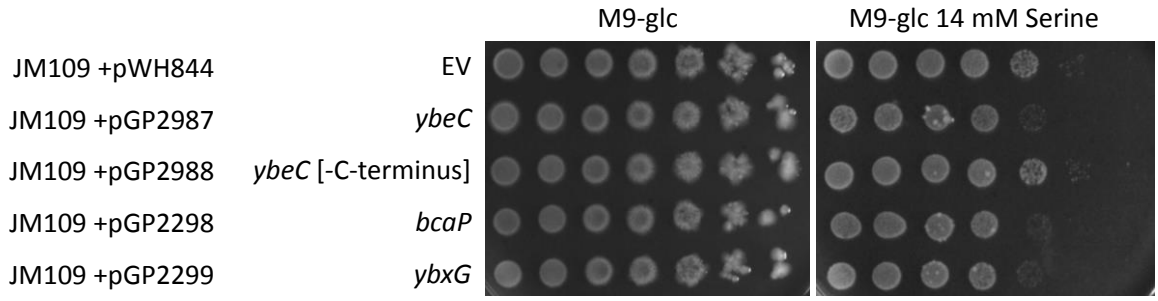


Figure 38: The drop dilution assay of *E. coli* strain harbouring the plasmids encoding for the *B. subtilis* serine importers. The genes encoding for serine transporters *ybeC*, *bcaP* and *ybxG* were cloned into a plasmid and transferred into *E. coli* JM109 cells. Additionally, the *ybeC* gene without the C-terminal part of the protein was tested. The plates were incubated at 37°C for two days.

The *E. coli* strains with the plasmid integrated gene *ybeC* shows again a growth disadvantage on M9-glc plates with serine. In contrast, the *ybeC* mutant without the C-terminal part of the protein growth like the strain with the EV, since the protein is not functional. Interestingly, the *bcaP* and *ybxG* strains also show a similar growth disadvantage than the strain with *ybeC*. The reason for that might be the expression in a different organism. The regulatory mechanism that control the expression and activity of the proteins might be only existing in *B. subtilis* but not in *E. coli*. Furthermore, the *E. coli* strain JM109 still harbours all serine importers of *E. coli*. A very severe growth disadvantage could be adjusted by regulating the native *E. coli* serine importers, like SstT (Ogawa *et al.*, 1997). Additionally, the expression of the plasmid coded genes is not induced by IPTG in these experiments. The basal, leaky transcription is enough to produce a few proteins that are responsible for this effect. However, these results show that the *E. coli* strains with the plasmid based *ybeC*, *bcaP* and *ybxG* take up more serine, which is in certain concentrations also toxic for *E. coli*.

The very high resistance of the *B. subtilis* triple deletion strain Δ*ybeC* Δ*bcaP* Δ*ybxG* to serine could indicate that all serine importers of *B. subtilis* might be deleted in this strain. To test this hypothesis, the gene *serA* was deleted in the triple deletion strain. It was already mentioned, that *serA* is involved in the biosynthesis of serine and a deletion of it leads to a serine auxotrophic strain. However, the deletion of *serA* was possible in the Δ*ybeC* Δ*bcaP* Δ*ybxG* deletion strain and the cells were viable. The resulting strain was then tested for its growth on C-glc medium with and without serine (Figure 39).

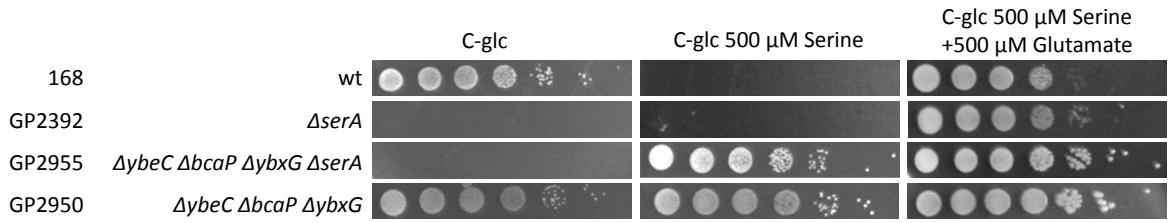


Figure 39: The growth of the strain GP2955 (Δ*ybeC* Δ*bcaP* Δ*ybxG* Δ*serA)* compared to the Δ*serA* deletion mutant and the triple deletion strain GP2950. The growth was tested on C-glc medium, on C-glc medium with 500 µM serine and on C-glc medium with 500 µM serine and 500µM glutamate. The plates were incubated at 37°C for two days

The single deletion strain Δ*serA* is not able to grow on C-glc medium without additional serine, since it is serine auxotrophic. The combined mutant Δ*ybeC* Δ*bcaP* Δ*ybxG* Δ*serA* shows also no growth on the C-glc medium, which means the gene *serA* was successfully deleted and the strain is indeed serine auxotroph. However, this strain is also still able to grow on C-glc plates with 500 µM serine like the strain without the *serA* deletion. This means, that the import of serine is still possible, although three serine importers are deleted. So YbeC, BcaP and YbxG are not the only membrane proteins that are able to take up serine from the medium. However, the amount of imported serine by the missing transporter is just enough that the cells do not suffer.

To conclude, the transporter BcaP is not only able to import valine, isoleucine and threonine into the cell, but also serine. The potential threonine transporter YbxG plays also a role in serine import, but the amount of serine transported into the cell by BcaP is higher. Nevertheless, YbeC seems to be the main importer of serine under the tested conditions, since a deletion of the *ybeC* gene leads to the highest resistance against high serine concentrations.

3.3.3. Threonine uptake function of YbeC, BcaP and YbxG

The protein BcaP was described to transport threonine into the cell and also YbxG could have a threonine import function (Belitsky, 2011; Commichau *et al.*, 2015). Therefore, the role of *bcaP*, *ybxG* and also *ybeC* in threonine import should be analysed in this chapter. YbeC could also import threonine, since threonine and serine are structurally similar, and the transport of both amino acids is often coupled. The growth of the single, double and triple deletion mutants of the transporters *ybeC*, *bcaP* and *ybxG* was tested in C-glc medium with 10 mM threonine (Figure 40).

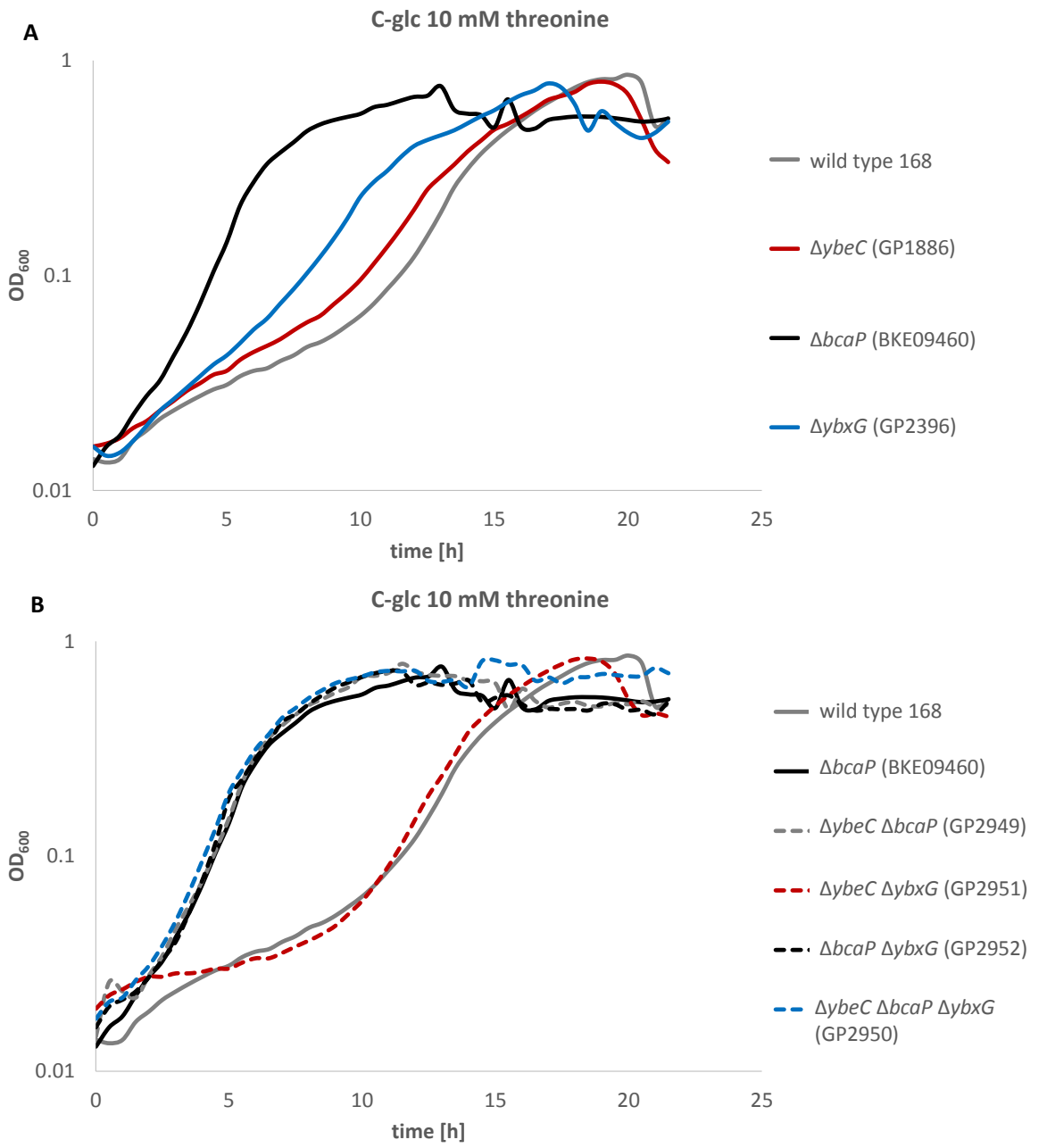


Figure 40: The growth of the potential threonine importer deletion strains in C-glc medium with 10 mM threonine. Precultures were prepared in LB medium. **A** The single deletion mutants of *ybeC, ybxG* and *bcaP* are compared to the wild type strain 168. **B** The growth of the double and triple deletion strains of *ybeC, ybxG* and *bcaP* are compared to the growth of the wild type strain 168 and the bcaP deletion strain. The growth was analysed at 37°C.

Interestingly, high concentrations of threonine are toxic for *B. subtilis*, since it inhibits growth and sporulation (Lamb and Bott, 1979b). The Δ*bcaP* deletion mutant has a clear growth advantage in comparison to the wild type strain 168, which means that BcaP is responsible for the uptake of threonine. However, also the Δ*ybxG* mutant can transport threonine, since it shows a better growth under these conditions. The *ybeC* deletion mutant in contrast grows just a little bit better than the wild type. This probably means, that a small amount of threonine can be imported to the cell by

YbeC, but since the effect is not as high as in the case of serine, the import is not as high as the import of serine. However, the double deletion strains that harbour a deletion in *bcaP* and also the triple deletion strain Δ*ybeC* Δ*bcaP* Δ*ybxG* grow, like the Δ*bcaP* single deletion, very good under these conditions. This indicates that BcaP is the main low-affinity transporter for threonine. In contrast, the strain Δ*ybeC* Δ*ybxG* has almost no growth advantage in comparison to the wild type, which indicates a weak threonine import function of the both proteins. It needs to be noted, that the growth of the Δ*ybeC* Δ*ybxG* deletion mutant is even worse than the growth of the Δ*ybxG* single deletion strain, but the reason for this is unclear.

To analyse if all threonine transporters are deleted in the strain Δ*ybeC* Δ*bcaP* Δ*ybxG*, the strain was combined with a deletion of *thrC*. The gene *thrC* encodes for the threonine synthase, a protein of the threonine biosynthesis pathway. A Δ*thrC* deletion mutant is auxotrophic for threonine. The created deletion mutant was viable and it was tested for its growth in C-glc medium with varying concentrations of threonine (Figure 41).

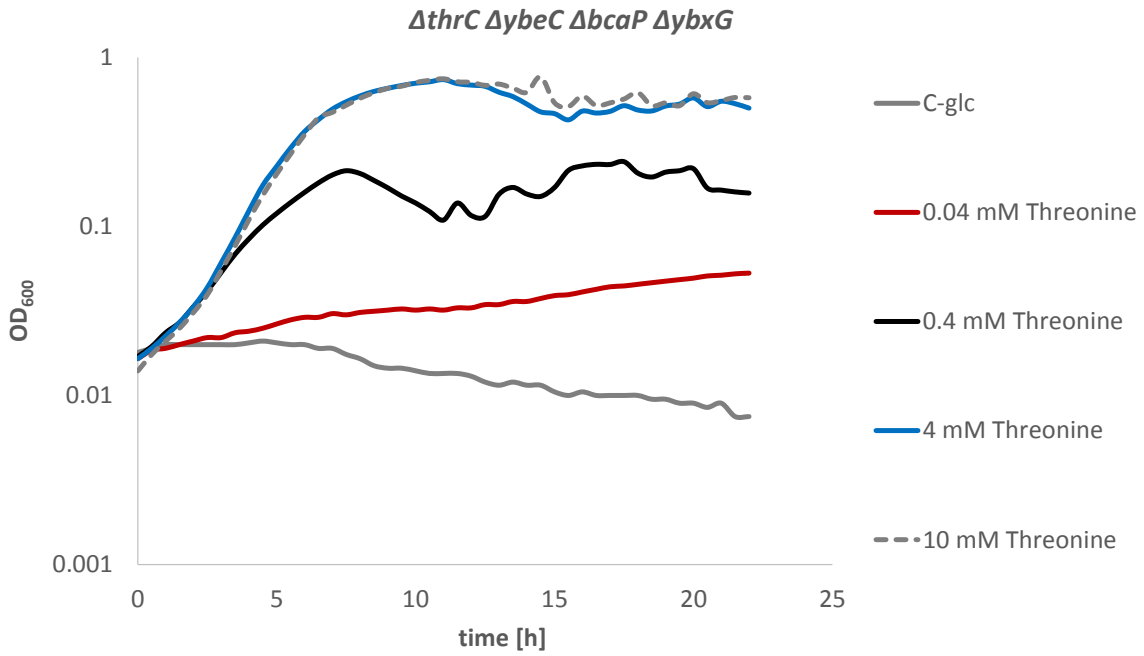


Figure 41: The growth of GP3037 (Δ*thrC* Δ*ybeC* Δ*bcaP* Δ*ybxG*) in C-glc medium with different concentrations of threonine. Precultures for this experiment were grown in LB medium. The growth was analysed in the plate reader at 37°C.

The strain cannot grow in C-glc medium, which indicates, that *thrC* was successfully deleted and the strain is auxotrophic for threonine. A concentration of 0.04 mM threonine is not enough to sustain a robust growth. This might be due to the reason that the strain needs a higher threonine concentration to efficiently take up the amino acid. If the threonine concentration is further increased to 0.4 mM, the strain grows better in comparison to the lower concentration, but not as

good as with 4 or 10 mM threonine. The 10 mM threonine concentration was previously shown to be toxic for the cell, but the strain Δ*thrC* Δ*ybeC* Δ*bcaP* Δ*ybxG* is resistant to it.

Furthermore, the gene *thrC* was deleted in all single and double deletion strains of the potential threonine transporters *bcaP, ybxG, ybeC*. The resulting strains were also tested in C-glc medium with different concentrations of threonine and the results are shown in Figure 42.

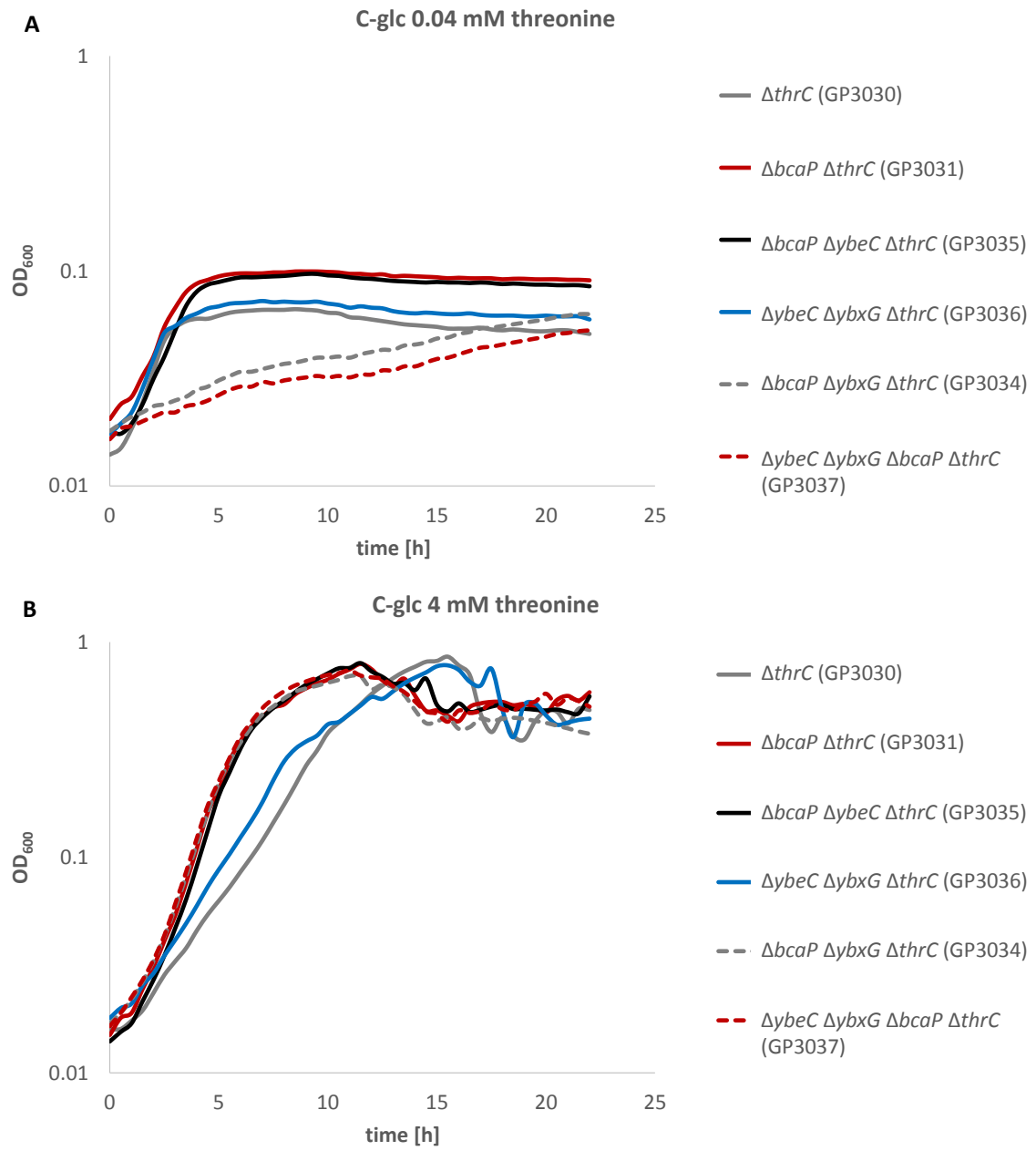


Figure 42: The growth of the potential threonine importer deletion strains in the background of Δ*thrC*. The strains were grown at 37°C in C-glc medium with 0.04 mM threonine (**A**) and 4 mM threonine (**B**).

Growth of the Δ*thrC* strains in 0.04 mM threonine is overall weak, and the Δ*thrC* Δ*bcaP* deletion strain grows the best in this medium. As already mentioned, the deletion of all three transporters and *thrC* leads to a reduced growth. Interestingly, this is also the case for the Δ*thrC* Δ*bcaP* Δ*ybxG*

deletion strain. The absence of the proteins BcaP and YbxG seems to have a more severe effect, than the absence of YbeC and BcaP or YbxG. This indicates, that if YbeC has a role in the uptake of threonine, it might be a rather small one. In contrast, the major role of BcaP in the uptake of threonine can be observed in the growth curves at 4 mM threonine. The only strain, in which the gene *bcaP* is not deleted (Δ*thrC* Δ*ybeC* Δ*ybxG*) grows the worst. This might be caused by the threonine concentration of 4 mM, that is already harmful for the cell. The remaining transporter BcaP imports high amounts of threonine into the cell. Therefore, BcaP seems to be the main transporter for threonine.

Interestingly, threonine can counteract the toxic effect of serine to a certain limit, if the two amino acids are present in the medium (Figure 43).

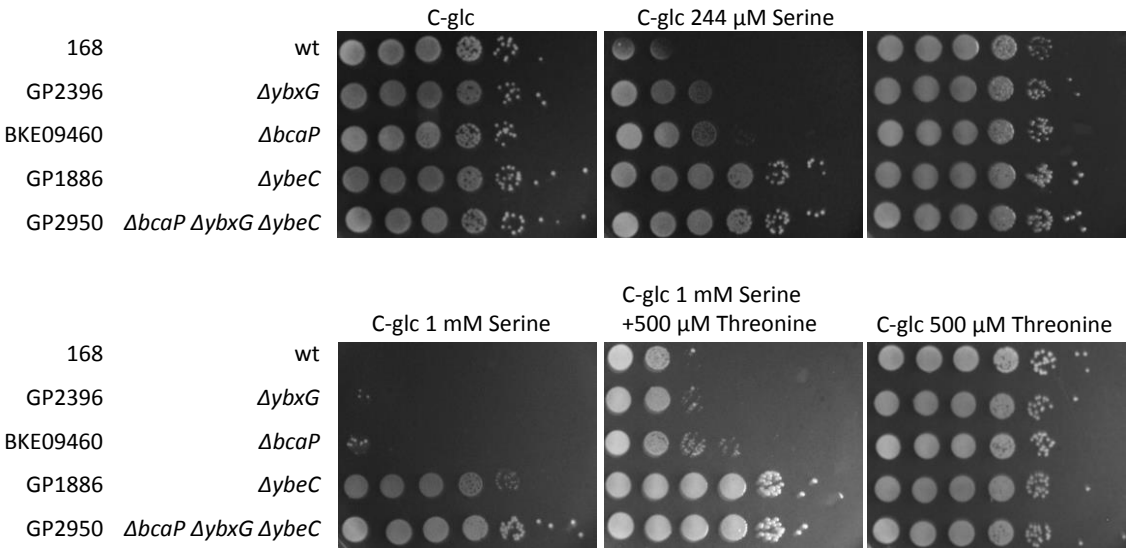


Figure 43: The drop dilution assay on different serine and threonine concentrations. The growth of the single deletion strain of *ybeC* (GP1886), *ybxG* (GP2396) and *bcaP* (BKE09460) is compared to the triple deletion strain GP2950. The C-glc plates contain 244 µM or 1 mM serine with and without 500 µM serine. The C-glc plates without additional amino acid serves as a control.

The wild type strain 168, the Δ*ybxG* and the Δ*bcaP* mutants show a growth defect on C-glc plates with 244 µM and 1 mM serine. However, the addition of 500 µM threonine to the medium with 244 µM serine can compensate this observed growth defect. This is also possible for 1 mM serine with 500 µM threonine, but the growth disadvantage is again visible. Furthermore, 500 µM threonine alone in C-glc plates is not toxic for the strains. This indicates, that serine and threonine are transported by the same importer into the cell. If only serine is present, the importer only transports serine, but if threonine is also present, the two amino acids are imported into the cell. Therefore, a lower amount of serine is imported into the cell and the toxic effect is less pronounced. Another possibility is that a higher threonine concentration in the cell can compensate directly the toxic effect of serine. This will be further analysed and discussed in chapter 3.4.

To conclude this chapter, the transporter BcaP is the main threonine importer of *B. subtilis*. However, the protein YbxG is also able to transport threonine into the cell. The previously identified serine transporter YbeC could also have a function in threonine import, but the amount of threonine transported by the protein is just very small. However, these are not all of the threonine importers in *B. subtilis,* since the deletion strain Δ*thrC* Δ*ybeC* Δ*bcaP* Δ*ybxG* is still viable, although it is auxotrophic for threonine.

3.4. The toxic effect of serine

High concentrations of serine are toxic for the growth of *B. subtilis*. Although, this observation is published in the literature, the mechanism itself and also the physiological role of it is unknown (Lachowicz *et al.,* 1996; Koo *et al.,* 2017). Therefore, the target of the serine toxicity in *B. subtilis* should be analysed further in this work.

It was already shown that a mutation in the *ybeC* gene, encoding for a serine importer can cause resistance to serine (chapter 3.3.1.). This indicates that the toxic effect of serine acts on something in the cell. However, the suppressor mutations in *ybeC* were not the only suppressor mutants that were isolated on high concentrations of serine (Figure 44).

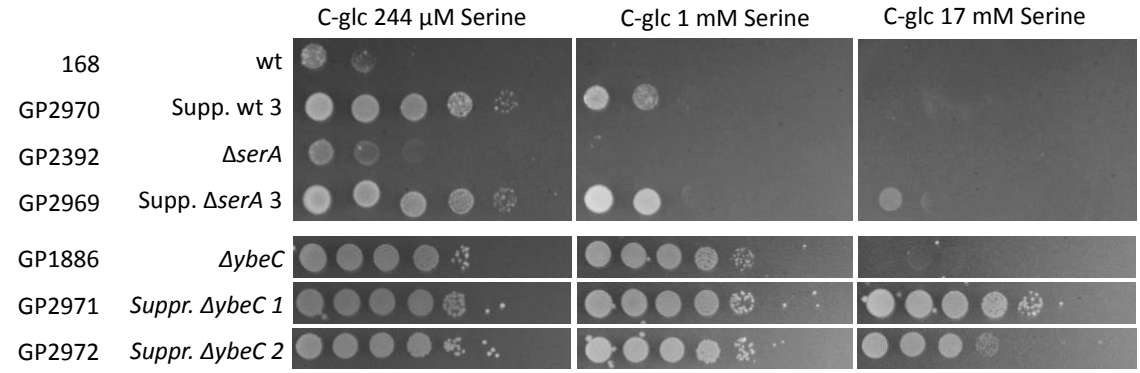


Figure 44: The drop dilution experiment of the isolated suppressor mutants in comparison to the parental strains. GP2970 was solated from the wild type strain 168, grown on C-glc with 244 µM serine. Strain GP2969 derives from the Δ*serA* strain GP2392, grown on 244 µM serine. The strains GP2971 and GP2972 were isolated from C-glc plates containing 10 mM and 17 mM serine and both derive from the Δ*ybeC* strain GP1886. A serial dilution was performed for all strains and the cells were plated on C-glc plates with 244 µM, 1 mM and 17 mM serine. The plates were incubated at 37°C for two days.

Suppressor mutants from the wild type strain 168 and the Δ*serA* strain (GP2392) were isolated, since they appear fast on C-glc plates with 244 µM and 1 mM serine. The suppressor wt 3 (GP2970) and the suppressor Δ*serA* 3 (GP2969) were isolated on 244 µM serine and a PCR and sequencing analysis of each *ybeC* gene showed no mutation. The *ybeC* deletion strain cannot grow on high serine concentrations and the emerge of suppressor mutants can also be observed. The suppressor mutants GP2971 and GP2972 were isolated on C-glc plates with 10 mM and 17 mM serine. All of

these isolated mutants grow better than their parental strains. To identify the suppressor mutation of each strain, WGS was applied to all suppressor strains (Table 12).

Table 12: The serine suppressor mutants and their identified mutations

Strain	Parental strain	Isolated on	Mutation found in WGS
GP2969	GP2392 (Δ*serA*)	244 µM serine	*thrR** (bp 90 deleted → frameshift)
GP2970	168	244 µM serine	Duplication /amplification of a genomic region, including *ilvA*
GP2971	GP1886 (Δ*ybeC*)	10 mM serine	*sdaAB* promoter mutation (-70 bp, C→A)
GP2972	GP1886 (Δ*ybeC*)	17 mM serine	*hom* promoter mutation (-56 bp, C→A)

The suppressor Δ*serA* 3 (GP2969) has a mutation in the gene *thrR*, encoding a transcriptional repressor of the genes of the threonine biosynthesis pathway (Rosenberg *et al.*, 2016). The mutation is a missing base pair at position 90 of the gene and leads to a frameshift and finally to a very short version of the protein. This probably inactivated protein is no longer able to repress the promoters of *thrD* and of the *hom-thrBC* operon (Figure 45). These genes are involved in threonine biosynthesis and the inactivation of the repressor probably leads to an increase of threonine biosynthesis.

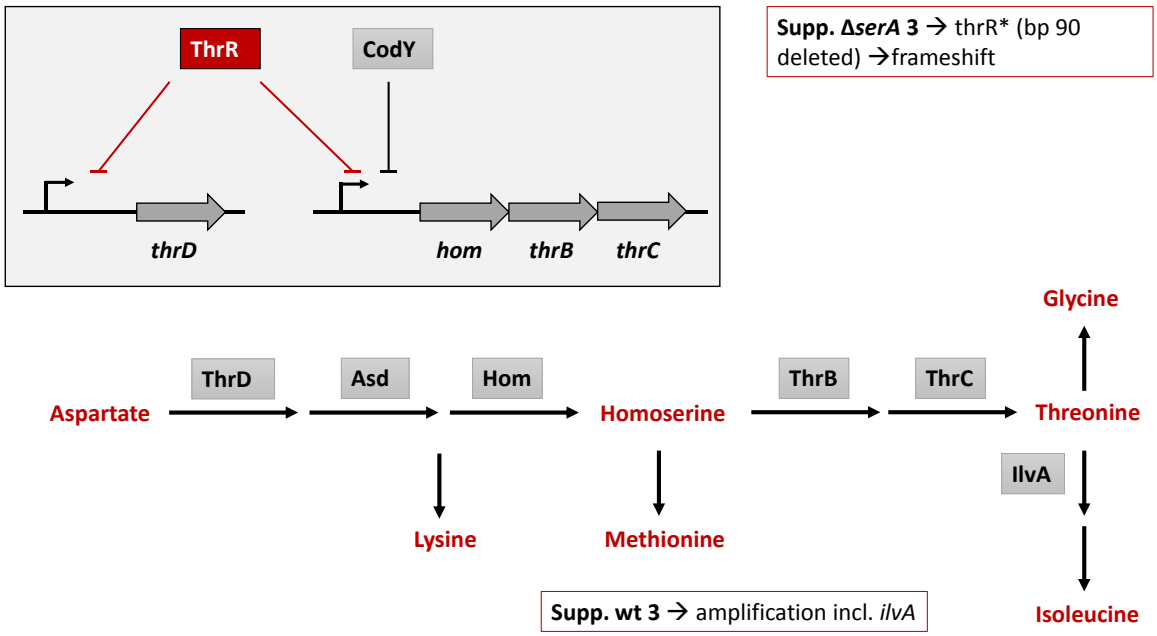


Figure 45: The suppressor mutations, that were found on high serine, are in genes that are involved in the threonine and isoleucine biosynthesis. The suppressor Δ*serA* 3 (GP2369) has a mutation in the transcriptional repressor gene *thrR* and the suppressor wt 3 (GP2970) shows amplification of the region including *ilvA*.

The second suppressor wt 3 (GP2970) has no mutation, but a duplication of a genomic region of 15.7 kb. This region includes 20 genes and shows a higher coverage in the WGS (Figure 46 A). The region is duplicated and inserted into the *ilvD* gene. The insertion borders can be amplified in the strain GP2970 with the primer pairs AK356/357 and AK353/354 and thereby confirm the insertion (Figure 46 B). However, the *ilvD* gene can still be amplified in the wild type size. Furthermore, the coverage across the duplication region varies, since the genes *yokABCD* show an even 3- fold higher coverage of around 183 reads in comparison to the coverage to the rest of the genome (around 60 reads) (Figure 46 C). This indicates that the duplication or even 3- fold amplification might be more complex.

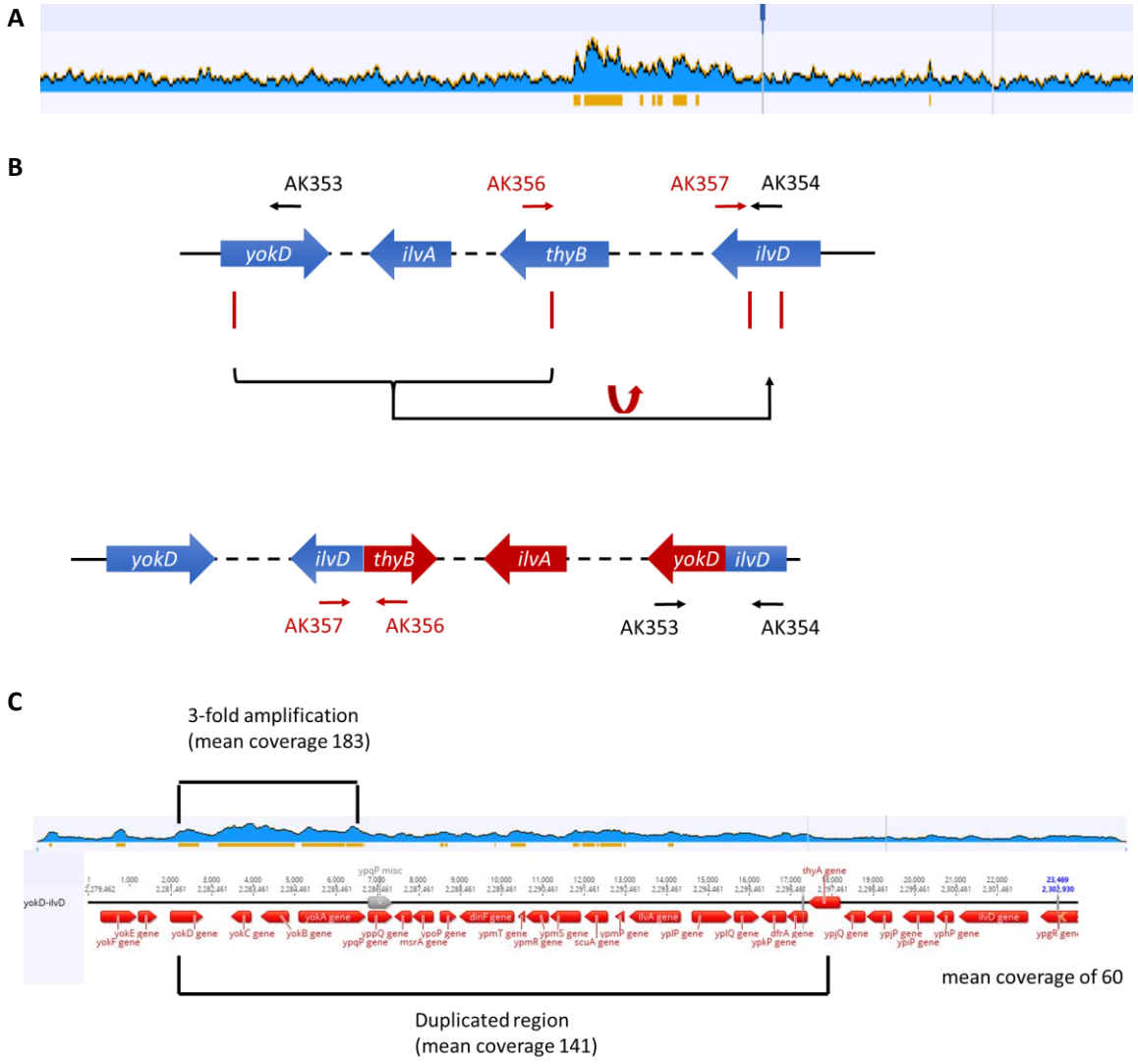


Figure 46: The duplication region in the suppressor mutant GP2970. A The duplication region was identified by WGS, since the coverage of reads is higher in this region. **B** The region from *yokD* to *thyB* is duplicated and inserted into the *ilvD* gene. The designed primer pairs AK356/357 and AK353/354 can amplifiy the boarders in the suppressor mutant. **C** The coverage of the genomic region including *yokABCD* is even higher and indicates that an additional amplification of this region might be possible.

The amplification region also includes *ilvA,* encoding the threonine dehydratase, which is involved in the production of isoleucine from threonine. To test, if the amplification of *ilvA* is the reason for the improved growth on C-glc plates with serine, an overexpression plasmid of *ilvA* was constructed (pGP2289). The gene *ilvA* was cloned into the vector pBQ200, which can be used for the constitutive overexpression. The plasmid pGP2289 was transformed into the wild type strain 168. This *ilvA* overexpression strain and a deletion mutant of *thrR* (BKE27910) (Koo *et al*., 2017) were compared with the growth of the suppressor mutants GP2969 and GP2970 (Figure 47).

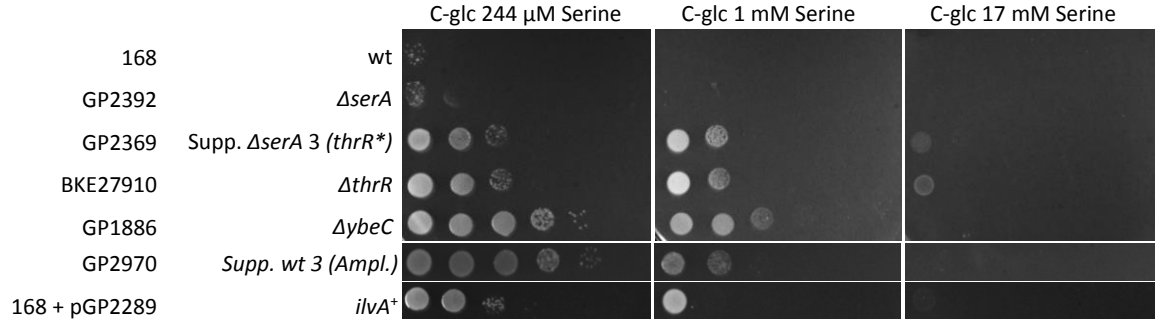


Figure 47: The drop dilution assay to test if the mutation in *thrR* and the amplification of the region including *ilvA,* influence the growth on C-glc serine medium. The mutations were found as suppressor mutants on high serine concentrations and are used as a comparison. The plates were incubated two days at 37°C.

It can be observed, that the suppressor Δ*serA* 3 (GP2369) shows the same growth as the Δ*thrR* deletion mutant. This leads to the assumption that the inactivation of ThrR results in a de-repression of the genes involved in the threonine pathway and finally in a growth advantage on high serine concentrations. The overexpression of *ilvA* leads also to a growth advantage on C-glc serine plates, but the suppressor wt 3 (GP2970) grows even better. This probably means that the amplification of another gene of the amplified region is also beneficial, beside *ilvA*. The genes in the amplified regions are listed in Table 13. Many unknown genes can be found in the amplification region. However, further analysis needs to be done. It is also possible, that a protein with a known function, has a second minor activity that rescues the serine toxicity phenotype. This was already observed for the threonine synthase ThrC, which has a minor threonine dehydratase activity (Rosenberg *et al.*, 2016).

The growth of the Δ*thrR* mutant and the *ilvA*$^+$ is not as good as the growth of the Δ*ybeC* mutant (Figure 47). The reduction of serine import seems to be of more advantage than deleting the repressor *thrR*. This indicates, that ThrR might not be the target of serine in the cell. To exclude a potential regulation of ThrR by serine, the expression of the genes, regulated by ThrR are analysed in the presence and absence of serine (Table 14). The strains BP558 and BP562 harbour a promoter *lacZ* fusion of the *hom* and the *thrD* promoter, integrated of into the *amyE* locus of the wild type

strain 168. The construction is based on the pAC5 plasmid. Furthermore, the strains BP557 and BP563 also harbour these *hom* and *thrD* promoter *lacZ* fusion constructs, but in a strain with an additional deletion in *thrR* (Rosenberg *et al.,* 2016).

Table 13: The genes of the amplification region including *ilvA*

Gene	Description of the function
yokD	Unknown
yokC	Unknown
yokB	Unknown
sprA	Excision of the SP-beta prophage
ypqP	Spore envelope polysaccharide biosynthesis
msrB	Regeneration of methionine, restoration of protein function after oxidative damage
msrA	Regeneration of methionine, restoration of protein function after oxidative damage
ypoP	Unknown
ypnP	Unknown
ypmT	Unknown
ypmS	Unknown
ypmR	Unknown
sco	Maturation of cytochrome c oxidase caa3
ypmP	Unknown
ilvA	biosynthesis of branched-chain amino acids
yplP	required for survival at low temperatures
ypkQ	Unknown
ypkP	Unknown
dfrA	biosynthesis of folate
thyB	biosynthesis of thymidine nucleotides

Table 14: The β-galactosidase activities of the *hom* and *thrD* promoters under the availability of serine

	Strain	C-glc	C-glc 244µM serine
β-galactosidase activity [Miller units]	**P_{hom}** (BP558)	283.1 ±42.6	302.8 ±46.6
	P_{hom} Δ*thrR* (BP557)	1427.8 ±239.2	1423.9 ±175.2
	P_{thrD} (BP562)	148.6 ±0.9	146.6 ±15.6
	P_{thrD} Δ*thrR* (BP563)	1502.7 ±284.4	1394 ±226.1

The activity of the promoter from the *hom* operon and the *thrD* promoter were analysed in C-glc medium with and without serine. There is no difference in promoter activity detectable in both strains if serine is added to the medium. The deletion of *thrR* leads to an increase of the *hom* operon and *thrD* expression. This can also be observed here, but the addition of serine also leads to no change in expression. These results indicate that the expression of both promoters is not directly influenced by serine and that the activity of the repressor ThrR is not regulated by serine. To conclude, ThrR is not the target of serine toxicity in *B. subtilis*.

The suppressors GP2971 and GP2972 derive from the *ybeC* deletion strain and were isolated on 10 and 17 mM serine. The suppressor strain GP2971 exhibits a mutation in the promoter of the operon *sdaAB-AA*, which encodes the L-serine deaminase. This protein is involved in the serine utilization by converting serine to pyruvate (Xu and Grant, 2013). The suppressor (GP2972) has a mutation in the *hom* operon promoter, that was already mentioned. Interestingly, the mutation is located in the binding side of the transcriptional repressor ThrR (Figure 48).

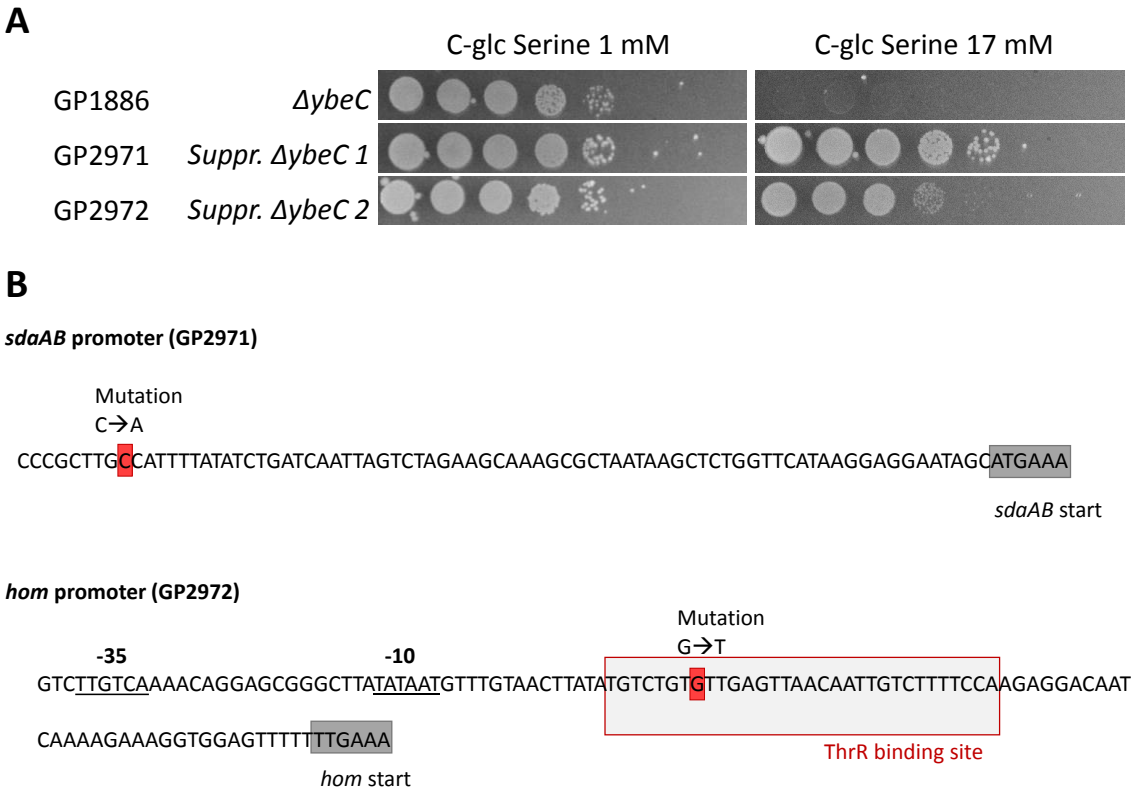


Figure 48: The suppressor mutations in the promoter regions of *sdaAB-AA* and the *hom* operon. A The Drop dilution assay of the suppressor mutants GP2971 and GP2972 in comparison to the parental strain GP1886 **B** The suppressor mutations can be found in the promoter regions of the *sdaAB-AA* operon and the *hom* operon. Furthermore, the mutation in front of the hom gene is in a binding site of the transcription repressor ThrR.

To analyse the effect of the mutation on the expression of the genes, *lacZ* fusions of the *sdaAB* and the *hom* promoters, with and without mutations, were constructed. The strain BP558 has a native *hom* promoter *lacZ*- fusion integrated into the *amyE* locus. The primerst MT24 and MT25 that were used for the construction of BP558, were used in this work to amplify the *hom* promoter with the mutation in the ThrR binding site (Rosenberg *et al*., 2016). This mutated *hom* promoter was cloned into the vector pAC5, to construct the plasmid pGP2296, which was further transformed into the strain 168 (GP2968). The native and the mutated *sdaAB* promoter was amplified with the primers AK379/380 and cloned into the pAC5 plasmids. The plasmids pGP2294 (*sdaAB* mutated) and pGP2295 (*sdaAB* native) were transformed into the wild type strain 168 to construct strains GP2966 (*sdaAB* mutated) and GP2967 (*sdaAB* native). The activity of these translational promoter-*lacZ* fusions was measured with a β-galactosidase activity. The strains was grown in different media to an OD_{600} of 0.5 and the β-galactosidase activity was measured as described in chapter 2.2.7. (Table 15).

Table 15: The β-galactosidase activity assay to analyse the influence of the promoter mutations on the expression of *sdaAB* and *hom*

Promoter	**P_{hom} (BP558)**	**P_{hom}* G56T (GP2968)**	**P_{sdaAB} (GP2967)**	**P_{sdaAB}* C70A (GP2966)**
β-galactosidase activity [Miller units]	273.6 ±34.3	973.3 ±83.2	7.4 ±2.2	368.4 ±47.8

These measurements show that the mutations of the promoter region of each operon lead to an increase in the expression. For the *sdaAB-AA* operon, these results indicate, that more copies of the L-serine deaminase are in the cell and more serine is converted to pyruvate. The toxic effect of serine is compensated by reducing the amount of serine in the cell. As already mentioned, the mutation in the *hom* operon promoter is in the binding site of the repressor ThrR and leads probably to a reduced binding of the protein to the DNA. Therefore, the expression of the promoter is not repressed and more enzymes from the threonine biosynthesis pathway are produced.

To summarize, two kinds of suppressor mutations could be identified for the growth on high serine concentrations. The first one leads either to the reduction of serine uptake by mutating the importer YbeC or to an increase of serine utilization by the upregulation of the *sdaAB-AA* expression. The second kind of mutation is the upregulation of threonine and isoleucine biosynthesis. Interestingly, the threonine biosynthesis is often upregulated by directly inactivating the repressor ThrR or by mutating the DNA binding targets of this protein. This link to the biosynthesis of threonine and especially to the *hom-thrB-thrC* operon is interesting, since it is known for *E. coli*, that serine can inhibit the homoserine dehydrogenase (ThrA) (Hama *et al.*, 1990).

However, also the genes *thrB* and *thrC* and their products could be potential targets. In *B. subtilis,* some amino acids can compensate the toxic effect of serine, if they are added additionally to the medium. Threonine, glycine, methionine, tryptophan, tyrosine are described to compensate low concentrations of 125 µM serine and furthermore, alanine, arginine, aspartate, glutamate and proline can compensate even 500 µM of serine. However, not all amino acids were tested for their potential compensation (Lachowicz *et al.*, 1996). The compensatory effect of some amino acids would also give another hint to whether the threonine pathway is affected by the serine toxicity. A drop dilution assay was performed with the wild type strain 168 on medium with 244 or 500 µM serine and one additional amino acid in a concentration of 500 µM (Figure 49).

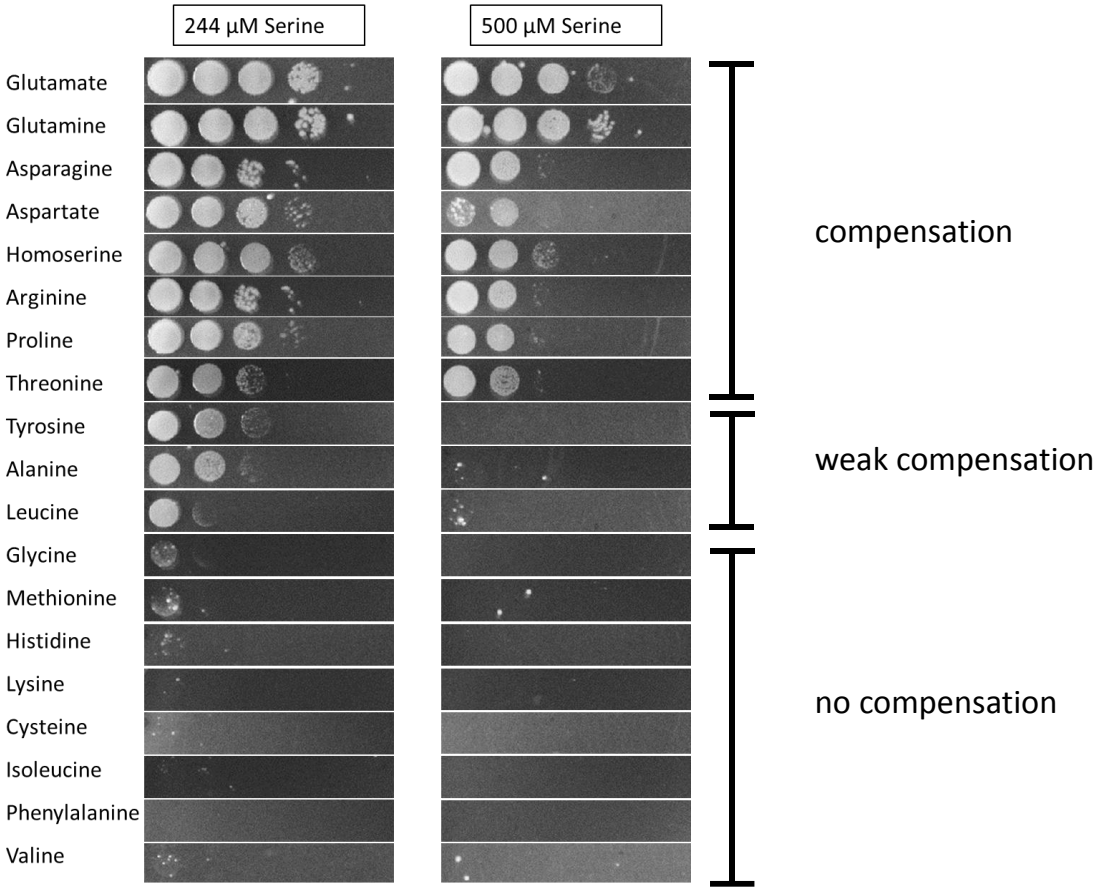


Figure 49: The drop dilution assay to test which amino acids can compensate the toxic effect of serine. The effect of 500 µM of each amino acid on the growth of the wild type strain 168 was analysed on plates with each 244 µM or 500 µM serine.

The addition of some amino acids lead to the compensation of the toxic effect of serine, since the wild type is able to grow on these plates. However, for some amino acids (tyrosine, alanine and leucine) the compensation is only possible with the low concentration of serine (244µM) and some amino acids are not able to compensate the effect of serine. It was previously mentioned (chapter 3.3.3.) that the compensation could be also due to the import of the amino acid by the same transporter as serine. The obtained results were illustrated in Figure 50.

Interestingly, aspartate and asparagine were detected to compensate the toxic serine effect. Aspartate is a precursor of the threonine biosynthesis and a high amount of it in the cell could also lead to a higher production of threonine. The addition of glutamate, glutamine, asparagine and proline might also lead to the same effect, because glutamate can be converted to α-ketoglutarate, an intermediate of the TCA cycle. Aspartate can then be synthesized from oxaloacetate. Furthermore, high glutamate concentrations are also needed for the transaminase reactions, that are involved in all amino acid biosynthesis pathways.

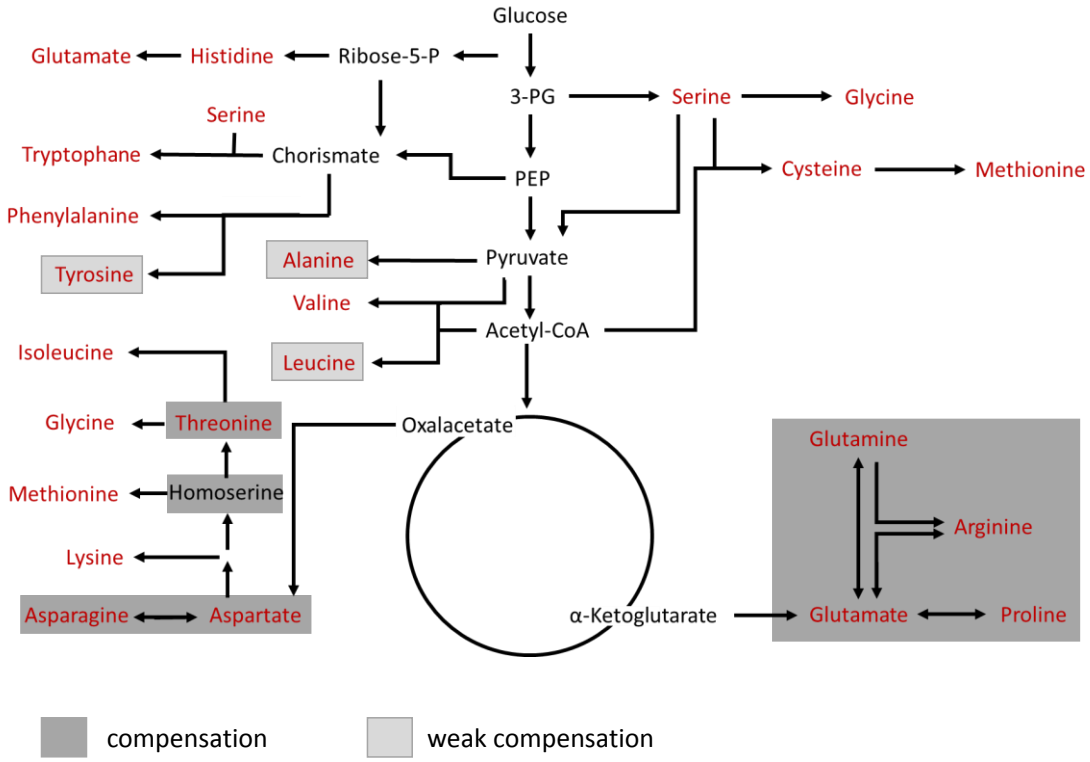


Figure 50: The amino acid biosynthesis pathways in *B. subtilis*. The amino acids that can compensate the toxic effect of serine are marked in grey.

It has to be noticed, that these mentioned amino acids were already detected in the screening for threonine importers (chapter 3.3.3.). Therefore, it is also possible, that some of these amino acids are also transported into cell by one of the serine/ threonine transporters. Furthermore, also threonine was detected, which could also be an effect of the shared transporter with serine. However, beside all amino acids, also homoserine was tested for its compensatory effect. Homoserine is formed by the homoserine dehydrogenase Hom and if this enzyme is affected by the serine concentration, the addition of homoserine can compensate the toxic effect. The obtained results support this theory, that the enzyme activity of the Hom protein must be altered by serine. To test this hypothesis, a Hom enzyme activity assay was performed. The reverse reaction of the homoserine dehydrogenase from L-homoserine and $NADP^+$ to L-aspartate 4-semialdehyde and NADPH was measured by the conversion of $NADP^+$ to NADH and the resulting change in absorbance

at 340 nm. The Hom protein was overexpressed from a plasmid in *E. coli* and purified. The first version of the protein was purified with a N-terminal SUMO-tag that could be cleaved by the SUMO protease (pGP2297). However, the cleavage was always only successful for half of the proteins and the protein solution showed no activity *in vitro*. As a second attempt a C-terminal strep-tagged construct was designed, but unfortunately this enzyme was also not active *in vitro* (data not shown).

Although it was not possible to measure the Hom activity *in vitro* and furthermore the serine influence on it, the results suggest a regulatory role of serine on the Hom activity. Some of the suppressor mutants that were isolated showed a link to the threonine pathway. It could be excluded that the transcriptional repressor ThrR of the threonine pathway is the serine target. Nevertheless, the operon *hom-thrBC* seems to be involved. The compensatory effect of homoserine on the serine toxicity strongly suggest, that the target of serine inhibition is the Hom enzyme. However, it could be shown, that the expression of the *hom-thrBC* operon is not influenced by the presence of serine.

3.5. The consequences for the *MiniBacillus* project (Blueprint 2.0)

The genes *ybeC*, *ybxG* and *bcaP* were identified to encode for serine/ threonine transporters. However, these are not the only serine and threonine importers in *B. subtilis*. The blueprint of the desired *MiniBacillus* strain includes the known amino acid importers, instead of the biosynthesis pathways. However, importers for serine were previously unknown in *B. subtilis*, therefore the biosynthesis pathway of serine was annotated to remain in the *MiniBacillus* strain (Reuß *et al.*, 2016). But the results of this work allow to keep one of the importers of serine, instead of the biosynthesis pathway. Although, YbeC seems to be the major serine importer, there is no need for this protein to remain in the final *MiniBacillu*s strain. An increased concentration of serine in the cell leads to the inhibition of the threonine pathway. In theory, one of the minor serine importers YbxG or BcaP should import enough serine into the cell to sustain good growth. The transporter BcaP will remain in the *MiniBacillus* strain anyway, since the transport of valine, isoleucine and also threonine is facilitated by this protein. To analyse if BcaP alone is able to sustain growth in LB-glc, growth curves were performed (Figure 51). The effect of the deletion of *ybeC* and *ybxG* was tested to confirm that the cell can survive with BcaP as the only known serine/ threonine transporter. Since the LB medium provides a lot of amino acids, the strains grow all similar. Even the combination with a Δ*serA* or Δ*thrC* mutant leads to no change in growth. These additional deletions were done to mimic the situation in the final *MiniBacillus* strain, which will be auxotrophic for most amino acids. To conclude, although we do not know all serine transporters in *B. subtilis*, the protein BcaP will probably import enough serine to sustain growth. The deletion of the major serine importer YbeC will probably have no effect on the growth of the strain. Furthermore, the current *MiniBacillus*

strain PG39 has already deleted *ybeC* and *ybxG*. However, the biosynthesis gene for serine are still in the genome, but the obtained results indicate, that the deletion of those will not lead to a serine shortage.

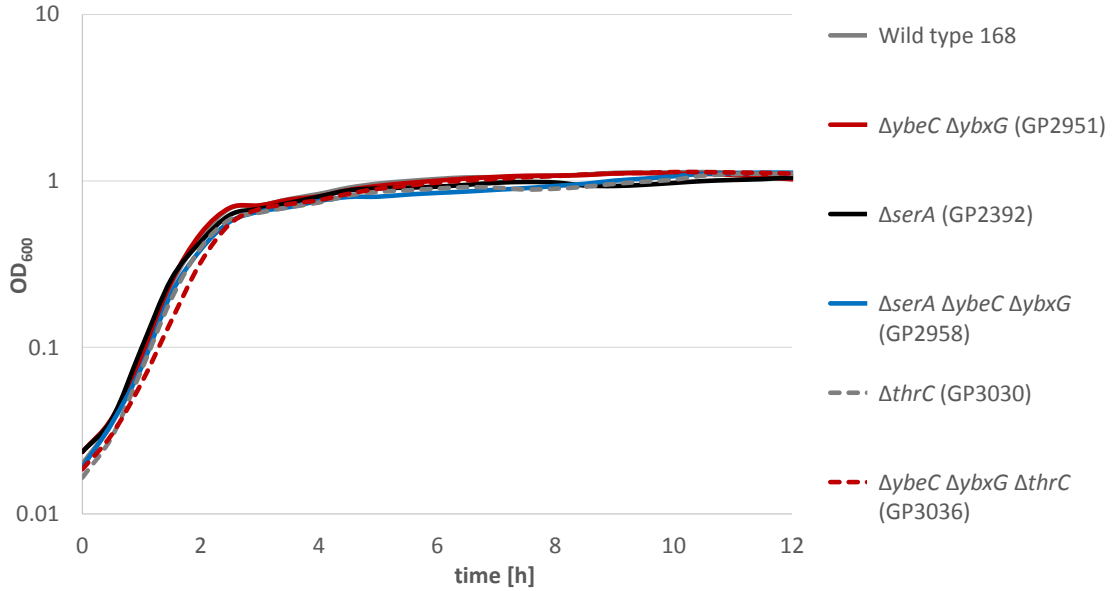


Figure 51: Growth curves in LB-glc medium to test if the lack of *ybeC* and *ybxG* might lead to a growth defect. The *serA* deletion leads to a serine auxotrophic strain and the *thrC* deletion to a threonine auxotrophic strain. The strains show no growth differences.

The results of this work can now contribute to an updated version of the *MiniBacillus* design, the blueprint 2.0. The genes encoding for the serine biosynthesis *serA, serB* and *serC* can be deleted and *bcaP* remains as the isoleucine, valine, threonine and serine importer. Furthermore, new data from *e.g.* the publication by Koo *et al.* (2018) can be included. They revealed new insights about the essentiality of genes by creating two deletion mutant libraries. Furthermore, the competence of the constructed deletion strains was tested, which is also from high importance for the *MiniBacillus* project. The number of genes in each category of the blueprint 2.0 are shown in Table 16.

Table 16: The comparison of the blueprint 1.0 and 2.0

Function	**Blueprint 1.0**		**Blueprint 2.0**	
	Protein genes	**RNA genes**	**Protein genes**	**RNA genes**
Information	197 (125)	119 (2)	201 (131)	119 (2)
Metabolism	218 (59)		215 (74)	
Cell division	81 (52)		84 (57)	
Integrity of the cell	16 (5)		17 (6)	
Other/ unknown	11 (2)		16 (7)	
Total	523 (243)	119 (2)	537 (275)	119 (2)

The total number of protein coding genes increases to 537 genes, from which 275 are essential. However, the number of RNA coding genes remains the same. The complete list of all included genes can be found in the supplementary information (chapter 6.5.). This new version of the *MiniBacillus* blueprint and the results of this work can contribute to improve the deletion process.

4. Discussion

4.1. The *MiniBacillus* project

One approach to understand the complexity of life is to reduce it. Several attempts in different organisms were performed. Especially, the minimal genome strains of *B. subtilis* show enhanced protein productivity and are therefore interesting for biotechnological applications (Morimoto *et al.*, 2008; Aguilar Suárez *et al.*, 2019). The *MiniBacillus* project attempts to create a minimal *B. subtilis* cell, in which every gene has an assigned function (Reuß *et al.*, 2016). An important goal in this project is to gain more knowledge about the organism and to utilize this information again to adapt the deletion process. During this work, a *MiniBacillus* strain with a genome reduction of 40.51% was created, which is the most extensive described reduction in a top-down approach and also for the model organism *B. subtilis*. The strain PG39 is still stable and shows good growth in the standard conditions LB-glc medium at 37°C. However, the maximal biomass that is reached in the growth curves is slightly reduced. But since the overall growth is stable, this will probably have no disadvantage for the *MiniBacillus* project. A slightly reduced growth of a genome reduced *B. subtilis* strain was already observed before, but this had no effect on the protein productivity (Ara *et al.*, 2007). Similarly, the strain PG39 could also show a similar or even better protein productivity than the ancestor strain PG10, although PG39 cannot reach the final biomass of PG10 in LB-glc medium (Aguilar Suárez *et al.*, 2019).

The multi-omics analysis is very important for the project, since it provides a multi-layered picture of the situation in the cell. Especially the transcriptome data has a high impact, since changes in the expression of genes caused by the deletions can be identified. The operons *paiAB* and *mhqNOP* were shown to be higher expressed in the strain PG10 in comparison to the ancestor strain Δ6 (Reuß *et al.,* 2017). Since PG10 was the last strain to be analysed with the multi-omics approach, a new set of data for the current strains would provide new insights. The proteome of the strain PG38 was analysed during this work. The data indicate, that the proteome of PG10 and PG39 are very similar. There is no evidence for the overexpression of a certain pathway. To get insights into every aspect of the strain and to ensure a stable strain, a complete multi-omics data set is necessary.

The described point mutation in the low-affinity phosphate transporter gene *pit*, lead to a high impact in the *MiniBacillus* strain (Reuß *et al.,* 2017). The phosphate level in the cell decreased and the PhoPR system was activated. This leads to the activation of genes for the acquisition of phosphate, like the phosphate ABC transporter for the high-affinity uptake of phosphate. On the other hand, the operons *tagAB* and *tagDEFGH* for the biosynthesis of teichoic acid are repressed (Allenby *et al.*, 2005). This might have caused the observed mutations in the *tagAB* promoter and

in the gene *yqgS*, encoding for the minor lipoteichoic acid synthase to counteract the constant regulation by the PhoPR system (chapter 3.1.3). Similarly, the phosphodiesterase / alkaline phosphatase PhoD would also be higher expressed in the strain PG18 with the *pit* mutation. PhoD is active during phosphate starvation conditions and degrades wall teichoic acid (Eder *et al.*, 1996). This could lead to an unwanted damage of the cell wall. Because of this reason, the analysis of the *MiniBacillus* strains by WGS is very important to detect such point mutations and to adapt the deletion process, before counteracting suppressor mutations accumulate.

A main problem of the *MiniBacillus* project is, that the deletion process becomes more difficult, since the deletion regions become smaller and thereby the deletion process decelerates. A solution for this might be the defragmentation approach to cluster functionally related genes together in one locus. The insertion of the glycolytic cassette shows, that the defragmentation approach in general is possible and that it could increase the deletion process. The clustering of functionally related genes was already used in metabolic engineering (Qi *et al.*, 2015). Metabolic engineering enables the construction of customized strains (Oesterle *et al.*, 2017). Such strains would be beneficial for biotechnology, since they can be easily modified, if a certain pathway should be deleted to increase the production of a certain product. The expression of certain pathways can also be regulated easily. But metabolic engineering is a complicated process, since the transcriptional units need to be considered. Some genes can be transferred with their natural promoter or terminator, but some genes have to be fused to a new promoter or terminator to sustain the correct transcription. If an artificial promoter is used, it has to maintain a certain level of expression and also a similar regulation that was given by the natural promoter. Otherwise the different regulation will lead to an imbalance in the cell. As an example, the higher expression of the serine biosynthesis genes, would lead to a growth inhibition by the increasing serine concentrations (Lachowicz *et al.*, 1996). Furthermore, the insertion of the modified DNA segments needs to be tested for functionality, before the natural locus is deleted. Several *B. subtilis* production strains were already constructed by metabolic engineering. By the insertion of several genes, a *B. subtilis* strain was created, that is able to produce D-lactic acid, a compound of poly lactic acid (Awasthi *et al.*, 2018). However, it is questionable if the defragmentation approach provides the final solution for the decrease of the deletion process.

A genomic approach of metabolic engineering is represented by the bottom-up genome reduction strain JCVI-syn3.0. The strain is synthesized. A first approach with a set of genes, which are known to be necessary for the strain, was unsuccessful. Therefore, the genome of the final minimized strain still contains 149 genes with unknown function (Hutchison *et al.*, 2016).

Since a main goal of the *MiniBacillus* project is to gain more knowledge about the cell, this work focuses additionally on the analysis of different pathways, like the TCA cycle or the

biosynthesis and uptake of amino acids. The results of this work and the new information from several publications were incorporated to a new blueprint 2.0, based on the original blueprint of a minimal cell (Reuß *et al*., 2016). Especially the work of Koo *et al*. (2017) identified many new essential genes and competence genes, which are important for the *MiniBacillus* strains. Furthermore, the missing gene of the biosynthesis of serine was discovered to be *ysaA*, which was therefore renamed to *serB*. The constant adaptation and re-evaluation of the blueprint is necessary to improve the deletion process. Similarly, the characterization of genes with unknown function is important to reduce the risk of dead ends in the *MiniBacillus* project.

4.2. The functions of the two citrate synthases

The role of the two citrate synthases of *B. subtilis* is unknown. They are differently regulated and the deletion of *citZ* has a greater impact on the growth in C-malate medium than the deletion of *citA* (chapter 3.2.1.) (Jin and Sonenshein, 1994a). The reaction of the citrate synthase is important, since it provides an important link to glycolysis (Jin and Sonenshein, 1994b). This could mean that the two citrate synthase enzymes catalyse the same reaction under different conditions to sustain a certain level of influx into the TCA cycle. Interestingly, the expression of the aconitase CitB and the isocitrate dehydrogenase gene Icd, which convert citrate further, are also regulated like the major citrate synthase CitZ (Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2002). The expression is repressed in the presence of glucose and glutamate. This means that the citrate which is produced under these conditions by the minor citrate synthase CitA, cannot be metabolized further. This would lead to an increase in the intracellular citrate concentration, which was shown to affect the ability to sporulate and is therefore a disadvantage for the cell (Craig *et al.*, 1997).

The activity assay of the two citrate synthases shows that the major citrate synthase CitZ can form more citrate than the minor citrate synthase CitA. This leads to the assumption, that oxaloacetate and acetyl-CoA might not be the ideal substrates for the CitA enzyme. Another paralogous protein of the citrate synthases is annotated in the genome of *B. subtilis*, the 2-methylcitrate synthase MmgD (Reddick and Williams, 2008). This enzyme catalyses the reaction of propionyl-CoA and oxaloacetate to 2-methylcitrate and CoA, which is similar to the citrate synthase reaction (Reddick *et al.*, 2017). The enzyme is part of the methylcitric acid cycle to produce pyruvate from propionate. For organisms like *E. coli,* this metabolic pathway is important to grow with propionate as the single carbon source (Brock *et al.*, 2002). In *B. subtilis*, the gene *mmgD* is encoded in the *mmg* operon, which is part of the mother cell metabolism. This operon is only active at a certain time during sporulation, mediated by the sigma factor σ^E controlled promoter. Additionally, the promoter is controlled by carbon catabolite repression through CcpA, which leads to a repression in the presence of glucose. The enzyme MmgD shows also citrate synthase activity

(Bryan *et al.*, 1996; Reddick *et al.*, 2017). This could mean that the citrate synthase CitA also favours other substrates and is therefore involved in another metabolism. This would also explain the observation, that CitA can only partially compensate the loss of the major citrate synthase CitZ.

Interestingly, even three citrate synthase genes were identified in *Saccharopolyspora erythraea*, a species of actinobacteria (Oliynyk *et al.*, 2007). The first gene *gltA-2* is highly similar to the *citZ* gene from *B. subtilis* and it is similarly repressed by a regulator of carbon catabolite repression. In contrast, the genes *citA* and *citA4* from *S. erythraea* are similar to the *citA* gene from *B. subtilis*. The *citA* and *citA4* genes are transcriptionally repressed by GlnR, a regulator of the nitrogen metabolism and DasR, a regulator of the amino-sugar metabolism (Yao *et al.*, 2014; Liao *et al.*, 2014). These similarities to the genes of *S. erythraea* might hint to a related regulation in *B. subtilis*. The fact that glutamate stimulates the expression of citA in glucose containing medium could also hintto a regulation by the nitrogen metabolism. In contrast, the expression of citZ is even higher repressed in medium containing glucose and glutamate, than in medium with only glutamate (Jin and Sonenshein, 1994a). However, this topic needs to be further analysed to elucidate the main function of the CitA protein.

4.3. The role of the TCA cycle in *B. subtilis*

The TCA cycle is not only a central point of carbon metabolism. It also provides important links to nitrogen metabolism and to several amino acid pathways. This work provides evidence, that the enzymes involved in this pathway are furthermore linked to other important processes in the cell.

The deletion of the complete TCA cycle is possible in *B. subtilis* and the involved enzymes are not necessary for the growth in LB-glc medium. Other organisms, like *Mycoplasma pneumoniae* harbour no TCA cycle. This is due to the fact, that the host provides all nutrients, which are imported into the cell (Manolukas *et al.*, 1988; Halbedel *et al.*, 2007). Accordingly, the TCA cycle deficient *B. subtilis* strain was not able to grow in C-glc minimal medium. Only the addition of glutamate led to the restoration of growth, since the TCA cycle deletion strain is glutamate auxotroph (chapter 3.2.3.). The glutamate auxotrophy was already observed for the single deletion mutants of the citrate synthase and the aconitase (Jin and Sonenshein, 1994b; Craig *et al.*, 1997).

Interestingly, the deletion of the TCA cycle led to a reduced competence of the strain. This link of the TCA cycle to the competence could be narrowed down to the deletion of the succinate dehydrogenase complex, encoded by *sdhCAB*. This complex has an additional function in the respiratory chain, since SdhC is part of the cytochrome b558 (Hederstedt and Rutberg, 1983; Baureder and Hederstedt, 2011). It was already mentioned, that a similar protein, the NADH dehydrogenase Ndh, is also important for competence, since the deletion strain shows no transformants (Koo *et al*., 2017). In contrast, the single deletion strains of *sdhC*, *sdhB* and *sdhA* are

transformable (Koo *et al.,* 2017). The deletion of *sdhC* has therefore not the same impact as the deletion of *ndh*, although both are involved in the electron transfer to menaquinone (Matsson *et al.*, 2000). Furthermore, the *ndh* deletion mutation facilitates the growth without a cell wall, as L-form cells. This was due to the reduction of oxidative stress (Kawaii *et al*., 2015). Similarly, other genes involved in the respiratory chain, like *qoxB*, encoding for the cytochrome aa3 quinol oxidase subunit I, also showed L-form cell formation (Santana *et al.*, 1992; Kawaii *et al*., 2015). Interestingly, this phenotype could also be observed for the TCA deletion strain, if no carbon source is added to the medium. Due to the similarity to Ndh and the role in the respiratory chain, the deletion of *sdhC* might be the reason for the observed phenotype. Furthermore, the deletion of the complete TCA cycle could also have an impact. The TCA cycle produces the major part of the reducing agents $FADH_2$, $NADH+H^+$ and $NADPH+H^+$, which are further used in the respiratory chain to produce the energy equivalent ATP (Nakamura *et al.*, 2011) (Figure 52).

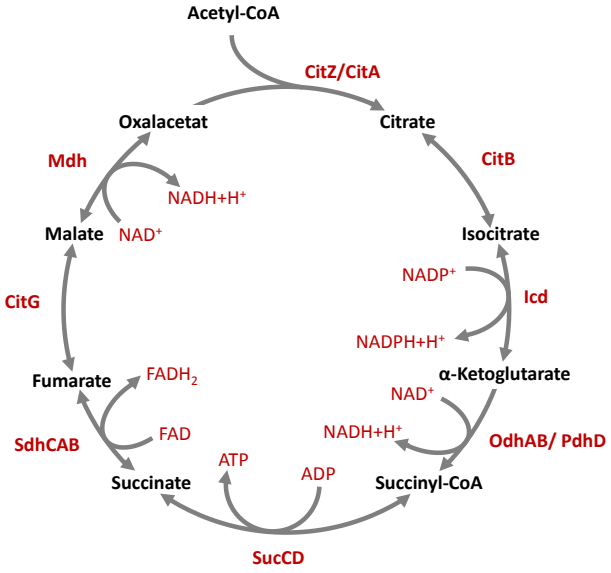


Figure 52: The TCA cycle of *B. subtilis*. The intermediates $NADH+H^+$, $NADPH+H^+$, $FADH_2$ and ATP are produces in different steps in the TCA cycle.

If the TCA cycle does not operate, less reducing agents are produced, which leads to a decrease of the activity of the respiratory chain. This is necessary to promote L-form growth since the oxidative stress is reduced (Kawai *et al.,* 2015). The addition of glucose leads to a the production of more NADH+H+ by the glycolysis, and thereby to an increase of the respiratory chain activity and the oxidative stress. This could prevent the formation of L-form cells. However, the L-form phenotype is probably combination of several effects. The observation that the addition of glucose can reverse the L-form formation indicates that the TCA cycle strain cannot produce enough peptidoglycan to sustain a stable cell wall. This might be due to the aspartate level in the cell. Aspartate can be formed from oxaloacetate, catalysed by the aspartate transaminase AspB (Dajnowicz *et al.*, 2017). A deletion mutant of this enzyme is auxotrophic for aspartate and asparagine. The aspartate

limitation leads to a decrease of 2-6-diaminopimelate (mDAP), a peptidoglycan precursor, which finally also reduces the peptidoglycan synthesis (Zhao *et al.*, 2018). In the TCA cycle deletion strain, less oxaloacetate is produced and therefore less aspartate by AspB. This might be the reason for the decrease in peptidoglycan formation. In contrast, the addition of glucose leads to the increased production of pyruvate that can be further metabolised by the pyruvate carboxylase PycA to oxaloacetate. This leads again to an increase in aspartate and furthermore in an increase of peptidoglycan production. Additionally, the reaction catalysed by AspB is a transaminase reaction, which needs high amounts of glutamate (Zhao *et al.*, 2018). Since the TCA cycle deletion mutant is also glutamate auxotroph, this might have also an effect on the production of aspartate. Interestingly, *Mycoplasma* cells have no TCA cycle and additionally no cell wall (Fraser *et al.*, 1995). They do not produce peptidoglycan and the TCA cycle is not necessary as a link to this pathway.

Furthermore, the reduced peptidoglycan synthesis could also have an effect on sporulation. The TCA cycle deletion strain is not able to form spores. Since spores are coated with a layer of peptidoglycan, the lack of peptidoglycan might be one of the reasons for the blockage of sporulation (Tocheva *et al.*, 2013). In previous studies, the block in sporulation was always narrowed to the accumulation of TCA intermediates, due to the deletion of single TCA cycle enzymes. The deletion of the aconitase CitB results in the accumulation of citrate, which builds chelating complexes with divalent ions like Mn^{2+} and Fe^{2+}. These ions are used to initiate the Spo0A phosphorelay and thereby sporulation (Craig *et al.*, 1997). This could also be shown for the *icd* mutant, but the addition of divalent ions and the lowering of the pH could lead to a complementation of the sporulation effect. Furthermore, the additional deletion of the major citrate synthase CitZ leads to a decrease of citrate in the cell and also to a compensation of the sporulation defect (Matsuno *et al.*, 1999). Since the complete TCA cycle was deleted, no intermediates should have accumulated that lead to a block in sporulation. However, the TCA cycle deletion strain is still unable to form spores and the reason for that still needs to be further investigated.

Spo0A plays also as role in genetic competence (Mirouze *et al.*, 2012). The level of phosphorylated Spo0A (Spo0A*) in the cell is an important indicator for the cell faith. At low Spo0A* concentrations the competence is inhibited, since ComK is repressed by the AbrB-Rok mechanism. At a certain intermediate level of Spo0A*, the competence is again possible. But if the Spo0A* reaches high levels, the competence is again inhibited, in this case by the SinI-SinR mechanism (Fujita *et al.*, 2005). In contrast, ComK can also regulate sporulation during competence. ComK activates the expression of RapH, a part of the Rap system, which dephosphorylates again Spo0F, a phosphotransferase of the sporulation initiation phosphorelay (Smits *et al.*, 2007). This might also be the reason for the observation, that high *comKS* levels have a negative effect on the sporulation

of the *citZ-icd-mdh* mutant. The lack of peptidoglycan synthesis cannot be the reason for the reduced spore formation in this case, since the sugar and carbon source mannitol is added to induce competence.

To conclude, the TCA cycle enzymes are not only important for central carbon metabolism, furthermore they also provide links to genetic competence, the cell morphology and sporulation. The blueprint of a minimal cell does not include the TCA cycle, since in the complex LB-glc medium, enough glucose is provided to gain energy from glycolysis and pentose-phosphate pathway (Reuß *et al.,* 2016). Furthermore, the genes of the TCA cycle and also the respiratory chain are repressed in the presence of glucose, whereas the genes of glycolysis and overflow metabolism are upregulated (Blencke *et al.*, 2003).. Although, the current *MiniBacillus* strain is already unable to form spores, competence is needed to sustain a stable working strain. Furthermore, the cell morphology can be critical, since this might have further impact on other cellular processes and the stability of the strain.

4.4. The serine/ threonine transporter of *B. subtilis*

High concentrations of serine in minimal medium have a growth inhibitory effect on *B. subtilis* cells (Lachowicz *et al.*, 1996). This indicates a potential complex regulation of serine uptake to sustain a non-toxic level of serine in the cell. Similarly, serine inhibits also the growth of *E. coli* cells in high concentrations (Hama *et al.*, 1990). Several serine uptake systems are described in *E. coli*, namely the serine-threonine system (SstT, TdcC), the osmotic shock sensitive alanine, serine, threonine and leucine system (CycA) and a high-affintiy import system, specific for L-serine (SdaC) (Robbins and Oxender, 1973; Hama *et al.*, 1988; Shao *et al.*, 1994; Ogawa *et al.*, 1998). This suggest that *B. subtilis* also harbours several serine uptake systems.

The results of this work indicate that the genes *ybeC*, *ybxG* and *bcaP* encode for serine/ threonine importers. YbeC seems to be a low-affinity serine transporter, which transports the major part of serine into cell. The *ybeC* deletion strain has a clear growth advantage on C-glc minimal plates with a serine concentration that inhibits the growth of the wild type cells. The low-affinity transporters for the uptake of valine and isoleucine are not characterized, but they are only expressed and active under high substrate conditions (Belitsky, 2015). Although there was no change in expression of YbeC in the absence of serine, the expression needs to be further investigated under different serine concentrations to characterize YbeC further. Many suppressor mutants can be isolated that mutated YbeC in a way, that destroys the protein or remove the C-terminal part. Especially the mutation in the C-terminus of the protein is very interesting, since the C-terminus is most likely located in the cytoplasma and could harbour a regulatory function. It could sense a signal from the cell and adapt the import of certain intermediates. In contrast, the C-

terminus could also transmit a signal to a regulatory mechanism in the cell. The L- and D-serine importer YhaO of enterohaemorrhagic *E. coli* (EHEC), is involved in the activation of a type II secretion system, which is essential for virulence (Connolly *et al.*, 2016; Pifer *et al.*, 2018). This suggests, that YbeC could also have a second regulatory function in the cell. The expression of the YbeC protein without the C-terminal part in *E. coli* shows, that it has not the same activity as the complete enzyme, since the deletion of the C-terminal part could also lead to a conformational change of the complete protein and thereby to an inactive protein.

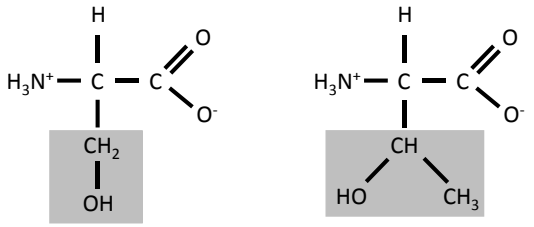
Previous studies suggested the protein SteT to be a serine/ threonine of from the L-amino acid transporter (LAT) family. The transport activity was tested by the construction of proteoliposomes with the desired transporter (Bartoccioni *et al.*, 2010; Rodríguez-Banqueri *et al.*, 2016). However, this protein showed no serine import activity under the tested conditions in this work. But *steT* could be important under different conditions or since it is annotated as a serine/ threonine exchanger, it could also export serine out of the cell.

The transporters YbxG and BcaP were identified to be also involved in the serine import in this work. The triple deletion mutant of *ybeC*, *ybxG* and *bcaP* is resistant to even very high concentrations of 100 mM serine. Interestingly, the single deletion of *bcaP* and *ybxG* leads not to the same growth advantage as the deletion of *ybeC*. Nevertheless, BcaP seems to have a greater impact than YbxG. Both proteins could be active as high-affinity transporters, or they are just minor low-affinity serine uptake systems. This needs to be further analyzed by testing the growth of the deletion strains in lower serine concentrations. Especially, the serine auxotrophic mutants need to be tested, since the serine uptake is here essential. Furthermore, the expression of the transporters at different serine and also threonine concentrations could be interesting. The expression of YbeC, BcaP and YbxG in *E. coli* shows almost the same growth inhibition. This could lead to the assumption that the transporters are differently regulated. Similarly, the valine and isoleucine transporter BrnQ has just a minor high-affinity transport activity, since its expression is lower than the expression of BcaP (Belitsky, 2015).

Not all serine importers of *B. subtilis* could be identified in this study, since the combination with the *serA* deletion is still possible. The serine auxotrophic strain with the deletion in the three identified serine transporters is still able to grow on plates with serine. This indicates, that serine can still be imported into the cell and that the transport is sufficient to sustain good growth and no excess of serine is imported to the cell that could be harmful. In the yeast like fungus *Pneumocystis carinii*, leucine, serine and glutamine are imported by the same transporter. But the transport is not dependent on sodium or the energy from the ATP hydrolysis and it is suspected to be a diffusion mechanism (Basselin *et al.*, 2001). This could also be possible for the detected rest import by the

deletion mutant of the three serine transporters and *serA*. Serine could diffuse into the cell by a channel protein, that is not energy dependent.

Furthermore, all three transporters have additionally a threonine import function. The two amino acids serine and threonine have both polar side chains and are very similar to each other (Figure 53).



Serine **Threonine**

Figure 53: The amino acids serine and threonine are very similar. Both molecules harbour a polar side chain.

Some serine/ threonine importers were already described in other organisms. The protein SstT from *E. coli,* is a Na+/ serine importer with an additional threonine import function (Ogawa *et al.*, 1998). Furthermore, a similar protein SstT from *Porphyromonas gingivalis* is known (Dashper *et al.*, 2001). The threonine import activities of the three serine/threonine importers YbeC, BcaP and YbxG of *B. subtilis* show also differences. Since high threonine concentrations were shown to be toxic for the cell, this effect was used in this work to test if the deletion of one transporter might lead to a growth advantage (Teas, 1950). Under the tested conditions, the deletion of *bcaP* led to the highest growth. This leads to the assumption, that BcaP is the low-affinity transporter for threonine. The deletion of *ybxG* and *ybeC* have just small effects and can possibly be considered as high-affinity transporter, but this needs to be further analysed. BcaP mediates additionally the high-affinity uptake of isoleucine and valine as a permease and it could be already shown that it is involved in the uptake of threonine (Belitsky, 2015). YbxG is a putative threonine transporter, since the deletion mutant shows resistance to the toxic threonine derivative 4-hydroxy-threonine (Commichau *et al.*, 2015). The three proteins YbeC, YbxG and BcaP are not the only threonine transporter, encoded in the genome of *B. subtilis*, since the combination with the threonine auxotrophic strain, the *thrC* deletion, is still possible. ThrC is the threonine synthase, that catalyses the last step in the threonine biosynthesis pathway from O-phospho-L-homoserine to threonine (Parsot, 1986). Interestingly, the triple importer deletion mutant with the *thrC* deletion cannot grow on very low concentrations of threonine. This might indicate, that all high-affinity threonine transporters are deleted, that would be active under low threonine conditions. If *ybeC* is still in the strain (Δ*thrC* Δ*bcaP* Δ*ybxG*), almost no difference to the deletion mutant of all three transporters can be observed. In contrast, if *ybxG* remains in the strain (Δ*thrC* Δ*bcaP* Δ*ybeC*), the strain growth better than the triple transporter

deletion strain. These results suggest, that YbxG might be important at low threonine concentrations and could be therefore a high-affinity transporter.

Most amino acids transporters are able to transport several amino acids. It was previously reported that the branched-chain amino acid permease BcaP from *Lactococcus lactis* seems to additionally transport methionine into the cell (Den Hengst *et al.*, 2006). The *B. subtilis* BcaP protein imports less isoleucine into the cell in the presence of the amino acids valine, serine, threonine, alanine and asparagine (Belitsky, 2015). A possible co-transport with serine was also tested in this work. The growth inhibitory effect of serine was used to identify amino acids that can compensate the toxicity. The amino acids threonine, asparagine, aspartate, glutamate, glutamine, arginine and proline were found to compensate the toxic effect of serine. Additionally, tyrosine, alanine and leucine could weakly compensate the effect. However, the compensation can be due to the import competition with another amino acid or due to the compensation of the toxic effect directly within the cell. Since alanine and asparagine were also found to reduce the isoleucine import function of BcaP, this transporter most likely imports these amino acids. The other identified amino acids are candidates for the co-transport by YbeC, YbxG or the unidentified serine or threonine importer. This can only be elucidated, if all serine and threonine transporters are known. Strains with only one serine/ threonine transporter can then be tested for the reduced toxicity of serine or threonine in the presence of the identified amino acids. Especially the combination with the data from the the threonine toxicity compensation experiment might be interesting. Amino acids that are found in both experiments with serine and threonine are most ikely co-transported into the cell. In contrast, those amino acids that can be only found in the serine or threonine competition experiment are counteracting the toxicity in the cell.

The results of this work indicate, that *B. subtilis* harbours at least three serine/ threonine importers. The reason for that might be that the uptake of the different co-transported amino acids needs to be adapted to the environmental conditions. The expression of the transporters can therefore be differentially regulated. BcaP expression is under control of the global regulator CodY and it is repressed under high branched-chain amino acid concentrations (Molle *et al.*, 2003). The regulation of YbeC is independent on the presence of serine in the cell (chapter 3.3.1.). Furthermore, the regulation of *ybxG* and *ybeC* need to be further analysed.

In the future, the missing serine/ threonine transporters need to be identified and a first approach therefore would be the screening of paralogous proteins of BcaP, YbeC and YbxG (Table 17). Several of these paralogous proteins are of known function, like GabP, the gamma-amino butyric acid permease and minor proline permease (Zaprasis *et al.*, 2014). Some proteins are also of unknown function, that still need to be characterized. Another candidate for a threonine

importer might also be SteT, since no serine uptake function of this serine/ threonine exchanger could be shown in this work.

Table 17: Paralogous proteins of the known serine/ threonine importers

Serine/ threonine importer	Paralogous proteins
BcaP	MtrA
YbxG	RocC, RocE, YbgF, YdgF, AapA, GabP, YtnA, HutM, YcbW
YbeC	YveA

4.5. The regulatory role of serine

Some amino acids were described to inhibit the cell growth and sporulation in high concentrations and are therefore toxic for *B. subtilis*. Especially, threonine, valine and isoleucine have an inhibitory effect (Lamb and Bott, 1979b; Lamb and Bott, 1979a). These toxic effects were used to analyze the regulatory mechanisms of amino acids in the cell. The threonine inhibitory effect was described as a block in valine biosynthesis. The cells starved of valine and their growth is therefore inhibited (Lamb and Bott, 1979b; Lamb and Bott, 1979a).

Serine can also inhibit the growth of *B. subtilis* if it is present in minimal medium as the single amino acid (Lachowicz *et al.*, 1996). Since the exact mechanism of toxicity is unknown, this work also analyzed the target of serine inhibition further. *B. subtilis* rapidly forms different suppressor mutants on minimal plates with serine. The first characterized mutations affect the import of serine, by the mutation of the previously characterized serine/ threonine importer *ybeC.* Several serine importers are known, but YbeC seems to be the low-affinity transporter, that is active under the tested high serine conditions. Different mutations in *ybeC* could be identified, that destroy the protein or remove the C-terminal part, that seems to be important for the active protein. The second type of mutations upregulated the serine degradation pathway to lower the intracellular serine concentration. Finally, the third type of mutations lead to an increase in the threonine/ isoleucine biosynthesis pathway. This leads to the assumption, that serine might have a function in the inhibition of the threonine pathway. Some amino acids, like aspartate and glutamate can compensate the toxic effect of serine (Lachowicz *et al.*, 1996). Furthermore, threonine can compensate the toxic effect, but more interestingly, also homoserine was able to compensate it. Therefore, the point of inhibition must be in the threonine pathway upstream of homoserine. The enzyme that catalyzes the reaction of L-aspartate semi-aldehyde to homoserine is the homoserine dehydrogenase Hom (Parsot and Cohen, 1988). The homoserine dehydrogenase is the target of the serine toxicity in *E. coli*, so this enzyme could also be the serine target in *B. subtilis* (Hama *et al.*, 1990; Hama *et al.*, 1991). The Hom enzyme from *B. subtilis* is partially inhibited by methionine,

isoleucine, threonine and casamino acids. However, the repression of CAA was higher, than the effect of any tested combination of aspartate derived amino acids (Yeggy and Stahly, 1980). This indicates, that there must be at least one additional amino acid that can inhibit the activity of the homoserine dehydrogenase. The results of this work indicate that the repressing amino acid could be serine.

The Hom protein harbors a C-terminal ACT-domain (Parsot and Cohen, 1988). ACT-domains are named after the enzymes in which they were discovered first: aspartate kinase-chorismate mutase-TyrA. These domains can be found in different proteins from bacteria, archaea and eukaryotes. Most of them are involved in amino acid related pathways and the binding of amino acids to the ACT-domain often regulate the protein activity (Aravind and Koonin, 1999). The first described crystal structure was the ACT-domain of the phosphoglycerate dehydrogenase (PGDH) from *E. coli*, which is responsible for the first step in the biosynthesis of serine from pyruvate. L-serine can bind to the ACT-domain of this protein and regulate its activity. However, the exact mechanism is unknown (Schuller *et al.*, 1995). Although the function of many ACT-domains from other organisms like *E. coli* is known, it is not much known about the ACT-domains from *B. subtilis* proteins. The L-serine dehydratase SdaAB-AA from *B. subtilis* also contains an ACT-domain in the β-chain encoded by *sdaAB*. It shows similarities to the ACT-domain in the PGDH of *E. coli* and furthermore the serine binding motif is also similar (Xu and Grant, 2013). The ACT-domain of the homoserine dehydrogenase from *B. subtilis* could also be bound by serine and the activity is therefore down regulated. To conclude, the reason for the toxicity of serine might be the binding of serine to the ACT-domain of the Hom protein and the resulting inhibition of the enzyme activity. This leads finally to a lack of threonine and the amino acids downstream of the pathway. In *Corynebacterium glutamicum*, the deletion of the C-terminus of the homoserine dehydrogenase leads to the inactivation of the threonine inhibitory effect on this protein (Archer *et al.*, 1991). To analyse if the ACT-domain of the Hom enzyme is responsible for the toxic serine effect, a strain could be constructed, which harbours the Hom protein without the ACT-domain. This strain can be then tested on C-glc plates with high concentrations of serine. If the strain is resistant to serine and grows better than the wild type strain, serine might bind to the ACT-domain of the homoserine dehydrogenase and change thereby its activity. The modified Hom, without ACT-domain protein needs to be still active. The deletion of the C-terminus could also result in the inactivation of the enzyme. However, this experiment could prove that serine inhibits the threonine biosynthesis.

The fact that serine and threonine are imported by the same proteins also supports this thesis. In natural conditions, both amino acids are imported into the cell. Since threonine has also a regulatory function in the cell and is toxic in high concentrations, the amount of serine that should be similar, seems to regulate the threonine biosynthesis (Lamb and Bott, 1979b). Therefore, the

amount of threonine does not increase by the biosynthesis and threonine cannot inhibit other pathways, like valine biosynthesis. But in C-glc minimal medium with serine, only serine is present and is transported into the cell. This leads to a decrease in threonine biosynthesis, which is essential under these conditions, since no threonine is available in the medium. Therefore, suppressor mutations accumulate in the uptake and degradation of serine or in the threonine pathway to upregulate it again.

Since isoleucine is produced out of threonine, the addition of isoleucine to the C-glc medium with serine could possibly also reverse the toxic effect, at least partially. This compensation could be shown for *E. coli* cells (Hama *et al.*, 1990). However, this effect was not visible in *B. subtilis* during this work (chapter 3.3.3.). This might be due to the fact, that high levels of isoleucine activate the global repressor CodY, which further represses the expression of the *hom* operon (Kriel *et al.*, 2014). The repression leads than again to a decrease of the threonine level and this is a disadvantage for the cell. Furthermore, isoleucine can inhibit the Hom enzyme activity in *B. subtilis* (Yeggy and Stahly, 1980). In contrast, the *ilvA*$^{+}$ mutation was found to counteract the toxic serine effect partially (chapter 3.4.). This could be due to the production of an intermediate level of isoleucine in the cell that is not too high to activate CodY, but enough to compensate the toxic effect partially. Another possibility is that the IlvA enzyme has a second minor function that is able to counteract the threonine auxotrophy. Similarly, the enzyme ThrC, which is normally involved in the threonine pathway, can partially take over the function if IlvA (Rosenberg *et al.*, 2016). This needs to be further investigated. An interesting experiment could also be to measure the intracellular amino acid concentrations of the wild type strain. This could be measured in minimal medium supplied with high concentrations of serine, threonine or isoleucine. This might indicate the changes of amino acid composition in the cell upon the excess of each amino acid.

In *E. coli*, several other effects of high intracellular serine concentration were observed. It could be shown, that peptidoglycan synthesis and cell division was inhibited (Zhang and Newman, 2008; Zhang *et al.*, 2010). In an attempt to create a serine producing *E. coli* strain, which tolerates high amounts of serine, several point mutations occurred. Beside mutations in serine importers and the homoserine dehydrogenase, also mutations in serine exporters occurred (Mundhada *et al.*, 2016; Mundhada *et al.*, 2017). So far are no serine exporters have been described in *B. subtilis*. The toxic effect of serine could also have other effects on the *B. subtilis* cell, except of the inhibition of the threonine pathway. Serine is often found to be a regulatory intermediate in the cell. L-serine shows a regulatory influence in mammalian cancer cells, since it is involved in cell proliferation by modulating the flux of glycolytic intermediates (Ye *et al.*, 2012; Newman and Maddocks, 2017). Furthermore, serine has an influence in the expression of respiratory genes in plants (Timm *et al.*, 2013).

To conclude, high concentrations of serine cause probably an inhibition of the homoserine dehydrogenase Hom of *B. subtilis*. This leads to a decrease of threonine in the cell. Serine acts therefore as a regulator of threonine biosynthesis, which is part of the cells complex regulatory mechanism to fit the needs of amino acids in the cell. This indicates an additional regulatory mechanism of amino acid homeostasis in the cell. The import of amino acids is tightly controlled by different transporter proteins, that are active under different amino acid concentrations or different lifestyles. Furthermore, the biosynthesis is often regulated by feedback inhibition of a biosynthetic enzyme by a produced amino acid. This works indicates a third level of regulation by amino acids that are not involved in the particular pathway. Furthermore, this adds an additional link of the import of an amino acid to its biosynthesis. Many genes for the biosynthesis of amino acids were shown to be higher expressed in the absence of casamino acids (Mäder *et al.*, 2002). This might indicate that even more, similar regulatory mechanism can be found in *B. subtilis*.
The biosynthesis pathways of all amino acids will be deleted in the *MiniBacillus* strain. Interestingly, the results of the toxicity of several amino acids indicate a highly regulated network of amino acid biosynthesis. The deletion of one amino acid biosynthesis pathway might have an effect on another pathway. Furthermore, it should be considered to not delete the degradation pathway of serine, before the biosynthesis of serine is deleted. This avoids the accumulation of serine in the cell.

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6. Appendix

6.1. Materials

6.1.1. Chemicals

Acetyl-CoA	Sigma-Aldrich, Munich
Acrylamide	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Agarose	Peqlab, Erlangen
Alanine	Roth, Karlsruhe
Ammonium iron (III) citrate	Sigma-Aldrich, Munich
Ammonium Peroxydisulfate	Roth, Karlsruhe
Antibiotics	Sigma-Aldrich, Munich
Arginine	Roth, Karlsruhe
Asparagine	Sigma-Aldrich, Munich
Aspartate	Sigma-Aldrich, Munich
B-Mercaptoethanol	Merck, Darmstadt
Bacto agar	Becton, Dickinson and Company, Heidelberg
BSA	Roth, Karlsruhe
Bromphenol blue	Serva, Heidelberg
$CaCl_2$	Sigma-Aldrich, Munich
CAA	Sigma-Aldrich, Munich
CDP*	Roche Diagnostics, Mannheim
Coomassie Brilliant Blue, G250	Roth, Karlsruhe
Cysteine	Sigma-Aldrich, Munich
Desthiobiotin	IBA, Göttingen
DMSO	Carl Roth, Karlsruhe
dNTPs	Roche Diagnostics, Mannheim

DTNB	Roth, Karlsruhe
DTT	Roth, Karlsruhe
Ethidium bromide	Roth, Karlsruhe
$FeCl_3$ x 6 H_2O	Sigma-Aldrich, Munich
FM™ 4-64 Dye (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide)	ThermoFisher, Braunschweig
D-Fructose-1,6-bisphosphate	Sigma-Aldrich, Munich
D-Glucose	Merck, Darmstadt
Glutamate	Sigma-Aldrich, Munich
Glutamine	Roth, Karlsruhe
Glycerine	Merck, Darmstadt
Glycine	Sigma-Aldrich, Munich
HDGreen™ plus	Intas, Göttingen
Histidine	AppliChem, Darmstadt
Homoserine	Sigma-Aldrich, Munich
Imidazole	Sigma-Aldrich, Munich
Isoleucine	Roth, Karlsruhe
Isopropyl ß-D-1-thiogalactopyranoside	Peqlap, Erlangen
KCl	Oxoid, Heidelberg
$KHCO_3$	Roth, Karlsruhe
Leucine	Sigma-Aldrich, Munich
Lysine	Sigma-Aldrich, Munich
Methionine	Sigma-Aldrich, Munich
$MgCl_2$	Sigma-Aldrich, Munich
$MgSO_4$ x 7 H_2O	Roth, Karlsruhe
$MnCl_2$ x 4 H_2O	Roth, Karlsruhe
$NADP^+$	Sigma-Aldrich, Munich

Ni^{2+}-nitrilotriacetic acid superflow	Qiagen, Hilden
Nutrient Broth	Merck, Darmstadt
ONPG	AppliChem, Darmstadt
Oxaloacetate	Sigma-Aldrich, Munich
Phenylalanine	Roth, Karlsruhe
Proline	Sigma-Aldrich, Munich
Serine	Sigma-Aldrich, Munich
Skim milk powder, fat-free	Roth, Karlsruhe
Sodium succinate	Fluka, Buchs, Switzerland
Sodium Dodecyl Sulfate	Roth, Karlsruhe
Strep-Tactin Sepharose	IBA, Göttingen
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Thiamine	Sigma-Aldrich, Munich
Threonine	AppliChem, Darmstadt
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe
Trypton	Oxoid, Heidelberg
Tween 20	Sigma-Aldrich, Munich
Tyrosine	Sigma-Aldrich, Munich
Valine	Roth, Karlsruhe
X-Gal	Peqlab, Erlangen
Yeast extract	Oxoid, Hampshire, U.K.

Other chemicals were purchase from Merck, Serva, Sigma or Roth.

6.1.2. Enzymes

FastAP™	ThermoFisher, Braunschweig
DreamTaq DNA polymerase	ThermoFisher, Braunschweig
Lysozym	Merck, Darmstadt
Phusion™ DNA polymerase	ThermoFisher, Braunschweig

Restriction nucleases	ThermoFisher, Braunschweig
T4-DNA ligase	ThermoFisher, Braunschweig

6.1.3. Materials

96-Well plates	Sarstedt, Nümbrecht
Centrifuge cups	Beckmann, München
Cuvettes (microliter, plastic)	Greiner, Nürtingen
Dialysis tube	Serva, Heidelberg
Falcon tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht
Gene Amp Reaction Tubes (PCR)	Perkin Elmer, Weiterstadt
Glass pipettes	Brandt, Wertheim
Microlitre pipettes (2 µl, 20 µl, 200 µl, 1000 µl, 5000µl	Eppendorf, Hamburg and Gilson, Düsseldorf
Petri dishes	Greiner, Nürtingen
Pipette tips	Sarstedt, Nümbrecht
Poly-Prep Chromatography Columns	Bio-Rad Laboratories GmbH, Munich
Polyvinylidene fluoride membrane (PVDF)	Bio-Rad Laboratories GmbH, Munich
Reaction tubes	Greiner, Nürtingen
Single-use syringes (5 ml, 10 ml)	Becton Dickson Drogheda, Ireland

6.1.4. Instruments/ Equipment

Autoclave	Zirbus technology, Bad Grund
Biofuge fresco	Heraeus Christ, Osterode
Fluorescence microscope Axioskop 40FL + AxioCam MRm	Zeiss, Göttingen
French pressure cell press	SLM Aminco, Lorch
GelDoc™ XR+	Biorad, Munich
Gel electrophoresis apparatus	PeqLab, Erlangen

Ice machine	Ziegra, Isernhagen
Heating block Dri Block DB3	Waasetec, Göttingen
Horizontal shaker 3006	GFL, Burgwedel
LabCycler	SensorQuest, Göttingen
Mini-Protean III System	Bio-Rad, Munich
Nanodrop ND-1000	ThermoFisher, Braunschweig
Open air shaker Innova 2300	New Brunswick, Neu-Isenburg
pH meter Calimatic	Knick, Berlin
Microplate Reader SynergyMx	BioTek, Bad Friedrichshall
Refrigerated centrifuge	Kendro, Hanau
Scale	Sartorius, Göttingen
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer	Amersham, Freiburg
Standard power pack	Bio-Rad, Munich
Thermocycler	Biometra, Göttingen
Ultra centrifuge, Sorvall Ultra Pro 80	ThermoFisher, Braunschweig
Ultrasonic device	Dr. Hielscher, Teltow
UV Transilluminator 2000	Bio-Rad, Munich
Vortex	Bender & Hobein, Bruchsal
Water desalination plant	Millepore, Schwalbach

6.1.5. Commercial systems

HDGreen DNA Stain	Intas, Göttingen
peqGOLD Bacterial DNA Kit	PeqLab, Erlangen
NucleoSpin Plasmid-Kit	Macherey-Nagel, Düren
Prestained Protein Marker (PageRuler)	ThermoFisher, Braunschweig
QIAquick PCR Purification Kit	Qiagen, Hilden

6.1.6. Software and webpages

Program	Provider	Application
AxioVision	Zeiss	Microscopy imaging
ChemoStar Imager	Intas	Western Blot imaging
Gen5™ Data Analysis Software	BioTek®	Plate reader analysis
Geneious 10.0.5	Biomatters	DNA analysis
ImageLab™ Software	BioRad	Geldoc imaging
Mendeley Desktop	PDFTron™ Systems Inc.	Reference Manager
Microsoft Office 365	Microsoft Inc.	Data processing
*Subti*Wiki 2.0	Michna *et al.,* 2016	*B. subtilis* database
Zen	Zeiss	Image processing

6.2. Bacterial strains

6.2.1. *B. subtilis* strains constructed in this work

Strain	**Genotype**	**Reference/ Construction**
GP2324	*trpC2 ybeC* (Δbp 340, stop aa 125) duplication (*yokD [bp 43-819] –thyA [bp 510-795]*)	Suppressor mutant of wt on high serine concentrations (WGS)
GP2325	*trpC2 ybeC* (Δbp 974, stop aa 350)	Suppressor mutant of wt on high serine concentrations (PCR)
GP2326	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::kan-lox*	LFH-PCR → GP1755
GP2327	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72*	GP2326 + 1% Xylose
GP2328	*trpC2 sacA::(phl-P_{xylA}-cre)*	pJK195 → 168
GP2329	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::kan-lox*	LFH-PCR → GP2327
GP2330	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::lox72*	GP2329 + 1% Xylose

GP2331	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::kan-lox*	LFH-PCR → GP2328
GP2332	*trpC2 sacA::(phl-P_{xylA}-cre) ΔodhAB::kan-lox*	LFH-PCR → GP2328
GP2333	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72*	GP2331 + 1% Xylose
GP2334	*trpC2 sacA::(phl-P_{xylA}-cre) ΔodhAB::lox72*	GP2332 + 1% Xylose
GP2335	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::lox72 ΔcitG::lox72 ΔsdhCAB::kan-lox*	PCR GP2342 → GP2352
GP2336	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitB::kan citA*-3xFLAG *spec*	GP1287 → GP2338
GP2337	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::lox72 ΔcitG::lox72 ΔsdhCAB::lox72*	GP2335 + 1% Xylose
GP2338	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitB::kan-lox*	LFH-PCR → GP2328
GP2339	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitB::lox72*	GP2338 + 1% Xylose
GP2340	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitG::kan-lox*	LFH-PCR → GP2328
GP2341	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitG::lox72*	GP2340 + 1% Xylose
GP2342	*trpC2 sacA::(phl-P_{xylA}-cre) ΔsdhCAB::kan-lox*	LFH-PCR → GP2328
GP2343	*trpC2 sacA::(phl-P_{xylA}-cre) ΔsdhCAB::lox72*	GP2342 + 1% Xylose
GP2344	*trpC2 sacA::(phl-P_{xylA}-cre) ΔsucCD::kan-lox*	LFH-PCR → GP2328
GP2345	*trpC2 sacA::(phl-P_{xylA}-cre) ΔsucCD::lox72*	GP2344 + 1% Xylose
GP2346	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::lox72 ΔcitG::kan-lox*	LFH-PCR → GP2330
GP2347	*trpC2 lacA::(C-yfp kan) citA-3xFLAG spec*	pGP888 → GP1287
GP2348	*trpC2 lacA::(P_{xyl} citZ kan) citA-3xFLAG spec*	pGP2261 → GP1287
GP2349	*trpC2 lacA::(P_{xyl} citR kan) citA-3xFLAG spec*	pGP2262 → GP1287

GP2350	*trpC2 lacA::(P_{xyl} citZ kan)*	pGP2261 → 168
GP2351	*trpC2 lacA::(P_{xyl} citR kan)*	pGP2262 → 168
GP2352	*trpC2 ΔcitRA::lox72 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔcitB::lox72 ΔcitG::lox72*	GP2346 + 1% Xylose
GP2353	*trpC2 ΔcitA::cat lacA::(P_{xyl} citR kan)*	pGP2262 → GP1282
GP2354	*trpC2 ΔaapA::tet ΔgltAB::erm*	BP123 → GP2377
GP2355	*trpC2 ΔsteT::cat ΔgltAB::erm*	BP123 → GP2378
GP2356	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet*	PCR GP791 → GP2333
GP2357	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::kan-lox*	PCR GP2342 → GP2356
GP2358	*trpC2 ΔtyrA::kan*	LFH → 168
GP2359	*trpC2 citA-3xFLAG spec ΔcitR::aphA3 ΔcitZ::erm*	GP1281 → GP1289
GP2360	*trpC2 ΔcitRA::erm-lox*	LFH → 168
GP2361	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh ΔsucCD::tet ΔsdhCAB::lox72*	GP2357 + 1% Xylose
GP2362	*trpC2 ΔtyrA::kan ΔybeC::cat*	GP1886 → GP2358
GP2363	*trpC2 ΔtyrA::kan ΔytnA::spec*	GP1885 → GP2358
GP2364	*trpC2 ΔserA::Tn917 (ermC) ΔytnA::spec*	GP1885 → 1A614
GP2365	*trpC2 ΔserA::Tn917 (ermC) ΔalsT::tet*	GP1888 → 1A614
GP2366	*trpC2 ΔserA::Tn917 (ermC) ΔybeC::cat*	GP1886 → 1A614
GP2367	*trpC2 ΔtyrA::kan ΔalsT::tet*	GP1888 → GP2358
GP2368	*trpC2 sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spc*	PCR GP718 → GP2361

GP2369	MGB874 *ΔtyrA:.kan*	PCR GP2358 → MGB874
GP2370	*trpC2 asnS* (Δbp 473, stop aa 195)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (WGS)
GP2371	*trpC2* Promoter *asnS* (Δbp -13)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (PCR)
GP2372	*trpC2 asnS* (Δbp 172, stop aa 58)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (PCR)
GP2373	*trpC2 ΔyodF::neo asnS* (bp 805 G → A, stop aa 269)	Suppressor mutant GP1887 on L-Aspartic acid β-hydroxamate (PCR)
GP2374	*trpC2 ΔgltT::ermC ΔyodF::neo*	GP1887 → GP2247
GP2375	*trpC2 ansA (Δbp 367, stop aa 157)*	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (WGS)
GP2376	*trpC2 ΔtyrA::kan yqiK (bp 677 T→G, aa 226 D→V)*	Suppressor mutant GP2358 on C-glc-tyrosin (WGS)
GP2377	*trpC2 ΔaapA::tet*	LFH → 168
GP2378	*trpC2 ΔsteT::cat*	LFH → 168
GP2379	*trpC2 ΔyfnA::kan*	LFH → 168
GP2380	*trpC2 yqiK (D226V)-cat*	LFH → GP2358
GP2381	*trpC2 ΔaapA::tet serA::Tn917 (ermC)*	1A614 → GP2377
GP2382	*trpC2 ΔsteT::cat serA::Tn917 (ermC)*	1A614 → GP2378
GP2383	*trpC2 ΔyfnA::kan serA::Tn917 (ermC)*	1A614 → GP2379
GP2384	*trpC2 ΔyqiK::cat*	LFH → 168

GP2385	*trpC2 ΔyveA::cat*	LFH → 168
GP2386	*trpC2 ΔyodF::kan tcyK* (bp 480 C→T, aa 159 V→I) P*(infC-rpmI-rplT-ysdA)* (Δbp -171)	Suppressor mutant GP1887 on L-Aspartic acid β-hydroxamate (WGS)
GP2387	*trpC2 ΔyveA::cat ΔgltT::ermC*	GP2247 → GP2385
GP2388	*trpC2 yqiK (D226V)-cat ΔtyrA::kan*	PCR GP2358 → GP2380
GP2389	*trpC2 ΔyqiK::cat ΔtyrA::kan*	PCR GP2358 → GP2384
GP2390	*trpC2 ΔyfnA::kan ΔgltAB::erm*	PCR BP123 → GP2379
GP2391	*trpC2 ΔysdA::kan*	LFH → 168
GP2392	*trpC2 ΔserA::zeo*	LFH → 168
GP2393	*trpC2 ΔaapA::tet ΔyfnA::kan*	GP2379 → GP2377
GP2394	*trpC2 ΔaapA::tet ΔyfnA::kan ΔsteT::cat*	GP2378 → GP2393
GP2395	*trpC2 ΔyhjB::tet*	LFH → 168
GP2396	*trpC2 ΔybxG::cat*	LFH → 168
GP2397	*trpC2 ΔyfnA::kan ΔsteT::cat*	GP2378 → GP2379
GP2398	*trpC2 ΔaapA::tet ΔsteT::cat*	GP2378 → GP2377
GP2399	*trpC2 ΔyhjB::tet ΔserA::Tn917 (ermC)*	1A614 → GP2395
GP2400	trpC2 Δ*yhjB::tet ΔtyrA::kan*	GP2358 → GP2395
GP2926	*trpC2 ΔyhjB::tet ΔgltT::erm*	GP2247 → GP2395
GP2927	*trpC2 ΔybxG::cat ΔserA::Tn917 (ermC)*	1A614 → GP2396
GP2928	*trpC2 ΔybxG::cat ΔtyrA::kan*	GP2358 → GP2396

GP2929	*trpC2 ΔybxG::cat ΔgltT::erm*	GP2247 → GP2396
GP2930	*trpC2 ΔydgF::cat*	LFH → 168
GP2931	*trpC2 ΔydgF::cat ΔgltT::erm*	GP2247 → GP2930
GP2932	*trpC2 ΔydgF::cat ΔtyrA::kan*	GP2358 → GP2930
GP2933	*trpC2 ΔydgF::cat ΔserA::Tn917 (ermC)*	1A614 → GP2930
GP2934	*trpC2 asnS* (Δbp 1014-1023, frameshift, stop aa 342)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (PCR)
GP2935	*trpC2 asnS (bp* 644 T→C, aa 215 H→R)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (PCR)
GP2936	*trpC2 asnS (*bp 369 G→T, stop aa 369) *yvoD* (bp 239 G→T, aa 239 A→D)	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (WGS)
GP2937	*trpC2 asnS (*Δbp 1207-1210, frameshift, stop aa 405*)*	Suppressor mutant 168 on L-Aspartic acid β-hydroxamate (PCR)
GP2938	*trpC2 ΔgltT::erm, asnS* (Δbp 900- 1229, in-frame deletion)	Suppressor mutant GP2247 on L-Aspartic acid β-hydroxamate (PCR)
GP2939	*trpC2 ΔgltT::erm, asnS (*duplication of bp 147-328; in-frame)	Suppressor mutant GP2247 on L-Aspartic acid β-hydroxamate (PCR)
GP2940	*trpC2 ΔybeC::cat ΔthrR::erm*	BKE27910 → GP1886
GP2941	*trpC2 ΔserA::zeo ΔybeC::cat*	GP1886 → GP2392
GP2942	*trpC2 ΔserA::zeo ΔthrR::erm*	BKE27910 → GP2392
GP2943	*trpC2 ΔyvoD::cat*	LFH → 168
GP2944	*trpC2 ΔyvoD::cat ΔgltT::erm*	GP2247 → GP2943

GP2945	*trpC2 ΔsteT::kan*	LFH → 168
GP2946	*trpC2 ΔybeC::cat ΔsteT::kan*	GP2946 → GP1886
GP2947	*trpC2 ΔybeC::cat xkdE::(N-yfp ermR)*	GP1171 → GP1886
GP2948	*trpC2 ΔybeC::cat xkdE::(P_{xyl}-ybeC ermC)*	GP3080 → GP1886
GP2949	*trpC2 ΔybeC::kan ΔbcaP::erm*	BKE09460 → GP2786
GP2950	*trpC2 ΔybeC::kan ΔbcaP::erm ΔybxG::cat*	GP2396 → GP2949
GP2951	*trpC2 ΔybeC::kan ΔybxG::cat*	GP2396 → GP2786
GP2952	*trpC2 ΔbcaP::erm ΔybxG::cat*	GP2396 → BKE09460
GP2953	*trpC2 ΔybeC::kan ΔbcaP::erm ΔserA::zeo*	GP2392 → GP2949
GP2954	*trpC2 ΔbcaP::erm ΔybxG::cat ΔserA::zeo*	GP2392 → GP2952
GP2955	*trpC2 ΔybeC::kan ΔbcaP::erm ΔybxG::cat ΔserA::zeo*	GP2392 → GP2950
GP2956	*trpC2 ΔybxG::cat ΔserA::zeo*	GP2392 → GP2396
GP2957	*trpC2 ΔybeC::kan ΔserA::zeo*	GP2392 → GP2786
GP2958	*trpC2 ΔybeC::kan ΔybxG::cat ΔserA::zeo*	GP2392 → GP2951
GP2959	*trpC2 ΔbcaP::erm ΔserA::zeo*	GP2392 → BKE09460
GP2960	*trpC2 amyE::(aapA-lacZ cat)*	pGP2273 → 168
GP2961	*trpC2 amyE::(ansB-lacZ cat)*	pGP2274 → 168
GP2962	*trpC2 amyE::(steT-lacZ cat)*	pGP2275 → 168
GP2963	*trpC2 amyE::(ytnA-lacZ cat)*	pGP2276 → 168

GP2964	*trpC2 amyE::(yfnA-lacZ cat)*	pGP2278 → 168
GP2965	*trpC2 amyE::(ybeC-lacZ cat)*	pGP2287 → 168
GP2966	*trpC2 amyE::(sdaAB* C70A-lacZ cat)*	pGP2294 → 168
GP2967	*trpC2 amyE::(sdaAB-lacZ cat)*	pGP2295 → 168
GP2968	*trpC2 amyE::(hom* G56T-lacZ cat)*	pGP2296 → 168
GP2969	*trpC2 ΔserA::zeo; thrR* (Δbp 90, stop aa 36)	Suppressor mutant GP2392 on C-glc-Serine (WGS)
GP2970	*trpC2* duplication (*yokD [bp 43-819] –thyA [bp 510-795]*)	Suppressor mutant 168 on C-glc-Serine (WGS)
GP2971	*trpC2 ΔybeC::kan* Promoter *sdaAB* [bp -70 C→A]*	Suppressor mutant GP1886 on C-glc-Serine (WGS)
GP2972	*trpC2 ΔybeC::kan* Promoter *hom* [bp -56 G→T]*	Suppressor mutant GP1886 on C-glc-Serine (WGS)
GP2973	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre)*	pJK195 → GP2618
GP2974	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72*	PCR GP2331 → GP2973 + 1% Xylose
GP2975	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet*	PCR GP791 → GP2974
GP3024	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72*	PCR GP2342 → GP2975 + 1% Xylose
GP3025	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec*	PCR GP718 → GP3024
GP3026	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat*	PCR GP1276 → GP3025
GP3027	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat ΔcitB::lox72*	PCR GP2338 → GP3026 +1% Xylose
GP3028	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔcitZ-icd-mdh::lox72 ΔsucCD::tet ΔsdhCAB::lox72 ΔcitG::spec ΔodhAB::cat ΔcitB::lox72 ΔcitRA::lox72*	PCR GP1753 → GP3027 +1% Xylose

GP3029	*trpC2 yvcA-P_{mtlA}-comKS-mls-hisI sacA::(phl-P_{xylA}-cre) ΔsdhCAB::lox72*	PCR GP2342 → GP2973 +1% Xylose
GP3030	*trpC2 ΔthrC::spec*	LFH → 168
GP3031	*trpC2 ΔbcaP::erm ΔthrC::spec*	GP3030 → BKE09460
GP3032	*trpC2 ΔybxG::cat ΔthrC::spec*	GP3030 →GP2396
GP3033	*trpC2 ΔybeC::kan ΔthrC::spec*	GP3030→GP2786
GP3034	*trpC2 ΔbcaP::erm ΔybxG::cat ΔthrC::spec*	GP3030 →GP2952
GP3035	*trpC2 ΔbcaP::erm ΔybeC::kan ΔthrC::spec*	GP3030 →GP2949
GP3036	*trpC2 ΔybeC::kan ΔybxG::cat ΔthrC::spec*	GP3030 →GP2951
GP3037	*trpC2 ΔybeC::kan ΔybxG::cat ΔbcaP::erm ΔthrC::spec*	GP3030 →GP2950
GP3038	*trpC2 ΔyvbW::cat*	LFH → 168
GP3039	*trpC2 ΔyecA::cat*	LFH → 168
GP3040	*trpC2 ΔybgF::cat*	LFH → 168
GP3041	*trpC2 ΔyodF::cat ΔgltT::erm*	GP2247 → GP1887
GP3042	*trpC2 ΔyodF::cat ΔtyrA:: kan*	GP2358 → GP1887
GP3043	*trpC2 ΔyvbW::cat ΔgltT::erm*	GP2247 → GP3038
GP3044	*trpC2 ΔyvbW::cat ΔtyrA:: kan*	GP2358 → GP3038
GP3045	*trpC2 ΔyecA::cat ΔgltT::erm*	GP2247 → GP3039
GP3046	*trpC2 ΔyecA::cat ΔtyrA:: kan*	GP2358 → GP3039
GP3047	*trpC2 ΔybgF::cat ΔgltT::erm*	GP2247 → GP3040

GP3048	*trpC2 ΔybgF::cat ΔtyrA:: kan*	GP2358 → GP3040
GP3049	*trpC2 ΔserA::zeo ybeC* (bp 1564 G→T, stop aa 522)	Suppressor mutant of GP2392 on high serine concentrations (PCR)
GP3050	*trpC2 ΔserA::zeo ybeC* (Δbp 307-1014, in-frame deletion)	Suppressor mutant of GP2392 on high serine concentrations (PCR)
PG29	PG18 *ΔmhqNOP*	pGP2093 → PG18
PG30	PG29 *Δ(yuzG-sufA)*	pGP2094 → PG29
PG31	PG30 restored wild type *pit* allele	pJOE3256 → PG30
PG32	PG31 *ΔytrH::pgi-fbaA-cat*	PCR product → PG31
PG33	PG32 *Δ(cat-ytrI-ytzJ)::ptsGHI-kan*	PCR product → PG32
PG34	PG33 *Δ(ycgQ-yckE)*	pGP2098→ PG33
PG35	PG34 *Δ(yvaM-yvbK)*	pGP2088 → PG34
PG36	PG35 *Δ(nhaX-yhaX)*	pGP2073 → PG35
PG37	PG36 *Δ(glpQ-ycbK)*	pGP2270 → PG36
PG38	PG37 *Δ(yqjF-yqjG)*	pGP2282 → PG37
PG39	PG38 *Δ(yddN-ydfM)*	pGP2283 → PG38

6.2.2. Isolated suppressor mutants of this work

Strain	Phenotype relevant mutation	Remarks
168, C-glc 244 µM serine		
GP2970	duplication (*yokD[bp 43-819] –thyA[bp 510-795]*)	WGS
168, C-glc 1 mM serine		
GP2324	*ybeC* (Δbp 340, stop aa 125) duplication (*yokD[bp 43-819] –thyA[bp 510-795]*)	WGS
GP2325	*ybeC* (Δbp 974, stop aa 350)	PCR *ybeC*
GP2392 (Δ*serA*::*zeo*), C-glc 244 µM serine		
GP2969	*thrR* (Δbp 90, stop aa 36)	WGS
GP3049	*ybeC* (bp 1564 G→T, stop aa 522)	PCR *ybeC*
GP3050	*ybeC* (Δbp 307-1014, in-frame deletion)	PCR *ybeC*
GP1886 (Δ*ybeC::cat*), C-glc 10 mM serine		
GP2971	Promoter *sdaAB* [bp -70 C→A]*	WGS
GP1886 (Δ*ybeC::cat*), C-glc 17 mM serine		
GP2972	Promoter *hom* [bp -56 G→T]*	WGS
168, C-glc L-aspartic acid β-hydroxamate		
GP2370	*asnS* (Δbp 473, stop aa 195)	WGS
GP2371	Promoter *asnS* (Δbp -13)	PCR *asnS*
GP2372	*asnS* (Δbp 172, stop aa 58)	PCR *asnS*
GP2934	*asnS* (Δbp 1014-1023, frameshift, stop aa 342)	PCR *asnS*
GP2935	*asnS (bp* 644 T→C, aa 215 H→R)	PCR *asnS*
GP2936	*asnS (*bp 369 G→T, stop aa 369) *yvoD* (bp 239 G→T, aa 239 A→D)	WGS
GP2937	*asnS (*Δbp 1207-1210, frameshift, stop aa 405*)*	PCR *asnS*
GP1887 (Δ*yodF::kan*), C-glc L-aspartic acid β-hydroxamate		
GP2373	*asnS* (bp 805 G → A, stop aa 269)	PCR *asnS*
GP2247 (Δ*gltT::erm*), C-glc L-aspartic acid β-hydroxamate		
GP2938	*asnS* (Δbp 900- 1229, in-frame deletion	PCR *asnS*
GP2939	*asnS (*duplication of bp 147-328, in-frame)	PCR *asnS*

6.2.3. *B. subtilis* strains used in this work

Strain	Genotype	Reference/ Construction
1A614	*trpC2 serA::Tn917 (ermC)*	Vandeyar and Zahler, 1986

1A773	*pheA1 trpC2 thrC::cat*	Vandeyar and Zahler, 1986
BKE09460	*trpC2 ΔbcaP::erm*	Koo *et al*., 2017
BKE27910	*trpC2 ΔthrR::erm*	Koo *et al*., 2017
BKG9	*trpC2 Δspo0A::kan*	Katrin Gunka, Medical Microbiology Göttingen
BP123	*trpC2 gltAB::ermC*	Victoria Keidel, AG Commichau
BP557	*trpC2 ΔthrR::ermC amyE::(P_{hom}* G56T-lacZ cat)*	Rosenberg *et al.,* 2016
BP558	*trpC2 amyE::(P_{hom}* G56T-lacZ cat)*	Rosenberg *et al.,* 2016
BP562	*trpC2 amyE::(P_{thrD}* G56T-lacZ cat)*	Rosenberg *et al.,* 2016
BP563	*trpC2 ΔthrR::ermC amyE::(P_{thrD}* G56T-lacZ cat)*	Rosenberg *et al.,* 2016
GP718	*trpC2 ΔcitG::spc amyE::(gltA-lacZ aphA3)*	Commichau, 2006
GP791	*trpC2 ΔsucCD ::tet*	Zschiedrich, 2014
GP1171	*trpC2 xkdE::*(N-*yfp ermC)*	Gunka, 2011
GP1173	*trpC2 lacA::(C-yfp kan)*	Diethmaier *et al.*, 2011
GP1276	*trpC2 ΔodhAB::cat*	Zschiedrich, 2014
GP1281	*trpC2 ΔcitZ::erm*	Zschiedrich, 2014
GP1282	*trpC2 ΔcitA::cat*	Zschiedrich, 2014
GP1283	*trpC2 ΔcitR::aphA3*	Zschiedrich, 2014
GP1284	*trpC2 ΔcitZ::erm ΔcitA::cat*	Zschiedrich, 2014
GP1285	*trpC2 ΔcitZ::erm ΔcitR::aphA3*	Zschiedrich, 2014

GP1286	*trpC2 ΔcitA::cat ΔcitR::aphA3*	Zschiedrich, 2014
GP1287	*trpC2 citA*-3xFLAG *spec*	Zschiedrich, 2014
GP1288	*trpC2 citA*-3xFLAG *spec ΔcitZ::erm*	Zschiedrich, 2014
GP1289	*trpC2 citA*-3xFLAG *spec ΔcitR::aphA3*	Zschiedrich, 2014
GP1752	*trpC2 dnaE-pgi-fbaA-cat-ytrI*	Zschiedrich, 2014
GP1753	*trpC2 ΔcitRA::aphA3-lox*	Zschiedrich, 2014
GP1755	*trpC2 ΔcitRA sacA::(phl-*P_{xylA}*-cre)*	Zschiedrich, 2014
GP1757	*trpC2 dnaE-pgi-fbaA-ptsGHI-aphA3-ackA ΔytrI-moaB::aphA3*	Zschiedrich, 2014
GP1885	*trpC2 ΔytnA::spec*	Reuß, 2017
GP1886	*trpC2 ΔybeC::cat*	Reuß, 2017
GP1887	*trpC2 ΔyodF::neo*	Reuß, 2017
GP1888	*trpC2 ΔalsT::tet*	Reuß, 2017
GP2247	*trpC2 ΔgltT::ermC*	Gundlach, 2017
GP2618	*trpC2 yvcA-*P_{mtlA}*-comKS-mls-hisI*	Martin Benda, PhD
GP2786	*trpC2 ΔybeC::kan*	Larissa Krüger, PhD
GP3080	*trpC2 xkdE::Pxyl-ybeC ermC*	Larissa Krüger, PhD
IIG-Bs168-1	*trpC2 manPA::ermC*	Rahmer *et al.,* 2015
MGB874	Genome reduced strain of 21%	Morimoto *et al*., 2008
PG10	*MiniBacillus* genome reduced strain of 34.54%	Reuß *et al.*, 2017

PG18	*MiniBacillus* genome reduced strain of 36.61%	Reuß, 2017
Δ6	deletion of 6 prophaes and AT-rich islands	Westers *et al.*, 2003

6.2.4. *E. coli* strains used in this work

Strain	Genotype	Reference/ Construction
JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15]	Yanisch-Perron *et al.* (1985)
XL1 blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[*F' proAB lacIq ZΔM15* Tn*10* (*Tetr*)]	Stratagene, Woodcock *et al.* (1989)
DH5α	*recA1 endA1 gyrA96 thi hsdR17rK-mK+relA1 supE44 Φ80ΔlacZΔM15* Δ(*lacZYA-argF*)*U169*	Sambrook *et al.*, 1989
BL21	*B*(*834*)*-derivate F-lon ompT hsdS*(*rB mB*) *gal dcm*[*DE3*]	Novagen, Sambrook *et al.*, 1989

6.3. Plasmids

6.3.1. Plasmids constructed in this work

Plasmid	Vector	Reference/ Construction
pGP1022	pJOE6743	modified pJOE (GeneArt)
pGP1023	pGP574/*Sac*I + *Bam*HI	PCR-Prod.: *citR*/*Sac*I + *Bam*HI with CZ238/CZ239
pGP1029	pGP380/ *Bam*HI + *Pst*I	PCR-Prod.: *citR*/*Pst*I + *Bam*HI with AK57/58
pGP1030	pGP382/ *Bam*HI + *Pst*I	PCR-Prod.: *citR*/*Pst*I + *Bam*HI with AK59/60
pGP2260	pGP172/*Sac*I + *Bam*HI	PCR-Prod.: *citR*/*Sac*I + *Bam*HI with CZ224/CZ225
pGP2261	pGP888/*Kpn*I + *Xba*I	PCR-Prod.: *citZ*/*KpnI* + *XbaI* with FM20/21

pGP2262	pGP888/*Kpn*I + *Xba*I	PCR-Prod.: *citR*/*KpnI* + *XbaI* with AK89/90
pGP2263	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod.: *yhfU* for insertion between *panB* and *birA* without promoters */ BamHI +NcoI* with AK21/69
pGP2264	pETM-11/ *Nco*I *+ Bam*HI	PCR-Prod.: *citR*/*Nco*I +*Bam*HI with AK76/99
pGP2265	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod.: deletion *yhfR-yhzC* (bp 1.108.708-1.116.817)
pGP2266	pGP1022/ *BamHI + NcoI*	LFH-Prod.: deletion *bglC-ycgJ* (371,726-340,585) with integration of *putP*
pGP2267	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod.: *yhfU* for insertion between *panB* and *birA* without promotors, with long *panB* site */ BamHI +NcoI* with AK21/69
pGP2268	pBluescript II SK(-)/ *Eco*RI + *Xba*I	LFH-Prod.: *ermR/ Eco*RI *+Xba*I, with AK101/ AK102, lox sites added to the resistance with primers
pGP2269	pGP1022/ *BamHI + XhoI*	LFH-Prod. AK119/146 and AK147/148 for deletion of *ermR* next to *ybgE*
pGP2270	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK206/207 and AK205/DR377 for the deletion of *alkA-ycbK*
pGP2271	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK252/253 and AK254/AK255 for the deletion of *ynfC-iseA*
pGP2272	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK258/259 and AK260/AK261 for the deletion of *pckA-mntA*
pGP2273	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *aapA/BamHI +EcoRI* with AK298/299
pGP2274	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *asnB/BamHI +EcoRI* with AK304/305
pGP2275	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *steT/BamHI +EcoRI* with AK300/301
pGP2276	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *ytnA/BamHI +EcoRI* with AK306/307
pGP2277	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *serA/BamHI +EcoRI* with AK308/309
pGP2278	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *yfnA/BamHI +EcoRI* with AK302/303

pGP2279	pBQ200/*BamHI + SalI*	PCR-Prod. *aapA/BamHI +SalI* with AK240/241
pGP2280	pBQ200/*BamHI + SalI*	PCR-Prod. *ytnA/BamHI +SalI* with AK246/247
pGP2281	pBQ200/*BamHI + SalI*	PCR-Prod. *yfnA/BamHI +SalI* with AK244/245
pGP2282	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK216/217 and AK215/DR393 for the deletion of *yqiF-yqiG*
pGP2283	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK220/DR527 and AK219/DR387 for the deletion of *yddN-ydfM*
pGP2284	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. DR401/DR402 and AK222/AK223 for the deletion of *yqzK-yqjT*
pGP2285	pBQ200/*BamHI + SalI*	PCR-Prod. *steT/BamHI +SalI* with AK242/243
pGP2286	pAC7/ *BamHI + EcoRI*	PCR-Prod. promoter *serA/BamHI +EcoRI* with AK308/309
pGP2287	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *ybeC/BamHI +EcoRI* with AK321/322
pGP2288	pGP1022/ *Bam*HI *+ Nco*I	LFH-Prod. AK313/314 and AK315/316 for the deletion of *yqgM-pstS*
pGP2289	pBQ200/*BamHI + SalI*	PCR-Prod. *ilvA/BamHI +SalI* with AK325/326
pGP2290	pBQ200/*BamHI + SalI*	PCR-Prod. *thrR/BamHI +SalI* with AK333/DT126
pGP2291	pAC7/ *BamHI + EcoRI*	PCR-Prod. promoter *thrR/BamHI +EcoRI* with AK334/335
pGP2292	pGP888/*BamHI+EcoRI*	PCR-Prod. *ypkP/BamHI +EcoRI* with AK360/361
pGP2293	pGP888/*BamHI+EcoRI*	PCR-Prod. *ypnP/BamHI +EcoRI* with AK360/361
pGP2294	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *sdaAB* mutated*/BamHI +EcoRI* with AK379/380
pGP2295	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *sdaAB/BamHI +EcoRI* with AK379/380
pGP2296	pAC5/ *BamHI + EcoRI*	PCR-Prod. promoter *hom* mutated*/BamHI +EcoRI* with MT24/25
pGP2297	pET-SUMOadapt/*Eco31I + XhoI*	PCR-Prod. *hom/Eco31I + XhoI* with AK349/350

pGP2298	pWH844/*BamH*I+ *Sal*I	PCR-Prod. *bcaP /BamHI + SalI* with AK392/393
pGP2299	pWH844/*BamH*I+ *Sal*I	PCR-Prod. *ybxG /BamHI + SalI* with AK394/395
pGP2300	pWH844/*BamH*I+ *Sac*I	PCR-Prod. *hom /BamHI + SalI* with AK387/388

6.3.2. Plasmids used in this work

Plasmid	Description	Reference
pAC5	Construction of translational *lacZ* fusions in *B. subtilis*, integrates into the *amyE* site	Martin-Verstraete *et al.*, 1992
pAC7	Construction of translational *lacZ* fusions in *B. subtilis*, integrates into the *amyE* site	Weinrauch *et al.*, 1991
pBlueskript SK(-)	Vector for cloning	Stratagene
pBQ200	Constitutive overexpression of proteins in *B. subtilis*	Martin-Verstraete *et al.*, 1992
pDG1726	Plasmid for the amplification of the spec cassette for LFH-PCR	Guérout-Fleury *et al.*, 1995
pDG780	Plasmid for the amplification of the kan cassette for LFH-PCR	Guérout-Fleury *et al*., 1995
pETM-11	Overexpression of C-terminal His-Tag fusion proteins in *E. coli*; the His-Tag can be cleaved off with the TEV Protease	Dümmler *et al.*, 2005
pET-SUMO adapt	Fusion of SUMO protein and a His(6) tag at the N-terminus of a protein for inducible overexpression via IPTG in *E. coli*	Hanington *et al.*, 2006
pGEM-cm	Plasmid for the amplification of the cat cassette for LFH-PCR	Guérout-Fleury *et al*., 1995
pGP172	Fusion of *Strep*-tag at the N-terminus of a protein for inducible overexpression via IPTG in *E. coli*	Merzbacher 2004
pGP2072	LFH-Prod. DR337/DR338 and DR339/DR340 for deletion of *yobQ-desR*	Reuß, 2017
pGP2073	LFH-Prod. DR343/DR344 and DR345/DR346 for deletion of *nhaX-yhaX*	Reuß, 2017

pGP2093	LFH-Prod. DR525/DR526 and DR527/DR528 for deletion of *mhqN-mhqP*	Reuß, 2017
pGP2094	LFH-Prod. DR531/DR532 and DR533/DR534 for deletion of *yuzG-sufA*	Reuß, 2017
pGP2514	Plasmid for amplification of kan-lox for the cre-lox system	Zschiedrich, 2014
pGP2515	N-terminal *Strep*-tag Fusion to CitZ for IPTG induced overexpression in *E. coli*	Zschiedrich, 2014
pGP2516	N-terminal *Strep*-tag Fusion to CitA for IPTG induced overexpression in *E. coli*	Zschiedrich, 2014
pGP2980	pGP886 with *ybeC*	Larissa Krüger, PhD thesis
pGP2987	pWH844 with *ybeC*	Larissa Krüger, PhD thesis
pGP2988	pWH844 with *ybeC* without C-terminus	Larissa Krüger, PhD thesis
pGP380	Expression of proteins in *B. subtilis* allows fusion to a Strep-tag at the N-terminus of the protein	Herzberg *et al.*, 2007
pGP382	Expression of proteins in *B. subtilis* allows fusion to a Strep-tag at the C-terminus of the protein	Herzberg *et al.,* 2007
pGP574	Fusion of *Strep*-tag at the C-terminus of a protein for inducible overexpression via IPTG in *E. coli*	Schilling *et al.*, 2006
pGP886	Integration vector (integrates in *ykdE*); allowing the expression of genes under the control of the xylose-inducible P*xylA* promoter in *B. subtilis*	Gerwig *et al.*, 2014
pGP888	Integration vector (integrates in *ganA*); allowing the expression of genes under the control of the xylose-inducible P*xylA* promoter in *B. subtilis*	Diethmaier *et al.,* 2011
pJK195	Integration of the xylose, inducible cre recombinase in the *sacA* locus	Kumpfmüller *et al.*, 2013
pJOE6743.1	Marker-free deletion in a *manPA* deletion strain	Wenzel and Altenbuchner, 2015
pJOE6981.2	Plasmid for the marker-free deletion of *spoIIIAH-yqhV* (2,532,960 -2,538,080)	Josef Altenbuchner, Stuttgart
pJOE8670.1	Plasmid for the marker-free deletion of *mta-rapB* (3,764,119-3,772,301)	Josef Altenbuchner, Stuttgart

pJOE9255	pJOE6743 via *BamH*I, deletes *ypsA-sspM*	Josef Altenbuchner, Stuttgart
pJOE9256	pJOE6743 via *BamH*I, deletes *uxaC-spoIISA*	Josef Altenbuchner, Stuttgart
pWH844	Fusion of His(6) tag at the N-terminus of a protein for inducible overexpression via IPTG in *E. coli*	Schirmer *et al.*, 1997

6.4. Oligonucleotides

6.4.1. Oligonucleotides constructed in this work

Name	Sequence	Purpose/ Reference
AK18	**[phos]**CCATCAAAAACCGGTCTGCCATACG	fwd, *yhfU* for LCR
AK19	**[phos]**TCATCCTCCTTTTGTAAACATCGTATCAGAAAG	rev, *yhfU* for LCR
AK20	**[phos]**TGTGTTGGTACAAGCCCGTTGATTTTG	fwd, upstream LCR of *yhfU,* in *panB*
AK21	**[phos]**TTTGGATCCACTTTATAGCCGCCCAGTACGCCG	rev, upstream LCR of *yhfU*
AK22	**[phos]**AAAGGATCCAAGCACGCAAAAAACGGCTCATGAGC	fwd, downstream LCR of *yhfU,* in *birA*
AK23	**[phos]** TTAGCCCAATTCGATATCGGCAGAATAG	rev, downstream LCR of *yhfU,* in *birA*
AK24	CTATTCTGCCGATATCGAATTGGGCTAACCATCAAAAACCG GTCTGCCATACG	bridging oligo LCR of upstream + *yhfU*
AK25	CTTTCTGATACGATGTTTACAAAAGGAGGATGATGTGTTGG TACAAGCCCGTTGATTTTG	bridging oligo LCR of *yhfU* + downstream
AK26	AAAGGATCCGATCCATAACGGAATGCTGAACCAGAC	fwd, downstream LCR of menaquinone, in *mntA*
AK27	**[phos]**GAAAAAAGCATTTTGCTATTTTGAATAAATGACACT G	rev, downstream LCR of menaquinone, in *mntA*
AK28	**[phos]** TTAACCATGCTGTGTGAAAACATCCATTTTGG	fwd, upstream LCR of menaquinone, in *menC*
AK29	TTTGAATTCCCTGGCGCCTCTTGACGATATG	rev, upstream LCR of menaquinone, upstream of *menC*

AK30	**[phos]** TTATCGGAAATAGCTGATCAATAATCCGATC	fwd, *menA* or LCR
AK31	**[phos]** ATCAAAAATTCCCTTCCCGTTTTTTCGACAATC	rev, *menA* for LCR, upstream
AK32	**[phos]**TTAAAATTTTCTTTTACCGATATATTTTGCGATGGC	fwd, *hepT-menH-hepS* for LCR
AK33	**[phos]** GCTGGCTGTCCCCGCTGTTAAAA	rev, *hepT-menH-hepS* for LCR, upstream
AK34	CAGTGTCATTTATTCAAAATAGCAAAATGCTTTTTTCTTATC GGAAATAGCTGATCAATAATCCGATC	briging oligo for the LCR of downstream+ *menA*
AK35	GATTGTCGAAAAAACGGGAAGGGAATTTTTGATTTAAAATT TTCTTTTACCGATATATTTTGCGATGGC	briging oligo for the LCR of *menA+ hepT-menH-hepS*
AK36	TTTTAACAGCGGGGACAGCCAGCTTAACCATGCTGTGTGAA AACATCCATTTTGG	briging oligo for the LCR of *hepT-menH-hepS+* upstream
AK37	**[phos]** CTTGCCTATGGAT**A**CAGATCGCG	Fwd, CCR primer *menA*
AK38	**[phos]** CGCGATCTG**T**ATCCATAGGCAAG	Rev, CCR primer *menA*
AK39	AAAGTCGACCGCTTGTAAACCGTTTTGTGAAAAAATTTTTA AAATAAAAAAG	amplification of pJOE6743 from the CDS ter
AK40	TTTGTCGACCTTGTAAACCGTTTTGTGAAAAAATTTTTAAAA TAAAAAAGGGG	rev CDS ter, construction of modif. pJOE
AK41	**[phos]** CATCCGGTGGATGACCTTTTGAATGAC	fwd CDS ter, construction of modif. pJOE
AK42	**[phos]** CAATACGCAAACCGCCTCTCCC	rev Insert pJOE, construction of modif. pJOE
AK43	**[phos]** TCTAGAGGATCCCCGGGTACCAT	fwd MCS of pJOE6743, construction of modif. pJOE
AK44	**[phos]GTGACACTATAGAA**GATCGATGAATTCGAGCTCGT ACGC	rev MCS of pJOE6743, construction of modif. pJOE
AK45	**[phos]CTAAATCGTATGC**CATCCGGTGGATGACCTTTTGAA TGAC	fwd CDS ter, construction of modif. pJOE
AK46	GTCATTCAAAAGGTCATCCACCGGATGGTAAAGCTTGCATG CCTAATACGAC	bridging oligo for the LCR of CDS term +DR207 fragment
AK47	GAGAGGCGGTTTGCGTATTGTCTAGAGGATCCCCGGGTAC C	bridging oligo for the LCR of DR207 fragment + MCS

AK48	CGAATTCATCGATCTTCTATAGTGTCACCTAAATCGTATGCC ATCCGGTGGATGAC	bridging oligo for the LCR of MCS + CDS term
AK49	**[phos]** GTAAAGCTTGCATGCCTAATACGAC	DR207 with phosphorylation
AK50	CAGTTGCGCAGCCTGAATGGC	Sequencing primer modif. pJOE
AK51	CGCGGGGAGAGGCGGTTTG	Sequencing primer modif. pJOE
AK52	CTCTTCGCTATTACGCCAATCTAGATCC	Sequencing primer pGP1022
AK53	GTTGGCCGATTCATTAATGCAGATCGATC	Sequencing primer pGP1022
AK54	AAAGCTAGCGTCAACATACGTAGAAGTTGTGATCTCTCC	fwd, downstream, LCR of menaquinone, in *metK*
AK55	**[phos]** CAACTGAGTTCATATGAAACCTTCCTTTATCG	rev, downstream LCR of menaquinone, in *metK*
AK56	CGATAAAGGAAGGTTTCATATGAACTCAGTTGTTATCGGAA ATAGCTGATCAATAATCCGATC	bridging oligo for the LCR of metK+ *menA*
AK57	AAAGGATCCATGGATTTCAAATGGCTTCACACCTTTG	fwd, *citR* for N-term. Strep-tag fusion in pGP380
AK58	TTTCTGCAGCTCCTAAAAATGAAAATGTGATAAAAAATCCA AGAAC	rev, *citR* for N-term. Strep-tag fusion in pGP380
AK59	AAAGGATCCCAGAGGGAGAATAGAAATGGATTTCAAATG	fwd, *citR* for C-term. Strep-tag fusion in pGP382
AK60	TTTCTGCAGAAAATGAAAATGTGATAAAAAATCCAAGAACT TTTTTTCTTTC	rev, *citR* for C-term. Strep-tag fusion in pGP382
AK61	AAAGAATCCCCCCAATGCCTTTTTTATAGTATATG	fwd, upstream (*comK*) of *yhfU* deletion region
AK62	AGAGATCGGCAGCTCCATCGTTTTC	rev, upstream (*comK*) of *yhfU* deletion region
AK63	CGGCTACACATCTGTCGGCAC	fwd, downstream (*yhfQ*) of *yhfU* deletion region
AK64	**CATATACTATAAAAAAGGCATTGGGGGGATTCTTT** CTTTTATTTCTTAGCAGCCGGCATCTCTTTTTG	rev, downstream (*yhfQ*) of *yhfU* deletion region
AK65	AAAGGTCTCATGGTATGGATTTCAAATGGCTTCACACCTTT GTG	fwd, *citR* for pET-SUMOadapt

AK66	TTTCTCGAGCTAAAAATGAAAATGTGATAAAAAATCCAAGA ACTTTTTTTC	rev, *citR* for pET-SUMOadapt
AK67	CGTATGGCAGACCGGTTTTTGATGGTTAGCCCAATTCGATA TCGGCAGAATAG	bridging oligo LCR of upstream + *yhfU*
AK68	CAAAATCAACGGGCTTGTACCAACACATCATCCTCCTTTTGT AAACATCGTATCAGAAAG	bridging oligo LCR of *yhfU* + downstream
AK69	TTTCCATGGAAGCACGCAAAAAACGGCTCATGAGC	fwd, downstream, LCR of *yhfU,* in *birA*
AK70	**GTATAATGTATGCTATACGAACGGTA**CTGAATAAATCAGT TGAATCTGTC	rev, upstream LFH of *citZ-icd-mdh*
AK71	**GTATAGCATACATTATACGAACGGTA**GTTGTTGCTACAAC CCCTTC	fwd, downstream LFH of *citZ-icd-mdh*
AK72	**CCTATCACCTCAAATGGTTCGCTG**CTAAAAATGAAAATGTG ATAAAAAATCCA	*citR* reverse, fusion of *lacA-cat-citR-lacA*
AK73	**CAGCCATATTGATGGTGAAAAAG**CCGTATCGTTCTGCTAA TAAGC	*lacA* reverse downstream, fusion of *lacA-cat-citR-lacA*
AK74	**CTTTCTGATACGATGTTTACAAAAGGAGGATGA**CATGAAA ACAAAACTGGATTTTCTAAAAATGAAGGA	downstream fwd *panB*, *yhfU* LFH without promoter
AK75	**CTATTCTGCCGATATCGAATTGGGCTAA**ATGCTGAAATTAA TCGACATGATGCATATTGCG	fwd, *yhfU* for LFH, without promoter
AK76	AAACCATGGAAATGGATTTCAAATGGCTTCACACCTTTGTG	fwd, *citR* for N-terminal His-tag fusion in pETM-11
AK77	**GTATAATGTATGCTATACGAACGGTA**CTATACCTGAATCTT CTAACGC	rev, upstream LFH *citB*
AK78	**GTATAGCATACATTATACGAACGGTA**CATCCTTCAAATGGT GCTTCG	fwd, downstream LFH *citB*
AK79	**GTATAGCATACATTATACGAACGGTA**CTGGAAGATCCTGA ACAGCT	fwd, downstream LFH *odhAB*
AK80	**GTATAATGTATGCTATACGAACGGTA**GTAAAAATCTTCCCA ATTCATTC	rev, upstream LFH *odhAB*
AK81	**GTATAATGTATGCTATACGAACGGTA**CACTTTACCTTCAGG AACAGATACCCC	rev, upstream LFH *sucCD*
AK82	**GTATAGCATACATTATACGAACGGTA**GCAGAGACACCTTC TGTCATGGGTG	fwd, downstream LFH *sucCD*
AK83	**GTATAATGTATGCTATACGAACGGTA**GACGACTAAATGCT GAATAAG	rev, upstream LFH *sdhCAB*

AK84	**GTATAGCATACATTATACGAACGGTA**GATTGCAGCCTTGAATAGAG	fwd, downstream LFH *sdhCAB*
AK85	**GTATAGCATACATTATACGAACGGTA**CGGAAGACATGGTAAAACCAAAGGCG	downstream LFH *citG*
AK86	**GTATAATGTATGCTATACGAACGGTA**CCATGGTGTCTCGTTCAATTCTGTATTCC	rev, upstream LFH *citG*
AK87	**CCTATCACCTCAAATGGTTCGCTG**GAGCACAGGCGTTTTGGTTGCTCC	rev, *citR* for the integration into *lacA* (*lacA-cat-citR*-fusion)
AK88	CGTAATGTACCATTTGTATTCCCCCTATCC	fwd, *citR* for the integration into *lacA* (*lacA-cat-citR*-fusion)
AK89	AAATCTAGAAATGGATTTCAAATGGCTTCACACCTTTG	fwd, *citR* for the integration into *lacA* with pGP888
AK90	TTTGGTACCCTCCTAAAAATGAAAATGTGATAAAAAATCCAAGAAC	rev, *citR* for the integration into *lacA* with pGP888
AK91	**GATCGGATTATTGATCAGCTATTTCCGATAA**CAACTGAGTTCATATGAAACCTTCCTTTATCG	rev, downstream, LCR of *menA,* in *metK*
AK92	**GATTGTCGAAAAAACGGGAAGGGAATTTTTGAT**TTAACCATGCTGTGTGAAAACATCCATTTTGG	fwd, upstream, LCR of *menA,* in *menC*
AK93	**CGATAAAGGAAGGTTTCATATGAACTCAGTTG**TTATCGGAAATAGCTGATCAATAATCCGATC	fwd, *menA* for LCR
AK94	**CCAAAATGGATGTTTTCACACAGCATGGTTAA**ATCAAAAATTCCCTTCCCGTTTTTTCGACAATC	rev, *menA* for LCR, binds upstream
AK95	AAAGAATCCCCCCAATGCCTTTTTTATAGTATATG	rev, upstream *yhfU* deletion region
AK96	AAAGGATCCAGAGATCGGCAGCTCCATCGTTTTC	fwd, upstream *yhfU* deletion region
AK97	TTTCCATGGCGGCTACACATCTGTCGGCAC	rev, downstream *yhfU* deletion region
AK98	**CATATACTATAAAAAAGGCATTGGGGGGATTCTTT**CTTTTATTTCTTAGCAGCCGGCATCTCTTTTTG	fwd, downstream *yhfU* deletion region
AK99	TTTGGATCCCTAAAAATGAAAATGTGATAAAAAATCCAAGAACTTTTTTTC	rev, *citR* for N-terminal His-tag fusion in pETM-11
AK100	AAAGGATCCCGATGGTTTCATCAATGCGCGTATATTGC	rev, upstream, LCR of *yhfU* (long)
AK101	AAAGAATTCTACCGTTCGTATAGCATACATTATACGAAGTTATGATCCTTTAACTCTGGCAACCCTCAAAATTG	fwd, erm resistance +lox71 for pBluescript

AK102	TTTTCTAGATACCGTTCGTATAATGTATGCTATACGAAGTTA TGCCGACTGCGCAAAAGACATAATCG	rev, erm resistance +lox71 for pBluescript
AK103	AAAGAATTCTACCGTTCGTATAGCATACATTATACGAAGTT ATCGGCAATAGTTACCCTTATTATCAAGATAAGAAAG	fwd, cat resistance +lox71 for pBluescript
AK104	TTTTCTAGATACCGTTCGTATAATGTATGCTATACGAAGTTA TCCAGCGTGGACCGGCGAG	rev, cat resistance +lox71 for pBluescript
AK105	AAAGGATCCGCCAAGCATATTAAGGATGCGATAAATGAG	fwd, downstream, defragmentation of *putP* (LFH)
AK106	**CGACTACCAAGATACAATGTCGCAATAAAG**CATTACACAG AAACAGCATCAATAATATAAGTGTC	rev, downstream, defragmentation of *putP* (LFH)
AK107	**GAGGATGCCCATTTAGTCCCGC**TTACATCGGCTGATAAAG ATCCCAGC	fwd, upstream, defragmentation of *putP* (LFH)
AK108	AAACCATGGGTACAATGAATGTCATTCAGGCAAAAATGGC	rev, upstream, defragmentation of *putP* (LFH)
AK109	CTTTATTGCGACATTGTATCTTGGTAGTCG	fwd, *putP* defragmentation of *putP* (LFH)
AK110	GCGGGACTAAATGGGCATCCTC	rev, *putP* defragmentation of *putP* (LFH)
AK111	CTTCAACCGCAACTGTACAGGTGC	rev, *putP* defragmentation of *putP* (LFH)
AK112	**GCACCTGTACAGTTGCGGTTGAAG**TTACATCGGCTGATAA AGATCCCAGC	fwd, upstream, defragmentation *putP* (LFH)
AK113	AAAGGATCCGCTGTTTTGCTTATGCAAAACAGCTTTTTTGTC	fwd, upstream, defragmentation *tcyP* (LFH)
AK114	GTGACCATACTCCTATCTATGTATTAGAGCATG	rev, upstream, defragmentation *tcyP* (LFH)
AK115	**CATGCTCTAATACATAGATAGGAGTATGGTCAC**GGTCAAA TTTACAAACAGTTCTTTCAGCAAATATTATC	fwd, *tcyP*, defragmentation *tcyP* (LFH)
AK116	**GGGCTGCAGACAGCCCGTTATGAT**GTTACGCTTCTTCAGCT TCAATCACTCTG	rev, *tcyP*, defragmentation *tcyP* (LFH)
AK117	ATCATAACGGGCTGTCTGCAGCCC	fwd, downstream, defragmentation *tcyP* (LFH)
AK118	TTTCCATGGCTTCGTCCACTGTGATCGTCAGCT	rev, downstream, defragmentation *tcyP* (LFH)
AK119	AAAGGATCCCCTGACAATCACAAACGTACCTGGATC	fwd, upstream, defragmentation *ybgE* (LFH)

AK120	**AATTGATCTTCCGCGCCCATGCAAAC**TTACTCCACAGTAAC ACTCTTCGCAAGGTT	rev, upstream, defragmentation *ybgE* (LFH)
AK121	GTTTGCATGGGCGCGGAAGATCAATT	fwd, *ybgE*, defragmentation *ybgE* (LFH)
AK122	**GCCGGAACACCATTTCGTCATCAAG**TCACACTTCCACTGTC CAGTTAAACGG	rev, *ybgE*, defragmentation *ybgE* (LFH)
AK123	CTTGATGACGAAATGGTGTTCCGGC	fwd, downstream, defragmentation *ybgE* (LFH)
AK124	TTTCTCGAGCCATACGTGAGGGTCCATTGCATGA	rev, downstream, defragmentation *ybgE* (LFH)
AK125	AAAGGATCCCCGTCAGTGAAACGGTAGGTTTTCC	fwd, downstream, defragmentation *mntH* (LFH)
AK126	CACGGGTGGTCGGTTATTGATTAAGTTTC	rev, downstream, defragmentation *mntH* (LFH)
AK127	**GAAACTTAATCAATAACCGACCACCCGTG**TTATCGAAACG TATCTACAATTAAAAACACATTCAAAGC	fwd, *mntH,* defragmentation *mntH* (LFH)
AK128	CATTTTCGGTTGACAAGAAACCGGGATG	rev, *mntH,* defragmentation *mntH* (LFH)
AK129	**CATCCCGGTTTCTTGTCAACCGAAAATG**CGGCCATTATCGG GCTGAAACAATTTCGT	fwd, upstream, defragmentation *mntH* (LFH)
AK130	TTTCTCGAGAGCGCCAATGCCAAAACGCCTGAA	rev, upstream, defragmentation *mntH* (LFH)
AK131	CAGCCTGACAAAAGCAATACCTATGTCG	fwd, sequencing of *yveA*
AK132	CTTTATTTATCATAATCAATGGCGGCTTTTGGG	rev, sequencing of *yveA*
AK133	GCAATGAGAACTCCCGGCCAATTG	rev, sequencing of *yveA*
AK134	CTATGAGCACACACAAAAAGAGCCCAC	fwd, sequencing of *ytnA*
AK135	CTTGCGGATCTCTGGCGAATGCT	fwd, sequencing of *yodF*
AK136	CTGCCGCGTATGGTTTTTTTATGCGC	rev, sequencing of *yodF*
AK137	GTCACACGTTCAATGGTGTTTGTTGTCATC	fwd, sequencing of *yodF*
AK138	CTCGAGGCCGTGTCAGTGATGAAGGGCC	rev, LFH deletion plasmid for *mntH* defragmentation

AK139	**GAGGGTTGCCAGAGTTAAAGGATC**CACGGGTGGTCGGTTATTGATTAAGTTTC	fwd, downstream, LFH for *mntH* defragmentation
AK140	**CGATTATGTCTTTTGCGCAGTCGGC**TTATCGAAACGTATCTACAATTAAAAACACATTCAAAGC	rev, *mntH*, LFH for *mntH* defragmentation
AK141	GATCCTTTAACTCTGGCAACCCTC	fwd, *mls* for LFH *mntH*
AK142	GCCGACTGCGCAAAAGACATAATCG	rev, mls for LFH *mntH*
AK143	**GCCGGAACACCATTTCGTCATCAA**GGTTTGCATGGGCGCGGAAGATCAATT	fwd, *ybgE*, defragmentation of *ybgE* (LFH)
AK144	**CGATTATGTCTTTTGCGCAGTCGG**CTCACACTTCCACTGTCCAGTTAAACGG	rev, *ybgE*, defragmentation of *ybgE* (LFH)
AK145	**GAGGGTTGCCAGAGTTAAAGGATC**TTACTCCACAGTAACACTCTTCGCAAGGTT	rev, upstream, defragmentation *ybgE* (LFH)
AK146	**CCGTTTAACTGGACAGTGGAAGTGTGA**TTACTCCACAGTAACACTCTTCGCAAGGTT	rev, upstream, defragmentation *ybgE* (deletion plasmid)
AK147	AAACTCGAGGAGCTGGAGAAAGATTGGGTTCCAAAG	fwd, *ybgE,* defragmentation *ybgE* (deletion plasmid)
AK148	TCACACTTCCACTGTCCAGTTAAACGG	rev, *ybgE,* defragmentation *ybgE* (deletion plasmid)
AK149	TTTGGATCCCGATACAAATTCCTCGTAGGCGCTC	rev, *kanR* for the ligation of glycolytic cassette fragment
AK150	AAAGGATCCAAGTCTACGAGGACCTTACTGATT	fwd, *nrnA* for the ligation of glycolytic cassette fragment
AK151	CCGTCAAACTGACAGTTGCCAAATGG	rev, sequencing *yodF*
AK152	**GTATAGCATACATTATACGAACGGTA**GATCCTAATCGCCATCTTCCAGCAG	fwd, P_{xylA}-*cre* and *xylR* for integration into *citB*
AK153	**CGAAGCACCATTTGAAGGATG**CTAACTTATAGGGGTAACACTTAAAAAAGAATCAATAACG	rev, P_{xylA}-*cre* and *xylR* for integration into *citB*
AK154	CATCCTTCAAATGGTGCTTCG	fwd, *citB* down
AK155	CTTGAGAAATCATATAATTGAATCTCATCCCATTG	fwd, sequencing *xylR* for integration into *citB*
AK156	CGCTGGAGTTTCAATACCGGAGATC	rev, sequencing *cre* for integration into *citB*

AK157	GCTCTCCTGAGAATGTCCGTCTTTC	fwd, Sequencing *asnS*
AK158	CATCGTCACACCTCTTACTGTAAAGGATTG	rev, Sequencing *asnS*
AK159	**AGCAGCAACTGTTCTTACATACTTTC**CCTTGGTATCATTGATGCGGC	rev, upstream pGP2270
AK160	CTTTCACTTCCATCATCTCTGTATCCC	fwd, upstream LFH *aapA*
AK161	**CCTATCACCTCAAATGGTTCGCTG**GGCCTCTAGACAATTTCTGTTGCTGG	rev, upstream LFH *aapA*
AK162	**CGAGCGCCTACGAGGAATTTGTATCG**AGGTGCAAACCCGCAGAGGAC	fwd, downstream LFH *aapA*
AK163	ACATCTCCCCTTATGACAAGACTTTCC	rev, downstream LFH *aapA*
AK164	CTCAGCCTGATCAAAATGCTCTTCCG	rev, sequencing of LFH *aapA*
AK165	GGAGGAGCATATGCAGACCGTTC	fwd, sequencing of LFH *aapA*
AK166	GTCGCAATTGCGTTTGCCGTATCATG	rev, downstream LFH *stet*
AK167	**CGAGCGCCTACGAGGAATTTGTATCG**GCCGGACTGCCTGTTTATTACGG	fwd, downstream LFH *stet*
AK168	**CCTATCACCTCAAATGGTTCGCTG**CAAGAGGCCTATCTCTTTTTTCAAACCG	rev, upstream LFH *stet*
AK169	GTTCCTTGCCCTTCGACTGGTTC	fwd, upstream LFH *stet*
AK170	CTTACCCGAAATGCGACGTGGTG	fwd, sequencing of LFH *stet*
AK171	GGATCTACGATGTCTTTCGTAATGGTTTTG	rev, sequencing of LFH *stet*
AK172	GATACACCGTGGTGCCTGGTTTTTG	rev, downstream LFH *yfnA*
AK173	**CGAGCGCCTACGAGGAATTTGTATCG**GGCTTTCATTTGTCATCTGGATCGCTG	fwd, downstream LFH *yfnA*
AK174	**CCTATCACCTCAAATGGTTCGCTG**CTGCGCACTCAATGTTTCAAGCGG	rev, upstream LFH *yfnA*
AK175	CCGATGTAGCTGCCGACTTTCG	fwd, upstream LFH *yfnA*

AK176	GCTGAACACCGCCGCATTGAC	fwd, sequencing of LFH *yfnA*
AK177	CGTCAGGCTCATGGAGCGTTC	rev, sequencing of LFH *yfnA*
AK178	CACATGTCAAAACAACTGAAGCAAAAGCTTC	rev, sequencing of LFH *yqiK* (mutated)
AK179	GCGCTGGCATTCCCGTAGTTG	rev, downstream LFH *yqiK* (mutated)
AK180	**CCTATCACCTCAAATGGTTCGCTG**TTGTTAGAAGGAGGCTG TTTGACGCAG	fwd, downstream LFH *yqiK* (mutated)
AK181	**CGAGCGCCTACGAGGAATTTGTATCG**CTATTCATTTTTCAG CAATGCCGAAGCC	rev, upstream LFH *yqiK* (mutated)
AK182	CGATATTCAGTCACAGCTCGTTTCTTC	fwd, upstream LFH *yqiK* (mutated)
AK183	CAGCATTCATGTGAATTCAGGGGTTG	fwd, sequencing of LFH *yqiK* (mutated)
AK184	GGTTTCACCTCATTCAGAAGATAGACAG	rev, sequencing *yqiK*
AK185	CATATTCACAAATGCCAAGTGTGTTGGC	fwd, sequencing *yqiK*
AK186	**CGAGCGCCTACGAGGAATTTGTATCG**CCGGTGGGCAAAAA TCTTTGTCATCG	rev, upstream LFH *yqiK* (deletion)
AK187	CATCAGGAAGAGGTGGTCATGTGAAAC	fwd, upstream LFH *yqiK* (deletion)
AK188	GGAAATGATCGGGTCGTATAGCCC	fwd, sequencing of LFH *yqiK* (deletion)
AK189	CATTCCCGTTTTGGAAATTGCTAAACCTG	fwd, sequencing *ybeC*
AK190	GTAACAGGAGTGTTCACCAACTATCC	rev, sequencing *ybeC*
AK191	GACGATTTCAGAGATCCGAAAGTCATATG	fwd, sequencing LFH *yveA*
AK192	CTTAAGGACTGGCATTACACAAGCGG	fwd, upstream LFH *yveA*
AK193	**CCTATCACCTCAAATGGTTCGCTG**CTTGTTTAGACATTCGCT TCCTCCTTTG	rev, upstream LFH *yveA*
AK194	**CGAGCGCCTACGAGGAATTTGTATCG**CCCAAAAGCCGCCA TTGATTATGATAAATAAAG	fwd, downstream LFH *yveA*

AK195	GCTGCTGAGAAGGATTCGCCC	rev, downstream LFH *yveA*
AK196	CCATGCCATTATTTTTCACCTCCCGG	rev, sequencing LFH *yveA*
AK197	CAGCCGATGCGGTTGTCCCAG	rev, sequencing LFH *ysdA*
AK198	GGCTTCTTCCCGGCTTGAAGC	rev, downstream LFH *ysdA*
AK199	**CGAGCGCCTACGAGGAATTTGTATCG**CTCATCGCTATTTAC TACAGCCCGTTTG	fwd, downstream LFH *ysdA*
AK200	**CCTATCACCTCAAATGGTTCGCTG**CCGCACAGATTAATCAA CACCAAATAAGC	rev, upstream LFH *ysdA*
AK201	CATGGACTACGGTAAGTTCCGATTTG	fwd, upstream LFH *ysdA*
AK202	GAATCAAGTCCCGTCAGGAAGCAC	fwd, sequencing LFH *ysdA*
AK203	GTCCGCACCAGGCCAAATTCC	rev, sequencing of *serA*
AK204	CAAGCTGTCAGATCATTGATTTATTAGGCTTTAC	fwd, sequencing of *serA*
AK205	**CTGTTTTCAGCTTCCTGTATTCCATGC**CATTTATTACTCCAC AGTAACACTCTTCGC	rev, upstream for pGP2270
AK206	GCATGGAATACAGGAAGCTGAAAACAG	fwd, downstream for pGP2270
AK207	AAACCATGGGATCATCAAAGTACTCTTCATTCCAAACGG	rev, downstream for pGP2270
AK208	CCTAATTTATAACCGATGCCCCATAACG	rev, sequencing of pGP2270 deletion
AK209	AAAGAATTCGTTGGTTCTTTGTATTCTGGGTGGGG	fwd, promoter 1+2 of *infC* for pAC5
AK210	AAAGAATTCGTAAAAGTTGTTCCGGATAAGTCGTCC	fwd, promoter 2 of *infC* for pAC5
AK211	TTTGGATCCATACCCTCATTAACCAATTGATCTTTGCTAATA AT	rev, promoter of *infC* for pAC5, AUU start codon
AK212	TTTGGATCCATACCCTCATTAACCAATTGATCTTTGCTAATC AT	rev, promoter of *infC* for pAC5, AUG start codon
AK213	CGATATCCAAATTGTAAGATTGACTGGGAC	rev, sequencing *infC-rpmI-rplT-ysdA* operon

AK214	CTGTATCAGTTTGTTCATGTGTCAGGC	fwd, sequencing *infC-rpmI-rplT-ysdA* operon
AK215	**GAGCTTATTAAGTGGTCATTAAATCAAACGTC**CAAGACTGTTTGGCGCGGTACTTTG	rev, upstream for pGP2282
AK216	GACGTTTGATTTAATGACCACTTAATAAGCTC	fwd, downstream for pGP2282
AK217	TTTCCATGGGACATGGAAGTGATCGGCGTTGC	rev, downstream for pGP2282
AK218	CCTGTTAACTACATTTGGGGAGGAAG	rev, sequencing of pGP2282
AK219	**AGCAGCAACTGTTCTTACATACTTTC**CCCTTGGTATCATTGATGCGGCC	rev, upstream for pGP2283
AK220	TTTCCATGGCGGACAAAACTTGCAAAACAGCCATAC	rev, downstream for pGP2283
AK221	CTCTTAAATCTGCCCCGTTCTCAAG	fwd, sequencing of pGP2284
AK222	AAACCATGGCTTCCAAAATCCCTGGCGGCTG	fwd, upstream for pGP2284
AK223	**TGTATGTCTCTGATTTGGAGGC**GCGGATGGTTCGACCGGTTG	rev, upstream for pGP2284
AK224	CTTCTCCCCCCTCATCCGAAG	fwd, sequencing of *tcyK* LFH
AK225	GAACCATTCGAATAAAACCGCTACAGC	fwd, downstream *tcyK* LFH
AK226	**CCGAGCGCCTACGAGGAATTTGTATCG**GACGACTATTCCAAAGAGC	rev, downstream *tcyK* LFH
AK227	**CCTATCACCTCAAATGGTTCGCTG**GTTATAAGCGAAAATAATATTGCCATGAATGCTG	fwd, upstream *tcyK* LFH
AK228	CCGGGTATGACATTGAAGTGATGAAAG	rev, upstream *tcyK* LFH
AK229	GCATTACTTGGCGGGGGATGTTC	rev, sequencing of *tcyK* LFH
AK230	CCTTCGTACCTGTATTTTCATTCCGTATATATG	fwd, sequencing of *glyA*
AK231	GTGCGGGCGGTTTATGAGTGC	rev, sequencing of *glyA*
AK232	ATTCATCCGCAAGCCTTGCAGGG	fwd, *glyA* deletion region

AK233	GTTAGCGGCGAAGTTGACAGAGG	fwd, *glyA* deletion region
AK234	CTAAATGTAAGCTTGGGATCGTCCATC	fwd, sequencing of *glyA*
AK235	CATGTTAAACGGCAGGATACCTGCG	fwd, upstream *serA* LFH
AK236	**CCTATCACCTCAAATGGTTCGCTG**GTCTGAGACCAATACTC GAAACATCG	rev, upstream *serA* LFH
AK237	**CGAGCGCCTACGAGGAATTTGTATCG**GTGTCTGTGAAGCT CATTGATCTGCC	fwd, downstream *serA* LFH
AK238	GTGCCAGCTGCTCCAAATCCG	rev, downstream *serA* LFH
AK239	GGAGTCAGAAGTTGATGATTCTATTGCC	rev, sequencing of *serA* LFH
AK240	AAAGGATCCCCGATAAAACTTTACTCATCTTTTTATTACTGG AG	fwd, *aapA* for pBQ200
AK241	TTTGTCGACTTATTTCACCTTATGTCCTCTGCGGG	rev, *aapA* for pBQ200
AK242	AAAGGATCCCATCATAGGAGGTTAAGGACATGCATAC	fwd, *steT* for pBQ200
AK243	TTTGTCGACCGTTTTATCAGCTTGCTTTTCGTTTTTTCATC	rev, *steT* for pBQ200
AK244	AAAGGATCCCTAGGAGGAACTTTTGATGAGTTCATTATTTA G	fwd, *yfnA* for pBQ200
AK245	TTTGTCGACACCGCCGGCTGAAAAGAGATTATTTG	rev, *yfnA* for pBQ200
AK246	AAAGGATCCCTAGGGGAGAAGAAGCATGCAAAAAC	fwd, *ytnA* for pBQ200
AK247	TTTGTCGACCTTTTTGTCAGCTGATATTTCGTTCGCTG	rev, *ytnA* for pBQ200
AK248	AAAGGTACC**A**ATGACAAAGATTTTTGCCCACCGGGG	fwd, *yqiK* for pGP172
AK249	TTTGGATCCCCTTCTAACAACTATTCATTTTTCAGCAATGC	rev, *yqiK* for pGP172
AK250	AAAGGTACC**A**ATGAGAAAAAATAGAATACTGGCCTTGTTT GTTC	fwd, *glpQ* for pGP172
AK251	TTTGGATCCGCTTTTAATAACCCTTTTTTACTTTGTGGAAAA G	rev, *glpQ* for pGP172

AK252	AAACCATGGCCTTAAATCTGAAGGGTGAAGATGAACTG	fwd, upstream of pGP2271
AK253	**CCGGGCGTTTTTCTTATATAACTGCG**CGAAAATATGAGGAGGCTGTTAAACATGTTG	rev, upstream of pGP2271
AK254	CGCAGTTATATAAGAAAAACGCCCGG	fwd, downstream of pGP2271
AK255	TTTGGATCCGCCGATTCGCAGTCTAGAGAAAATCC	rev, downstream of pGP2271
AK256	CCGCCGCACTTTGACATTCAACG	rev, sequencing of pGP2271
AK257	GAATTTGATCCGTCGGCATATCTTCTATTC	fwd, sequencing of pGP2271
AK258	AAACCATGGCATACGTAGAAGTTGTGATCTCTCCG	fwd, upstream pGP2272
AK259	**CAAAATGGATGTTTTCACACAGCATGGTTAAG**GAGTTCATATGAAACCTTCCTTTATCGTTTTTTG	rev, upstream pGP2272
AK260	CTTAACCATGCTGTGTGAAAACATCCATTTTG	fwd, downstream pGP2272
AK261	TTTGGATCCCCTGGCGCCTCTTGACGATATG	rev, downstream pGP2272
AK262	GACTTGAATCGGCTGTATGGGATATTTATG	rev, sequencing pGP2272
AK263	GATGTTAAAACCGCACAAGTTTCCGC	fwd, sequencing pGP2272
AK264	AAAGGATCCCAGCGCCCACTTCAGGAAGTTC	fwd, upstream deletion plasmid *fadE-mrgA*
AK265	**GTTACCCTAAATAAGAGGAAAGCATCCAC**GAGGCTGAGAAATATACTGTCTGATTGG	rev, upstream deletion plasmid *fadE-mrgA*
AK266	GTGGATGCTTTCCTCTTATTTAGGGTAAC	fwd, downstream deletion plasmid *fadE-mrgA*
AK267	TTTCCATGGGTAAAAAAAGTGGCGAGCTTTGGTGAC	rev, downstream deletion plasmid *fadE-mrgA*
AK268	GCTGACTGTTACGCTATACAACGGAG	rev, sequencing deletion plasmid *fadE-mrgA*
AK269	CAAGATCTTCGTTTACTTCCACAACCG	fwd, sequencing deletion plasmid *fadE-mrgA*
AK270	**AATGACGGGCGGAACCGGTTTTC**GTTTTTCGCCACTTTCTCCCTCATAC	rev, upstream deletion plasmid *yflD-yfhF*

AK271	GAAAACCGGTTCCGCCCGTCATT	fwd, downstream deletion plasmid *yflD-yfhF*
AK272	TTTCCATGGGAGAAGCCCTTGCGCTGAAGC	rev, downstram deletion plasmid *yflD-yfhF*
AK273	CCGTCATAGCGGTATTTTCTAAACGC	rev, sequencing deletion plasmid *yflD-yfhF*
AK274	CGACCAAGAAGCGAGCCCATTC	*fwd,* upstream *ydgF* LFH
AK275	**CGAGCGCCTACGAGGAATTTGTATCG**CATCAATAAGACTC AAAACTCCTGCCTC	*rev,* upstream *ydgF* LFH
AK276	**CCTATCACCTCAAATGGTTCGCTG**GTCTTTCGTCATGTCGTC TGTCACTTTATG	*fwd,* downstream *ydgF* LFH
AK277	GCCTGTTCCCTTGAGATATGCTTGAAG	*rev,* downstream *ydgF* LFH
AK278	CCAATCCCTTTTCGAGCAGCTTTTTC	*rev,* sequencing *ydgF* LFH
AK279	GATTTTATCTGGGCATCCGTACGCTTC	*fwd,* sequencing *ydgF* LFH
AK280	CAATTATGTGAAAGGTGTGCTGATTAGATTG	fwd, sequencing *ybgF* LFH
AK281	GATTATATGTTTGTGATGGACTACGAAGAGG	*fwd,* upstream *ybgF* LFH
AK282	**CGAGCGCCTACGAGGAATTTGTATCG**CTCATTCAAAATAA AAAGAACCTGCCTCC	*rev,* upstream *ybgF* LFH
AK283	**CCTATCACCTCAAATGGTTCGCTG**GAGTTCATCTTTTTCCAA CTTTCTATCAGCG	*fwd,* downstream *ybgF* LFH
AK284	CTATCCAACACATATTAGATACATACCCGC	*rev*,downstream *ybgF* LFH
AK285	CATATGGGCTGAACACCTTTCTCTTTTTGC	*rev,* sequencing *ybgF* LFH
AK286	GGGCGCAACTGAATTTACTCTGATG	fwd, sequencing *yhjB* LFH
AK287	GTTGTGATAATTGAAGCTCCCTCCGG	*fwd,* upstream *yhjB* LFH
AK288	**CGAGCGCCTACGAGGAATTTGTATCG**CCTAAGCATAAAAA AAGCAATCTGGACACC	*rev,* upstream *yhjB* LFH
AK289	**CCTATCACCTCAAATGGTTCGCTG**CACCGAAGATGATGATG AGAGCTGC	*fwd,* downstream *yhjB* LFH

AK290	CGTATTCGCCACCACAAACACGATC	*rev,* downstream *yhjB* LFH
AK291	CGGACAATCAAAAAGACGAGAATGGAAC	*rev,* sequencing *yhjB* LFH
AK292	GTACCAAAATTCAAAGTCTCCTTATTTCAGAAG	fwd, sequencing *ybxG* LFH
AK293	CAAAGGGAAGGCGCAAAACTATAACC	*fwd,* upstream *ybxG* LFH
AK294	**CGAGCGCCTACGAGGAATTTGTATCG**CTTTATTTGCCACTC CTCTTCCTCCTC	*rev,* upstream *ybxG* LFH
AK295	**CCTATCACCTCAAATGGTTCGCTG**GAACAAGCAGCTGAATA ACGATAAAAAAGAG	*fwd,* upstream *ybxG* LFH
AK296	CGGCGAGGAAAGAATGAAAACATGGC	*rev,* upstream *ybxG* LFH
AK297	CGCAATCCACCTTTAAAGTCGTGAAC	*rev,* sequencing *ybxG* LFH
AK298	AAAGGATCCCCAAAATTGTCTTTGCTAGAATTGCCTATCAA	rev, *aapA* promoter for pAC5
AK299	TTTGAATTCGATCATGACAAATTACCCAAATATAACCCCTTA AG	fwd, *aapA* promoter for pAC5
AK300	AAAGGATCCTCTTTTTTCAAACCGTTGTCTTCAGTATGCAT	fwd, *steT* promoter for pAC5
AK301	TTTGAATTCCTCATCTCCCCTCTGTACCGG	rev, *steT* promoter for pAC5
AK302	AAAGGATCCAATGTTTCAAGCGGTTTTTTTCTAAATAATGA ACTCAT	fwd, *yfnA* promoter for pAC5
AK303	TTTGAATTCCATGGTCAGCATCTCCTTTATAACCG	rev, *yfnA* promoter for pAC5
AK304	AAAGGATCCTTGTTAAAAACCCCGACAAATCCACACAT	fwd, *asnB* promoter for pAC5
AK305	TTTGAATTCCACGATGTTGACCTTCCATGGGAG	rev, *asnB* promoter for pAC5
AK306	AAAGGATCCCGGTGCAGCTCTTGTTTTTGTTTTTGCAT	fwd, *ytnA* promoter for pAC5
AK307	TTTGAATTCCGGTATATGCCTGAAGAGCTGAGC	rev, *ytnA* promoter for pAC5
AK308	AAAGGATCCTCATTTTGTCTGAGACCAATACTCGAAACAT	rev, *serA* promoter for pAC5/ pAC7

AK309	TTTGAATTCCCCTGCCCCGAAGATTGATCATATG	fwd, *serA* promoter for pAC5/ pAC7
AK310	AAACTCGAGTATGGCAGAAGTGTCGGGTGCG	fwd, upstream deletion plasmid *papB-ptsI*
AK311	**CACCATTAATCCAAGCCCCGT**GGCTGCTGGTTTTATTTTAG GAGTCTTAAAC	rev, upstream deletion plasmid *papB-ptsI*
AK312	CGAAACGCAATCAAGCCATCCGC	fwd, sequencing deletion plasmid *papB-ptsI*
AK313	AAACCATGGGCGTACACGGTTTCGAGCTGATC	fwd, upstream pGP2288
AK314	GCCCGCCGGATAAAAGAAGCTG	rev, upstream pGP2288
AK315	**CAGCTTCTTTTATCCGGCGGGC**CATCATGTGAGCGTTTTTTT TTAGTTATCAGAAG	fwd, downstream pGP2288
AK316	TTTGGATCCCAGTCAATTTGGATTAGGAGTAAAAACAGGA	rev, downstream pGP2288
AK317	GTATTCAAACTGCATTAGAGAAGTCATCAAAC	fwd, sequencing pGP2288 insertion
AK318	CTAAACCCGTTAACATCAAAACAAACACCC	rev, sequencing of pGP2288 insertion
AK319	GGTGTCCAGATTGCTTTTTTTATGCTTAGG	fwd, sequencing *yhjCB*
AK320	GCCTTTTGAATCATACTGCCCGTTCC	rev, sequencing *yhjCB*
AK321	AAAGAATTCCCGTTTTGGAAATTGCTAAACCTGTTGTG	fwd, *ybeC* promoter for pAC5
AK322	TTTGGATCCGAAAACGTTCCCATTCTTCGATGCAATTGATTC AT	rev, *ybeC* promoter for pAC5
AK323	AAAGGATCCGCAAGAGAATGTCATCATACATGAAAGGTG	fwd, *ybeC* for pBQ200
AK324	TTTGTCGACTTATTCTTTTCCGGCAGCAGCTTCTG	rev, *ybeC* for pBQ200
AK325	AAAGGATCCAGGGATTTAAAACAAGAAAGGAATCTGTACA TG	rev, *ilvA* for pBQ200
AK326	TTTGTCGACTTAGATTAGCAAATGGAACAAGTCCTCATCC	fwd, *ilvA* for pBQ200
AK327	CACCAATCATCACTTCGTCCCCG	fwd, downstream *yvoD* LFH

AK328	**CCGAGCGCCTACGAGGAATTTGTATCG**CAGCGGTTGGAAAGGAAGCAATATGAG	rev, downstream *yvoD* LFH
AK329	**CCTATCACCTCAAATGGTTCGCTG**GACCGGCCAGAAAGATCTTCTTCATC	fwd, upnstream *yvoD* LFH
AK330	CGAAATCTTGCAGTCATCATCGAAGTG	rev, upnstream *yvoD* LFH
AK331	GGCAAGCAATACGACCGGCTC	rev, sequencing *yvoD* LFH
AK332	CAAATGCCCCGTCAGGCACATC	fwd, sequencing *yvoD* LFH
AK333	AAAGGATCCGGTGCCAGGCTCTCTATTTTAAAGGG	rev, *thrR* for pBQ200
AK334	AAAGGATCCTCTTGATTTTTTGAAACATCCTTCATTTTCGCAC	fwd, promoter *spo0B-obg-thrR-pheA* operon
AK335	TTTGAATTCGCAATATGCGGAGTAAACACCTAGAATG	rev, promoter *spo0B-obg-thrR-pheA* operon
AK336	CTTCAGTTTTTCGCTTCTAAAAAAGGAGTAGG	rev, sequencing *spo0B* promoter
AK337	GTGTTTTTTGTATCTAAAATCCATCAGGGTG	fwd, sequencing *spo0B* promoter
AK338	GGGCGTTATGAACAATTTCTCAATACAGC	rev, sequencing *hom* operon
AK339	GCTGTGATGTCGTCTGCGGTTATC	fwd, sequencing *hom* operon
AK340	CTGGAAGACGTCAAAGTAAAGGGG	rev, sequencing *hom* operon (in *hom*)
AK341	CGAAGAGATCAAGCGGCGACAG	rev, sequencing of *thrC*
AK342	CATTTGGCAGGGATAATAGTGGACAAG	fwd, sequencing of *thrC*
AK343	CCCGATCAGCTTCATTGTATAGCCG	fwd, sequencing of *hom* operon (in *hom*)
AK344	GAAGTGTTACCTGTTGACGCGCAC	fwd, sequencing of *hom* operon (in *thrC*)
AK345	CGAAAGGCAGCAAGGTCGTAGC	rev, sequencing *hom* operon (in *thrC*)
AK346	TTTGAATTCCGATCACATCTTACTCGAAAACGGAAAG	fwd, *serA* promoter for pAC5/ pAC7 (bigger region)

AK347	GCCGGATAATGCGCTGCTGAAAC	fwd, sequencing of *codY*
AK348	AGAACTACATTTCTCGCCTTGATATAAGCC	rev, sequencing of *cod*
AK349	TTTCTCGAGTTAGCTCCAACCGTTCCCTTCTACAC	fwd, *hom* for pET-SUMOadapt
AK350	AAAGGTCTCATGGTTTGAAAGCGATTCGTGTAGGGCTTTTAG	rev, *hom* for pET-SUMOadapt
AK351	AAAGGATCCTTAGCTCCAACCGTTCCCTTCTACAC	fwd, *hom* for pGP172
AK352	TTTGGTACCATTGAAAGCGATTCGTGTAGGGCTTTTAG	rev, *hom* for pGP172
AK353	CCCATCCTATAGAAGATAAAGAGGAATGTAC	rev, sequencing of amplification boarder *yokD-ilvD*
AK354	CGTTGGAATGGGACTCGGGC	rev, sequencing of amplification boarder *yokD-ilvD*
AK355	GATGCGCTGACTGTTACAGGAAAAAC	rev, sequencing of amplification boarder *yokD-ilvD*
AK356	CGACAGCCGGCCCTTCGTG	fwd, sequencing of amplification boarder *ilvD-thyA*
AK357	GGAAGCGGTCTAACATCTCTTTCCAG	fwd, sequencing of amplification boarder *ilvD-thyA*
AK358	AAAGAATTCTTATTTTTCAGTAAACAGCTTCATTGCTTTCCAG	fwd, *ypnP* for pGP888
AK359	TTTGGATCCAATGAAAGCATACGATTTTACACAGGGGAAC	rev, *ypnP* for pGP888
AK360	AAAGAATTCTTATGCACGTAATTGTTTTTCATTGGCAGGTA	fwd, *ypkP* for pGP888
AK361	TTTGGATCCATTGGTTCGCTACAGCCTTCTAGTGG	rev, *ypkP* for pGP888
AK362	AAAGGATCCTTTGATAGGCAATTCTAGCAAAGACAATTTTGG	fwd, *aapA* for pGP888
AK363	TTTGAATTCTTATTTCACCTTATGTCCTCTGCGGG	rev, *aapA* for pGP888
AK364	AAAGAATTCTCAGCTTGCTTTTCGTTTTTTCATCCCG	fwd, *steT* for pGP888
AK365	TTTGGATCCAATGCATACTGAAGACAACGGTTTGAAAAAAGAG	rev, *steT* for pGP888

AK366	CCGCTTCATAGAAAGGTTCAGCTTTTAAAATG	fwd, downstream *ypnP* LFH
AK367	**CGAGCGCCTACGAGGAATTTGTATCG**GCAATGAAGCTGTTTACTGAAAAATAAGTGAAG	rev, downstream *ypnP* LFH
AK368	**CCTATCACCTCAAATGGTTCGCTG**CCCCTGTGTAAAATCGTATGCTTTCATC	fwd, upstream *ypnP* LFH
AK369	GATTGGAACCATATTGCTGAAAAAGAGCTG	rev, upstream *ypnP* LFH
AK370	CAATGCTCGCTGATAAATCCCGCTC	fwd. Sequencing *ypnP* LFH
AK371	CTGAATGATCACGCTGAGCTGATTTATG	rev, sequencing *ypnP* LFH
AK372	CGGGGGAACGATTCAGCCATATG	fwd, downstream *ypkP* LFH
AK373	**CGAGCGCCTACGAGGAATTTGTATCG**GCTTATATACCTGCCAATGAAAAACAATTACG	rev, downstream *ypkP* LFH
AK374	**CCTATCACCTCAAATGGTTCGCTG**GAAGGCTGTAGCGAACCAATTAAAATCC	fwd, upstream *ypkP* LFH
AK375	CTCCCAATGGCGTTCTTGGCG	rev, downstream *ypkP* LFH
AK376	GTCAGCAATTGTTAATCGATCCCTCC	fwd, sequencing *ypkP* LFH
AK377	GCTGTGGTTCTTAAAAGGAGATACGAATG	rev, sequencing *ypkP* LFH
AK378	CTACGACGTCCACATATCTCGGG	fwd, sequencing of *ptsGHI* LFH
AK379	AAAGAATTCCGTATATAGTTTCAGCAGAATAGAAGGGC	fwd, promoter of *sdaAB* for pAC5
AK380	TTTGGATCCTTCATGCTATTCCTCCTTATGAACCAGAG	rev, promoter of *sdaAB* for pAC5
AK381	GAAAGTGTCCCCAGCTATCTAGATTTTC	fwd, sequencing of *ilv*
AK382	GCAAAAACAGCCCATAAATAAACTGAAAATTGTC	rev, sequencing of *ilvD*
AK383	GACGAATCTTCCTATATGACAGGGCAG	fwd, sequencing *bcaP*
AK384	CTATAGATTCATTTGCCAAGAACAGAAAAGAG	rev, sequencing *bcaP*

AK385	CGGGTGTGAATTATTAGGTAAGCTGTTC	fwd, sequencing *ybxG*
AK386	GCTTGCTAAAATAGGGAAAATCCATACGC	rev, sequencing *ybxG*
AK387	AAAGAGCTCTTGAAAGCGATTCGTGTAGGGCTTTTAG	fwd, *hom* for pGP574
AK388	TTTGGATCCGCTCCAACCGTTCCCTTCTACAC	rev, *hom* for pGP574
AK389	CGAGTGATACGTTTTGCAATAATAGGAACC	fwd, LFH of *pgi*
AK390	**CCTATCACCTCAAATGGTTCGCTG**GGAAGATTAATGTGAG AAAGCTGACTGG	rev, LFH of *pgi*
AK391	CACTATCGTCCCTATAATGAAAAATAAAAACCG	fwd, sequencing of *pgi* LFH
AK392	AAAGGATCCATGAAAGGGAGCGTTTTTAGGAAGAAAAGC	fwd, *bcaP* for pWH844
AK393	TTTGTCGACGGTTATTGATTTAATTTTGAATGCTTTCTTGAA TACAG	rev, *bcaP* for pWH844
AK394	AAAGGATCCGTGGCAAATAAAGAATTAAAGAGGGGCC	fwd, *ybxG* for pWH844
AK395	TTTGTCGACTTATTCAGCTGCTTGTTCGCTTTTTGTCAAATTG	rev, *ybxG* for pWH844
AK396	CTGAGGAACGCTTGGCATGATCTATAC	fwd, downstream *thrC* LFH
AK397	**CGAGCGCCTACGAGGAATTTGTATCG**GTAAAAGGAGCGG CCCGTGTATG	rev, downstream *thrC* LFH
AK398	**CCTATCACCTCAAATGGTTCGCTG**GATAAGTCCTTTCCACA TTAGCTCCAAC	fwd, upstream *thrC* LFH
AK399	GCCGAAGCGAAGGAAAATGGATGC	rev, upstream *thrC* LFH
AK400	CATTGATGATCCAGATGTTGATGTCGTC	rev, sequencing *thrC* LFH
AK401	CATATGATGGAAAAGTTCTACAAGGAGTG	fwd, downstream *yvbW* LFH
AK402	**CGAGCGCCTACGAGGAATTTGTATCG**CGCAAGCATCAGTA AATAAGAAACCCTC	rev, downstream *yvbW* LFH
AK403	**CCTATCACCTCAAATGGTTCGCTG**GTCGTTTTTCATCCTCTT CTACCTCTCC	fwd, upstream *yvbW* LFH

AK404	GATTTTGAAGCAGTAAATCCAGAAGACCG	rev, upstream *yvbW* LFH
AK405	CAGCGAAAAAACGATTCACAGATCAGTTAATTAC	rev, sequencing *yvbW* LFH
AK406	CAGCTATTTTCACAAAAACCTTTAATTGAGTAATG	fwd, sequencing *yvbW* LFH
AK407	GCTTACTGAAAACGGCTCGAAGGTC	fwd, upstream *yecA* LFH
AK408	**CGAGCGCCTACGAGGAATTTGTATCG**CATGATGCCCCCTCT CTGATTGATG	rev, upstream *yecA* LFH
AK409	**CCTATCACCTCAAATGGTTCGCTG**CGTTTTGTGATCAAGCT TTTCCATTTATCCG	fwd, downstream *yecA* LF
AK410	CGTTTTCTCTGTTTCTTTGTAGCTTGCATAC	rev, downstream *yecA* LFH
AK411	CATCACCTGTCATCGCTTCATGATCAC	fwd, sequencing *yecA* LFH
AK412	CGGTCCGCCATGCTGTAGAAACG	rev, sequencing *yecA* LFH
AK413	AAATCTAGAAGTGACAGACGACATGACGAAAGAC	fwd, *ydgF* for pGP888
AK414	TTTGGTACCCTTATTGATGCTTCGCTTTTCTCACTTTATAAAT C	rev, *ydgF* for pGP888
AK415	AAATCTAGAAATGCAAGGGAATCTGACTGCACTTC	fwd, *yodF* for pGP888
AK416	TTTGGTACCGTTTTTTTATGCGCCCTTTTGATTTGGATTAG	rev, *yodF* for pGP888

6.4.2. Oligonucleotides used in this work

Name	Sequence	Purpose
cat check fwd	CTAATGTCACTAACCTGCCC	Sequencing out of *cat* resistance cassette
cat check rev	GTCTGCTTTCTTCATTAGAATCAATCC	Sequencing out of *cat* resistance cassette
cat fwd (kan)	CGGCAATAGTTACCCTTATTATCAAG	Amplification of *cat* resistance cassette
cat rev (kan)	CCAGCGTGGACCGGCGAGGCTAGTTACCC	Amplification of *cat* resistance cassette

CZ114	CAGAATTAAAACAAGCATGGC	rev, downstream *citZ-icd-mdh* deletion
CZ116	CATGTCCTAGCTTATCAGAAC	fwd, upstream *citZ-icd-mdh* deletion
CZ119	GAAAACAATATGCAACTTTAAATC	rev, downstream *sucCD* deletion
CZ120	GATACAACAGCAGTTGCTTTG	rev, downstream *sdhCAB* deletion
CZ123	CTCATTTTCTCTTCATTTCATGC	fwd, upstream *sdhCAB* deletion
CZ126	CAGCGAACCATTTGAGGTGATAGGGAACGATGACC TCTAATAATTG	Sequencing out of *phleo* resistance cassette
CZ127	CGATACAAATTCCTCGTAGGCGCTCGGGTAGTATTT TTTGAGAAGATCAC	Sequencing out of *phleo* resistance cassette
CZ128	CCAAAGTGAAACCTAGTTTATC	Sequencing *phleo* resistance
CZ129	CGAGACTTTGCAGTAATTGATC	Sequencing *phleo* resistance
CZ135	CAACAGTCGTAAGCGAAAATG	Check PCR *sdhCAB* deletion
CZ140	GTATACGAAGAGAGATTAGAAG	rev, downstream *citB* deletion
CZ143	GCTGTTTATCTTCTCCTGAAG	fwd, upstream *citB* deletion
CZ145	GGGTATGGCTGACGGCAAAG	Check PCR *odhAB* deletion
CZ151	CAAGAACATTTTAATTAGTTTACATC	Check PCR *odhAB* deletion
CZ154	GTTTGTCTGTCCATTGGGTTC	Check PCR *citRA* deletion
CZ171	GAAAATACGACACAACGATCAG	Check PCR *citRA* deletion
CZ200	TACCGTTCGTATAGCATACATTAATACGAAGTTATCC TTCCCAGCGAACCATTTGAGGTGATAGGTAAG	Amplification of *kan*-lox from pGP2514
CZ201	TACCGTTCGTATAATGTATGCTATCGAAGTTATGTA ACGATCGATACAAATTCCTCGTAGGCGCTCGGGAC	Amplification of *kan*-lox from pGP2514
CZ202	CTACATAAGAGGACATTCGAC	amplification of the glycolytic cassette upstream fragement

CZ203	GCGCGCCTTCACTTGACAACATCGATATTGGCTGTATCAAC	amplification of the glycolytic cassette upstream fragement
CZ204	TTGTCAAGTGAAGGCGCGCTATCGTACAATACAGCTTGGAAATAGAGGAGGTCAATTCTTATGTTTAAAGC	Amplification of *ptsGHI*
CZ205	CCTATCACCTCAAATGGTTCGCTGGTTAAATTGGTTTGACATACTATC	Amplification of *ptsGHI*
CZ236	GTTTTAGTTCCAGCAGCCAG	Check PCR *citZ-icd-mdh* deletion
DR343	TTTGGATCCCAAGCGCCGCATATGACTG	check of deletion with pGP2073
DR348	CCTGTCCTTGGATAGCGTAC	check of deletion with pGP2073
DR377	TTTGGATCCCTGACAATCACAAACGTACCTGG	construction of pGP2270
DR381	CGTGCCGGTTGAAGTGCAT	check of deletion with pGP2270
DR387	TTTGGATCCGTTCGAATCCTGCCTGTGGA	construction of pGP2283
DR391	GAATATGGCAAGCCTATGTTACATTAT	check of deletion with pGP2283
DR393	TTTGGATCCCGGACTCTCTATTATCCTCGTAA	construction of pGP2282
DR397	GGGTATCTTTTTGATCGTATTATGCT	check of deletion with pGP2282
DR402	TTTGGATCCCGCAGTCGGAAAAAGCACGA	construction of pGP2284
DR491	CCATTACGTTTTTCCACCAGTCTT	check of deletion with pGP2088
DR492	GGGCGCTCAAATCTTCCACA	check of deletion with pGP2088
DR529	CGTACGAAATCAGAGCCGCAA	check of deletion with pGP2093
DR530	GTAAATAGACTGCCAGCGTCCT	check of deletion with pGP2093
DR535	CCCGAAAATCCGCGCGC	check of deletion with pGP2094
DR536	CCAGAAGAAAAACTGTCAGAGATTG	check of deletion with pGP2094

DR591	TGTACGAGACCTCCTTCCATG	check of deletion with pGP2098
DR592	AAAAGGAGACTTTTTCTCAGCTGATC	check of deletion with pGP2098
DR607	TGCGGAAGTAAGCTCTTTCTCTG	check of deletion with pJOE9256
DR608	TGGCTGCTGTGATGAACTTTGTC	check of deletion with pJOE9256
DR615	CGAGCGCCTACGAGGAATTTGTATCGAAGTCTACG AGGACCTTACTGATT	Amplification of *nrnA*
DR616	ATAGACATGTGCGTCCTGATCC	Amplification of *nrnA*
FC121	CATTTATAGTAAAAAGAGAAAGGCTGTATTAAGCAA GCC	Check PCR *citZ-icd-mdh* deletion
FC50	GTCATATCCTAGCAGGCCTCCGG	Check PCR *citG* deletion
FC53	CGACCAAAATTGCCACACGGCCG	Check PCR *citG* deletion
FC60	GAACGTTTCTCTCAGGAAGTTCCTCG	Check PCR *sucCD* deletion
FM86	CTGCCTTAAGAGCATCGCATGAGGTA	Check PCR *sucCD* deletion
FM111	CAACAGATAGGTTTCTCAAAAGGAGGGG	Check PCR *citB* deletion
FM165	AAAGAGCTCTGATCTGAAGGGGGATTTTGGAGAAT GG	Check PCR *citB* deletion
FM172	TTGTCCATCCCTCACTCAAGGATCTC	Check PCR *sdhCAB* deletion
JR154	GCAAAATCAACTTCGCCTGCA	Sequencing of *thrR*
JR155	CGATGGAAAATGAAGAAGTGCCAT	Sequencing of *thrR*
kan check fwd	CATCCGCAACTGTCCATACTCTG	Sequencing out of *kan* resistance cassette
kan check rev	CTGCCTCCTCATCCTCTTCATCC	Sequencing out of *kan* resistance cassette
kan-fwd	CAGCGAACCATTTGAGGTGATAGG	Amplification of *kan* resistance cassette

kan-rev	CGATACAAATTCCTCGTAGGCGCTCGG	Amplification of *kan* resistance cassette
M13_puc_for	GTAAAACGACGGCCAGTG	Sequencing of pBQ200
M13_puc_rev	GGAAACAGCTATGACCATG	Sequencing of pBQ200
MD113	CCGAGCGCCTACGAGGAATTTGTATCGTTCTGTTTCCGGCCCAATACC	sequencing *sdaCAB* suppressors
ML84	CTAATGGGTGCTTTAGTTGAAGA	*Cat* check up-fragment
ML85	CTCTATTCAGGAATTGTCAGATAG	*Cat* check down-fragment
ML107	GCTTCATAGAGTAATTCTGTAAAGG	sequencing pAC7 plasmid
ML244	CTAATACGACTCACTATAGGGAGAGGATATGTGCACTTCGCTGCTGCTCAAT	sequencing *serA* deletion
MT24	AAAAGAATTCATGAATATGCGGGCGCAGAAGCT	amplification of *hom* promoter
MT25	AAAAGGATCCTTCAAAAAAACTCCACCTTTCTTTTGATTGTCC	amplification of *hom* promoter
pAC5F	GCGTAGCGAAAAATCCTTTTC	sequencing pAC5 plasmid
SH71	AACGGTGGTATATCCAGTG	sequencing pWH844
spec check fwd	GTTATCTTGGAGAGAATATTGAATGGAC	Sequencing out of *spec* resistance cassette
spec check rev	CGTATGTATTCAAATATATCCTCCTCAC	Sequencing out of *spec* resistance cassette
spec-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG	Amplification of *spec* resistance cassette
spec-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG	Amplification of *spec* resistance cassette
T7-Prom.	TAATACGACTCACTATAGGG	Sequencing of pGP574 and pET-SUMOadapt
T7-Term.	GCTAGTTATTGCTCAGCGG	Sequencing of pGP574 and pET-SUMOadapt
Tc fwd2	GCTTATCAACGTAGTAAGCGTGG	Sequencing out of *tet* resistance cassette

Tc rev	GAACTCTCTCCCAAAGTTGATCCC	Sequencing out of *tet* resistance cassette
Tc check fwd	CGGCTACATTGGTGGGATACTTGTTG	Amplification of *tet* resistance cassette

6.5. Supplementary information

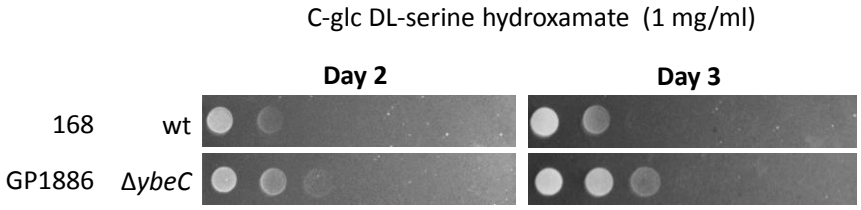

Figure 54. The growth of the wild type strain 168 and the Δ*ybeC* strain on C-glc with DL-serine hydroxamate (1 mg/ml). A serial dilution of the wild type strain and the strain GP1886 was performed in C-glc liquid medium and the cells were plated on C-glc plates with 1 mg/ml DL-serine hydroxamate.

Table 18: The complete list of genes, included in the *MiniBacillus* blueprint 2.0

	Gene	BSU no.	Essential	Function
Information				
DNA replication				
	dnaA	BSU00010	yes	Replication initiation protein
	dnaB	BSU28990	yes	Initiation of chromosome replication
	dnaC	BSU40440	yes	Replicative DNA helicase
	dnaD	BSU22350	yes	Initiation of chromosome replication
	dnaE	BSU29230	yes	DNA polymerase III (alpha subunit)
	dnaG	BSU25210	yes	DNA primase
	dnaI	BSU28980	yes	Primosome component (helicase loader)
	dnaN	BSU00020	yes	DNA polymerase III (beta subunit), beta clamp
	dnaX	BSU00190	yes	DNA polymerase III (gamma and tau subunits)
	holA	BSU25560	yes	DNA polymerase III, delta subunit
	holB	BSU00310	yes	DNA polymerase III (delta subunit)
	ligA	BSU06620	yes	DNA ligase (NAD dependent)
	priA	BSU15710	yes	Primosomal replication factor Y
	polC	BSU16580	yes	DNA polymerase III (alpha subunit)
	rtp	BSU18490	no	Replication terminator protein
	ssbA	BSU40900	yes	Single-strand DNA-binding protein
	yabA	BSU00330	no	Inhibitor of DnaA oligomerization
	polA	BSU29090	no	DNA polymerase I
Chromosome maintenance				
	scpA	BSU23220	yes	DNA segregation/condensation protein

	Gene	BSU no.	Essential	
	scpB	BSU23210	no	DNA segregation/condensation protein
	smc	BSU15940	yes	SMC protein
	parE	BSU18090	yes	Subunit of DNA topoisomerase IV
	parC	BSU18100	yes	Subunit of DNA topoisomerase IV
	spoIIIE	BSU16800	no	ATP-dependent DNA translocase
	sftA	BSU29805	no	DNA translocase
	codV	BSU16140	no	Site-specific integrase/recombinase
	ripX	BSU23510	no	Site-specific integrase/recombinase
	gyrB	BSU00060	yes	DNA gyrase (subunit B)
	gyrA	BSU00070	yes	DNA gyrase (subunit A)
	topA	BSU16120	yes	DNA topoisomerase I
	hbs	BSU22790	yes	Nonspecific DNA-binding protein HBsu
Transcription				
	rpoA	BSU01430	yes	RNA polymerase alpha subunit
	rpoB	BSU01070	yes	RNA polymerase beta subunit
	rpoC	BSU01080	yes	RNA polymerase beta' subunit
	sigA	BSU25200	yes	RNA polymerase sigma factor SigA
	rpoE	BSU37160	no	RNA polymerase delta subunit
	helD	BSU33450	no	DNA 3'-5' helicase IV
	greA	BSU27320	no	Transcription elongation factor
	nusA	BSU16600	yes	Transcription termination factor
	yhdL	BSU09510	yes	control of SigM activity
RNA folding and degradation				
	cspD	BSU21930	no	Cold shock protein
	cspB	BSU09100	no	Major cold shock protein
	rny	BSU16960	no	RNase Y
	rnjA	BSU14530	yes	RNase J1
	pnpA	BSU16690	no	Polynucleotide phosphorylase
	nrnA	BSU29250	no	Oligoribonuclease (nano-RNase)
	rnc	BSU15930	yes	Processing and degradation of RNA molecules
	yqfG	BSU25320	yes	unknown
Aminoacyl-tRNA synthetases				
	alaS	BSU27410	yes	Alanine-tRNA synthetase
	argS	BSU37330	yes	Arginyl-tRNA synthetase
	asnS	BSU22360	yes	Asparagyl-tRNA synthetase
	aspS	BSU27550	yes	Aspartyl-tRNA synthetase
	cysS	BSU00940	yes	Cysteine-tRNA synthetase

	Gene	BSU no.	Essential	Function
	gatC	BSU06670	yes	Production of glutamyl-tRNA^{Gln}
	gatA	BSU06680	yes	Production of glutamyl-tRNA^{Gln}
	gatB	BSU06690	yes	Production of glutamyl-tRNA^{Gln}
	gltX	BSU00920	yes	Glutamyl-tRNA synthetase
	glyS	BSU25260	yes	Glycyl-tRNA synthetase (beta subunit)
	glyQ	BSU25270	yes	Glycyl-tRNA synthetase (alpha subunit)
	hisS	BSU27560	yes	Histidyl-tRNA synthetase
	ileS	BSU15430	yes	Isoleucyl-tRNA synthetase
	leuS	BSU30320	yes	Leucyl-tRNA synthetase
	lysS	BSU00820	yes	Lysyl-tRNA synthetase
	metS	BSU00380	yes	Methionyl-tRNA synthetase
	pheT	BSU28630	yes	Phenylalanyl-tRNA synthetase (beta subunit)
	pheS	BSU28640	yes	Phenylalanyl-tRNA synthetase (alpha subunit)
	proS	BSU16570	yes	Prolyl-tRNA synthetase
	serS	BSU00130	yes	Seryl-tRNA synthetase
	thrS	BSU28950	no	Threonyl-tRNA synthetase (major)
	trpS	BSU11420	yes	Tryptophanyl-tRNA synthetase
	tyrS	BSU29670	yes	Tyrosyl-tRNA synthetase (major)
	valS	BSU28090	yes	Valyl-tRNA synthetase
Ribosomal proteins				
	rplA	BSU01030	no	Ribosomal protein L1
	rplB	BSU01190	yes	Ribosomal protein L2
	rplC	BSU01160	yes	Ribosomal protein L3
	rplD	BSU01170	yes	Ribosomal protein L4
	rplE	BSU01280	yes	Ribosomal protein L5
	rplF	BSU01310	yes	Ribosomal protein L6
	rplI	BSU40500	no	Ribosomal protein L9
	rplJ	BSU01040	yes	Ribosomal protein L10
	rplK	BSU01020	no	Ribosomal protein L11
	rplL	BSU01050	yes	Ribosomal protein L12
	rplM	BSU01490	yes	Ribosomal protein L13
	rplN	BSU01260	yes	Ribosomal protein L14
	rplO	BSU01350	no	Ribosomal protein L15
	rplP	BSU01230	yes	Ribosomal protein L16
	rplQ	BSU01440	yes	Ribosomal protein L17
	rplR	BSU01320	yes	Ribosomal protein L18
	rplS	BSU16040	yes	Ribosomal protein L19

Gene	BSU no.	Essential	Function
rplT	BSU28850	yes	Ribosomal protein L20
rplU	BSU27960	yes	Ribosomal protein L21
rplV	BSU01210	yes	Ribosomal protein L22
rplW	BSU01180	yes	Ribosomal protein L23
rplX	BSU01270	yes	Ribosomal protein L24
rpmA	BSU2794	yes	Ribosomal protein L27
rpmB	BSU15820	no	Ribosomal protein L28
rpmC	BSU01240	no	Ribosomal protein L29
rpmD	BSU01340	yes	Ribosomal protein L30
rpmE	BSU37070	no	Ribosomal protein L31
rpmF	BSU15080	no	Ribosomal protein L32
rpmGA	BSU24900	no	Ribosomal protein L33a
rpmGB	BSU00990	no	Ribosomal protein L33b
rpmH	BSU41060	no	Ribosomal protein L34
rpmI	BSU28860	no	Ribosomal protein L35
rpmJ	BSU01400	no	Ribosomal protein L36
rpsB	BSU16490	yes	Ribosomal protein S2
rpsC	BSU01220	yes	Ribosomal protein S3
rpsD	BSU29660	yes	Ribosomal protein S4
rpsE	BSU01330	yes	Ribosomal protein S5
rpsF	BSU40910	no	Ribosomal protein S6
rpsG	BSU01110	yes	Ribosomal protein S7
rpsH	BSU01300	yes	Ribosomal protein S8
rpsI	BSU01500	yes	Ribosomal protein S9
rpsJ	BSU01150	yes	Ribosomal protein S10
rpsK	BSU01420	yes	Ribosomal protein S11
rpsL	BSU01100	yes	Ribosomal protein S12
rpsM	BSU01410	yes	Ribosomal protein S13
rpsN	BSU01290	yes	Ribosomal protein S14
rpsO	BSU16680	yes	Ribosomal protein S15
rpsP	BSU15990	yes	Ribosomal protein S16
rpsQ	BSU01250	yes	Ribosomal protein S17
rpsR	BSU40890	yes	Ribosomal protein S18
rpsS	BSU01200	yes	Ribosomal protein S19
rpsT	BSU25550	no	Ribosomal protein S20
rpsU	BSU25410	no	Ribosomal protein S21

	Gene	BSU no.	Essential	Function
rRNA and tRNA				
	rrnO-16S- trnO-Ala- trnO-Ile- rrnO-23S- rrnO-5S			
	trnSL-Ser1			
	rrnA-16S- trnA-Ile- trnA-Ala- rrnA-23S- rrnA-5S			
	trnSL-Met1- trnSL-Glu1			
	rrnJ-16S- rrnJ-23S- rrnJ-5S- trnJ-Ala- trnJ-Arg- trnJ-Gly- trnJ-Leu1- trnJ-Leu2- trnJ-Lys-			
	trnJ-Pro- trnJ-Thr- trnJ-Val- rrnW-16S- rrnW-23S- rrnW-5S			
	rrnI-16S- rrnI-23S- rrnI-5S-trnI-Ala- trnI-Arg- trnI-Asn- trnI-Gly- trnI-Pro- trnI-Thr			
	rrnH-16S- rrnH-23S- rrnH-5S- rrnG-16S- rrnG-23S- rrnG-5S			
	trnSL-Gln2- trnSL-Glu2 - trnSL-Thr1- trnSL-Tyr1 - trnSL-Val1			
	trnS-Asn- trnS-Gln- trnS-Glu- trnS-Leu1- trnS-Leu2- trnS-Lys- trnS-Ser			
	trnE-Arg- trnE-Gly- rrnE-16S- rrnE-23S- rrnE-5S- trnE-Asp- trnE-Met			
	rrnD-16S- rrnD-23S- rrnD-5S - trnD-Asn- trnD-Asp- trnD-Cys- trnD-Gln- trnD-Glu-			
	trnD-Gly- trnD-His- trnD-Leu1- trnD-Leu2- trnD-Met- trnD-Phe- trnD-Ser- trnD-Thr-			
	trnD-Trp- trnD-Tyr- trnD-Val			
	trnSL-Gly1			
	trnSL-Val2			
	trnSL-Arg1			
	trnSL-Gln1			
	trnSL-Arg2			
	rrnB-16S- rrnB-23S- rrnB-5S - trnB-Ala- trnB-Arg- trnB-Asn- trnB-Asp- trnB-Glu- trnB-			
	Gly1- trnB-Gly2- trnB-His- trnB-Ile2- trnB-Leu1- trnB-Leu2- trnB-Lys- trnB-Met1- trnB-			
	Met2- trnB-Met3- trnB-Phe- trnB-Pro- trnB-Ser1- trnB-Ser2- trnB-Thr- trnB-Val			
	trnSL-Ala1			
	trnQ-Arg			
	trnY-Asp- trnY-Glu- trnY-Lys- trnY-Phe			
rRNA/ tRNA maturation and modification				
	rnpA	BSU41050	yes	Protein component of RNase P
	rnpB	BSU_misc_RNA_35	yes	RNA component of RNase P
	rnz	BSU23840	yes	RNase Z
	rph	BSU28370	no	RNase PH
	rbfA	BSU16650	no	Ribosome-binding factor A
	rimM	BSU16020	no	16S rRNA-processing protein, RNase
	cca	BSU22450	yes	tRNA nucleotidyltransferase
	fmt	BSU15730	yes	Methionyl-tRNA formyltransferase

	Gene	BSU no.	Essential	Function
	folD	BSU24310	yes	Methylenetetrahydrofolate dehydrogenase
	rlmCD	BSU06730	no	rRNA methyltransferase
	ysgA	BSU28650	yes	Similar to rRNA methylase
	mraW	BSU15140	no	SAM-dependent methyltransferase
	cspR	BSU08930	yes	Similar to tRNA (Um34/Cm34) methyltransferase
	trmD	BSU16030	yes	tRNA methyltransferase
	trmU	BSU27500	yes	tRNA(5-methylaminomethyl-2- thiouridylate) methyltransferase
	yrvO	BSU27510	yes	Cysteine desulfurase
	yacO	BSU00960	no	Putative 23S rRNA methyltransferase
	ksgA	BSU00420	no	rRNA adenine dimethyltransferase
	rluB	BSU23160	no	Pseudouridine synthase
	ypuI	BSU23200	no	rRNA pseudouridine 2633 synthase
	tilS	BSU00670	yes	$tRNA^{Ile}$ lysidine synthetase
	tsaB	BSU05920	yes	tRNA modification
	tsaD	BSU05940	yes	tRNA modification
	tsaC	BSU36950	no	tRNA modification
	gidA	BSU41010	no	tRNA modification
	thdF	BSU41020	no	GTP-binding protein, putative tRNA modification GTPase
	truA	BSU01480	no	Pseudouridylate synthase I, universally conserved protein
	tsaE	BSU05910	no	P-loop ATPase
	trmFO	BSU16130	no	tRNA:m(5)U-54 methyltransferase
	miaA	BSU17330	no	tRNA isopentenylpyrophosphate transferase
	yaaJ	BSU00180	no	tRNA-specific adenosine deaminase
	ylyB	BSU15460	no	Similar to pseudouridylate synthase
	ypsC	BSU22170	no	rRNA modification
Ribosome maturation/ assembly				
	ydiD	BSU05930	no	Similar to ribosomal protein alanine N-acetyltransferase
	ylxS	BSU16590	no	Similar to 30S ribosomal subunit maturation protein
	prp	BSU27950	yes	Maturation ofL27
	engA	BSU22840	yes	GTPase, ribosome 50S subunit assembly

	Gene	BSU no.	Essential	Function
	era	BSU25290	yes	GTP-binding protein
	obg	BSU27920	yes	GTP-binding protein
	rbgA	BSU16050	yes	Assembly of the 50S subunit of the ribosome
	yqeH	BSU25670	yes	Assembly/stability of the 30S subunit of the ribosome, assembly of the 70S ribosome
	ysxC	BSU28190	yes	Assembly of the 50S subunit of the ribosome Elongation
Translation factors				
	efp	BSU24450	no	Elongation factor P
	frr	BSU16520	yes	Ribosome recycling factor
	fusA	BSU01120	yes	Elongation factor G
	infA	BSU01390	yes	Translation initiation factor IF-1
	infB	BSU16630	yes	Translation initiation factor IF-2
	infC	BSU28870	yes	Translation initiation factor IF-3
	prfA	BSU37010	yes	Peptide chain release factor 1
	prfB	BSU35290	yes	Peptide chain release factor 2
	tsf	BSU16500	yes	Elongation factor Ts
	tufA	BSU01130	yes	Elongation factor Tu
	lepA	BSU25510	no	Elongation factor 4
Translation/ others				
	map	BSU01380	yes	Methionine aminopeptidase
	ywkE	BSU37000	no	Similar to N5-glutamine methyltransferase that modifies peptide release factors
	rplGB	BSU01090	no	Similar to ribosomal protein L7 family
	spoVC	BSU00530	yes	Putative peptidyl-tRNA hydrolase
	ssrA	BSU_MISC_RNA_55	no	tmRNA
	smpB	BSU33600	no	tmRNA-binding protein
Protein secretion				
	scr	BSU_misc_RNA_2	yes	Signal recognition particle RNA
	ffh	BSU15980	yes	Signal recognition particle component
	ftsY	BSU15950	yes	Signal recognition particle
	yidC2	BSU23890	no	Sec-independent membrane protein translocase
	secA	BSU35300	yes	Preprotein translocase subunit (ATPase)
	secE	BSU01000	yes	Preprotein translocase subunit

	Gene	BSU no.	Essential	Function
	secY	BSU01360	yes	Preprotein translocase subunit, universally conserved protein
	secG	BSU33630	no	Preprotein translocase subunit
	sipS	BSU23310	no	Signal peptidase I
	prsA	BSU09950	yes	Protein secretion (posttranslocation molecular chaperone)
	csaA	BSU19040	no	Molecular chaperone involved in protein secretion
	lgt	BSU34990	no	Prolipoprotein diacylglyceryl transferase
	lspA	BSU15450	no	Signal peptidase II
Proteolysis/ quality control/ chaperones				
	htrB	BSU33000	no	Serine protease
	groES	BSU06020	yes	Chaperonin, universally conserved protein
	groEL	BSU06030	yes	Chaperonin
	dnaJ	BSU25460	no	Activation of DnaK
	dnaK	BSU25470	no	Molecular chaperone
	grpE	BSU25480	no	Activation of DnaK
	tig	BSU28230	no	Trigger factor (prolyl isomerase)
Metabolism				
Central carbon metabolism				
Glycolysis				
	ptsG	BSU13890	no	PTS glucose permease, EIICBA(Glc)
	ptsH	BSU13900	no	HPr, general component of the PTS
	ptsI	BSU13910	no	Enzyme I, general component of the PTS
	pgi	BSU31350	no	Glucose-6-phosphate isomerase
	pfkA	BSU29190	no	Phosphofructokinase
	fbaA	BSU37120	no	Fructose 1,6-bisphosphate aldolase
	tpi	BSU33920	no	Triose phosphate isomerase
	gapA	BSU33940	yes	Glyceraldehyde-3-phosphate dehydrogenase
	pgk	BSU33930	No	Phosphoglycerate kinase
	pgm	BSU33910	yes	Phosphoglycerate mutase
	eno	BSU33900	yes	Enolase
	pyk	BSU29180	no	Pyruvate kinase
	pdhA	BSU14580	yes	Pyruvate dehydrogenase (E1 alpha subunit)
	pdhB	BSU14590	no	Pyruvate dehydrogenase (E1 beta subunit)
	pdhC	BSU14600	no	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)

	Gene	BSU no.	Essential	Function
	pdhD	BSU14610	no	Dihydrolipoamide dehydrogenase E3 subunit of both pyruvate and 2-oxoglutarate dehydrogenase complexes
Transhydrogenation cycle				
	ytsJ	BSU29220	no	Malic enzyme
	malS	BSU29880	no	Malate dehydrogenase (decarboxylating)
Pentose phosphate pathway				
	ykgB	BSU13010	no	6-Phosphogluconolactonase
	rpe	BSU15790	no	Ribulose 5-phosphate 3-epimerase
	tkt	BSU17890	no	Transketolase
	zwf	BSU23850	no	Glucose-6-phosphate dehydrogenase
	gndA	BSU23860	no	NADP-dependent phosphogluconate dehydrogenase
	ywlF	BSU36920	no	Ribose-5-phosphate isomerase
	ywjH	BSU37110	no	Transaldolase
Recycling of acetate				
	acsA	BSU29680	no	Acetyl-CoA synthetase
Respiration/ energy				
	ndh	BSU12290	no	NADH dehydrogenase
Cytochrome aa3				
	qoxD	BSU38140	no	Cytochrome aa3 quinol oxidase (subunit IV)
	qoxC	BSU38150	no	Cytochrome aa3 quinol oxidase (subunit III)
	qoxB	BSU38160	no	Cytochrome aa3 quinol oxidase (subunit I)
	qoxA	BSU38170	no	Cytochrome aa3 quinol oxidase (subunit II)
Cytochrome maturation				
	resC	BSU23130	yes	Part of heme translocase, required for cytochrome c synthesis
	resB	BSU23140	yes	Part of heme translocase, required for cytochrome c synthesis
ATPase				
	atpC	BSU36800	no	ATP synthase, F_1 (subunit epsilon)
	atpD	BSU36810	no	ATP synthase, F_1 (subunit beta)
	atpG	BSU36820	no	ATP synthase, F_1 (subunit gamma)
	atpA	BSU36830	no	ATP synthase, F_1 (subunit alpha)
	atpH	BSU36840	no	ATP synthase, F_1 (subunit delta)
	atpF	BSU36850	no	ATP synthase, F_o (subunit b)
	atpE	BSU36860	no	ATP synthase, F_o (subunit c)

	Gene	BSU no.	Essential	Function
	atpB	BSU36870	no	ATP synthase, F_o (subunit a)
	atpI	BSU36880	no	ATP synthase (subunit i)
Amino Acids				
Asp, Glu				
	gltT	BSU10220	no	Major H^+/Na^+-glutamate symport protein
Arg				
	rocE	BSU40330	no	Amino acid permease
Pro				
	putP	BSU03220	no	High-affinity proline permease
Trp				
	trpP	BSU10010	no	S protein of tryptophan ECF transporter
Met				
	metQ	BSU32730	no	Methionine ABC transporter (binding lipoprotein)
	metP	BSU32740	no	Methionine ABC transporter, permease
	metN	BSU32750	no	Methionine ABC transporter (ATP-binding protein)
His				
	hutM	BSU39390	no	Histidine permease
Cys				
	tcyP	BSU09130	no	Cystine transporter
Gly				
	glyA	BSU36900	yes	Serine hydroxymethyltransferase
Ile, Val, Thr, Ser				
	bcaP	BSU09460	no	Branched-chain amino acid transporter
Lys				
	yvsH	BSU33330	no	Putative lysine transporter
Chorismate for aromatic amino acids, menaquinone, and folate				
	aroA	BSU29750	no	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase/chorismate mutase isozyme 3
	aroB	BSU22700	no	3-Dehydroquinate synthase
	aroC	BSU23080	no	3-Dehydroquinate dehydratase
	aroD	BSU25660	no	Shikimate dehydrogenase
	aroE	BSU22600	yes	3-Phosphoshikimate 1-carboxyvinyltransferase

	Gene	BSU no.	Essential	Function
	aroF	BSU22710	yes	Chorismate synthase
	aroK	BSU03150	yes	Shikimate kinase
Phe, Tyr				
	pheA	BSU27900	no	Prephenate dehydratase
	hisC	BSU22620	no	Histidinol-phosphate aminotransferase/ tyrosine and phenylalanine aminotransferase
	aroH	BSU22690	no	Chorismate mutase (isozymes 1 and 2)
	tyrA	BSU22610	no	Prephenate dehydrogenase
Asn				
	asnB	BSU30540	no	Asparagine synthase (glutamine hydrolyzing)
Ala				
	alaT	BSU31400	no	Alanine aminotransferase
Leu				
	yvbW	BSU34010	no	Putative leucine permease
Gln				
	glnA	BSU17460	no	Glutamine synthetase
Nucleotides				
PRPP				
	prs	BSU00510	yes	Phosphoribosylpyrophosphate synthetase
Pyrimidine biosynthesis				
	pyrAA	BSU15510	no	Carbamoyl-phosphate synthetase (glutaminase subunit)
	pyrAB	BSU15520	no	Carbamoyl-phosphate synthetase (catalytic subunit)
	pyrB	BSU15490	no	Aspartate carbamoyltransferase
	pyrC	BSU15500	no	Dihydro-orotase
	pyrD	BSU15540	no	Dihydro-orotic acid dehydrogenase (catalytic subunit)
	pyrE	BSU15560	no	Orotate phosphoribosyltransferase
	pyrF	BSU15550	no	Orotidine 5'-phosphate decarboxylase
	cmk	BSU22890	yes	Cytidylate kinase (CMP, dCMP)
	pyrG	BSU37150	yes	CTP synthase (NH3, glutamine)
	yncF	BSU17660	no	dUTPase
	thyB	BSU21820	no	Thymidylate synthase B
	tmk	BSU00280	yes	Thymidylate kinase
	pyrH	BSU16510	yes	Uridylate kinase

	Gene	BSU no.	Essential	Function
Purine biosynthesis				
	purF	BSU06490	no	Glutamine phosphoribosyldiphosphate amidotransferase
	purD	BSU06530	no	Phosphoribosylglycinamide synthetase
	purN	BSU06510	no	Phosphoribosylglycinamide formyltransferase
	purS	BSU06460	no	Phosphoribosylformylglycinamidine synthase
	purQ	BSU06470	no	Phosphoribosylformylglycinamidine synthase
	purL	BSU06480	no	Phosphoribosylformylglycinamidine synthase
	purM	BSU06500	no	Phosphoribosylaminoimidazole synthetase
	purE	BSU06420	no	Phosphoribosylaminoimidazole carboxylase (ATP dependent)
	purK	BSU06430	no	Phosphoribosylaminoimidazole carboxylase (ATP dependent)
	purC	BSU06450	no	Phosphoribosylaminoimidazole succinocarboxamide synthase
	purB	BSU06440	no	Adenylsuccinate lyase
	purH	BSU06520	no	Phosphoribosylaminoimidazole carboxamide formyltransferase
	guaB	BSU00090	yes	IMP dehydrogenase
	guaA	BSU06360	no	GMP synthase (glutamine hydrolyzing)
	gmk	BSU15680	yes	Guanylate kinase (GMP:dATP, dGMP:ATP)
	purA	BSU40420	no	Adenylosuccinate synthetase
	adk	BSU01370	yes	Adenylate kinase
Pyrimidine/purine biosynthesis				
	nrdE	BSU17380	yes	Ribonucleoside diphosphate reductase (major subunit)
	nrdF	BSU17390	yes	Ribonucleoside diphosphate reductase (major subunit)
	nrdI	BSU17370	yes	Ribonucleoside diphosphate reductase
	ndk	BSU22730	no	Nucleoside diphosphate kinase
	hprT	BSU00680	yes	Hypoxanthine phosphoribosyltransferase
Lipids				
Malonyl-CoA synthesis				
	accC	BSU24340	yes	Acetyl-CoA carboxylase (biotin carboxylase subunit)
	accB	BSU24350	yes	Acetyl-CoA carboxylase (biotin carboxyl carrier subunit)

	Gene	BSU no.	Essential	Function
	accA	BSU29200	yes	Acetyl-CoA carboxylase (alpha subunit)
	accD	BSU29210	yes	Acetyl-CoA carboxylase (beta subunit)
	birA	BSU22440	yes	Biotin protein ligase
Acyl carrier				
	acpS	BSU04620	yes	Acyl carrier protein synthase, 4=-phosphopantetheine transferase
	acpA	BSU15920	yes	Acyl carrier protein
Aceto-acyl-Acp synthesis				
	fabD	BSU15900	yes	Malonyl-CoA—acyl carrier protein transacylase
	fabHA	BSU11330	no	Beta-ketoacyl–acyl carrier protein synthase III
β-Ketoacyl-Acp chain elongation				
	fabG	BSU15910	yes	Beta-ketoacyl–acyl carrier protein reductase
	fabF	BSU11340	yes	Beta-ketoacyl–acyl carrier protein synthase II
	fabI	BSU11720	no	Enoyl-acyl carrier protein reductase
	ywpB	BSU36370	yes	β-Hydroxyacyl (acyl carrier protein) dehydratase
Phosphatidic acid synthesis				
	plsC	BSU09540	yes	Acyl-ACP:1-acylglycerolphosphate acyltransferase
	plsX	BSU15890	yes	Acyl-ACP:phosphate acyltransferase
	plsY	BSU18070	yes	Acylphosphate:glycerol-phosphate acyltransferase
	gpsA	BSU22830	yes	Glycerol-3-phosphate dehydrogenase (NAD)
Phosphatidylglycerol phosphate synthesis				
	cdsA	BSU16540	yes	Phosphatidate cytidylyltransferase
	pgsA	BSU16920	yes	Phosphatidylglycerophosphate synthase
Phosphate				
	pit	BSU12840	no	Low-affinity phosphate transporter
Cofactors				
ECF transporter (general comonent) for riboflavin, biotin, thaimine, tryptophan				
	ybxA	BSU01450	no	ATP-binding A1 component of ECF transporters
	ybaE	BSU01460	no	ATP-binding A2 component of ECF transporters
	ybaF	BSU01470	no	Transmembrane T component ofECF transporters

	Gene	BSU no.	Essential	Function
NAD				
	nadD	BSU25640	yes	Nicotinamide-nucleotide adenylyltransferase
	nadE	BSU03130	yes	NH_3-dependent NAD^+ synthetase
	nadF	BSU11610	yes	NAD kinase
	niaP	BSU02950	no	Nicotinate transporter
	pncB	BSU31750	yes	Putative nicotinate phosphoribosyltransferase
Riboflavin/FAD				
	ribC	BSU16670	yes	Riboflavin kinase/FAD synthase
	ribU	BSU23050	no	Riboflavin ECF transporter, S protein
Pyridoxal phosphate				
	pdxS	BSU00110	no	Pyridoxal-5'-phosphate synthase (synthase domain)
	pdxT	BSU00120	no	Pyridoxal-5'-phosphate synthase (glutaminase domain)
Biotin				
	yhfU	BSU10370	no	S protein of biotin ECF transporter Thiamine
Thaimine, TPP				
	yloS	BSU15800	no	Thiamine pyrophosphokinase
	thiT	BSU30990	no	S protein of thiamine ECF transporter
Lipoate				
	gcvH	BSU32800	no	Glycine cleavage system protein H, 2-oxo acid dehydrogenase
	lipM	BSU24530	no	Octanoyltransferase
	lipL	BSU37640	no	GcvH:E2 amidotransferase
	lipA	BSU32330	Yes	Lipoic acid synthase
CoA				
	ykpB	BSU14440	no	Putative ketopantoate reductase
	panD	BSU22410	no	Aspartate 1-decarboxylase
	panC	BSU22420	no	Pantothenate synthase
	panB	BSU22430	no	3-Methyl-2-oxobutanoate hydroxymethyltransferase
	ywaA	BSU38550	no	Branched-chain amino acid aminotransferase
	coaA	BSU23760	no	Probable pantothenate kinase
	yloI	BSU15700	yes	Coenzyme A biosynthesis bifunctional protein CoaBC
	ylbI	BSU15020	yes	Pantetheine-phosphate adenylyltransferase

	Gene	BSU no.	Essential	Function
	ytaG	BSU29060	yes	Dephospho-CoA kinase
SAM				
	metK	BSU30550	yes	S-Adenosylmethionine synthetase
Folate				
	folE	BSU22780	yes	GTP cyclohydrolase I
	phoB	BSU05740	no	Alkaline phosphatase A
	folB	BSU00780	yes	Dihydroneopterin aldolase
	folK	BSU00790	yes	2-Amino-4-hydroxy-6-hydroxymethyl-dihydropteridine diphosphokinase
	sul	BSU00770	yes	Dihydropteroate synthase
	folC	BSU28080	yes	Folyl-polyglutamate synthetase
	dfrA	BSU21810	yes	Dihydrofolate reductase
	pabB	BSU00740	no	p-Aminobenzoate synthase (subunit A)
	pabA	BSU00750	no	p-Aminobenzoate synthase (subunit B)/ anthranilate synthase (subunit II)
	pabC	BSU00760	no	Aminodeoxychorismate lyase
	gsaB	BSU08710	no	Formate dehydrogenase
Heme biosynthesis				
	hemE	BSU10120	no	Glutamate-1-semialdehyde aminotransferase
	hemH	BSU10130	no	Uroporphyrinogen decarboxylase (uroporphyrinogen III)
	hemY	BSU10140	no	Ferrochelatase
	ctaA	BSU14870	no	Protoporphyrinogen IX oxidase
	ctaB	BSU14880	no	Heme A synthase
	hemL	BSU28120	no	Heme O synthase (major enzyme)
	hemB	BSU28130	no	Glutamate-1-semialdehyde aminotransferase
	hemD	BSU28140	no	Porphobilinogen synthase
	hemC	BSU28150	no	Uroporphyrinogen III synthase
	hemX	BSU28160	no	Hydroxymethylbilane synthase
	hemA	BSU28170	no	Glutamyl-tRNA reductase
	hemQ	BSU37670	no	Heme-binding protein
Menaquinone				
	menA	BSU38490	yes	Probable 1,4-dihydroxy-2-naphthoate octaprenyltransferase
	menH	BSU22750	yes	Menaquinone biosynthesis methyltransferase
	menC	BSU30780	yes	O-Succinylbenzoate-CoA synthase
	menE	BSU30790	yes	O-Succinylbenzoate-CoA ligase

	Gene	BSU no.	Essential	Function
	menB	BSU30800	yes	Naphthoate synthase
	menD	BSU30820	yes	2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase/ 2-oxoglutarate decarboxylase
	ytxM	BSU30810	no	Similar to prolyl aminopeptidase
	menF	BSU30830	no	Menaquinone-specific isochorismate synthase
Metals and iron-sulfur clusters				
Sodium export				
	mrpA	BSU31600	yes	Na^+/H^+ antiporter subunit
	mrpB	BSU31610	yes	Na^+/H^+ antiporter subunit
	mrpC	BSU31620	yes	Na^+/H^+ antiporter subunit
	mrpD	BSU31630	yes	Na^+/H^+ antiporter subunit
	mrpE	BSU31640	yes	Na^+/H^+ antiporter subunit
	mrpF	BSU31650	yes	Na^+/H^+ antiporter subunit
	mrpG	BSU31660	yes	Na^+/H^+ antiporter subunit
Potassium				
	kimA	BSU04320	no	High-affinity potassium transporter
Iron				
	efeB	BSU38260	no	Heme peroxidase in elemental iron uptake
	efeO	BSU38270	no	Lipoprotein, elemental iron uptake system (binding protein)
	efeU	BSU38280	no	Elemental iron uptake system (permease)
	yfmF	BSU07490	no	Iron/citrate ABC transporter (ATP-binding protein)
	yfmE	BSU07500	no	Iron/citrate ABC transporter (permease)
	yfmD	BSU07510	no	Iron/citrate ABC transporter (permease)
	yfmC	BSU07520	no	Iron/citrate ABC transporter (binding protein)
	yhfQ	BSU10330	no	Iron/citrate ABC transporter (solute-binding protein)
	pfeT	BSU13850	no	Iron efflux pump
Magnesium				
	mgtE	BSU13300	yes	Primary magnesium transporter
	mntH	BSU04360	no	Manganese transporter (proton symport)
Zinc				
	znuA	BSU02850	no	ABC transporter for zinc (binding protein)

	Gene	BSU no.	Essential	Function
	znuC	BSU02860	no	ABC transporter for zinc (ATP-binding protein)
	znuB	BSU02870	no	ABC transporter for zinc (permease)
Copper				
	ycnJ	BSU03950	no	Copper transporter
Fe-S cluster				
	sufB	BSU32670	yes	Synthesis of Fe-S clusters
	sufU	BSU32680	yes	Iron-sulfur cluster scaffold protein
	sufD	BSU32700	yes	Synthesis of Fe-S clusters
	sufS	BSU32690	yes	Cysteine desulfurase
	sufC	BSU32710	yes	ABC transporter (ATP-binding protein)
	fra	BSU05750	no	Frataxin-like protein
	yutI	BSU32220	no	Putative iron-sulfur scaffold protein
Cell division				
Cell wall synthesis				
Synthesis of D-glutamate				
	racE	BSU28390	yes	Glutamate racemase
Synthesis of D-Ala-D-Ala				
	alr	BSU04640	yes	Alanine racemase
	ddl	BSU04560	yes	D-Alanine-D-alanine ligase
Synthesis of m-diaminopimelate				
	dapG	BSU16760	yes	Aspartokinase I (alpha and beta subunits)
	asd	BSU16750	yes	Aspartate-semialdehyde dehydrogenase
	dapA	BSU16770	yes	Dihydrodipicolinate synthase
	dapB	BSU22490	yes	Dihydrodipicolinate reductase (NADPH)
	ykuQ	BSU14180	yes	Similar to tetrahydrodipicolinate succinylase
	patA	BSU14000	yes	Aminotransferase
	ykuR	BSU14190	yes	N-Acetyl-diaminopimelate deacetylase
	dapF	BSU32170	yes	Diaminopimelate epimerase
Isoprenoid biosynthesis				
	dxs	BSU24270	yes	1-Deoxyxylulose-5-phosphate synthase
	ispC	BSU16550	yes	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
	ispD	BSU00900	yes	2-C-Methyl-D-erythritol 4-phosphate cytidylyltransferase

	Gene	BSU no.	Essential	Function
	ispE	BSU00460	yes	4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase
	ispF	BSU00910	yes	2-C-Methyl-D-erythritol-2,4-cyclodiphosphate synthase
	ispG	BSU25070	yes	Similar to peptidoglycan acetylation, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase
	ispH	BSU25160	yes	(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase
	fni	BSU22870	no	Isopentenyl diphosphate isomerase
Undecaprenyl phosphate biosynthesis				
	yqiD	BSU24280	no	Geranyltransferase
	uppS	BSU16530	yes	Probable undecaprenyl pyrophosphate synthetase
	bcrC	BSU36530	no	Undecaprenyl pyrophosphate phosphatase
	hepT	BSU22740	yes	Heptaprenyl diphosphate synthase component II
	hepS	BSU22760	yes	Heptaprenyl diphosphate synthase component I
Peptidoglycan biosynthesis				
	glmS	BSU01780	yes	Glutamine:fructose-6-phosphate transaminase
	glmM	BSU01770	yes	Phosphoglucosamine mutase
	gcaD	BSU00500	yes	UDP-N-acetylglucosamine pyrophosphorylase
	murAA	BSU36760	yes	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
	murB	BSU15230	yes	UDP-N-acetylenolpyruvoylglucosamine reductase
	murC	BSU29790	yes	UDP-N-acetylmuramoyl-L-alanine synthetase
	murD	BSU15200	yes	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
	murE	BSU15180	yes	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimelate synthetase
	murF	BSU04570	yes	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine synthetase

	Gene	BSU no.	Essential	Function
	mraY	BSU15190	yes	Phospho-N-acetylmuramoyl-pentapeptide transferase (meso-2,6-diaminopimelate)
	murG	BSU15220	yes	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
	amj	BSU04230	no	Lipid II flipase
Peptidoglycan polymerization				
	ponA	BSU22320	no	Penicillin-binding protein 1A/1B Penicillin-binding
PG cross-links, cell separation				
	pbpB	BSU15160	yes	Penicillin-binding protein 2B
	pbpA	BSU25000	no	Penicillin-binding protein 2A
	lytE	BSU09420	no	Cell wall hydrolase (major autolysin) for cell elongation and separation
	lytF	BSU09370	no	Gamma-D-glutamate-meso-diaminopimelate muropeptidase (major autolysin)
Wall teichoic acid				
	tagO	BSU35530	yes	Undecaprenyl phosphate-GlcNAc-1-phosphate transferase
	mnaA	BSU35660	yes	UDP-N-acetylglucosamine 2-epimerase
	tagA	BSU35750	yes	UDP-N-acetyl-D-mannosamine transferase
	tagB	BSU35760	yes	Putative CDP-glycerol:glycerol phosphate glycerophosphotransferase
	tagD	BSU35740	yes	Glycerol-3-phosphate cytidylyltransferase
	tagF	BSU35720	yes	CDP-glycerol:polyglycerol phosphate glycerophosphotransferase
	tagH	BSU35700	yes	ABC transporter for teichoic acid translocation (ATP-binding protein)
	tagG	BSU35710	yes	ABC transporter for teichoic acid translocation (permease)
	tagU	BSU35650	no	Phosphotransferase, attachment of anionic polymers to peptidoglycan
Lipoteichoic acid				
	dgkB	BSU06720	yes	Diacylglycerol kinase
	pgcA	BSU09310	no	Alpha-phosphoglucomutase
	gtaB	BSU35670	no	UTP-glucose-1-phosphate uridylyltransferase

	Gene	BSU no.	Essential	Function
	ltaS	BSU07710	no	Lipoteichoic acid synthase
	ugtP	BSU21920	no	UDP-glucose diacylglycerol glucosyltransferase
Teichuronic acid				
	tuaB	BSU35600	yes	Biosynthesis of teichuronic acid
Coordination				
Divisome				
	divIC	BSU00620	yes	Cell division initiation protein (septum formation)
	ftsL	BSU15150	yes	Cell division protein (septum formation)
	divIB	BSU15240	yes	Cell division initiation protein (septum formation)
	ftsZ	BSU15290	yes	Cell division initiation protein (septum formation)
	ftsW	BSU14850	yes	Cell division protein
	ezrA	BSU29610	no	Negative regulator of FtsZ ring formation
	sepF	BSU15390	no	Part of the divisome
	gpsB	BSU22180	no	Removal of PBP1 from the cell pole after completion of cell pole maturation
	yvcK	BSU34760	no	Correct localization ofPBP1, essential for growth under gluconeogenic conditions
	yvcL	BSU34750	no	Involved in Z-ring assembly
	ftsA	BSU15280	yes	Formation of Z-ring
Division site selection				
	divIVA	BSU15420	no	Cell division initiation protein (septum placement)
	minC	BSU28000	no	Cell division inhibitor (septum placement)
	minD	BSU27990	no	Cell division inhibitor (septum placement)
	noc	BSU40990	no	DNA-binding protein, spatial regulator of cell division to protect the nucleoid, coordination of chromosome segregation and cell division
	minJ	BSU35220	no	Topological determinant of cell division
Elongasome				
	mreD	BSU28010	yes	Cell shape-determining protein, associated with the MreB cytoskeleton
	mreC	BSU28020	yes	Cell shape-determining protein, associated with the MreB cytoskeleton

	Gene	BSU no.	Essential	Function
	mreB	BSU28030	yes	Cell shape-determining protein
	rodA	BSU38120	yes	Control of cell shape and elongation
	mreBH	BSU14470	no	Cell shape-determining protein
	rodZ	BSU16910	yes	Required for cell shape determination
	mbl	BSU36410	yes	MreB-like protein
Coordination of cell divison and DNA replication				
	walJ	BSU40370	no	Coordination of cell division and DNA replication
Signalling				
	walK	BSU40400	yes	Two-component sensor kinase
	walR	BSU40410	yes	Two-component response regulator
	cdaA	BSU01750	no	Diadenylate cyclase
	gdpP	BSU40510	no	c-di-AMP-specific phosphodiesterase
Integrity of the cell				
Protection				
	ytbE	BSU29050	yes	Putative aldo/keto reductase
	katA	BSU08820	no	Vegetative catalase 1
	sodA	BSU25020	no	Superoxide dismutase
	ahpC	BSU40090	no	Alkyl hydroperoxide reductase (small subunit)
	ahpF	BSU40100	no	Alkyl hydroperoxide reductase (large subunit)/NADH dehydrogenase
	trxA	BSU28500	yes	Antioxidative action by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange
	yumC	BSU32110	yes	Ferredoxin-NAD(P)$^+$ oxidoreductase
	trxB	BSU34790	yes	Thioredoxin reductase (NADPH)
Repair/ Genome integrity				
	hlpB	BSU10660	yes	HNH nuclease-like protein, rescues AddA recombination intermediates
	mutY	BSU08630	no	A/G-specific adenine glycosylase
	polY1	BSU23870	no	Translesion synthesis DNA polymerase Y1
	mutM	BSU29080	no	Formamidopyrimidine-DNA glycosidase
	mfd	BSU00550	no	Transcription repair-coupling factor
	recD2	BSU27480	no	5'–3' DNA helicase replication fork progression
	rnhB	BSU16060	no	RNase HII, endoribonuclease
	recA	BSU16940	no	Homologous recombination and DNA repair

	Gene	BSU no.	Essential	Function
	pcrA	BSU06610	yes	ATP-dependent DNA helicase
Other/ unknown				
	ppaC	BSU40550	yes	Inorganic pyrophosphatase
	ylaN	BSU14840	yes	Unknown
	yitI	BSU11000	no	Unknown
	yitW	BSU11160	no	Unknown
	yqhY	BSU24330	no	Unknown
	ykwC	BSU13960	no	Putative beta-hydroxy acid dehydrogenase
	ylbN	BSU15070	no	Unknown
	ypfD	BSU22880	no	Similar to ribosomal protein S1
	yugI	BSU31390	no	Similar to polyribonucleotide nucleotidyltransferase
	floT	BSU31010	no	Similar to flotillin 1, orchestration of physiological processes in lipid microdomains
	yyaF	BSU40920	no	GTP-binding protein/GTPase
	yezG	BSU06811	yes	Antitioxin
	yneF	BSU17910	yes	Unknown
	yqeG	BSU25680	yes	Unknown
	wapI	BSU39220	yes	Immunity protein
	yxxD	BSU39290	yes	Antitioxin

7. Curriculum Vitae

Personal information

Name	Anika Klewing
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Education

2015-2019 **Ph.D. thesis**
Georg-August-Universität Göttingen, Prof. Dr. Jörg Stülke
at the GGNB program Microbiology and Biochemistry
Title of thesis: "*MiniBacillus*- the construction of a minimal organism"

2013-2015 **Master of Science, Microbiology & Biochemistry**
Georg-August-Universität Göttingen, Prof. Dr. Jörg Stülke
Title of thesis: "Investigation of the impactof YqhY on the acetyl-CoA carboxylase activity in *Bacillus subtilis*"

2010-2013 **Bachelor of Science, Biology**
Georg-August-Universität Göttingen, Prof. Dr. Jörg Stülke
Area of specification: Molecular life sciences
Title of thesis: "Genomminimierung in *Bacillus subtilis*: Konsequenzen des Verlustes des Citratzyklus"

2003-2010 **Higher School Certificate**
Franziskus Gymnasium, Lingen