Control of glutamate homeostasis in the Gram-positive model organism *Bacillus subtilis*

Dissertation

to acquire the doctoral degree in mathematics and natural science

"Doctor rerum naturalium"

at the Georg-August-Universität Göttingen

in the doctoral program *Microbiology & Biochemistry*

at the Georg-August University School of Science (GAUSS)

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Göttingen 2015

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I hereby declare that the doctoral thesis entitled,

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Danksagung

An erster Stelle möchte ich mich bei Fabian dafür bedanken, dass ich die Ehre hatte, seine erste Doktorandin sein zu dürfen. Würde es eine Prüfung für die Premiere als Doktorvater geben, du hättest sie mit Bravour bestanden. Danke für deine Geduld, all deine aufschlussreichen Zeichnungen und vor allem dafür, dass du mich in die Welt der *Bacillus*-Forschung geführt hast.

Ein großer Dank geht auch an Jörg. Danke für all die hilfreichen Einfälle. Du warst nicht nur während der Thesis Committees ein Anprechpartner für mich. In diesem Zusammenhang möchte ich mich auch ganz herzlich bei Kai Tittmann bedanken, für die netten und informativen Gespräche bei den Thesis Committee Meetings. Desweiteren danke ich Ivo Feussner, Rolf Daniel und Michael für die Teilnahme an meiner Prüfungskommission.

Bei Till Ischebeck bedanke ich mich sehr für die GC-MS Messungen, die so schöne Ergebnisse geliefert haben. Katrin, du hast mir die beste Einführung in die *Bacillus*-Grundlagenforschung gegeben und mich mit prompten Antworten auf jegliche meiner Fragen beeindruckt. Danke, dass ich auch noch von deinem Wissen profitieren durfte, nachdem du zu den eher difficilen Organismen abgewandert bist.

Miriam, mit einer Lab-Rotation hat alles angefangen. Ich danke dir natürlich für all die anregenden Diskussionen über *Bacillus subtilis*. Doch vielmehr freue ich mich über das, was darüber hinaus entstanden ist. Danke für eine immer offene Tür, für Hilfe in der größten Not, ohne dich hätte meine Arbeit nicht das jetzige Format angenommen. Danke für Lachanfälle und Wein-Abende in doppelter Bedeutung – Willkommen in der Liebrechtstraße. Cedric, schön, dass du dazu gestoßen bist. Was hätte ich nur ohne die Kaffee-Versorgung der letzten Wochen gemacht.

Ohne dich, Sabine, wären es für mich lange Tage mit *lacZ* Messungen und Western Blots geworden. Ich bin sehr froh, dass ich mit dir zusammen arbeiten durfte. Danke für deine aufmunternden Worte und manch süße Unterstützung, wenn ich auf die Unterzuckerung zustrebte.

Natürlich bedanke ich mich auch bei meinen weiteren ehemaligen Studenten. Vicky, Markus, Richard und Martin, es war schön mit euch zu arbeiten und ich danke euch, dass ihr die Forschung so vorangetrieben habt. Martin, dein erfrischender Humor und deine Sicht der Dinge sind großartig. Es war eine super Zeit mit dir im Labor, wir haben uns über jede Suppressormutante gefreut und der Laborsong bleibt mir im Ohr.

Es hat mich glücklich gemacht, den Weg vom Bachelor, über den Master bis hin zum Doktor mit euch zusammen zu bestreiten, Jan und Steffi. Steffi, du warst mein Ruhepol, meine Kopfschmerzen-bekämpferin und Tagungsreisegefährtin. Was hätte ich nur ohne dich gemacht. Ihr Lieben aus dem S2 Labor, Julia, Katrin und Johannes, ich bin immer gerne bei euch vorbeigekommen.

Die Feiern mit nur einem Glas Wein waren die besten, Dominik. Ich danke natürlich auch allen anderen lieben (ehemaligen) Kollegen dieser tollen Abteilung, die ich kennenlernen durfte: Andrea, Arne, Bärbel, Christina, Chris, Daniel, Felix, Jan G. und Jan K. und Silvia, es hat Spaß gemacht mit euch. Joni,

schön, dass du die letzten Wochen noch ein bisschen frischen Wind in unser Labor gebracht hast. Deine Gesangseinlagen sind spitze. Bingyao, Nora und Raphael, mit euch habe ich entspannte Kaffeepausen verbracht und ab und an meine Spanisch- und Chinesischkenntnisse testen können.

Meinen Freunden und meiner Familie danke ich für all ihre Unterstützung in jeglicher Hinsicht. Mama und Papa, ich danke euch dafür, dass ihr mich meinen Weg habt gehen lassen, mit der Gewissheit, dass ihr immer hinter mir steht, egal in welche Richtung meine Schritte gehen.

Bernard, du hast deinen ganz eigenen Namen für *B. subtilis* gefunden. Mit dir kann ich über Gott und die Welt reden (ausgeschlossen Forschung), um die Welt reisen und die Welt um mich herum vergessen. Dafür danke ich dir von ganzem Herzen.

Table of contents

List of publications

GUNKA, K., STANNEK, L., CARE, R. A. & COMMICHAU, F.M. (2013) Selection-driven accumulation of suppressor mutants in *Bacillus subtilis*: the apparent high mutation frequency of the cryptic *gudB* gene and the rapid clonal expansion of *gudB*$^{+}$ suppressors are due to growth under selection. *PloS one*, 8, e66120.

STANNEK, L., EGELKAMP, R., GUNKA, K. & COMMICHAU, F.M. (2014) Monitoring intraspecies competition in a bacterial cell population by cocultivation of fluorescently labelled strains. *Journal of Visualized experiments*, 83, e51196.

DORMEYER, M., EGELKAMP, R., THIELE, M.J., HAMMER, E., GUNKA, K., STANNEK, L., VÖLKER, U. & COMMICHAU, F.M. (2015) A novel engineering tool in the *Bacillus subtilis* toolbox: inducer-free activation of gene expression by selection-driven promoter decryptification. *Microbiology*, 161, 354–361.

ZAPRASIS, A., HOFFMANN, T., STANNEK, L., GUNKA, K., COMMICHAU, F.M. & BREMER, E. (2014) The γ-aminobutyrate permease GabP serves as the third proline transporter of *Bacillus subtilis*. *Journal of Bacteriology*, 196, 515–526.

STANNEK, L., GUNKA, K., CARE, R.A., GERTH, U. & COMMICHAU, F.M. (2015a) Factors that mediate and prevent degradation of the inactive and unstable GudB protein in *Bacillus subtilis*. *Frontiers in Microbiology*, 5, 758.

STANNEK, L., THIELE, M.; ISCHEBECK, T.; GUNKA, K.; HAMMER, E., VÖLKER, U. & COMMICHAU, F.M. (2015b) Evidence for synergistic control of glutamate biosynthesis by glutamate dehydrogenases and glutamate in *Bacillus subtilis*. *Environmental Microbiology,* 17, 3379-3390.

Abbreviations

% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
ADP	adenosine diphosphate
ATP	adenosine triphosphate
B.	*Bacillus*
B2H	bacterial two-hybrid
C	carbon
CAF	ammonium ferric citrate
CcpA	catabolite control protein A
CDP-*Star*	disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate
CFP	cyan fluorescent protein
CR	cryptic
DAS	downstream activating sequence
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dpi	days post inoculation
E.	*Escherichia*
e.g.	*exempli gratia*
et al.	*et alii*
Fig.	figure
fwd.	forward
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
Glc	glucose
GOGAT	glutamate-oxoglutarate amidotransferase or glutamate synthase
GS	glutamine synthetase
i.e.	*id est*
IPTG	isopropyl-β-D-thiogalactopyranoside
k_m	Michaelis Menten constant
LB	Luria Bertani (medium)
LFH	long-flanking homology
max.	maximum
MMR	multiple mutation reaction
mRNA	messenger RNA
N	nitrogen
n. d.	not determined
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OD_x	optical density (measured at wavelength λ = x nm)
P	promoter
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	*pondus hydrogenii*

PLP	pyridoxal 5'-phosphate (active form of vitamin B6)
PVDF	polyvinylidene difluoride
rev.	reverse
RNA	ribonucleic acid
RNase	ribonuclease
S	Succinate
S.	*Saccharomyces*
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SP	sporulation medium
SPABBATS	shortest pathway between the basal and target sets
SPINE	Strep-protein interaction experiment
Tab.	table
TCA cycle	tricarboxylic acid cycle
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP	yellow fluorescent protein
σ^L	sigma factor L

Units

bp	base pair
°C	degree Celsius
Da	Dalton
g	gram
h	hour
kb	kilo base pair (10^3 bases)
l	liter
m	meter
M	molar (mol/l)
min	minute
rpm	rounds per minute
s	second
U	units
V	volt
W	watt

Prefixes

k	kilo	10^3
m	milli	10^{-3}
µ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}

Nucleotides

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

1 Summary

Glutamate is a central metabolite in any living organism because it is the major amino group donor for nitrogen containing compounds. To ensure a constant glutamate supply, *Bacillus subtilis* tightly regulates glutamate metabolism in dependence of the available carbon and nitrogen sources. The only glutamate synthesizing reaction in *B. subtilis* is the reductive amination of 2-oxoglutarate by the glutamate synthase GOGAT. The glutamate dehydrogenases (GDHs) of *B. subtilis* are strictly devoted to glutamate degradation. The genome of the *B. subtilis* laboratory strain 168 contains the *rocG* and *gudB*^{CR} genes, encoding the active GDH RocG and the highly instable and inactive GDH GudB^{CR}, respectively. While transcription of the *rocG* gene is tightly regulated, the *gudB*^{CR} gene is constitutively expressed. Upon deletion of the gene encoding RocG, *B. subtilis* forms suppressor mutants on complex medium. The suppressor mutants synthesize a stable and active GudB^{+} enzyme, thereby keeping the glutamate metabolism in balance. The aim of this work was to shed more light on the complex regulation of glutamate metabolism and to dissect the role of the GDHs within *B. subtilis*.

It has been unclear, why the *gudB*^{CR} gene encoding the inactive GDH GudB^{CR} is stably inherited over many generations in the *B. subtilis* laboratory strain 168. Here, for the first time a plausible explanation for the stable inheritance of the *gudB*^{CR} allele under laboratory growth conditions is provided. Compared to a strain synthesizing the active GDHs RocG and GudB^{+}, the laboratory strain producing only RocG has a selective growth advantage when glutamate is scarce. By contrast, with excess of exogenous glutamate the strain expressing the GDHs RocG and GudB^{+} rapidly outcompetes a strain synthesizing only RocG. Thus, the level of GDH activity strongly influences fitness of the bacteria depending on the availability of glutamate.

In the present work, a mechanism for the degradation of the inactive GDH GudB^{CR} is also proposed. It could be demonstrated, that the arginine kinase McsB and the cognate phosphatase YwlE affect the stability of the GudB^{CR} protein. Furthermore, interaction-studies suggest that the ClpCP protease complex is involved in the degradation of the GudB^{CR} protein. Finally, in this work it could be demonstrated that like the GDH RocG also GudB^{+} acts as a trigger enzyme that controls the DNA-binding activity of GltC, which is the transcriptional activator of the GOGAT-encoding *gltAB* genes. Moreover, the essential role of glutamate for the GDH-dependent control of GltC was demonstrated. Only when the internal glutamate concentrations are sufficiently high, the GDH inhibits the DNA binding activity of GltC. Thus, the GDHs function as sensors of the cellular glutamate pool and regulate the glutamate synthesis through interaction with GltC. The finding of the synergistic control of glutamate biosynthesis by glutamate and the GDHs deepens our understanding of the regulation of glutamate metabolism in *B. subtilis*.

2 Introduction

2.1 *Bacillus subtilis*

Since its first description in 1835, *Bacillus subtilis*, formerly known as *Vibrio subtilis* (Gordon, 1981), has become the leading paradigm for research on Gram-positive bacteria. The primary habitat of the rod-shaped bacterium is the soil. Soil bacteria have to cope with different challenging conditions like nutrient starvation, changing water supply, fluctuating temperatures or osmotic stress. *B. subtilis*, which belongs to the Firmicutes, has evolved a broad spectrum of mechanisms to withstand these challenges (e.g. Hecker & Völker, 1998; Budde et al., 2006). In order to compete with other organisms, *B. subtilis* produces and secretes a variety of both proteases and antibiotics such as the well-known surfactin, providing access to a variety of nutrient sources (Babasaki et al., 1985; Vollenbroich et al., 1997; Hamoen et al., 2003; Bais et al., 2004). As these traits, among others, are also beneficial for plants, *B. subtilis* falls into the category of plant growth promoting rhizobacteria, which turns this bacterium into a valuable biofertilizer (for a review see Lucy et al., 2004; Cazorla et al., 2007). Indeed, *B. subtilis* can be found within the rhizosphere (Pandey & Palni, 1997; Fall et al., 2004). Recently, it was proven that plant polysaccharides trigger root-associated biofilm formation (Beauregard et al., 2013). In times of very harsh conditions, *B. subtilis* forms endospores resistant to a broad range of environmental challenges (Piggot & Hilbert, 2004; Galperin et al., 2012). Formation of these metabolically dormant endospores, which can germinate upon improvement of the environmental conditions, ensures a last resort for long-term survival. Moreover, *B. subtilis* is capable of taking up and integrating foreign DNA; it is genetically competent (Hamoen et al., 2003).

These features make *B. subtilis* not only interesting for academic research (e.g. Sonenshein et al., 2002). Nowadays, *B. subtilis* is also extensively used as an industrial workhorse in biotechnology (Harwood, 1992). In contrast to *Escherichia coli*, *B. subtilis* has the GRAS (generally regarded as safe) status. Without producing toxic by-products such as endotoxins, *B. subtilis* secretes high amounts of protein directly into the growth medium. For instance, bacilli have a well-known reputation in the production of enzymes like alkaline proteases used in washing agents (Simonen & Palva, 1993; Chu, 2007; Degering et al., 2010) and vitamins, like riboflavin (Bretzel et al., 1999; Hao et al., 2013). Lately, *B. subtilis* was engineered for the production of the B6 vitamer pyridoxine (Commichau et al., 2014; Commichau et al., 2015).

In 1997, researchers from the European-Japanese joint research program published the whole genome sequence of *B. subtilis* (Kunst et al., 1997) and since 2009 an updated sequence has been publically available (Barbe et al., 2009). This facilitated the research on *B. subtilis* significantly. In proteomic analyses, for instance, research groups currently still try to assign functions to the about 4100 genes of *B. subtilis* (Hahne et al., 2010; Becher et al., 2011).

2.1.1 *B. subtilis* strains used in research

B. subtilis has been a research object for more than a century. *B. subtilis* strain 168 is the strain used in laboratories worldwide. This strain was one of the first genetically competent *B. subtilis* strains transformed by John Spizizen and his colleagues (Spizizen, 1958). *B. subtilis* strain 168 is a descendant from the *B. subtilis* Marburg strain, that was treated with X-rays in 1947 by Burkholder and Giles (Burkholder & Giles, 1947). The wild-type Marburg parent strain got lost, but besides the tryptophane-auxotroph strain 168, descendants like strains 160 and 166, the threonine auxotroph strain 23 and the nicotinic acid requiring strain 122 are maintained in the *Bacillus* Genetic Stock Center in Columbus, Ohio (http://www.bgsc.org/). Another *B. subtilis* strain often used in research is the biofilm-forming NCIB 3610, a close derivative of the wild-type Marburg strain (for a review see Cairns et al., 2014; Gerwig et al., 2014). Zeigler and his colleagues stressed the importance of understanding the relation between the different strains, especially when data from several *B. subtilis* studies are compiled. A sequence analysis approach was performed to shed light on the history of the early *B. subtilis* legacy strains. Inter alia, the comparative sequence analysis revealed that the laboratory strain 168 has a different equipment to degrade glutamate than the wild NCIB 3610 strain (Belitsky & Sonenshein, 1998; Zeigler et al., 2008).

2.2 The relevance of glutamate

In any living organism, the amino acid glutamate is an outstanding metabolite. Accounting for nearly 40 % of the internal metabolite pool, glutamate is the most abundant metabolite in *E. coli* cells, for instance (Bennett et al., 2009). In the amino acid pool of *B. subtilis* and *Pseudomonas aeruginosa*, glutamate adding up to 75 % is also the most abundant amino acid (Whatmore et al., 1990; Frimmersdorf et al., 2010). The high abundance of glutamate within the cells is no longer surprising, considering the different functions glutamate fulfills. This proteinogenic amino acid is the major amino group donor for nitrogen-containing compounds like nucleotides or other amino acids. In *Saccharomyces cerevisiae* and *B. subtilis* more than 80 % of the nitrogen embedded in nitrogen-containing metabolites is derived from glutamate (Wohlheuter et al., 1973; Magasanik, 2003). In *B. subtilis* its function of being the major amino group donor is reflected by the involvement of glutamate in 37 detected transamination reactions so far (Oh et al., 2007). An important amino acid synthesized from glutamate as precursor is the osmoprotectant proline, which is produced in high concentrations during osmotic stress (Kempf & Bremer, 1998; Brill et al., 2011).

Besides glutamate´s relevant function for the cell, the metabolic pathway of glutamate biosynthesis is striking as well. Glutamate is synthesized from 2-oxoglutarate, a derivative of the tricarboxylic acid cycle (TCA) and the nitrogen-containing compound glutamine. Thus, the biosynthesis

of glutamate represents a very important metabolic branch point; it links the carbon metabolism with the nitrogen metabolism (Commichau et al., 2006).

Glutamate plays another vital role in human life. Already in 1908 L-glutamate was detected to be responsible for the umami taste, often referred to as the fifth taste (Rolls, 2000; McCabe & Rolls, 2007; Sano, 2009). Since then, L-glutamate is produced industrially and commercialized as a flavor enhancer for food products. L-glutamate is mainly produced by *Corynebacterium glutamicum* and research is continuously ongoing to improve the production strains (Peters-Wendisch et al., 2001; Georgi et al., 2005; Witthoff et al., 2015). Today, L-glutamate holds number one in amino acid production in terms of volume (ca. two million tons in 2007; (Sano, 2009)), and the demand is increasing each year.

As it is the major excitatory neurotransmitter in the human body, glutamate plays an exceptional role in the brain (Meldrum, 2000). Several studies link changed levels of glutamate in the cortex or the plasma to major depressive disorders (Sanacora et al., 2004; Mitani et al., 2006). Recently, the glutamate N-methyl-D-aspartate receptor was found to be a promising target for antidepressants, like ketamine (for a review see Rasmussen et al., 2013; Ionescu et al., 2014; Kavalali & Monteggia, 2014).

2.3 Glutamate metabolism in *B. subtilis*

In order for it to ensure high growth rates under any condition, *B. subtilis* has developed a complex control system to keep catabolism and anabolism in balance. For efficient glutamate biosynthesis, signals from both the carbon and the nitrogen cycle are processed (for a review see Gunka & Commichau, 2012).

In *B. subtilis*, glutamate is exclusively synthesized by the combined action of the glutamate synthase (GOGAT) and the glutamine synthetase (GS) (Fig. 2.1). In the ATP-dependent reaction, the GS incorporates ammonium into glutamate, leading to the production of glutamine. One glutamine molecule and one molecule of 2-oxoglutarate are needed for the synthesis of two glutamate molecules. This NADPH-consuming reaction is catalyzed by the GOGAT. While one glutamate molecule produced in this reaction stays in the cycle, the second can be used for anabolism where it serves as a nitrogen donor (Belitsky, 2002).

By the degradation of glutamate to 2-oxoglutarate *B. subtilis* is able to use glutamate as an additional carbon source to gain energy (Belitsky & Sonenshein, 1998; Gunka et al., 2013). This NAD^+-fuelled deamination reaction is performed by the enzyme glutamate dehydrogenase (GDH), which has a sole purpose, glutamate degradation (Fig. 2.1). The GDH of *B. subtilis* has a high K_m value of 18 mM for ammonium (Gunka et al., 2010). Consequently, the *B. subtilis* GDH is unable to synthesize glutamate *in vivo* (Belitsky & Sonenshein, 1998). This also implies that ammonium assimilation occurs solely via the activity of the GS-GOGAT cycle in *B. subtilis*. In contrast, the GDH of *E.coli* has a sevenfold

lower K_m value for ammonium. Therefore, *E. coli* can switch between the GS-GOGAT- and the GDH-dependent pathway to form glutamate. Under high intracellular ammonia concentrations and in energy-poor environments, *E. coli* can use the ATP-independent GDH pathway to synthesize glutamate via the incorporation of ammonia into 2-oxoglutarate (Reitzer, 2003). Under conditions where nitrogen is a limiting factor, glutamate is synthesized via the GS-GOGAT cycle because the GDH of *E. coli* has a lower affinity for ammonium than the GS, too (Reitzer, 2003).

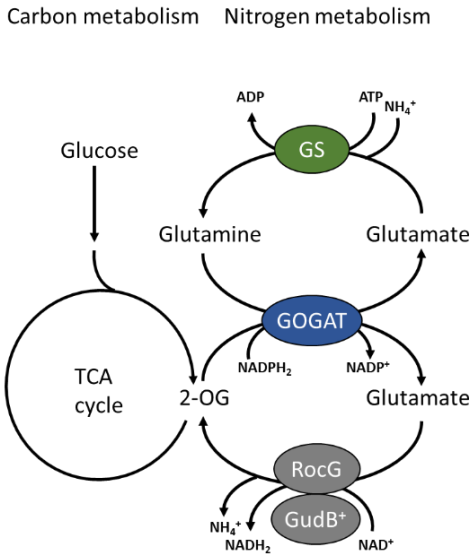

Fig. 2.1 The glutamate biosynthesis links the carbon to the nitrogen cycle
Glutamate is synthesized by the GOGAT and the GS. The GDHs RocG and GudB$^+$ are strictly devoted to glutamate degradation; 2-OG, 2-oxoglutarate (adapted from Gunka & Commichau, 2012).

Interestingly, the laboratory *B. subtilis* strain 168 encodes two GDHs, the active enzyme RocG and the inactive enzyme GudB^{CR} (Belitsky & Sonenshein, 1998). The latter is inactive and highly unstable, but due to a mutagenesis process the protein can become active (GudB$^+$) under specific growth conditions (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The importance of this protein will be pointed out in chapter 2.4.4.

2.4 Control of glutamate homeostasis in *B. subtilis*

Ammonium assimilation, glutamate biosynthesis, and glutamate degradation are regulated in an elaborative system to assure the glutamate homeostasis under any situation. Nitrogen and glutamate metabolism in *B. subtilis* is not only controlled by its global regulatory protein TnrA and the transcriptional repressor GlnR, but also by the GS and the GDH, which are so called trigger enzymes (Commichau et al., 2007a). These enzymes exert different functions, e. g. they are active in metabolism and in gene regulation (Wray et al., 2001). In the last years, it became clear that such enzymes exist more often than expected and perform important regulatory functions in metabolic pathways (Martin-Verstraete et al., 1998; Alén & Sonenshein, 1999; for a review see Commichau & Stülke, 2008).

2.4.1 Regulation of ammonium uptake

B. subtilis can use ammonia as nitrogen source, when the preferred nitrogen source glutamine is not present. Inorganic nitrogen in form of ammonia is either taken up by diffusion or by the AmtB transporter under low pH conditions (Detsch & Stülke, 2003). The AmtB transporter is encoded in the *nrgAB* operon together with the regulatory P_{II} protein GlnK. As a P_{II} protein GlnK is involved in sensing and transmitting signals from the nitrogen status of the cell (Atkinson & Fisher, 1991; Wray et al., 1994). Expression of the *nrgAB* genes is regulated by the key transcription factor of the nitrogen metabolism TnrA. During nitrogen-limited conditions, TnrA activates transcription of the *nrgAB* genes and ammonia is taken up. Under these conditions, TnrA also forms a complex with GlnK and AmtB (Fig. 2.2; Heinrich et al., 2006). Recently, it was observed that deletion of *glnK* results in a strong increase of ammonium leakage into the medium (Fedorova et al., 2013). This indicates a function of GlnK in controlling the opening status of AmtB. In *E. coli* GlnK is associated with AmtB upon high extracellular ammonia concentrations and negatively affects AmtB ammonium transport activity (Coutts et al., 2002). Furthermore, the crystal structure of the AmtB-GlnK complex in *E. coli* has already revealed that insertion of a T-loop of GlnK into the cytoplasmic exit pore of the AmtB subunits blocks ammonia conductance under nitrogen excess conditions (Conroy et al., 2007). Recently, a model was suggested in which not glutamine but the internal 2-oxoglutarate level controls the AmtB status (Kim et al., 2012). If ammonium concentrations are low within the cell, less ammonium is assimilated and less glutamine is synthesized. In turn, as no glutamate is synthesized either, 2-oxoglutarate concentrations increase within the cell. Increases of 2-oxoglutarate concentrations accompany disassociation of GlnK from AmtB, which becomes active in ammonium transport (Radchenko et al., 2010). Thus, 2-oxoglutarate and AmtB serve as a control variable and controller, being part of an internal feedback mechanism which ensures a need-based activation of ammonium uptake in *E. coli* (Kim et al., 2012).

2.4.2 Assimilation of ammonia via the GS-GOGAT cycle

As stated earlier, ammonia assimilation and glutamate synthesis strictly depend on the activity of the GS and the GOGAT in *B. subtilis* (Fig. 2.1; see chapter 2.3). Therefore, in addition to the ammonium uptake itself, the activity of these enzymes also has to be tightly regulated.

The GS is expressed under nitrogen limiting conditions. It is encoded in the *glnRA* operon together with the transcriptional repressor GlnR. Once the preferred nitrogen source glutamine is present in sufficient amounts, the GS is feedback-inhibited by glutamine. Three distinct classes of GS are known, GSI, GSII and GSIII enzymes (Brown et al., 1994). Bacteria and archaea synthesize GSI enzymes, which are divided into the isoenzymes GSI-α and GSI-ß (Brown et al., 1994; Murray et al., 2013). *B. subtilis* synthesizes the GSI-α enzyme. As of now, the structures of all catalytic and regulatory

states of the *B. subtilis* GS are available (Murray et al., 2013). Similar to other GSI-α enzymes, two stacked hexamers form the dodecameric GSI-α of *B. subtilis* (Krajewski et al., 2005; Murray et al., 2013). The data evinced that the subunits of the enzyme undergo large conformational changes during catalysis to form the active site. Upon glutamine binding, transition to the active state is not possible anymore. The subunits are locked in their closed state preventing release of glutamine and new substrate binding (Murray et al., 2013). Moreover, it was proven that the residue Arg62, involved in Arg62-Glu304-H bond linkage, plays a crucial role in feedback inhibition of GSI-α (Murray et al., 2013). The GSI-β of *E. coli* is not subject to feedback inhibition by glutamine. In this variant of the GS the specific Arg62 residue is missing, explaining the difference in the regulatory mechanism of *E. coli* GSI-β and *B. subtilis* GSI-α (for a review see Fisher, 1999; Murray et al., 2013).

Feed-back inhibited GS (FBI-GS) interacts with TnrA and GlnR regulating their activity (Fig. 2.2). TnrA and GlnR are homologs which so far have been assigned to belong to the MerR family of transcriptional regulators. Regulators of the MerR family have a winged-helix-turn-helix motif (HTH) and a C-terminal coiled coil domain (Wray et al., 1996; Brown et al., 2003). Analyzing structures of TnrA and GlnR in complex with DNA revealed that TnrA and GlnR form a separate family of transcriptional regulators. The C-terminal coiled coils used by MerR proteins for tight dimer formation are missing. Instead, TnrA and GlnR only form weak dimers via hydrophobic amino acid residues in the winged-HTH motif and the N-terminal domain. The highly disordered C-terminal domains of TnrA and GlnR in their DNA-bound form serve as nitrogen sensors instead (Schumacher et al., 2015).

Under excess nitrogen conditions transcription of the *glnRA* operon is repressed by GlnR. In a physiologically logic manner FBI-GS acts as a chaperone and stabilizes GlnR-DNA complexes, thereby regulating its own and TnrA synthesis (Fig. 2.2*;* Wray et al., 2001; Fisher & Wray, 2008). The cavity present in the dodecameric form of the GS was shown to serve as a chaperone for GlnR (Schumacher et al., 2015). Under nitrogen limitation, the C-terminal domain of GlnR has autoinhibitory functions inhibiting dimerization of GlnR. Under excess nitrogen, the C-terminal tail of GlnR is folded into a helix within the GS cavity allowing GlnR to form dimers and be active as transcriptional repressor (Schumacher et al., 2015).

Additionally, FBI-GS inactivates TnrA by a direct protein-protein interaction inhibiting the DNA-binding activity of TnrA (Wray et al., 2001). Structural analyses revealed that interaction of the TnrA helix with the GS causes drastic changes of the GS dodecamer resulting in the formation of a GS tetradecamer. Consequently, the GS becomes catalytically inactive and the TnrA dimer needed for *nrgAB* expression is disrupted (Schumacher et al., 2015).

Recently, a novel function has been assigned to the binding of TnrA to GlnK, too. Apparently, GlnK competitively counteracts binding of the non-feedback inhibited GS to TnrA. *In vitro* experiments revealed that especially under high glutamine concentrations, TnrA inhibits the GS activity.

Furthermore, in a *glnK* mutant GS activity was strongly impaired compared to wild-type cells, indicating constitutive binding of TnrA to the active GS (Fedorova et al., 2013). Structural data of the TnrA/GS complex provided insight into the TnrA-mediated inactivation of the GS. TnrA allosterically inhibits the GS by interacting with the active site loops which thereupon freeze in their inactive state (Schumacher et al., 2015). Thus, binding of TnrA to GlnK under nitrogen limitation is one of the many build-in mechanisms to control nitrogen metabolism in *B. subtilis* (Fedorova et al., 2013).

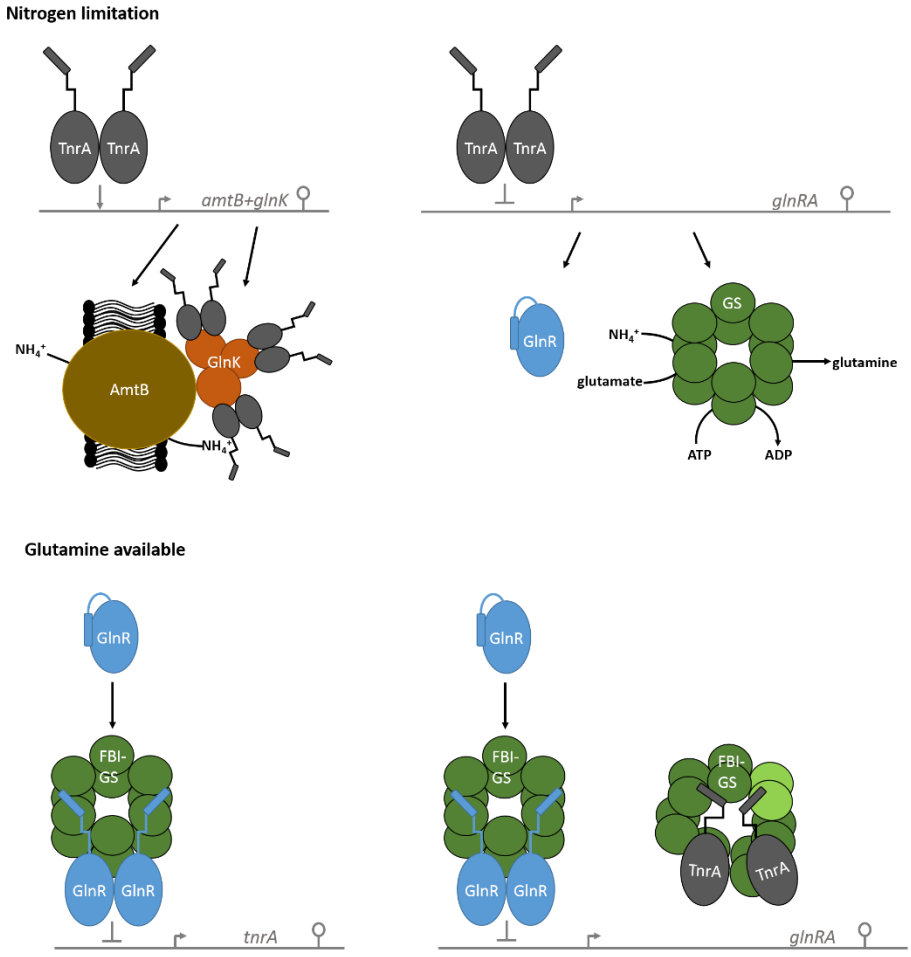

Fig. 2.2 Regulation of ammonium assimilation.
Under nitrogen-limiting conditions, TnrA in its dimeric form activates expression of the *nrgAB* genes, formely known as *amtB* and *glnK* genes, respectively, and slightly represses *glnRA* expression. At the same time, TnrA binds to the PII protein GlnK, which prevents binding of TnrA to the active GS. GlnK also interacts with the ammonium transporter AmtB. The GS and the transcriptional repressor GlnR are expressed under nitrogen limitation. GS is active, synthesizing glutamine and GlnR is autoinhibited. In the presence of the preferred nitrogen source glutamine, the GS is feedback-inhibited. Interaction of FBI-GS with autoinhibited GlnR relieves autoinhibition of GlnR, enabling GlnR to dimerize and repress *tnrA* and *glnRA* expression. Interaction of FBI-GS with dimerized TnrA leads to disruption of the TnrA dimer and oligomeric conversion of the GS dodecamer to a GS-TnrA tetradecamer.

Furthermore, TnrA regulates the expression of the *gltAB* genes, encoding the GOGAT. Under nitrogen limiting conditions, TnrA binds downstream of the *gltAB* promoter and represses transcription of *gltAB* (Fig. 2.3; Belitsky et al., 2000). In contrast, when ammonium and glucose, which is degraded to 2-oxoglutarate in the glycolysis and in the TCA cycle, are available, the genes are highly

transcribed. TnrA is feedback-inhibited by the GS. At the same time, GltC, the LysR-type transcriptional activator of the *gltAB* operon, binds to the *gltAB* promoter and activates transcription (Bohannon & Sonenshein, 1989). This leads to gene expression of the *gltAB* operon. Hence, expression of GOGAT, the enzyme at the interface of the nitrogen and carbon metabolism, occurs upon signals from both, the nitrogen and the carbon availability. Only if both substrates, 2-oxoglutarate and glutamine, are present the GOGAT is synthesized to produce glutamate, thereby keeping the glutamate homeostasis in balance.

Moreover, the synthesis of the GOGAT, as an iron-containing enzyme, is regulated in an iron-dependent manner. Under conditions of iron deficiency FsrA, a small non-coding RNA, hybridizes directly with the leader region of the *gltAB* operon resulting in translational repression of the GOGAT (Smaldone et al., 2012).

2.4.3 Glutamate degradation via the active glutamate dehydrogenase RocG

To prevent a futile cycle of glutamate biosynthesis and degradation, another intrinsic control mechanism is present in *B. subtilis*. Regulation of the expression of the GDH RocG itself is highly regulated depending on nutrient availability. Transcription of *rocG* is positively regulated by the two transcription factors AhrC and RocR (Gardan et al., 1997; Miller et al., 1997) and the alternative sigma factor σ^L (Débarbouillé et al., 1991). When the preferred carbon source glucose is present together with ammonium, transcription of *rocG* is severely repressed by the activity of CcpA (Belitsky et al., 2004). This pleiotropic transcription factor of carbon catabolite repression binds to the promoter region and the *rocG* coding region preventing binding of the sigma factor σ^L. At the same time, CcpA binds within the *sigL* gene, causing a roadblock in transcription (Choi & Saier, 2005). Thus, RocG is not active, when glutamate levels are low and when glucose is available.

However, under low glucose and high arginine or ornithine concentrations, *rocG* and the genes of the *rocABC* and *rocDEF* operon, which are involved in the arginine uptake and degradation pathway, are highly transcribed (Calogero et al., 1994; Gardan et al., 1997). Under these conditions the operon-specific transcriptional activator RocR binds to a downstream activating sequence or an upstream activating sequence of the *rocG* gene and the *rocABC* and *rocDEF* operon, respectively (Gardan et al., 1995, 1997; Belitsky & Sonenshein, 1999; Ould Ali et al., 2003). Consequently, the aforementioned proteins are synthesized, enabling *B. subtilis* to grow with arginine as single carbon source. Thus, signals from both the carbon and the nitrogen metabolism regulate the synthesis of the catabolically active GDH RocG.

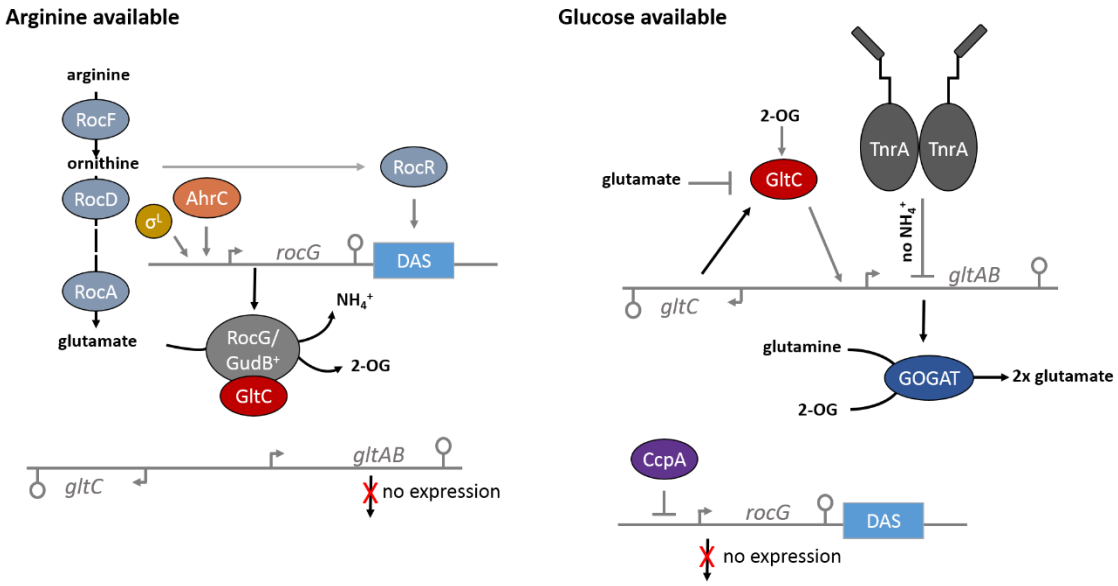

Fig. 2.3 Regulation of glutamate biosynthesis and degradation.
In the presence of glucose GltC activates *gltAB* expression. 2-oxoglutarate (2-OG) stimulates GltC binding to the promoter, whereas binding is inhibited by glutamate. If a product, e.g. ammonium, for the GOGAT reaction is missing, TnrA represses *gltAB* expression. When glucose is available, CcpA binds to the *rocG* promoter inhibing *rocG* expression. Ornithine, a product of the arginine degradation pathway, activates the transcriptional regulator RocR. Upon binding to a downstream activating sequence (DAS) of *rocG*, its expression is induced. AhrC and sigma factor σ^L additionally activate expression of *rocG*. In the presence of high glutamate concentrations, RocG or GudB$^+$ degrade glutamate and inactivate the transcriptional activator GltC by a direct protein-protein interaction, preventing *gltAB* expression (adapted from Gunka & Commichau, 2012).

2.4.3.1 Trigger enzyme activity of RocG

In the elaborative system which controls glutamate synthesis and degradation, another level preserving the glutamate homeostasis is observable. It has been demonstrated that RocG controls the DNA-binding activity of GltC. Under high glutamate concentrations RocG was found to bind to GltC. This interaction inhibits GltC in activating the transcription of the *gltAB* genes (Fig. 2.3; Commichau et al., 2007a). The exact mechanism of GltC inhibition by RocG is still unknown.

However, RocG has the attributes of being a trigger enzyme; it is active in metabolism and in gene regulation (Commichau & Stülke, 2008). The regulation described above also reveals that the key enzymes of the glutamate synthesis and degradation, GOGAT and RocG, respectively, are synthesized under mutual exclusive conditions. Surprisingly, high-level GDH activity does not cause a permanent inhibition of GltC in medium without any or with low levels of glutamate (Gunka et al., 2010). As addition of arginine to the medium re-establishes GltC inhibition by RocG, it was speculated that arginine or a metabolite from the arginine degradation pathway serves as co-factor for RocG to synergistically control GltC (Gunka et al., 2010). However, the co-factor still has to be identified (see 11.2.4.)

2.4.4 Importance of the cryptic glutamate dehydrogenase GudB^{CR}

As mentioned earlier, the laboratory strain *B. subtilis* 168 encodes not only the gene for the active GDH RocG, but also the gene for a second GDH GudB^{CR}. Although the *gudB^{CR}* gene is

constitutively transcribed, no active protein is formed (Belitsky & Sonenshein, 1998; Gunka et al., 2012). This is due to the presence of a perfect 18 bp-long direct repeat, which lies in the region encoding for the active center of the protein. Deletion of the only active GDH RocG in the strain 168 results in an unbalanced glutamate homeostasis for several reasons. First of all, the GOGAT is constitutively expressed, as its transcriptional regulator GltC is deregulated due to the absence of RocG. Second, glutamate cannot be degraded anymore. Either glutamate itself or a product from the arginine degradation pathway seems to be toxic for the cell (Belitsky & Sonenshein, 1998). A toxic effect of D-glutamate accumulation in *B. subtilis* cells was already demonstrated (Kimura et al., 2004). Phenotypically, the unbalanced glutamate homeostasis upon *rocG* deletion becomes noticeable as a strong growth defect on complex medium or on minimal medium containing glutamate as only carbon source (Gunka et al., 2012). A *rocG* deletion strain suffering from an unbalanced glutamate homeostasis shows a translucent background growth on complex medium. However, suppressor mutants, in which the growth defect is abolished, appear quickly and with a very high frequency (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The mutation frequency at which suppressor mutants appear is $1x10^{-4}$, the highest observed mutation frequency for *B. subtilis* so far (Gunka et al., 2012). Similar to the wild-type suppressor mutants can grow on complex medium and on minimal medium containing only metabolites from the arginine degradation pathway like arginine or ornithine.

Sequence analyses of the *gudB* gene shows that in the suppressor mutants one part of the perfect direct repeat is always precisely excised (Belitsky & Sonenshein, 1998; Gunka et al., 2012). This deletion renders the $GudB^+$ protein active (Belitsky & Sonenshein, 1998). RocG and $GudB^+$ show a sequence identity of 74 %. Furthermore, also the structures and the enzymatic activities of the GDHs are very similar (Gunka et al., 2010). Nonetheless, the inactive $gudB^{CR}$ gene is stably inherited in the laboratory strain (Zeigler et al., 2008). Interestingly, the parental Marburg strain or the wild-type strain NCIB 3610 encode two active GDHs, RocG and $GudB^+$ (Zeigler et al., 2008). The reason for the stable inheritance of the inactive $gudB^{CR}$ in the laboratory strain is still unknown. It is speculated that *B. subtilis* cells encoding only the highly regulated *rocG* gene might have a growth advantage under certain conditions encountered in the laboratory compared to cells encoding the constitutively transcribed $gudB^+$ in addition.

2.4.4.1 Proteolysis of the inactive $GudB^{CR}$

Organisms from all domains of life are well-appointed with control networks for protein quality (for a review see Wickner, 1999; Bhattacharyya et al., 2014). Misfolded proteins tend to form protein aggregates having detrimental effects on physiological functions. In humans, protein aggregation is closely related to neurodegenerative diseases as Alzheimer´s and Parkinson´s disease (Narhi et al., 1999; for a review see Ross & Poirier, 2004; Greenbaum et al., 2005). Thus, proteolytic degradation of

misfolded proteins is required for the cells viability (Kock et al., 2004). Several different proteases involved in degradation of misfolded proteins exist in microorganisms.

In *B. subtilis*, the most important protease responsible for degradation of misfolded proteins is the ATP-dependent two-component ClpCP protease (Krüger et al., 2000). To a lesser extend the ClpXP protease complex mediates proteolysis, too. Whereas the Lon protease is the key protease for misfolded proteins in *E. coli* (Gottesman, 1996), it is only slightly involved in proteolysis in *B. subtilis*. The ClpCP and ClpXP proteases consist of the proteolytic subunit ClpP and the AAA+ ATPases ClpC and ClpX, respectively. The proteolytic subunit is composed of a chamber formed by two heptameric rings containing the proteolytic active sites responsible for protein degradation. The ATPases interact as hexameric rings with the protease. ClpC and ClpX act as chaperones, which recognize the substrates being helped by adapter proteins (Battesti & Gottesman, 2013). These proteins are equally responsible for the unfolding of the proteins. In an ATP-dependent manner the unfolded substrates are translocated into the proteolytic chamber of the protease. A known adaptor protein for the ClpCP protease is McsB. A substrate for MscB was shown to be the heat-shock regulator CtsR (Elsholz et al., 2010b). Fuhrmann et al. assigned another function to McsB. Phosphoproteomic analyses indicated that McsB acts as an arginine kinase, which phosphorylates proteins on arginine residues (Fuhrmann et al., 2009). Lately, in a global phosphoproteomic study phosphorylation of the inactive GudB^{CR} protein on four different arginine residues by McsB was demonstrated. The cognate arginine phosphatase of McsB was found to be YwlE (Elsholz et al., 2012). Regarding the influence of the ClpCP protease on GudB^{CR} stability, a slight influence of ClpP on the stability of GudB^{CR} was observed during glucose-limited stationary phase (Gerth et al., 2008). However, clear evidence for the involvement of Clp-proteases in the degradation of GudB^{CR} is missing and the precise proteolytic machinery responsible for the degradation of the GudB^{CR} protein still remains to be solved.

2.4.5 Suppressor mutants balancing glutamate metabolism

The activation of the *gudB^{CR}* gene is not the only suppressor mutation observable in *B. subtilis* cells with a disturbed glutamate metabolism. Depending on the cause for the imbalance mutations in different genes involved in the glutamate metabolism can restore growth. In a *rocG gudB^{CR}* double mutant growth-restoring suppressor mutants appear on complex medium, too. Similar to the *rocG* single mutant a *rocG gudB^{CR}* double mutant is unable to degrade glutamate. Additionally, the GOGAT is constitutively active in glutamate synthesis and the 2-oxoglutarate pool is depleted (Commichau et al., 2007a). As the gene encoding the second GDH GudB^{CR} cannot be activated upon deletion in the double mutant, the growth defect has to be resolved by different mutations. Indeed, suppressor mutants were found to have a two base pair deletion in the *gltB* gene, inactivating the GOGAT in a *rocG gudB^{CR}* double mutant (Commichau et al., 2008). The formation of additional glutamate is

prevented. However, glutamate would still accumulate in the strain. Interestingly, another mutation present in the repressor AnsR was detected implicating an increased *ansAB* expression (Sun & Setlow, 1993). A new metabolic pathway for glutamate degradation due to higher levels of the aspartase AnsB was predicted to restore glutamate homeostasis in the described suppressor mutant strain (Flórez et al., 2011). Grown on minimal medium containing ammonium as only nitrogen source a second mutation restoring the reading frame of the *gltB* gene occurred in the above described mutant. This mutation resulted in a functional GltB and the strain regained the ability to synthesize glutamate via the GS-GOGAT pathway.

2.5 Industrial relevance of GDH and GS-inactivation/mutation

Having the complete genome sequence in hand, genetic engineering was facilitated tremendously (Kunst et al., 1997). Nowadays, researchers are making extensive efforts to identify the minimal gene set needed to sustain life (Kobayashi et al., 2003; Ara et al., 2007; Juhas et al., 2014). In the end, industrially attractive products, as certain enzymes, can be synthesized in cell factories which have predictable and tightly regulated metabolic pathways. As the following examples demonstrate, it is not unusual to adjust glutamate metabolism in order to improve productivity.

The research team of Takuya Morimoto (Morimoto et al., 2008) was one of the first who demonstrated that microbial genome reduction is indeed a good approach to obtain industrially relevant strains. The *B. subtilis* strain MGB874 with a 20.7 % reduced genome exhibited an impressively increased production efficiency of recombinant extracellular proteases and cellulases in comparison to the wild-type strain (Morimoto et al., 2008). Interestingly, in a follow-up study, it was discovered that the deletion of the *rocDEF-rocR* region led to improved cell yields and a higher specific productivity in strain MGB874 (Manabe et al., 2011). The high cell yield of MGB874 is attributed to decreased expression of *rocG* due to deletion of *rocR.* Complete deletion of *rocG* increased the cell yield even more (Manabe et al., 2011). However, a *rocG* deficient MGB784 strain is characterized by significantly decreased specific productivities of alkaline cellulase Egl-237 (Manabe et al., 2011, 2013). RocG is known for preventing acidification of the growth medium by its glutamate degrading activity. The deamination of glutamate is an ammonia-releasing reaction increasing the external pH, which positively affects the production of the α-amylase AmyK38 (Manabe et al., 2012). Cultivation of strain MGB784ΔrocG under constant pH conditions at pH 7.2 solved the problem and led to the highest reported level of alkaline cellulase Egl-237 production so far (Manabe et al., 2013).

B. subtilis species are also used in traditional Japanese soybean fermentation to produce so-called natto soybeans (Nishito et al., 2010; Kubo et al., 2011). Secondary fermentation, a process during which high ammonia levels are released, is regarded as problematic in natto production. A study uncovered, that inactivation of glutamate degradation by deletion of the two active GDH genes *rocG*

and *gudB*$^{+}$ in the natto producing *B. subtilis* strain r22 reduced the ammonia production by 50 % (Kada et al., 2008). However, this strain has a growth defect (Belitsky & Sonenshein, 1998). A strain with retained GDH activity was shown to be the optimal strategy to produce natto with decreased ammonia production. Therefore, Chen et al. determined the glutamate binding pocket of GudB^{+} of strain *B. subtilis* natto r22. Amino acid residues relevant for substrate binding were identified and mutation of these residues resulted in a lower substrate binding affinity reducing the activity of the GudB^{+}. Ammonium production was lowered and the GDH activity was still high enough ensuring growth (Chen et al., 2013).

Also with respect to industrial glutamate production new findings were made. In the genome of *Bacillus methanolicus*, which is related to *B. subtilis*, two genes encoding a GOGAT, GltAB and GltA, were found (Heggeset et al., 2012). This thermotolerant bacterium can form L-glutamate and L-lysine from methanol and is regarded as an alternative to *C. glutamicum* for industrial glutamate production (for a review see Brautaset et al., 2007). Recently, Krog et al. provided mounting evidence that both GOGATs are active in *B. methanolicus* and contribute to the production of L-glutamate (Krog et al., 2013). Future observations with respect to the regulation might explain the potential of *B. methanolicus* of producing up to 59 g/liter of L-glutamate (Brautaset et al., 2010).

2.6 Survival of the fittest – adaptation strategies

Rapid and appropriate adaptation to changing environmental conditions sustains life, not only with respect to microorganisms, but to any living organism. Consequently, adaptation and natural selection are key factors driving evolution forward. In general, there are three major mechanisms contributing to adaptation. All of these mechanisms are found within the complex regulatory network of the glutamate metabolism.

Transcriptional regulation of gene expression is indispensable for adaptation – it is an ubiquitous phenomena in bacteria. For instance, several transcription regulatory proteins – GlnR, TnrA, GltC – are involved in the adjustment of the glutamine and glutamate metabolism (for a review see Gunka & Commichau, 2012). Both transcriptional control and (post)translational control are fundamental to regulate gene expression and therefore contribute to adaptation. By adjusting the translation of mRNA into proteins, protein levels within the cell are fine-tuned. An example is the expression of the *gltAB* genes in an iron-dependent manner (Smaldone et al., 2012).

As a last resort, one other mechanism exists in nature, which might lead to adaptation as shown for the inactive *gudB*^{CR} gene: mutations. This mechanism was first described by Charles Darwin in his book „The origin of species“ of 1859 (Darwin, 1859). Simply by observing the varieties of organisms – without understanding the underlying mechanism – he discovered how mutation of an organism over sufficiently long periods may result in creating new species favored by natural selection. With today´s

knowledge, we can explain that changes in the DNA sequence form the basis of evolution and that mutations are the tenet for natural selection.

2.7 The role of mutations for adaptability

Although mutations constitute the only heritable adaptation to changing environmental conditions, genomic alteration does not always have positive consequences. Mutations can be beneficial, which would lead to adaptation, neutral or as mostly, harmful. Since decades it has been discussed controversially, if mutations occur spontaneously and randomly or directed (Roth et al., 2006). Performing a fluctuation test in 1943, Luria and Delbrück were the first to deliver evidence for the occurrence of spontaneous mutations in *E. coli* grown under non-selective conditions (Luria & Delbrück, 1943). In this study *E. coli* cells were grown in separate cultures and equal amounts were plated on agar plates containing the T1 phage virus. By analyzing the number of bacterial colonies growing on the agar plate conclusions were drawn about the mechanism by which the mutations occurred. Either the mutations referring resistance against the phages were induced upon contact of the bacteria with the phages or the mutations occurred spontaneously already prior to plating. A highly variable number of mutants on each plate supports the latter theory. Depending on the point in time at which a random mutation resulting in resistance occurs in the culture, more or less resistant colonies will be found on the plates in the end (Luria & Delbrück, 1943). While the system of Luria and Delbrück selects preexisting mutations, an approach introduced by Cairns and Foster allows the detection of beneficial mutations occurring under stressful conditions (Luria & Delbrück, 1943; Cairns & Foster, 1991). Basically, *E. coli* cells impaired in lactose utilization due to a -1 frameshift in the *lac* gene were plated on medium containing lactose as only energy source. After some days, the appearance of colonies with a compensating *lac*$^{+}$ mutation was observed. Strikingly, mutants that had regained the ability to use lactose as carbon source appeared to a much higher frequency when grown under selective pressure. This indicated the presence of an adaptive mutation mechanism (Cairns et al., 1988; Cairns & Foster, 1991).

In general, bacteria have to find a balance concerning the mutation rate. Bacteria living under constant environmental conditions have a lower mutation rate than bacteria found in challenging habitats (Saint-Ruf & Matic, 2006; Li et al., 2014). An increase in mutation rate might be beneficial, but the long-term benefit is questionable. Deleterious mutations occur with a much higher frequency than beneficial mutations as shown for *E. coli* (Kibota & Lynch, 1996; Imhof & Schlotterer, 2001). Well-balanced mutation rates secure high probabilities of survival for bacteria. I.e. mutation rates need to be sufficiently high to allow an evolution of genes, while simultaneously being sufficiently low for ensuring genomic integrity under non-selective conditions. On the one hand, bacteria have evolved high-fidelity repair mechanisms to keep the mutation rate low (for a review see Kunkel & Erie, 2005).

On the other hand, several interesting features were discovered which allow the bacteria a certain scope for adaptation.

2.7.1 Scope for adaptation

2.7.1.1 Cryptic genes and direct repeats

As observed by the mutation of the *gudB*^{CR} gene, activation of otherwise phenotypically silent genes provides one means for adaptation (Hall et al., 1983; Masel, 2006). These silent genes, also known as cryptic genes, can be activated by mutagenic processes, recombination, or by insertion elements. Under certain environmental conditions, a selection for mutants haboring the activated genes can occur (Hall et al., 1983; Hall, 1989). As previously mentioned, a perfect direct repeat of 18 bp renders the *gudB*^{CR} gene inactive (Belitsky & Sonenshein, 1998). Direct repeats or tandem repeats are DNA sequences in the genome, either intra- or intergenic, which are repeated several times in a head-to-tail manner (for a recent review see Zhou et al., 2014a). Direct repeats are found in genomes throughout the bacterial domain and represent so called mutational hotspot DNA sequences (Mrázek et al., 2007). Being prone to mutations, direct repeats are important for bacterial adaptation by spontaneous mutagenesis (Kassai-Jáger et al., 2008). For humans, these unstable DNA regions bear the danger of provoking illnesses, such as the Huntington´s disease (Hannan, 2010). Strand-slippage mispairing during DNA replication and recombination events are the proposed molecular mechanisms of direct repeat variation (Zhou et al., 2014a). So far, Gunka et al. could observe a bias for excision of the first part of the direct repeat present in the *gudB*^{CR} gene (Gunka et al., 2012). To what extend DNA strand slippage plays a role for the mutagenesis process of the *gudB*^{CR} gene, is not completely understood yet.

2.7.1.2 Transcription associated mutagenesis

Since decades, transcription is discussed as a process affecting the maintenance of genome integrity. Several causes for and factors involved in transcription associated mutagenesis are known. Different studies in prokaryotes and eukaryotes have already revealed the importance of transcription for mutagenesis processes (Datta & Jinks-Robertson, 1995; Kim et al., 2007; Park et al., 2012).

One mechanism involved in transcription associated mutagenesis is R-loop formation. During transcription, the non-transcribed strand can occasionally form R-loops. R-loops are RNA:DNA hybrids, in which the nascent transcript is stably base-paired to the template DNA. Formation of RNA:DNA hybrids implicates the generation of unstable ssDNA, which is more susceptible to damage than dsDNA. To countervail the deleterious effects of R-loop formation, the activity of RNase H enzymes is required to cleave RNA in RNA:DNA hybrids (Itaya et al., 1999). Deletion of RNase H II in *B. subtilis* leads to an increase in spontaneous mutation rate (Yao et al., 2013).

Data exist which show a correlation between genes located on the lagging strand and an increased mutation rate. The cause are head on collisions, a conflict arising between the transcription machinery and replication fork progression for genes which are not co-oriented with replication (Srivatsan et al., 2010). As the replication rate is faster than transcription rate also co-directional encounters between replication and transcription machinery are inevitable. Generally co-directional conflicts are not as detrimental as head-on collisions. In *E. coli*, comparisons revealed that head-on encounters but not co-directional collisions drastically slow down replication fork progression (French, 1992). If gene evolution is accelerated in genes encoded on the lagging strand where replication and transcription converge, is controversely discussed (Chen & Zhang, 2013; Paul et al., 2013). Additionally, a correlation between a high mutation rate and the rate of transcription as well as the gene length was also demonstrated for *B. subtilis* (Pybus et al., 2010; Paul et al., 2013). Also in *S. cerevisiae* transcription associated mutagenesis is directly proportional to gene expression (Kim et al., 2007). Several studies detected that the genome is organized in a way to avoid head-on collisions. Not only in *B. subtilis* but also in other bacteria like *Mycoplasma pneumoniae* or *Mycoplasma genitalium* a strong bias is observed for the so-called core genes, many of them being essential, to be encoded on the leading strand (Kunst et al., 1997; McLean et al., 1998; Rocha & Danchin, 2003; Price et al., 2005). In all bacteria, highly transcribed ribosomal RNA genes are for instance codirectionally orientated to replication direction (Guy & Roten, 2004). Interestingly, in *B. subtilis*, under the 17 % of genes encoded on the lagging strand many are involved in stress responses (Paul et al., 2013).

In case of RNA polymerase stalling at nucleotide lesions in the template strand, the transcription-repair coupling factor Mfd comes into action. The transcription repair coupling factor Mfd removes the stalled RNA polymerases from the DNA strand and recruits the DNA excision repair machinery (Ayora et al., 1996). *B. subtilis* Mfd deficiency strains exhibit a lower mutation rate compared to their parental strains (Ross et al., 2006). Deletion of *mfd* in a delta *rocG* background resulted in a strongly reduced mutation frequency of the *gudB*^{CR} gene under selective growth conditions (Gunka et al., 2012). However, the absence of other proteins involved in DNA repair or recombination such as RecJ and RecU, UvrAB or MutSL did not affect the mutation rate of *gudB*^{CR} (Gunka et al., 2012). To understand the molecular mechanism underlying the excision of the perfect direct repeat of the *gudB*^{CR} gene other proteins involved still have to be discovered.

2.8 Objectives

B. subtilis wild-type strains synthesize two active GDHs, RocG and GudB^{+}. The latter enzyme is inactive in the laboratory strain 168 (Belitsky & Sonenshein, 1998). Although the *gudB*^{CR} gene encoding for the inactive GudB^{CR} enzyme mutates with a very high frequency and becomes active under specific growth conditions, it is stably inherited in the laboratory strain 168 (Zeigler et al., 2008; Gunka et al.,

2012). In this work, the selective advantage for the laboratory strain 168 for keeping the inactive *gudB*^{CR} will be analyzed. For this purpose, co-cultivation experiments between strains synthesizing the active or the inactive *gudB* gene will be performed in different media.

As stated above, a *rocG* deletion strain spontaneously activates the *gudB*^{CR} gene when cells are grown under high glutamate concentrations. RocG^{-} GudB^{+} cells proliferate quickly under these growth conditions (Gunka et al., 2012). The spontaneous mutation of the direct repeat in the *gudB*^{CR} gene provides a basic principle for the development of an industrially attractive novel genetic system which allows inducer-free activation of gene expression. In this work, such an alternative expression system leading to the synthesis of industrially relevant substances will be developed and established.

The GudB^{CR} protein, although inactive, is constantly synthesized and rapidly degraded in the *B. subtilis* laboratory strain 168 (Gunka et al., 2012). Thus, GudB^{CR} represents a good example to study protein-turnover in cells. Until now, the proteolytic machinery has not been discovered. A screening system to uncover proteins involved in proteolysis of the inactive GudB^{CR} protein will be developed in this work. Putative factors influencing the stability of the GudB^{CR} protein will be analyzed in their specific role in the proteolytic mechanism of the GudB^{CR} protein.

It has been shown that the active GudB^{+} enzyme takes over the function of RocG regarding glutamate degradation. In this work, it will be investigated if GudB^{+} is also capable of interacting with and regulating the activity of the transcriptional activator GltC in a similar way as RocG does (Gunka et al., 2010). Furthermore, this work aims at deepening our understanding of the regulatory mechanism between the GDHs and GltC.

3 Selection-driven accumulation of *gudB*$^{+}$ suppressor mutants in *Bacillus subtilis*

The results described in this chapter were published in:

GUNKA, K., **STANNEK, L.**, CARE, R. A. & COMMICHAU, F.M. (2013) Selection-driven accumulation of suppressor mutants in *Bacillus subtilis*: the apparent high mutation frequency of the cryptic *gudB* gene and the rapid clonal expansion of *gudB*$^{+}$ suppressors are due to growth under selection. *PloS ONE*, 8 (6): e66120.
doi: 10.1371/journal.pone.0066120

Author´s contribution:

The study was designed and interpreted by KG, LS and FMC. Intra-species competition experiments and the monitoring of the emergence of the *gudB* allele by a blue-white screening system were done by LS. Isolation of *gudB*$^{-}$ mutants, Western blotting, analysis of direct repeat integrity, fluorescence microscopy and analysis of growth of microcolonies were performed by KG. RAC contributed substantially to the generation of strains and plasmids. The paper was written by KG and FMC.

Abstract

Soil bacteria like *Bacillus subtilis* can cope with many growth conditions by adjusting gene expression and metabolic pathways. Alternatively, bacteria can spontaneously accumulate beneficial mutations or shape their genomes in response to stress. Recently, it has been observed that a *B. subtilis* mutant lacking the catabolically active glutamate dehydrogenase (GDH), RocG, mutates the cryptic *gudB*^{CR} gene at a high frequency. The suppressor mutants express the active GDH GudB, which can fully replace the function of RocG. Interestingly, the cryptic *gudB*^{CR} allele is stably inherited as long as the bacteria synthesize the functional GDH RocG. Competition experiments revealed that the presence of the cryptic *gudB*^{CR} allele provides the bacteria with a selective growth advantage when glutamate is scarce. Moreover, the lack of exogenous glutamate is the driving force for the selection of mutants that have inactivated the active *gudB* gene. In contrast, two functional GDHs are beneficial for the cells when glutamate was available. Thus, the amount of GDH activity strongly affects fitness of the bacteria depending on the availability of exogenous glutamate. At a first glance the high mutation frequency of the cryptic *gudB*^{CR} allele might be attributed to stress-induced adaptive mutagenesis. However, other loci on the chromosome that could be potentially mutated during growth under the selective pressure that is exerted on a GDH-deficient mutant remained unaffected. Moreover, we show that a GDH-proficient *B. subtilis* strain has a strong selective growth advantage in a glutamate-dependent manner. Thus, the emergence and rapid clonal expansion of the active *gudB* allele can be in fact explained by spontaneous mutation and growth under selection without an increase of the mutation rate. Moreover, this study shows that the selective pressure that is exerted on a maladapted bacterium strongly affects the apparent mutation frequency of mutational hot spots.

Introduction

The high abundance of glutamate in many living organisms suggests that this metabolite fulfills fundamental tasks in the cell (Fisher & Magasanik, 1984; Hu et al., 1999; Bennett et al., 2009; Frimmersdorf et al., 2010). Indeed, glutamate delivers the majority of amino groups for biosynthesis of nitrogen-containing building blocks (Magasanik, 2003; Wohlheuter et al., 1973). Moreover, beside its important role in anabolism, glutamate serves as an osmoprotectant in some archaea and bacteria (Martin et al., 1999; Saum et al., 2006). The Gram-positive model organism *Bacillus subtilis*, however, needs glutamate in high amounts to synthesize proline, which serves as a compatible solute to protect cells growing under high external osmotic pressure (Brill et al., 2011).

In *B. subtilis* glutamate is exclusively synthesized by the combined action of the glutamine synthetase (GS) and the glutamate synthase (GOGAT) that are encoded by the *glnA* and *gltAB* genes, respectively (for a recent review Gunka & Commichau, 2012). The glutamate dehydrogenase (GDH) RocG, which is encoded by the *rocG* gene, strictly degrades glutamate *in vivo* (Fig. 3.1A; Belitsky & Sonenshein, 1998). The inability of RocG to synthesize glutamate in the background of a *B. subtilis* cell is caused by the very low affinity of the enzyme for ammonium (Commichau et al., 2008; Gunka et al., 2010).

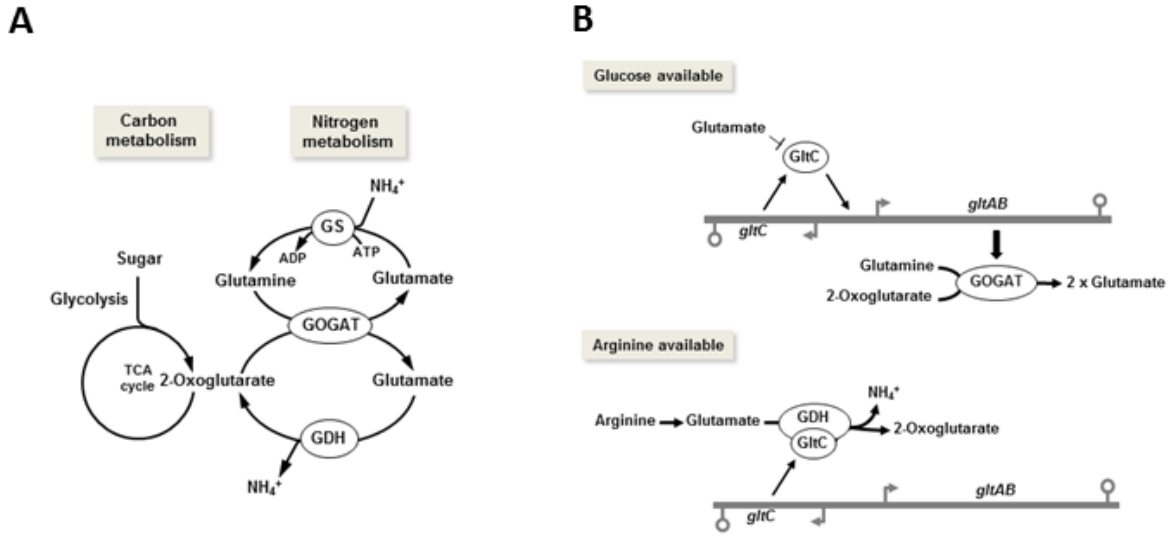

Fig. 3.1 Glutamate biosynthesis and degradation in *B. subtilis*.
(A) The link between carbon and nitrogen metabolism. GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase. **(B)** In the presence of glucose GltC activates the *gltAB* operon and the synthesized GOGAT converts 2-oxoglutarate and glutamine to glutamate. In the presence of arginine the GDH RocG is synthesized and the catabolically active enzyme binds to GltC and inhibits its DNA-binding activity.

As glutamate synthesis and degradation link carbon to nitrogen metabolism, this important metabolic intersection has to be tightly controlled. Indeed, in *B. subtilis* and in many other organisms, glutamate biosynthesis and degradation are subject to dual control by signals derived from carbon and nitrogen (Commichau et al., 2006; Leigh & Dodsworth, 2007; Sonenshein, 2007). During growth of

B. subtilis cells in the presence of their preferred carbon source glucose and ammonium as the single source of nitrogen, the transcription factor GltC activates the expression of the *gltAB* genes and the encoded GOGAT synthesizes glutamate (Fig. 3.1B; Bohannon & Sonenshein, 1989; Wacker et al., 2003). At the same time, transcription of the *rocG* gene encoding the catabolically active GDH, RocG is strongly inhibited by the pleiotropic transcription factor CcpA (Belitsky et al., 2004). This carbon source-dependent transcriptional activation and inhibition of the *gltAB* and *rocG* genes, respectively, allows the bacteria to produce glutamate, which is needed in high amounts to achieve high growth rates when external glutamate is scarce. If glutamate is provided to the cell, the slight inhibition of the transcription activator GltC results in a twofold reduced expression of the *gltAB* genes, and exogenously provided together with endogenously formed glutamate is incorporated into biomass (Commichau et al., 2007b; Picossi et al., 2007). In the presence of arginine or related amino acids such as ornithine, which can be degraded to glutamate, the GDH-encoding *rocG* gene is strongly induced (Belitsky & Sonenshein, 1998; Belitsky et al., 2004). This has two implications for the cells. First, the bifunctional GDH RocG directly binds to and prevents GltC from transcription activation of the *gltAB* genes, encoding the glutamate-synthesizing GOGAT (Fig. 3.1B; Commichau et al., 2007a; Commichau & Stülke, 2008). Second, the catabolically active GDH enables the bacteria to utilize glutamate as an additional carbon source that is fed into the tricarboxylic acid cycle (see Fig. 3.1A). This elegant regulatory mechanism allows the bacteria to accurately adjust glutamate metabolism depending on the available carbon and nitrogen sources.

The genome of the *B. subtilis* laboratory strain 168, which is used worldwide in basic research and industry, contains two GDH-encoding genes, *rocG* and *gudB*^{CR} (Kunst et al., 1997). However, only the *rocG* gene encodes a functional GDH, whereas the *gudB*^{CR} gene is cryptic and encodes the enzymatically inactive GDH, GudB^{CR} (formerly designated as GudB, Belitsky & Sonenshein, 1998; Zeigler et al., 2008). The GDH GudB^{CR} is enzymatically inactive and extremely unstable because it contains a duplication of three amino acids in its active center (Belitsky & Sonenshein, 1998; Gerth et al., 2008; Gunka et al., 2012). The duplication of these amino acids in GudB^{CR} is due to a perfect 9 bp-long direct repeat (DR) that is present in the cryptic *gudB*^{CR} gene. In contrast to the laboratory strain 168, the genomes of closely related "wild" wild-type *B. subtilis* strains such as ATCC 6051 and NCIB 3610 encode two functional GDHs, RocG and GudB (Zeigler et al., 2008). It is tempting to speculate that the *gudB*$^{+}$ gene became cryptic during domestication of a *B. subtilis* "wild" wild-type strain (Burkholder & Giles, 1947; Zeigler et al., 2008).

We are interested in the control of glutamate homeostasis in *B. subtilis*. As described above, due to its outstanding role in cellular metabolism, the intracellular pool of glutamate has to be tightly adjusted for cellular vitality. Indeed, disruption of the arginine-degradative pathway by inactivation of the *rocG* gene causes a severe growth defect of the bacteria on rich medium (Belitsky & Sonenshein,

1998; Gunka et al., 2012). Although the reason for the growth defect remains to be elucidated, glutamate homeostasis is obviously completely out of balance because the lack of GDH activity causes a block in glutamate catabolism and results in the overexpression of the *gltAB* genes, encoding the glutamate-synthesizing GOGAT (Belitsky & Sonenshein, 2004; Commichau et al., 2007b). Interestingly, the growth defect of a *rocG* mutant is suppressed by the emergence of mutants that have activated the cryptic *gudB*^{CR} gene by the precise deletion of one part of the perfect DR repeat that is present in the gene (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The decryptification of the *gudB*^{CR} gene occurs at a very high frequency of 10^{-4} and the resulting *gudB* suppressor mutants synthesize the enzymatically and regulatory active GDH GudB, which may control as RocG the activity of the transcription factor GltC (see above; Commichau et al., 2007a; Gunka et al., 2012).

In this work we addressed the question of how GDH activity affects fitness of the bacteria. Moreover, we show that the availability of glutamate is the driving force for the selection of mutants expressing the active *gudB*$^{+}$ and inactive *gudB*^{CR} alleles, respectively. Suppressor mutants that have decryptified the *gudB*^{CR} gene and synthesize the enzymatically active GDH, GudB have an extremely strong growth advantage over cells lacking a functional GDH. Thus, the rapid emergence and clonal expansion of the active *gudB*$^{+}$ allele in a population of cells can be explained rather by spontaneous mutation than by adaptive mutagenesis.

Materials and Methods

Construction of plasmids and bacterial strains

The plasmids (Tab. S 13.3) of this study were constructed using oligonucleotides that are listed in Tab. S 13.4. Plasmid DNA was extracted using the Nucleospin Extraction Kit (Machery and Nagel, Germany). Commercially available restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. PCR products and DNA fragments isolated from agarose gels were purified using the PCR purification Kit (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method (SeqLab, Göttingen, Germany). The plasmid pAC5 was used to express *gudB* alleles from the *amyE* locus in *B. subtilis* (Stülke et al., 1997). Plasmids pCFPbglS and pYFPbglS served as templates for PCR to amplify the fluorophore-encoding *cfp* and *yfp* genes, respectively (Bisicchia et al., 2010). The plasmid pGP1870 was used for the construction of a *gudB-gfp* fusion (Rothe et al., 2013) (Tab. S 13.3).

The *B. subtilis* strains used in this study are derivatives of strain 168 *trp*$^{-}$. All strains were constructed by transformation according to the two-step protocol (Kunst & Rapoport, 1995) using chromosomal or plasmid DNA (Tab. S 13.1 and Tab. S 13.3). Transformants were selected on SP plates supplemented with the appropriate antibiotics. Chromosomal DNA was isolated as described previously (Kunst & Rapoport, 1995).

Correct integration of DNA constructs into the *amyE* locus of the *B. subtilis* chromosome was verified by monitoring amylase activity. The activity of this enzyme was detected after growth on plates containing nutrient broth (7.5 g/l), 17 g Bacto agar/l (Difco) and 5 g hydrolyzed starch/l (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

Growth conditions

E. coli or *B. subtilis* were grown in LB and SP medium or in C minimal medium supplemented with carbon sources, nitrogen sources and auxotrophic requirements (at 50 mg/l) as indicated (Sambrook et al., 1989; Kunst & Rapoport, 1995; Commichau et al., 2007a). CSE medium is C minimal medium supplemented with 0.6% (w/v) succinate and 0.8% (w/v) glutamate together with ammonium as basic sources of carbon and nitrogen, respectively (Wacker et al., 2003). C-Glc medium is C minimal medium supplemented with 0.5% (w/v) glucose. LB and SP plates were prepared by the addition of 17 g Bacto agar/l (Difco) to LB and SP (8 g nutrient broth/l, 1 mM $MgSO_4$, 13 mM KCl, supplemented after sterilization with 2.5 µM ammonium ferric citrate, 500 µM $CaCl_2$, and 10 µM $MnCl_2$), respectively. When required, media were supplemented with antibiotics at the following concentrations: kanamycin (10 µg/ml), chloramphenicol (5 µg/ml) and spectinomycin (150 µg/ml).

Competition experiments

For the competition experiment, the bacteria were grown over night in LB medium at 28°C, diluted to an OD_{600} of 0.05 in either C-Glc or CE-Glc minimal medium, and mixed 1:1 with the competitor strain in 20 ml of media in a 100 ml flask. The cultures were incubated at 37°C with agitation. The cells obtained by sampling at defined time points were diluted in a 0.9% saline solution up to 10^{-3} and 100 µl of the dilutions were plated on SP medium agar plates. The plates were incubated over night at 37°C and the surviving cells were visualized by stereo fluorescence microscopy. Each competition experiment was repeated at least four times. Transcription of the fluorophore genes is driven by the constitutively active *gudB* promoter (Gunka et al., 2012) (Tab. S 13.3).

Isolation of *gudB*$^{-}$ mutants

Strain GP801 (*ΔrocG gudB*$^{+}$) synthesizing a single active GDH, GudB was first grown over night in LB medium at 30°C. Next day this culture was used to inoculate CSE-Glc minimal medium supplemented with succinate/glucose and ammonium/glutamate as carbon and nitrogen sources, respectively, to an approximate OD_{600} of 0.1. After propagation of strain GP801 for 16 h at 37°C the cells were diluted a second time to an OD_{600} of 0.05 in CS-Glc medium containing 10-fold less glucose, and ammonium as the single nitrogen source. After growth for 8 h a sample was taken and the cells were propagated on C-Glc medium supplemented with 0.5% glucose. The remaining cells were again diluted in CS-Glc medium containing 0.05% glucose and ammonium as carbon and nitrogen sources,

respectively, and further incubated for up to 48 h. Samples taken after 41 h and 48 h of incubation were treated as the first sample.

Western blotting

For Western blot analyses proteins present in 15 µg cell free crude extract were separated by 12% SDS PAGE and transferred onto polyvinylidene difluoride membranes (BioRad) by electroblotting. RocG and GFP polyclonal antibodies were diluted 1:15000 and 1:10000, respectively and served as primary antibodies (Commichau et al., 2007a). MBL, Medical & Biological Laboratories). The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (Commichau et al., 2007a).

Analysis of direct repeat integrity

Deletions of single repeat units of 9 bp-long perfect and imperfect direct repeats that are present on the *B. subtilis* chromosome (Fig. S 13.3 and Tab. S 13.5) were detected by colony PCR. Briefly, we designed oligonucleotides that hybridize 20 – 120 bp upstream and 20 – 120 bp downstream of the tandem repeats (Tab. S 13.4). The oligonucleotides were used to generate 80 – 140 b long DNA fragments by colony PCR. The deletion of a single repeat unit in a gene containing a 9 bp-long tandem repeat would give rise to a 9 bp smaller PCR product as illustrated for the *gudB* locus (Fig. S 13.2). To monitor the integrity of direct repeats, we grew the bacteria overnight in LB medium. Next day, an aliquot of the preculture was collected for colony PCR and the remaining cells were used to inoculate SP liquid medium to an OD_{600} of about 0.1. After 42 h of incubation, we collected another aliquot for colony PCR. Template DNA for PCR was generated by heating 100 µl of a *B. subtilis* cell suspension (usually about 1.5 X 10^8 CFU/ml) for 10 min at 98°C. The cell titre was determined by counting the colony forming units obtained from serial dilutions that were propagated on SP medium agar plates. 2.5 µl of the heated cells served as template DNA in a 50 µl PCR. PCR products were separated by 15% polyacrylamide (PAA) gel electrophoresis using TAE running buffer and DNA molecules were visualized by ethidium bromide staining. Template DNAs that were isolated from cell populations harboring either the inactive *gudB*^{CR} or the active *gudB*$^{+}$ allele, or both alleles gave rise to 111 bp and 102 bp DNA species (Fig. S 13.2).

Fluorescence microscopy

For fluorescence microscopy, cells were grown in LB medium to optical densities as indicated, harvested, and resuspended in phosphate-buffered saline (pH 7.5; 50 mM). Fluorescence images were obtained with an Axioskop 40 FL fluorescence microscope, equipped with digital camera AxioCam MRm and AxioVision Rel (version 4.8) software for image processing (Carl Zeiss, Göttingen, Germany)

and Neofluar series objective at ×100 primary magnification. The applied filter set was eGFP HC-Filterset (band-pass [BP] 472/30, FT 495, and long-pass [LP] 520/35; AHF Analysentechnik, Tübingen, Germany) for GFP detection. All images were taken at the same exposure times. The overlays of fluorescent and phase-contrast images were prepared for presentation with Adobe Photoshop Elements, version 8.0 (Adobe Systems, San Jose, CA). Pictures of developing *B. subtilis* colonies or aged colonies were taken with a stereo fluorescence microscope Lumar.V12 (Zeiss, Jana) equipped with the ZEN lite 2011 (blue edition) software. The applied filter sets were Lumar 46, 47 and 38 for YFP, CFP and GFP detection, respectively (Zeiss, Jena). Images were taken for up to 120 h at room temperature.

Growth of microcolonies on agarose slides

To prepare single cells of *B. subtilis* for outgrowth into microcolonies, LB precultures were grown over night at 30°C. Next day, the precultures were used to inoculate 10 ml SP liquid medium at an OD_{600} of about 0.05. At mid-exponential growth phase the cultures were diluted to an OD_{600} of 0.035 using SP liquid medium that has been 30-fold diluted with C minimal medium and the cells were spotted onto microscope slides for fluorescence microscopy (Commichau et al., 2007a; de Jong et al., 2011).

Monitoring the emergence of the *gudB* allele by a blue-white screening system

The emergence of the *gudB* allele encoding the enzymatically and regulatory active GDH GudB can be monitored indirectly using a translational *gltA-lacZ* fusion (Gunka et al., 2010). In cells lacking a functional GDH, the transcription factor GltC constitutively activates the transcription of the *gltA-lacZ* fusion. By contrast, in cells expressing either *rocG* or *gudB* GltC is unable to activate the *gltA* promoter because both active GDHs, either RocG or GudB, can bind to and inactivate GltC (Commichau et al., 2007a). Thus, colonies synthesizing the inactive GudB^{CR} enzyme or the active GDH GudB can be distinguished on SP agar plates supplemented with X-Gal to monitor the *gltA-lacZ* fusion. The amount of *gudB*^{CR} and *gudB* clones in a growing culture was determined by plating a countable number of cells on SP-X-Gal plates.

Results

GDH activity determines fitness of *B. subtilis* depending on the availability of glutamate

The laboratory *B. subtilis* strain 168 synthesizes only the enzymatically active GDH, RocG. The second GDH, GudB, which is encoded by the cryptic *gudB*^{CR} gene, is enzymatically inactive. So far it has remained unclear why the inactive *gudB*^{CR} gene is stably inherited in strain 168 in the lab over many passages. However, bacteria, which are equipped with reduced or elevated GDH activity might have a selective growth advantage when exogenous glutamate is scarce and present in excess, respectively.

To address this question we performed an intraspecies competition experiment with strains BP40 (*rocG*$^{+}$ *gudB*^{CR}) and BP52 (*rocG*$^{+}$ *gudB*$^{+}$) (Fig. 3.2A). Strain BP40 synthesizes only the active GDH RocG, while BP52 produces two active GDHs, RocG and GudB (Belitsky & Sonenshein, 1998; Gunka et al., 2012). To identify the survivors during and after co-cultivation of BP40 and BP52 by counting yellow and blue colonies, the strains were labelled with the fluorophore-encoding genes *yfp* and *cfp*, respectively (Fig. 3.2A and Fig. 3.2B).

Populations of the two strains that were mixed in a 1:1 ratio were grown for a maximum of 24 h either in C-Glc minimal medium containing glucose and ammonium as source of carbon and nitrogen, respectively, or in CE-Glc medium containing glutamate as the additional nitrogen source. The competition experiment revealed that strain BP40 (*rocG*$^{+}$ *gudB*^{CR} *yfp*), which is isogenic to the laboratory strain 168, outcompeted strain BP52 (*rocG*$^{+}$ *gudB*$^{+}$ *cfp*) expressing two functional GDHs, in the absence of exogenous glutamate (Fig. 3.2C). Thus, a reduced amount of glutamate-degrading enzyme activity provides the bacteria with a selective growth advantage when the supply with external glutamate is low. Indeed, a *B. subtilis* strain expressing only *rocG* grew faster with a generation time of 58 min than a strain synthesizing two active GDHs (generation time of 83 min) in the absence of glutamate. It is safe to assume that high GDH activity is a drain for the intracellularly formed glutamate that could otherwise be used for anabolic purposes (see Fig. 3.1A). By contrast, when external glutamate was available, strain BP52 (*rocG*$^{+}$ *gudB*$^{+}$ *cfp*) equipped with high amount of GDH activity outcompeted strain BP40 (*rocG*$^{+}$ *gudB*^{CR} *yfp*), which expressed a single GDH-encoding gene. Under these growth conditions, high-level of GDH activity is obviously advantageous for the cell because two catabolically active GDHs, RocG and GudB, degrade glutamate faster than a single enzyme and the liberated 2-oxoglutarate may serve together with glucose as an extra source of energy. The fact that a *B. subtilis* strain synthesizing two active GDHs grew slower with exogenous glutamate (generation time of 60 min) than a strain synthesizing a single GDH (generation time of 53 min) is in line with this idea. Very similar observations were made when the experiments were repeated with reciprocally labelled strains BP41 (*rocG*$^{+}$ *gudB*^{CR} *cfp*) and BP156 (*rocG*$^{+}$ *gudB*$^{+}$ *yfp*). Thus, neither the *cfp* gene nor the yfp gene influenced the outcome of the competition experiment.

Moreover, we excluded that either of the two fluorophores CFP and YFP affected growth of the parent strain 168 (*rocG*$^{+}$ *gudB*^{CR}) or that of derivatives of strain GP804 (*rocG*$^{+}$ *gudB*$^{+}$), which has been used for the competition experiment (Fig. S 13.1). Thus, our results indicate that the amount of GDH activity strongly determines the fitness of the bacteria depending on the supply with external nitrogen. Moreover, the adaptation of *B. subtilis* for fast growth in glucose-ammonium minimal medium during its domestication seems to be indeed the reason for the inactivation of the *gudB*$^{+}$ allele (see below Burkholder & Giles, 1947; Zeigler et al., 2008). However, the stable inheritance of the cryptic *gudB*^{CR}

allele in the laboratory strain 168 suggests that synthesizing at least one active GDH (RocG) is sufficient for optimal growth of the bacteria on complex medium.

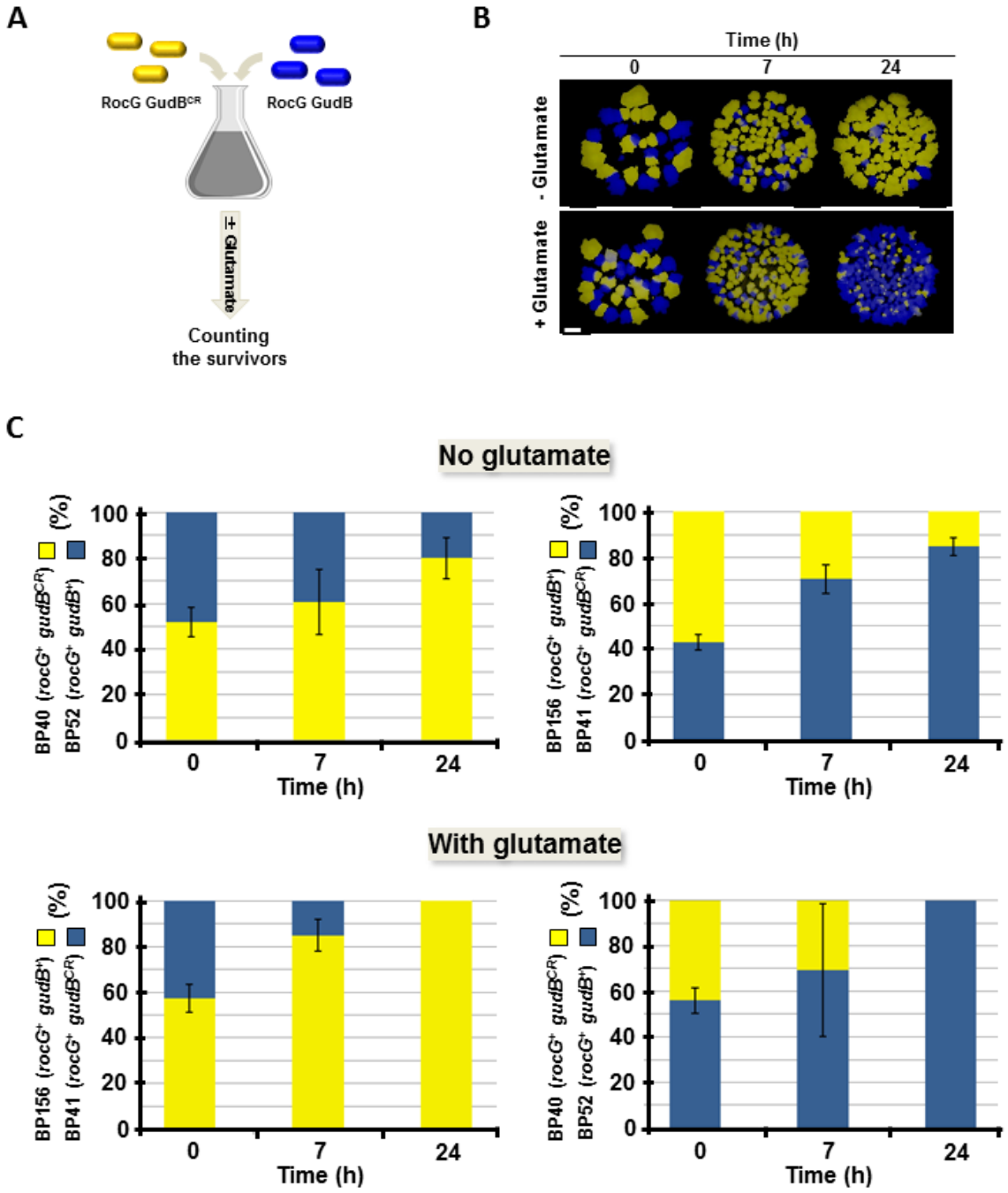

Fig. 3.2 Intraspecies competition experiment to identify the selective advantage for keeping the *gudB*^{CR} allele in the laboratory strain 168.
(A) Mixed populations of strains BP40 (*rocG*⁺ *gudB*^{CR} *amyE::yfp*) and BP52 (*rocG*⁺ *gudB*⁺ *amyE::cfp*) or BP41 (*rocG*⁺ *gudB*^{CR} *amyE::cfp*) and BP156 (*rocG*⁺ *gudB*⁺ *amyE::yfp*) were grown for up to 24 h in C minimal medium supplemented with glucose and ammonium, and in minimal medium supplemented with glucose, ammonium and glutamate. **(B)** Prior to co-cultivation (0 h), and after 7 h and 24 h of growth dilutions of cells were plated on complex medium. The surviving cells that emerged after 12 h of incubation were identified by fluorescence microscopy and counted. Exposure time, 0.6 s; Scale bar, 1 mm. **(C)** Outcome of the competition experiment. The bars represent standard deviations for at least four independently repeated experiments.

Lack of exogenous glutamate is the driving force for the selection of mutants that have inactivated the *gudB* gene

As described above, "wild" wild-type isolates of *B. subtilis* express the two functional GDH-encoding genes *rocG* and *gudB*, while the *gudB* allele is cryptic in the laboratory strain 168 (Belitsky & Sonenshein, 1998; Zeigler et al., 2008). It has been suggested that the *gudB* gene became cryptic during adaptation of *B. subtilis* for efficient growth with a poor nitrogen source such as ammonium (see above; Burkholder & Giles, 1947). Indeed, we have previously shown that only mutants of the laboratory strain 168, devoid of any glutamate-degrading GDH activity can grow in CS medium containing succinate and ammonium as poor sources of carbon and nitrogen, respectively, even though this strain possesses the genetic equipment for glutamate biosynthesis under these conditions (Commichau et al., 2007b). Here we wanted to address the question whether poor carbon and nitrogen supply results in the selection of mutants, which have specifically inactivated the *gudB* gene encoding the active GDH, GudB (Fig. 3.3A). For this purpose, we cultivated strain GP801 (*ΔrocG gudB*$^{+}$) synthesizing only the active GDH GudB in C minimal medium supplemented with succinate and low amounts of glucose (0.05%), and the poor nitrogen source ammonium. During growth for a maximum of 48 h we took samples at three different time points as indicated in Fig. 3.3B. The five samples that contained potential *gudB*$^{-}$ mutants, lacking GDH activity were propagated on CS agar plates. The strains GP801 (*ΔrocG gudB*$^{+}$) and GP754 (*ΔrocG gudB*^{CR}) served as negative control and positive controls, respectively. The CS plates were incubated for 48 h until single colonies appeared. As the cells in the five potential *gudB*$^{-}$ mutants grew as fast as the positive control it can be excluded that the inactivation of *gudB*$^{+}$ occurred on the CS plates (data not shown).

Next we isolated single colonies of the five potential *gudB*$^{-}$ isolates that were grown on the CS agar plates and evaluated growth of the isolates at conditions that require either the presence or the absence of the functional GDH, GudB (Fig. 3.3B and Fig. 3.3C). Strain GP754 (*ΔrocG gudB*^{CR}) and the parent strain GP801 (*ΔrocG gudB*$^{+}$) served as controls. The *gudB*$^{-}$-1 isolate, which was isolated early during cultivation, showed the phenotype of the GDH-proficient parent strain GP801 because it grew only poorly with glucose and ammonium as carbon and nitrogen sources, respectively. On the other hand, this isolate grew as well as the parent strain GP801 with glutamate as the single carbon and nitrogen source. It has been previously reported that *B. subtilis* is only capable of utilizing glutamate when *gudB* is encoding the enzymatically active GDH GudB (Belitsky & Sonenshein, 1998). Thus, isolate *gudB*$^{-}$-1 must still express the active *gudB*$^{+}$ gene. Indeed, sequencing and Western blot analyses revealed that the *gudB*$^{+}$ gene was intact and GudB was synthesized (Fig. 3.3D and Tab. 3.1). It is very likely to assume that either the inactivated *gudB*$^{-}$-1 allele was mutated back to *gudB*$^{+}$, encoding an active GDH or that the *gudB*$^{-}$-1 got lost during passaging of the isolates on rich medium. The *gudB*$^{-}$-2 isolate, which was isolated at the same time as the isolate *gudB*$^{-}$-1, as well as the isolates *gudB*$^{-}$-3, -4,

and -5 from later time points grew like the GDH-deficient control strain GP754 (*ΔrocG gudB*^{CR}) with glucose and ammonium as carbon and nitrogen sources, respectively (Fig. 3.3C). Moreover, the fact that the isolates *gudB*$^{-}$-2, -3, -4, and -5 were not able to utilize glutamate as the single carbon source indicated that the *gudB*$^{+}$ gene was inactivated.

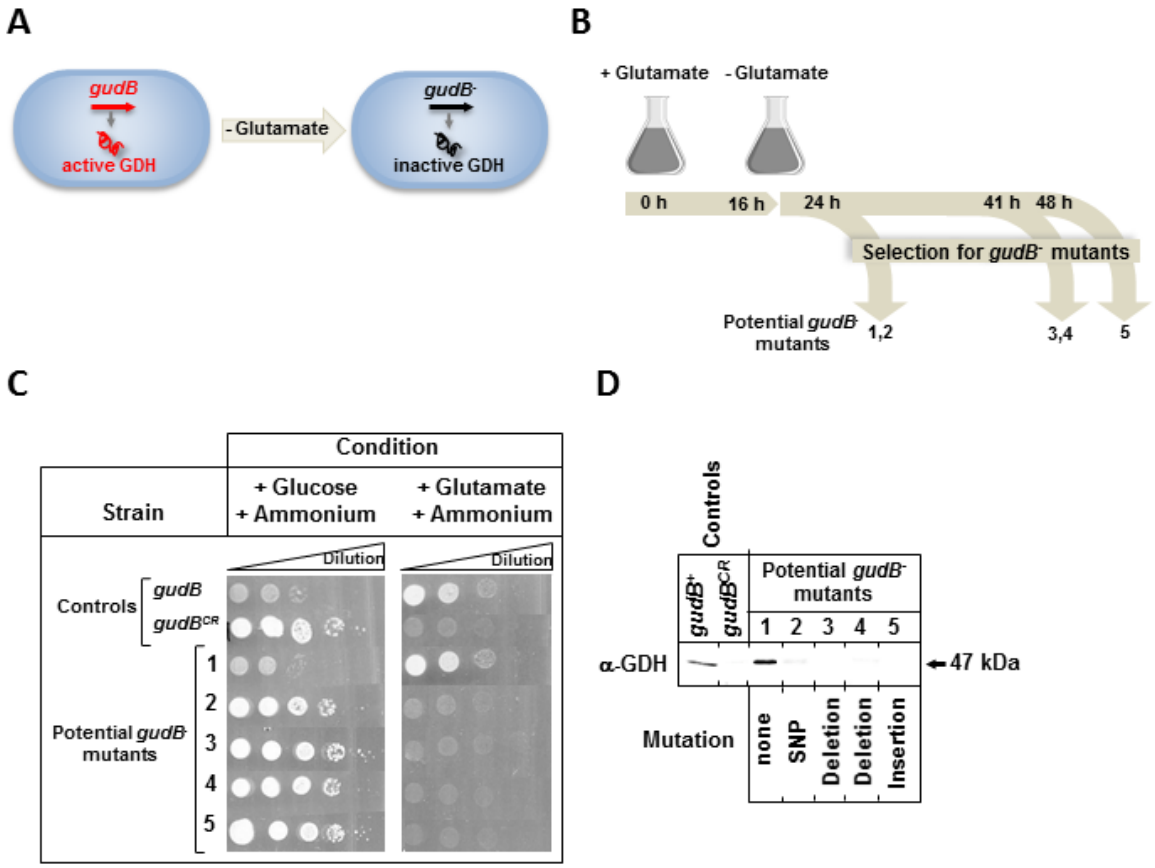

Fig. 3.3 Isolation of *B. subtilis* mutants that have inactivated the gudB gene.
(A) Lack of exogenous glutamate is the driving force allowing the selection of mutants with inactivated *gudB* alleles. **(B)** Prior to growth in the absence of glutamate, the *B. subtilis rocG*$^{-}$ mutant strain GP801 (*ΔrocG gudB*$^{+}$) expressing only the active GDH, GudB was grown in C minimal medium supplemented with glucose and glutamate as carbon and nitrogen sources (plus glutamate), respectively. During growth in minimal medium lacking glutamate (no glutamate) samples were taken at indicated time points. **(C)** 5 µl were plated from serial dilutions (from non-diluted till 10^{-6}) of cell suspensions of the *gudB*^{CR} and *gudB*$^{+}$ control strains GP754 (*ΔrocG gudB*^{CR}) and GP801 (*ΔrocG gudB*$^{+}$), respectively, and the isolated *gudB*$^{-}$ mutants for phenotypic analyses. The dilutions were spotted on minimal medium agar plates supplemented either with glucose and ammonium or with glutamate and ammonium. The plates were incubated for 48 h at 37°C. **(D)** Western blot analysis to monitor synthesis of the GDH, GudB in the *gudB*$^{-}$ isolates using GDH-specific antibodies. Results of the sequence analysis of the *gudB*$^{-}$ alleles are summarized below (see Tab. 3.1).

Indeed, all four mutants had acquired different mutations, such as point mutations, deletions and insertions (see Tab. 3.1). As revealed by Western blot analysis, these mutations resulted in the absence of a functional GDH in the four *gudB*$^{-}$ mutants (Fig. 3.3D). Thus, cultivation of *B. subtilis* in the absence of exogenous glutamate results in the emergence of mutants that have inactivated the single GDH-encoding gene to prevent degradation of glutamate.

Tab. 3.1 Mutations in the *gudB* gene of the *gudB*$^{-}$ isolates and biochemical consequences for the GudB mutant proteins.

Strain	Genotype	Time point of isolation (h)	Mutation	AA exchange	Western blotting signal
GP754	*gudB*^{CR}	-	-	-	no
GP801	*gudB*$^{+}$	-	Δ280-288	Δ94*VKA*96	yes
BP42	*gudB*$^{-}$*-1*	24	Δ280-288	Δ94*VKA*96	yes
BP44	*gudB*$^{-}$*-2*	24	Δ280-288, T896G	Δ94*VKA*96; *L*299*R*	no
BP48	*gudB*$^{-}$*-3*	41	Δ280-288, Δ766	Δ94*VKA*96; 256-277, 22 different amino acids; Δ278-419	no
BP46	*gudB*$^{-}$*-4*	41	Δ280-288, Δ673-738	Δ94*VKA*96, Δ225*VVQGFGNAG SYLAKFMHDAGAK*246	no
BP47	*gudB*$^{-}$*-5*	48	Δ280-288, insertion of C1222 and T1223	Δ94*VKA*96; 406-437, 32 different amino acids	no

In the active *gudB*$^{+}$ allele the bases from 280 till 288 are deleted.

Exogenous nitrogen strongly affects emergence and clonal expansion of the active *gudB*$^{+}$ allele in a population of cells, which originates from a *ΔrocG gudB*^{CR} strain

The presence of the *gudB*$^{+}$ allele in a population of *ΔrocG* cells, encoding the enzymatically and regulatory active GDH GudB can be monitored indirectly using a translational *gltA-lacZ* fusion (Gunka et al., 2010). In cells lacking a functional GDH, the transcription factor GltC constitutively activates the transcription of the *gltA-lacZ* fusion. By contrast, during growth in SP rich medium GltC is unable to activate the *gltA* promoter in cells expressing either *rocG*$^{+}$ or *gudB*$^{+}$ because both active GDHs, either RocG or GudB can bind to and inactivate GltC (Commichau et al., 2007a). Thus, colonies synthesizing the inactive GudB^{CR} enzyme or the active GDH GudB can be distinguished on SP agar plates supplemented with X-Gal to monitor the activity of a *gltA* promoter-*lacZ* fusion.

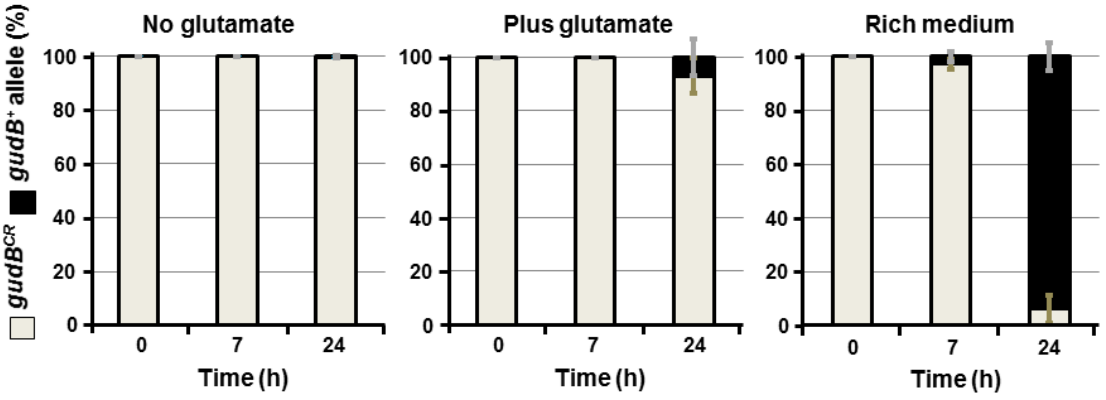

Fig. 3.4 Effect of glutamate supply on the clonal expansion of *gudB* mutants.
The *B. subtilis ΔrocG* mutant strain GP754 (*ΔrocG gudB*$^{-}$) was grown in C minimal medium supplemented with glucose and ammonium (no glutamate), glucose and ammonium/glutamate (plus glutamate), and in SP (rich) medium. The bars represent standard deviations for four independently repeated experiments (see Tab. S 13.6). The amount of *gudB*^{CR} and *gudB*$^{+}$ mutants at the indicated time points are shown by light brown and black bars, respectively.

Using this approach we studied the emergence and clonal expansion of the *gudB*$^{+}$ allele in cells that were grown in complex and minimal medium with different quantities of nitrogen (Fig. 3.4). For this purpose strain GP754 (*ΔrocG gudB*^{CR} *amyE::*(*gltA-lacZ*)) was grown over night in C-Glc medium at

37°C and used to inoculate either 10 ml SP rich medium or CE-Glc and C-Glc medium to an approximate OD_{600} of 0.1. C-Glc is minimal medium that contained glucose and ammonium as carbon and nitrogen sources, respectively. CE-Glc medium contained in addition to ammonium, glutamate as a nitrogen source. All cultures were grown for up to 24 h. Samples were taken and diluted to an appropriate cell titre, allowing to count single colonies (between 48 and 219 colonies per plate) on SP medium agar plates that were supplemented with X-Gal. Blue and white colonies that appeared after incubation of the plates overnight express the cryptic *gudB*^{CR} and the active *gudB*$^{+}$ allele, respectively (see Tab. S 13.6 for raw data of the experiment).

We observed that prior to growth under selective pressure (time point 0 h) all cells plated from each culture expressed the cryptic *gudB*^{CR} allele (Fig. 3.4). This was also true for all the cells that were cultivated for up to 24 h in C-Glc medium (no exogenous glutamate). Obviously, cells that express the cryptic *gudB*^{CR} allele have a selective growth advantage over cells that express the active *gudB*$^{+}$ gene when no exogenous glutamate is provided to the cells. In the culture containing CE-Glc medium the active *gudB*$^{+}$ allele did not appear after 7 h of growth but about 4% of the cells in this culture expressed the active *gudB*$^{+}$ allele after 24 h of growth (Fig. 3.4). Moreover, already 2% of the cells that were grown for 7 h in rich medium expressed the active *gudB*$^{+}$ allele and almost the complete cell population synthesized the active GDH, GudB after 24 h of cultivation. Thus, the suppressor mutants that have acquired the active *gudB*$^{+}$ gene by spontaneous mutation obviously had a strong selective growth advantage with excess glutamate that is present in CE-Glc and in rich medium, and the bacteria expressing this allele rapidly outcompeted those cells that had retained the *gudB*^{CR} allele (Fig. 3.4). The rapid propagation of *gudB*$^{+}$ mutants in the cell population is obviously driven by their capability of utilizing glutamate in addition to glucose as a carbon source. In contrast, the cells that express the cryptic *gudB*^{CR} allele have a selective growth advantage over cells that expressed the mutated *gudB*$^{+}$ gene in the absence of glutamate (Fig. 3.4). This suggests that the selective pressure acting on the *ΔrocG gudB*^{CR} mutant strain GP754 lacking GDH activity is rather low when the supply with external nitrogen is low. Moreover, the few *gudB*$^{+}$ alleles that might have emerged by spontaneous mutation of the *gudB*^{CR} allele in the population of cells obviously did not provide the bacteria with a selective advantage when exogenous glutamate was absent. Taken together, our observation suggests that external supply with glutamate strongly affects the clonal expansion of the *gudB*$^{+}$ gene in a population of cells but not its emergence.

A GFP-based system to monitor the state of the *gudB* allele

As mentioned above, the inactive GudB^{CR} protein is extremely unstable and subject to rapid proteolytic degradation (Gerth et al., 2008). In contrast to this, the enzymatically active GDH, GudB is stable (Gunka et al., 2012). These biochemical properties of the GudB variants stimulated us to develop a GFP-based system that would allow us to determine the state of the *gudB* allele in single cells and in

an aging colony of *B. subtilis* (Fig. 3.5A). For this purpose, we fused the *gfp* gene in frame either to the 5' or the 3' ends of the *gudB*^{CR} and *gudB*$^{+}$ alleles. The 3' and the 5' gene fusions were integrated into the chromosome by a Campbell-type and double homologous recombination into the *amyE* gene, respectively. Previously, we have shown that the decryptification frequency of *gudB*^{CR} was not affected when the gene was expressed from the *amyE* locus (Gunka et al., 2012).

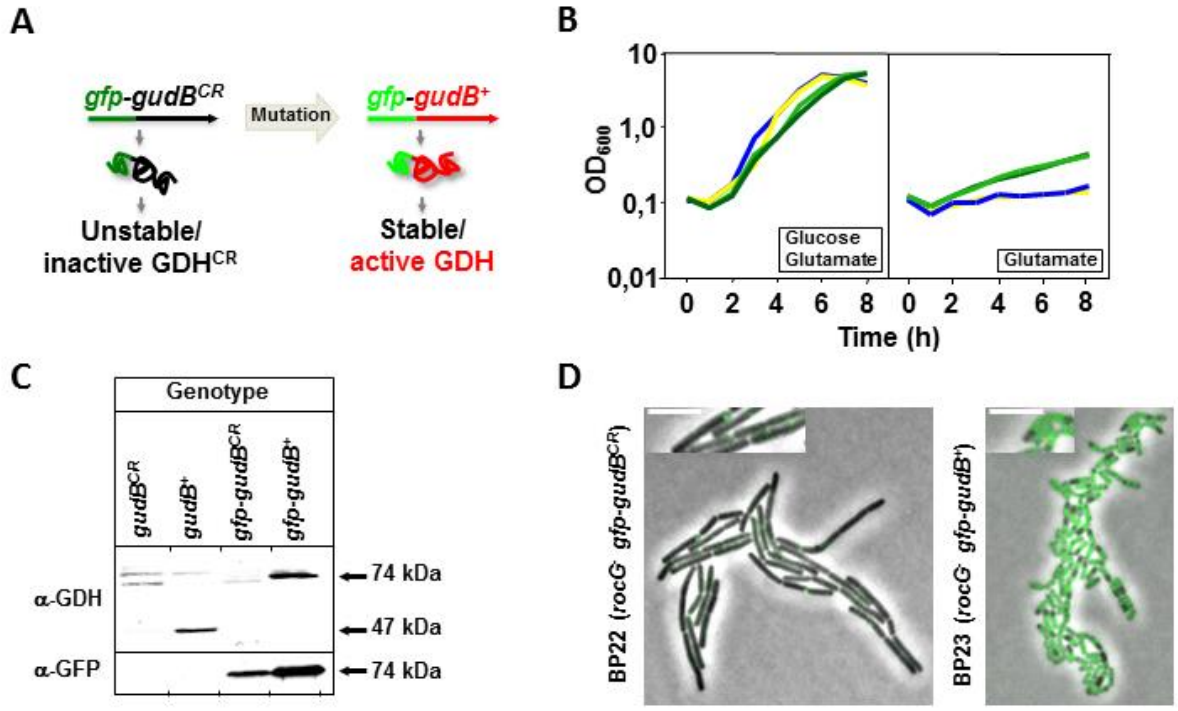

Fig. 3.5 A GFP-based system to monitor the state of the *gudB* allele in *B. subtilis*.
(A) The principle of the system is based on the stabilities of the inactive and active GudB^{CR} and GudB proteins, respectively. **(B)** Growth assay to confirm the enzymatic activity of the GFP-GudB fusion protein. C minimal medium supplemented with glucose and glutamate as the carbon and nitrogen sources, respectively, served as the positive control. Strains GP1165 (*ΔrocG gudB*⁺) and BP23 (*ΔrocG gfp-gudB*⁺) synthesizing the active GudB (dark green) and GFP-GudB (light green) alleles, respectively, were capable of catabolizing glutamate. The strains GP1163 (*ΔrocG gudB*^{CR}) and BP22 (*ΔrocG gfp-gudB*^{CR}) synthesizing the inactive *gudB*^{CR} (yellow) and *gfp-gudB*^{CR} (blue) alleles, respectively, did not grow with glutamate as the single source of carbon and nitrogen. **(C)** Western blot analysis to evaluate the stabilities of the inactive and active GudB^{CR} and GudB variants in strains GP754 (*ΔrocG gudB*^{CR}) and GP801 (*ΔrocG gudB*⁺), respectively, using polyclonal antibodies raised against GDH and GFP. The corresponding GFP-GudB^{CR} and GFP-GudB fusion proteins are synthesized in strains BP22 (*ΔrocG gfp gudB*^{CR}) and BP23 (*ΔrocG gfp-gudB*⁺), respectively. **(D)** Fluorescence of microcolonies of strains BP22 and BP23 that express the cryptic *gfp-gudB*^{CR} and the active *gfp-gudB*⁺ fusion genes, respectively. Exposure time, 1 s; scale bar, 5 µm.

The *in vivo* activities of the different fusion proteins were examined by growth experiments (Fig. 3.5B). The fusion of *gfp*, either to the 3' or the 5' end of the *gudB* variants did not affect growth of *B. subtilis* on complex and CE-Glc minimal medium containing glucose and glutamate as carbon and nitrogen sources, respectively. However, when glutamate was provided to the cells as the single carbon source, only strains GP1165 (*rocG*⁻ *gudB*⁺) and BP23 (*rocG*⁻ *gfp-gudB*⁺) synthesizing GudB and GFP-GudB were capable of utilizing glutamate (Fig. 3.5B). This observation is in perfect agreement with previous results showing that the capability of *B. subtilis* of growing with glutamate strictly depends on the presence of the active GDH, GudB (see Fig. 3.3; Belitsky & Sonenshein, 1998). Interestingly, fusion of the *gfp* gene to the 3' end of the *gudB*⁺ allele renders the encoded GudB-GFP fusion protein

inactive (Fig. S 13.4). It has been shown that the C-termini of the six identical subunits are buried within the structures of the active GDHs, RocG and GudB (Gunka et al., 2010). Therefore it is likely that the 27 kDa GFP protein disrupts the integrity of the GudB structure and the activity of the enzyme.

As the system to monitor the state of the *gudB* allele in a population of cells or in single cells of *B. subtilis* is based on protein stability, we evaluated the stabilities of the GFP-GudB^{CR} and GFP-GudB variants in strains BP22 (*rocG*$^{-}$ *gfp-gudB*^{CR}) and BP23 (*rocG*$^{-}$ *gfp-gudB*$^{+}$), respectively, by Western blotting and fluorescence microscopy (Fig. 3.5C and Fig. 3.5D). The isogenic strains GP754 (*rocG*$^{-}$ *gudB*^{CR}) and GP801 (*rocG*$^{-}$ *gudB*$^{+}$) synthesizing the native GudB^{CR} and GudB variants, respectively, served as controls for the Western blotting experiment. Like the native GudB^{CR} variant, the GFP-tagged GudB^{CR} protein was unstable and cells showed a polar fluorescence signal (Fig. 3.5C and Fig. 3.5D). By contrast, the GFP-GudB fusion and the native GudB protein were stable and active, and cells expressing the *gfp-gudB*$^{+}$ allele were strongly fluorescent. Thus, the GFP-based system is a powerful tool to assess the state of the *gudB* allele in single cells of *B. subtilis*.

Application of the GFP-based system to monitor the state of the *gudB* allele in single cells in an aging colony of *B. subtilis*

We next used the GFP-based system to visualize the emergence and clonal expansion of the *gudB*$^{+}$ allele in single cells that were derived from *B. subtilis ΔrocG* mutant strain BP22 lacking the active GDH RocG but expressing the potentially mutable cryptic *gfp-gudB*^{CR} allele (see Fig. 3.6A). For this purpose, we grew the strain overnight in C minimal medium supplemented with glucose and ammonium. As shown above, under these growth conditions the proliferation of suppressor mutants expressing the decryptified *gudB*$^{+}$ gene is very low (see Fig. 3.4). This culture was then used to inoculate SP rich medium to an approximate OD_{600} of 0.05. The isogenic strain BP23 (*ΔrocG gfp-gudB*$^{+}$) expressing the active GFP-GudB fusion protein served as a positive control. The emergence of *gudB*$^{+}$ suppressor mutants was then followed over time for up to 32 h by fluorescence microscopy (Fig. 3.6A). As a complementary approach to visualize the state of the *gudB* in cells obtained from the same culture, we performed colony PCR using a primer pair that hybridizes close to the DR repeat in the *gudB* gene. The 111 bp and 102 bp long DNA species derived from a *gudB*^{CR} and a *gudB*$^{+}$ mutant, respectively, can easily be distinguished by polyacrylamide (PAA) gel electrophoresis (see Fig. S 13.2 and Materials and Methods). As shown in Fig. 3.6A, single cells that were obtained from the control strain BP23 (*ΔrocG gfp-gudB*$^{+}$) showed a strong fluorescence signal that was evenly distributed over the cells. This confirmed our previous observation that the active *gfp-gudB*$^{+}$ fusion gene that is missing one part of the 9 bp long DR is stably expressed in *B. subtilis* (see Fig. 3.5C). By contrast, all cells of strain BP22 (*ΔrocG gfp-gudB*^{CR}) expressing the cryptic *gfp-gudB*^{CR} allele showed a polar fluorescence signal within the first 8 h of cultivation. Thus, the majority of cells expressed the *gudB*^{CR} gene during this time.

Indeed, the colony PCR on cells that were obtained from the same culture revealed that the complete population expressed the cryptic *gfp-gudB*^{CR} allele because only the 111 bp but not the 102 bp long DNA species was visible by PAA gel electrophoresis (Fig. 3.6A). After 16 h of incubation the cells expressing the mutable *gfp-gudB*^{CR} allele still showed a polar fluorescence signal. However, the colony PCR revealed that few cells expressed the decryptified *gudB* gene as the 102 bp long DNA species weakly appeared. This indicates that the mutagenesis of the cryptic *gudB*^{CR} allele must have taken place during the first 16 h of cultivation and those cells expressing the active *gudB*$^{+}$ allele started to outcompete cells that did not harbor the active *gudB*$^{+}$ allele. By looking at samples after 24 h and 32 h of cultivation, we observed that all cells were strongly fluorescent. Thus, most of the cells that have emerged from strain BP22 (*ΔrocG gfp-gudB*^{CR}) seemed to express the active *gfp-gudB*$^{+}$ allele. Indeed, the colony PCR revealed that the cryptic *gudB*^{CR} allele completely disappeared from the cell population (Fig. 3.6A).

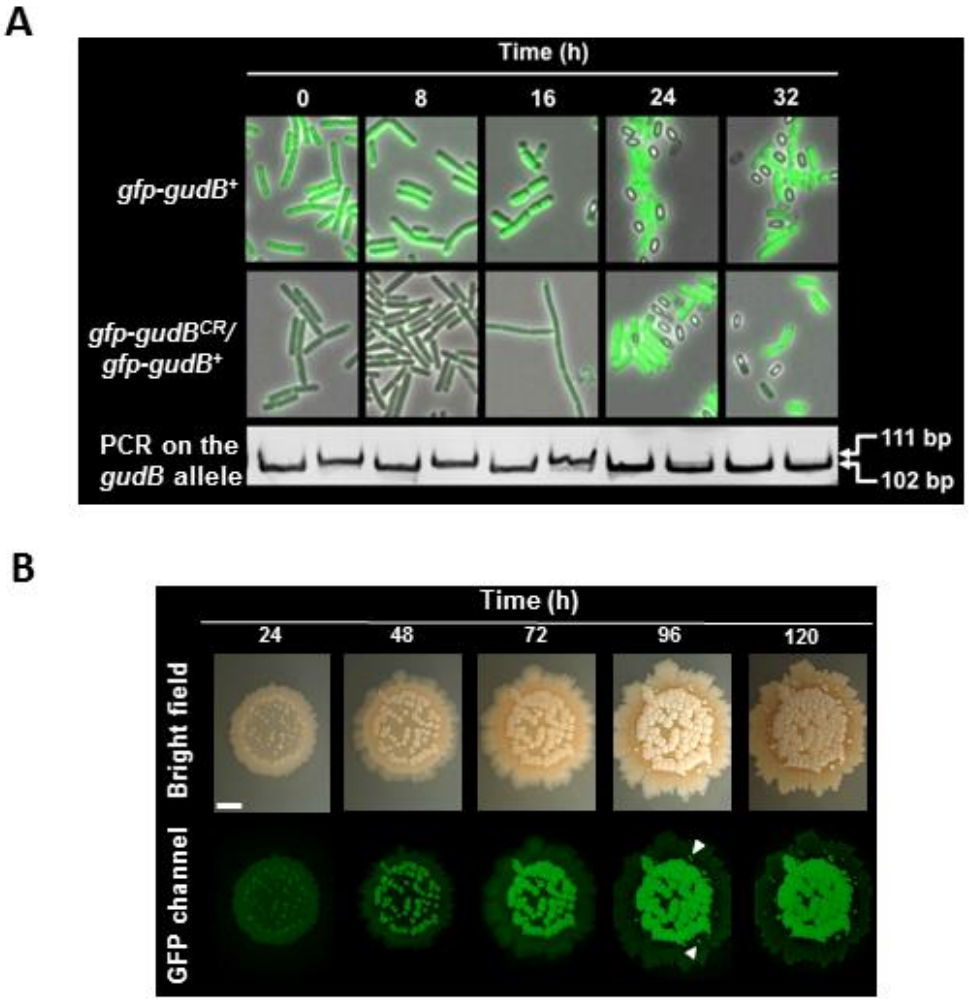

Fig. 3.6 Direct visualization of the emergence and clonal expansion of the decryptified *gudB*$^{+}$ allele in *B. subtilis*.
(A) Decryptification of the *gudB*^{CR} allele and clonal expansion of the *gudB*$^{+}$ mutants over time in complex medium. Exposure time, 0.6 s; scale bar, 5 µm. The state of the DR in the *gudB*^{CR} allele in strain BP22 (*ΔrocG gfp-gudB*^{CR}) was analyzed by colony PCR and the DNA species were visualized by PAA gel electrophoresis (Fig. S 13.2). Strain BP23 (*ΔrocG gfp-gudB*$^{+}$) expressing the active *gudB*$^{+}$ allele served as the positive control. **(B)** Emergence of *gfp-gudB*$^{+}$ suppressor mutants in a developing colony of strain BP22 (*ΔrocG gfp-gudB*^{CR}) on rich medium. The white arrows indicate late suppressors. Exposure time, 2 s; scale bar, 2 mm.

Previously, it has been claimed that each suppressor mutant that has emerged from a *rocG*$^{-}$ mutant lacking GDH activity will have mutated the cryptic *gudB*^{CR} (Belitsky & Sonenshein, 1998; Gunka et al., 2012). However, there are several examples showing that mutations that accumulate to suppress a strong phenotype might occur at different loci on the chromosome (Flórez et al., 2011). To verify that each suppressor of a *B. subtilis ΔrocG* mutant lacking the GDH RocG mutates the *gudB*^{CR} gene and expresses the functional GDH GudB, we spotted cells of strain BP22 (*ΔrocG gfp gudB*^{CR}) on rich medium and followed the emergence and clonal expansion of suppressor mutants in an aging colony. As shown in Fig. 3.6B, each emerging papilla, even papilla that appeared later, became fluorescent.

Obviously, the selective pressure that is exerted on the *rocG* mutant lacking a GDH results in the rapid proliferation of suppressors that have specifically mutated the *gudB*^{CR} gene as no phenotypically different suppressor emerged. Taken together, our fluorescence microscopic method, which is based on the stability of GFP-labelled GudB variants, is a powerful tool to monitor the emergence and clonal expansion of the *gudB*$^{+}$ allele in single cells and in an aging colony of *B. subtilis*.

Other perfect DRs present on the *B. subtilis* chromosome remain unaffected in suppressor mutants that have mutated the cryptic *gudB*^{CR} allele

Until now one factor, the Mfd protein that links transcription with DNA repair, was shown to be involved in the decryptification of the *gudB*^{CR} gene (Gunka et al., 2012). However, the observation that the cryptic *gudB*^{CR} gene is rapidly mutated with a high frequency of 10^{-4} in a *B. subtilis rocG*$^{-}$ mutant raised the question whether other loci on the *B. subtilis* chromosome that could be potentially mutated are modified by the same factor(s) during growth under strong selective pressure that is exerted on the *rocG*$^{-}$ mutant. To address this question, we studied the integrity of other DRs by colony PCR in a cell population that was derived from the *rocG*$^{-}$ mutant strain GP747 (*rocG*$^{-}$ *gudB*^{CR}). For this purpose the cells were cultivated in SP rich medium, conditions that result in the accumulation and clonal expansion of cells expressing the active *gudB*$^{+}$ allele in the population (see Materials and Methods). In addition to the DR of the *gudB*^{CR} gene, 15 other DRs were identified using the tandem repeat database for bacteria (http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php). The genes containing the DRs virtually cover the whole chromosome and the DRs are either in frame or not in frame but with the same total length 18 bp and a unit size of 9 bp as the DR in the *gudB*^{CR} gene (Fig. S 13.3). Among the 15 genes are at least five that show expression profiles similar to that of the *gudB*^{CR} gene (Tab. S 13.5; Blom et al., 2011; Buescher et al., 2012). As shown in Fig. 3.7, during growth under selection only those cells accumulated that harbor the active *gudB*$^{+}$ gene as none of the 15 other DRs was mutated in the population of *gudB*$^{+}$ cells. Thus, once the cryptic *gudB*^{CR} allele is mutated the strong selective pressure that is exerted on the *rocG*$^{-}$ mutant leads to the rapid proliferation of cells synthesizing the active GDH, GudB (see Discussion). Moreover, even if the other DRs were mutated by spontaneous or

adaptive mutagenesis in some *gudB*$^{+}$ cells of the population, these suppressor mutants synthesizing a functional GDH probably outcompeted those cells with mutations in other loci containing DRs due to their strong selective growth advantage.

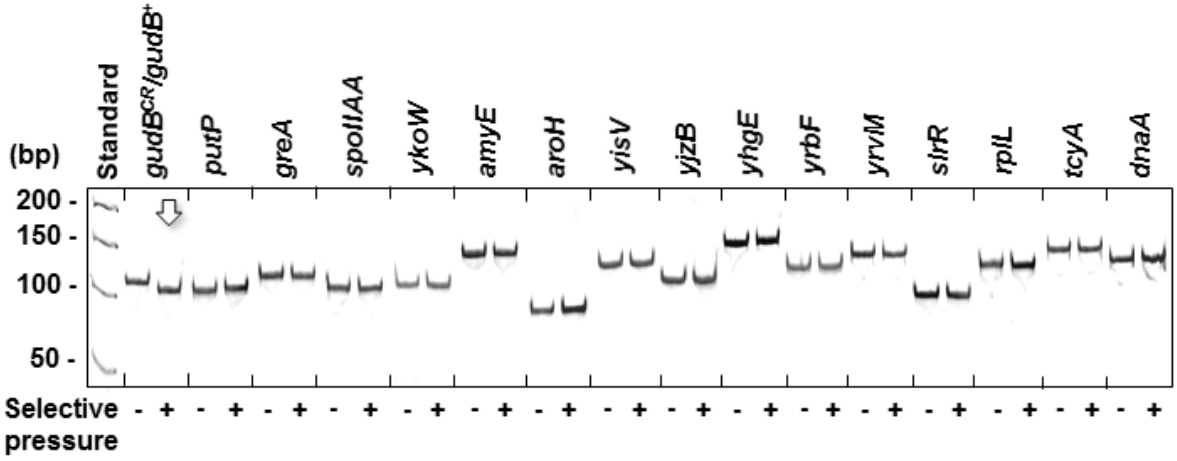

Fig. 3.7 Stabilities of DRs during growth under strong selective pressure that is exerted on a *B. subtilis rocG- gudB*^{CR} mutant. The stabilities of DRs in a population of *gudB*$^{+}$ cells that originated from the *rocG*$^{-}$ mutant GP747 were analyzed by colony PCR.

The accumulation of a mutated *gudB*$^{+}$ allele depends on the selective growth advantage it provides to a cell

We have observed that in the background of a *rocG*$^{-}$ mutant strain, the native *gudB*^{CR} gene, the *gfp-gudB*^{CR} fusion gene but not the *gudB*^{CR}*-gfp* fusion gives rise to an active GDH and provides the cell with a selective advantage (Fig. S 13.4). To address the question whether the strong selective pressure that is exerted on the *rocG*$^{-}$ mutant results in the adaptive mutagenesis or even directed mutagenesis of the DR present in the *gudB*^{CR} gene, we thought to analyze the stability of the DRs in the native *gudB*^{CR} allele and the *gudB*^{CR}*-gfp* allele in cells, originating from a *rocG*$^{-}$ mutant (Fig. 3.8A).

For this purpose we introduced the *gudB*^{CR}*-gfp* allele together with the *gudB* promoter into the *amyE* locus of the *rocG*$^{-}$ mutant strain GP747 giving strain BP31. Previously, we have shown that the decryptification of the *gudB*^{CR} gene does not depend on its position on the chromosome. The modification of the 9 bp-long DRs in the two alleles during growth under strong selective pressure (growth in SP rich medium) that is exerted on the *rocG*$^{-}$ mutant was analyzed by colony PCR (see Materials and Methods). The DNA molecules derived from the *gudB*^{CR} and *gudB*^{CR}*-gfp* alleles were made distinguishable by introducing a *Sac*I restriction site into the *gudB*^{CR}*-gfp* allele without changing the amino acid sequence in the translated protein (Fig. 3.8B and Fig. S 13.5). Digestion of PCR products derived from this allele would shorten them by 42 bp (see schemes in Fig. 3.8B and Fig. 3.8C). Strain BP31 (*ΔrocG gudB*^{CR} *amyE::*(*gudB*$^{CR}{}_{SacI}$*-gfp*)) was grown in rich medium to select for cells expressing the *gudB*$^{+}$ alleles and PCR products were generated from cell samples that were collected prior to growth and after growth under selective pressure.

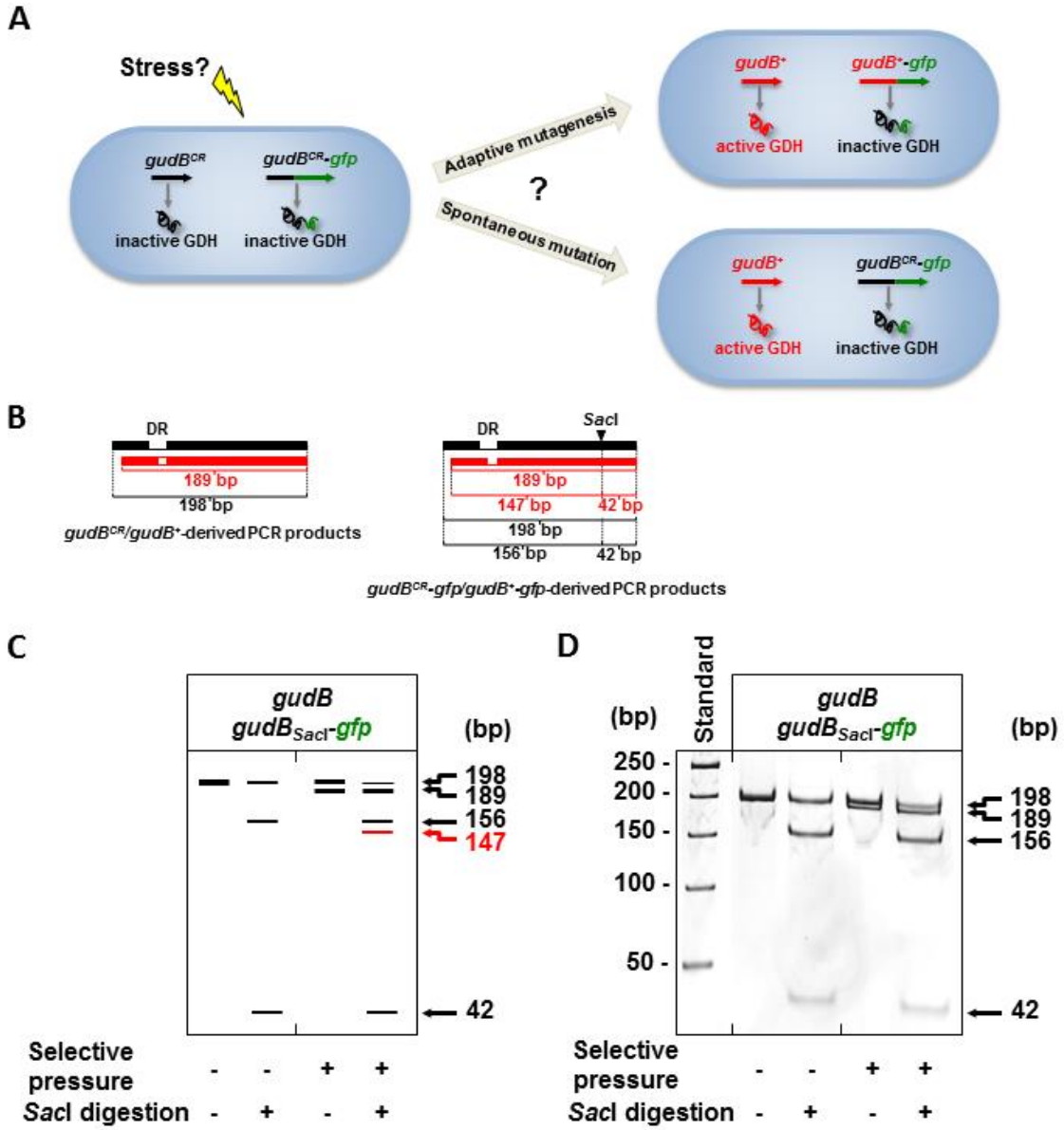

Fig. 3.8 Stabilities of DRs present in the native gudB^{CR} and in the *gudB^{CR}SacI-gfp* alleles.
(A) In addition to the native *gudB^{CR}* allele, a second *gudB^{CR}-gfp* fusion that could be potentially mutated during growth of a *B. subtilis ΔrocG* mutant under selective pressure was introduced into the *amyE* locus on the chromosome. **(B)** DNA species comprising the 9 bp DR were amplified by colony PCR using *gudB*-specific oligonucleotides (see Materials and Methods). To distinguish the DNA species derived from the two *gudB^{CR}* alleles, a *Sac*I site was introduced into the *gudB^{CR}-gfp* allele by exchanging G at position 402 by C. **(C)** Schematic illustration of the fragment pattern of DNA species obtained from cells collected prior to selective growth and after selection. The same samples were treated with *Sac*I. The emergence of a 147 bp DNA species shown by red letters would indicate the decryptification of the *gudB^{CR}-gfp* allele. **(D)** Fragment pattern of DNA species obtained from real samples.

The DNA molecules were analyzed by PAA gel electrophoresis. A single DNA fragment was observed in the PCR sample generated from cells prior to growth under selective pressure (Fig. 3.8D). Two bands occurred upon *Sac*I digestion, indicating that DNA molecules derived from the native *gudB^{CR}* allele and the *gudB^{CR}$_{SacI}$-gfp* allele were present in the mixture. In the PCR mixture that was generated from cells collected after growth under selective pressure, we identified two DNA species that might have been derived from cryptic and decryptified *gudB^{+}* and *gudB^{+}$_{SacI}$-gfp* alleles (Fig. 3.8D). After SacI digestion only four instead of five DNA species occurred. If both alleles were mutated, we would

have expected to observe 198 bp and 189 bp DNA species derived from the native allele, and 156 bp, 147 bp and 42 bp DNA species derived from the *gudB*$^{CR}_{SacI}$-*gfp* allele (Fig. 3.8D). However, the 147 bp DNA molecule that could have originated from the decryptified *gudB*$^{+}_{SacI}$-*gfp* allele was missing (Fig. 3.8C and Fig. 3.8D). We excluded that the *Sac*I recognition site interferes with the activation of the *gudB*$^{CR}_{SacI}$ allele (data not shown). Thus, only the native allele whose mutated form provides the bacteria with a strong selective growth advantage spread in the culture, while the mutated *gudB*$^{CR}_{SacI}$-*gfp* allele did not (Fig. 3.8D).

Discussion

Soil bacteria, such as *B. subtilis* live in a constantly changing environment. In principle, there are two different possibilities of how a living cell can respond and adapt to an environmental stimulus, *i.e*., a change in nutrient supply. On one hand bacteria can adjust their metabolism either by differential regulation of gene expression or by controlling the flux through central metabolic pathways (Wang et al., 2010; Buescher et al., 2012; Nicolas et al., 2012). On the other hand, mutational events may cause the accumulation of beneficial mutations and provide the bacteria with a selective growth advantage under a specific environmental condition (Cairns et al., 1988; Barrick et al., 2009; Blount et al., 2012). Although the accumulation of mutations can also be detrimental for the bacteria, it is often the last option to ensure survival or growth in a specific environment if the regulatory infrastructure of the cell is exhausted. Indeed, recently we found that in a *rocG*$^{-}$ mutant strain, lacking GDH activity, the cryptic *gudB*^{CR} gene is rapidly mutated with a high frequency of 10^{-4}, and the suppressor mutants synthesize the enzymatically and regulatory active GDH, GudB (Belitsky & Sonenshein, 1998; Gunka et al., 2012). Although mutation frequencies in the range of 10^{-4} and even higher have been described in many other bacteria it is the highest frequency that has been described so far for *B. subtilis* (Moxon et al., 2006; Gunka et al., 2012). One attractive explanation for the high mutation frequency of the *gudB*^{CR} allele might be that the lack of GDH activity due to the inactivation of the *rocG*$^{+}$ gene causes the stress-induced mutation of the *gudB*^{CR} gene. The lack of GDH activity has several implications for the bacteria. First, in the absence of a functional GDH, the transcription factor GltC is highly active and constitutively activates transcription of the GOGAT-encoding genes (Commichau et al., 2007a, b). Second, a *rocG*$^{-}$ mutant cannot fully metabolize nitrogen sources such as arginine and ornithine that end up at the level of glutamate (Belitsky & Sonenshein, 1998). Moreover, either the accumulation of glutamate or of intermediates of the arginine-degradation pathway may be toxic for the cell. This seems to be indeed the case, as we have observed that *B. subtilis* is unable to grow with arginine in the absence of a functional GDH (data not shown). Finally, a very recent study has revealed that a *B. subtilis* mutant lacking GDH activity is more sensitive to β-lactam antibiotics (Lee et al., 2012). Altogether, it seems to be an attractive idea that the pleiotropic phenotype of a *rocG* mutant might cause the stress-induced activation of the *gudB*^{CR} gene.

An observation 25 years ago suggested that bacteria respond to "stress" by directly modifying particular genes, and thereby speed up their own evolution (Cairns et al., 1988). This idea has been faced with skepticism as it implies the existence of a stress-sensing machinery, which transduces the selective pressure that is exerted on a maladapted organism to a specific locus on the chromosome (Cairns et al., 1988; Koonin & Wolf, 2009). However, the existence of a molecular machinery that can anticipate which genomic alteration would provide the cell with a selective growth advantage is hard to imagine. Indeed, the genetic system developed by Cairns that suggested genomic adaptation by directed mutagenesis can be fully explained by spontaneous mutation and growth under selection (Roth et al., 2006). However, non-spontaneous but stress-induced adaptive mutagenesis is exceedingly well-documented in both bacterial and human cells and many factors that are involved in stress-induced adaptive mutagenesis have been identified in the meantime (Foster, 2007; Rosenberg, 2011; Burch et al., 2011; Rosenberg et al., 2012). Recently, a large network comprising more than 90 genes was shown to be involved in stress-induced mutagenesis as a result of DNA double-strand breaks in *E. coli* (Al Mamun et al., 2012). This observation illustrates the complexity of how environmental or endogenous "stress" exerted on maladapted cells may stimulate factors, which in turn enable the cells to accelerate their own evolution. However, recent microarray analyses did not reveal that any DNA-modifying factors, which might be required for the decryptification of the *gudB*^{CR} gene, are induced by "stress" due to the lack of GDH activity in a *B. subtilis rocG*$^{-}$ mutant strain (Gunka et al., 2012; Lee et al., 2012; Manabe et al., 2013).

A plausible explanation for the rapid emergence and clonal expansion of the active *gudB*$^{+}$ allele in a population of cells could be that, if the allele once occurred by spontaneous mutation, those cells that harbor the *gudB*$^{+}$ allele have a strong selective growth advantage over the parent strain. Indeed, *rocG*$^{-}$ mutants expressing the *gudB*$^{+}$ allele showed a selective growth advantage in a strictly glutamate-dependent manner. The presence of exogenous glutamate strongly enhances growth of *gudB*$^{+}$ suppressors (see Fig. 3.4). Obviously, cells synthesizing the enzymatically active GDH GudB can use glutamate as an additional source of energy, which drives the rapid clonal expansion of the suppressors. By contrast, a *B. subtilis* mutant devoid of GDH activity has a growth advantage when exogenous glutamate is not available. Under these conditions the endogenously synthesized glutamate can be used for anabolism instead of being degraded by a GDH and fed into carbon metabolism (Fig. 3.1A). These interpretations are in perfect agreement with the results of our growth experiments and the competition experiments, showing that the fitness of *B. subtilis* equipped with different levels of GDH activity is determined by the availability of glutamate (see Fig. 3.2C). Thus, the rapid emergence of the active *gudB*$^{+}$ allele can be explained by spontaneous mutation and growth under selection. However, the 9 bp-long DR that is present in the cryptic *gudB*^{CR} gene seems to be a crucial element for the high mutation frequency of the gene. Indeed, mutations affecting the integrity

of the DR in the *gudB*^{CR} gene without changing the coding sequence resulted in a 15-fold reduced mutation frequency (Gunka et al., 2012). It is well-documented that DRs present in the genomes of both pro- and eukaryotes are hypermutable loci (Moxon et al., 2006; Bichara et al., 2006; Kovtun & McMurray, 2008). Thus, the DR in the *gudB*^{CR} allele is a mutational hot spot that is essential for the rapid decryptification of the *gudB*^{CR} allele in the background of a *rocG*$^{-}$ mutant.

There are several other prominent genetic systems that seemed to show stress-induced mutagenesis (Roth et al., 2006). One example is the Rif^{R} system describing the accumulation of rifampicin-resistant (Rif^{R}) mutants in aging, non-growing colonies of enteric bacteria (Taddei et al., 1997; Bjedov et al., 2003; Wrande et al., 2008). Rif^{R} mutants have a selective growth advantage due to the accumulation of mutations in the *rpoB* gene, encoding the β-subunit of the RNA polymerase. This phenomenon has been first explained by stress-induced mutagenesis. However, as we have observed for the *gudB* system in this study, the accumulation of the Rif^{R} mutants is due to growth under selection and not stress-induced mutagenesis (Wrande et al., 2008). There are two similarities between our system and the Rif^{R} system. First, both the Rif^{R} and *gudB*$^{+}$ mutants appear with an uneven spatial distribution in the aging colony (see Fig. 3.6). This observation suggests that the emergence of the beneficial mutations is rather spontaneous and driven by selection. In contrast, a globally enhanced mutation rate would result in the accumulation suppressor mutants that are evenly distributed over the aging colony. Second, neither the Rif^{R} nor the *gudB*$^{+}$ mutants show an increased frequency of second site mutations (Fig. 3.7 and Fig. 3.8). Taken together, our system and many other prominent genetic systems that appeared to support the idea of stress-induced adaptive mutagenesis can in fact be explained by growth under selection (Roth et al., 2006; Wrande et al., 2008). The present study also revealed that the strength of the selective pressure that is exerted on a maladapted bacterium strongly affects the apparent mutation frequency of a mutational hot spot.

Our current research focus is aimed at the understanding of the molecular mechanism of the decryptification of the *gudB*^{CR} gene and the role of the transcription-coupling repair factor Mfd, the only protein that has been identified to be involved in this process (Gunka et al., 2012). Moreover, it will be interesting to study the role of transcription in the mutation of the *gudB*^{CR} gene. Finally, using the GFP-based system, which is based on the stability of the GudB variants, we aim to identify the proteolytic machinery that is involved in the rapid degradation of the inactive GDH, GudB, which is the most-unstable protein in *B. subtilis* (Gerth et al., 2008).

Acknowledgments

We are grateful to J. Stülke and C. Wrede for helpful comments and critical reading of the manuscript. R. R. T. Eijlander and O. P. Kuipers are acknowledged for providing their microscopy facility. We thank R. Egelkamp for his help with some experiments.

4 Monitoring intraspecies competition in a bacterial cell population

The results described in this chapter were published in:

STANNEK, L., EGELKAMP, R., GUNKA, K. & COMMICHAU, F.M. (2014) Monitoring intraspecies competition in a bacterial cell population by cocultivation of fluorescently labelled strains. *Journal of Visualized Experiments*, 83, e51196.
doi: 10.3791/51196

Author´s contribution:

The study was designed and interpreted by LS, KG and FMC. Intra-species competition experiments and evaluation were done by LS. RE performed video recording together with LS and FMC. The paper was written by LS and FMC.

Abstract

Many microorganisms such as bacteria proliferate extremely fast and the populations may reach high cell densities. Small fractions of cells in a population always have accumulated mutations that are either detrimental or beneficial for the cell. If the fitness effect of a mutation provides the subpopulation with a strong selective growth advantage, the individuals of this subpopulation may rapidly outcompete and even completely eliminate their immediate fellows. Thus, small genetic changes and selection-driven accumulation of cells that have acquired beneficial mutations may lead to a complete shift of the genotype of a cell population. Here we present a procedure to monitor the rapid clonal expansion and elimination of beneficial and detrimental mutations, respectively, in a bacterial cell population over time by co-cultivation of fluorescently labelled individuals of the Gram-positive model bacterium *Bacillus subtilis*. The method is easy to perform and very illustrative to display intraspecies competition among the individuals in a bacterial cell population.

5 Inducer-free activation of gene expression by selection-driven promoter decryptification in *Bacillus subtilis*

The results described in this chapter were published in:

DORMEYER, M., EGELKAMP, R., THIELE, M.J., HAMMER, E., GUNKA, K., **STANNEK, L.**, VÖLKER, U. & COMMICHAU, F.M. (2015) A novel engineering tool in the *Bacillus subtilis* toolbox: inducer-free activation of gene expression by selection-driven promoter decryptification. *Microbiology*, 161, 354–361. doi: 10.1099/mic.0.000001

Author´s contribution:

The study was designed and interpreted by LS and FMC. MD performed most of the experiments including fluorescence microscopy, Western blotting, isolation of the PdxST enzyme complex and PdxST enzyme activity measurements. MT determined promoter activities and contributed together with RE and KG by constructing several plasmids. EH and UV identified proteins by mass spectrometry and evaluated the results. FMC wrote the paper.

Inducer-free activation of gene expression by selection-driven promotor decryptification in *Bacillus subtilis*

Abstract

Bacillus subtilis is a Gram-positive bacterium that is easy to manipulate genetically. Several methods for genome engineering have been developed that helped to extend our understanding of how the *B. subtilis* cell operates. Consequently, the bacterium has become one of the best-studied organisms. *B. subtilis* has also been engineered for industrial applications. Moreover, great progress has been achieved in promoter engineering to improve the performance of production strains. To expand the toolbox for engineering *B. subtilis*, we have constructed a system for the inducer-free activation of gene expression. The system relies on spontaneous mutational activation of a cryptic promoter and selection-driven enrichment of bacteria harboring the mutated promoter. The synthetic promoter is cryptic due to a perfect direct repeat, separating the binding motifs of the RNA polymerase housekeeping sigma factor. The promoter can be fused to genes for industrial applications and to a growth promoting gene that, upon mutational activation of the promoter, allows enrichment of the engineered bacteria due to a selective growth advantage.

Introduction

The Gram-positive soil-dwelling bacterium *Bacillus subtilis* has the remarkable ability to adapt to and to survive in a variety of ecological niches (Schilling et al., 2007; Buescher et al., 2012; Nicolas et al., 2012). Moreover, *B. subtilis* is capable of forming robust endospores allowing survival in unfavorable environments (McKenney et al., 2013). In the course of its domestication the genetic competence of *B. subtilis* was considerably enhanced, making manipulations of the genome much easier (Burkholder & Giles, 1947). Consequently, *B. subtilis* became one of the best-studied bacteria (Sonenshein et al., 2002). Because the organism is "generally regarded as safe" it is an attractive host for industrial applications (Schallmey et al., 2004). Indeed, the bacterium was engineered and used for the production of vitamins and enzymes (Perkins et al., 1999; Manabe et al., 2013; Commichau et al., 2014). *B. subtilis* has also been subjected to systematic genome reduction to identify the essential gene set that is needed to sustain life and to obtain minimal cell factories for industrial use (Juhas et al., 2014; http://mini*Bacillus*.org). Several methods have been developed for biochemical studies and for genome engineering (Herzberg et al., 2007; Kumpfmüller et al., 2013). Moreover, great progress has been achieved in promoter engineering to expand the transcriptional capacities of engineered organisms and to tightly control gene expression (Brautaset et al., 2009; Blazeck & Alper, 2013; Radeck et al., 2013; Vogl et al., 2014). Current knowledge about the control of gene expression has undoubtedly helped to enhance the production levels of many engineered bacteria.

Populations of rapidly growing bacteria such as *B. subtilis* can reach high cell densities, and small fractions of the populations always accumulate spontaneous mutations. Some mutations may become important for bacteria if they ensure survival of unpredictable environmental changes. DNA sequences such as direct repeats (DRs) are mutational hotspots that impact the potential of an organism to adapt to the environment by spontaneous mutagenesis (Zhou et al., 2014a). Several micro-organisms can exploit the instability of DRs to reversibly shut down genes, and to activate or to modulate gene expression (Belitsky & Sonenshein, 1998; Martin et al., 2005; Flores et al., 2013). To further expand the toolbox for engineering *B. subtilis*, we have designed a novel system for inducer-free activation of gene expression.

The system is based on spontaneous mutational activation of a synthetic core promoter that is cryptic due to a perfect DR, separating the binding motifs of the RNA polymerase housekeeping sigma factor σ^A (Fig. 5.1). The cryptic promoter is fused to a gene that when expressed provides the bacteria with a selective growth advantage. By adjusting the growth condition, those cells that have spontaneously "decryptified" the synthetic promoter and express the growth-promoting gene together with additional genes encoding proteins that are of interest for industrial applications can easily be enriched.

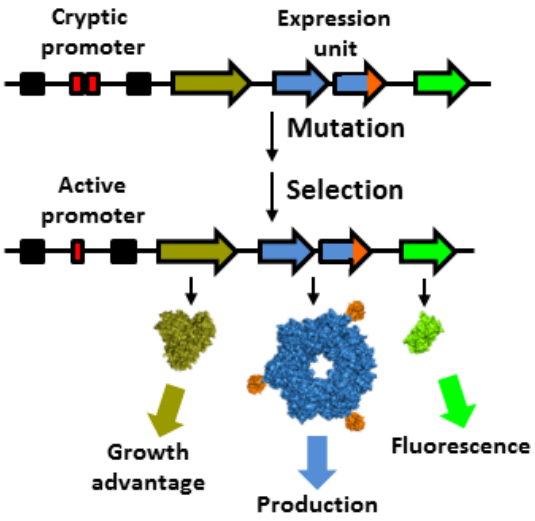

Fig. 5.1 Principle of the expression system.
A cryptic promoter is fused to an artificial expression unit, that when expressed, provides the bacteria with a selective growth advantage. Those cells that have spontaneously "decryptified" the synthetic promoter and express the growth-promoting gene together with additional genes encoding proteins that are of interest for industrial applications can be enriched during growth under selection.

Methods

Chemicals, media and DNA manipulation

Primers were purchased from Sigma-Aldrich (Germany), see Tab. S 13.4 for the sequences. Chromosomal DNA was isolated from *B. subtilis* using the DNeasy Blood & Tissue Kit (Qiagen, Germany). Plasmids were isolated using the Nucleospin Extract Kit (Macherey and Nagel, Germany). DNA fragments generated by PCR were purified using the PCR Purification Kit (Qiagen, Germany). Phusion DNA polymerase, restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (Germany) and used according to the manufacturer's instructions. Miscellaneous chemicals and media were purchased from Sigma-Aldrich (Germany), Carl Roth (Karlsruhe, Germany) and Becton Dickinson (Heidelberg, Germany). Plasmids were sequenced using Sanger sequencing services by SeqLab Sequence Laboratories (Göttingen, Germany).

Plasmid construction

To allow expression of genes in single copy from the *lacA* locus of the *B. subtilis* chromosome, we constructed the plasmid pBP106. The plasmid pBP106 (Fig. S 13.6) contains the *aphA3* kanamycin resistance gene and has a small multiple-cloning site containing six recognition sites for single-cutting restriction enzymes. The *aphA3* kanamycin resistance gene was amplified from plasmid pDG780 (Guérout-Fleury et al., 1995) using the oligonucleotides KG47 and FC200 (Tab. S 13.4 and Tab. S 13.3). The PCR product was digested with the enzymes BglII and *Sma*I, and ligated to the plasmid pGP882 (Diethmaier et al., 2011) cut with the enzymes BamHI and *Sma*I. The active P^{+} and the cryptic P^{CR} promoters were generated by hybridization of the oligonucleotide pairs ST5/ST6 and ST7/ST8,

respectively. To obtain *P⁺-lacZ* and *P^CR-lacZ* fusions, the promoters were ligated to plasmid pAC6 (Stülke et al., 1997) digested with the enzymes EcoRI and BamHI. The plasmids harboring the *P⁺-lacZ* and *P^CR-lacZ* fusions were designated as pBP300 and pBP311, respectively. For integrating the *P^CR-gudB* fusion into the *amyE* locus of the *B. subtilis* chromosome we constructed the plasmid pBP302. For this purpose the *gudB⁺* allele was amplified using the primer pair KG92/KG134 and chromosomal DNA of strain GP801. The PCR product was cut with BamHI and ligated to plasmid pBP302 digested with the same enzyme. The correct orientation of the *gudB* gene was confirmed by PCR. Plasmid pBP168 for the integration of the *P^CR-gudB* fusion into the *lacA* locus of the *B. subtilis* chromosome was constructed as follows. The *P^CR-gudB* fusion was amplified from plasmid pBP302 by PCR using the oligonucleotides LS32 and LS33. The PCR product was digested with the enzymes PstI and *Mfe*I and ligated to the plasmid pBP106 cut with the enzymes PstI and EcoRI (Fig. S 13.6 and Fig. S 13.7). For integrating the *P^CR-gudB-gfp* fusion into the *lacA* locus we constructed the plasmid pBP169. The *gfp* gene was amplified by PCR using oligonucleotides LS34 and LS35 and the plasmid pSG1154 (Lewis & Marston, 1999) as a template. The PCR product was digested with XbaI and EcoRI and ligated to plasmid pBP168 cut with the same enzymes. Plasmid pBP172 was constructed to integrate the entire artificial *P^CR-gudB-pdxST-Strep-gfp* fusion into the *lacA* locus. The *pdxST-Strep* genes were amplified by PCR using the oligonucleotides LS36 and LS37 and chromosomal DNA of the *B. subtilis* wild-type strain 168. The PCR product was digested with SalI and BglII and ligated to plasmid pBP169 cut with the same enzymes giving plasmid pBP172 (see Fig. S 13.7 and Fig. S 13.8).

Strains and cultivation conditions

E. coli or *B. subtilis* (Tab. S 13.2 and Tab. S 13.1) were grown in LB and SP medium or in C minimal medium supplemented with carbon sources, nitrogen sources and auxotrophic requirements (at 50 mg/l) as indicated (Sambrook et al., 1989). C-Glc medium is C minimal medium supplemented with 0.5% (w/v) glucose (Wacker et al., 2003; Commichau et al., 2007a). LB and SP plates were prepared by the addition of 17 g Bacto agar/l (Difco) to LB and SP (8 g nutrient broth/l, 1 mM $MgSO_4$, 13 mM KCl, supplemented after sterilization with 2.5 µM ammonium ferric citrate, 500 µM $CaCl_2$, and 10 µM $MnCl_2$), respectively. When required, media were supplemented with antibiotics at the following concentrations: ampicillin, (100 µg/ml), kanamycin (10 µg/ml), chloramphenicol (5 µg/ml) and spectinomycin (150 µg/ml). The chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was dissolved in N,N-dimethylformamide to a final concentration of 80 mg/ml and agar plates were supplemented with 80 µg/ml X-Gal. Deletion of the *gudB* gene including its native promoter was achieved by transformation with a deletion cassette constructed by Long-Flanking Homology (LFH) PCR using oligonucleotides (Tab. S 13.4) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes (Guérout-Fleury et al., 1995), as described previously (Wach, 1996). *B. subtilis* was transformed with plasmid and chromosomal DNA according to

a previously described two-step protocol (Kunst & Rapoport, 1995).

β-Galactosidase assay

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in SP medium. Cells were harvested at OD_{600} of 0.6–0.8. β-Galactosidase specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (Kunst & Rapoport, 1995). One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol per min at 28°C.

Isolation of suppressor mutants

To isolate suppressor mutants with the "decryptified" P^{CR} promoters that express the *gudB* gene from the artificial operons, strains BP205, BP206 and BP216 lacking native *rocG* and *gudB* genes (see Tab. S 13.1) were propagated on SP complex medium. The plates were incubated for 48 h at room temperature. Emerging papilla were isolated and characterized by fluorescence microscopy and Western blotting. Moreover, the excision of one part of the DR of the cryptic P^{CR} promoters was confirmed by DNA sequencing (Fig. S 13.9).

Fluorescence microscopy

For fluorescence microscopy, cells were grown in LB medium to optical densities as indicated, harvested, and resuspended in PBS (pH 7.5; 50 mM). Fluorescence images were obtained with an Axioskop 40 FL fluorescence microscope, equipped with an AxioCam MRm and AxioVision Rel (version 4.8) software for image processing (Carl Zeiss, Göttingen, Germany) and Neofluar series objective at 6100 primary magnification. The applied filter set was eGFP HC-Filterset (band-pass [BP] 472/30, FT 495, and long-pass [LP] 520/35; AHF Analysentechnik, Tübingen, Germany) for eGFP detection. All images were taken at the same exposure times. The overlays of fluorescent and phase-contrast images were prepared for presentation with Adobe Photoshop Elements, version 8.0 (Adobe Systems, San Jose, CA, USA). Pictures of *B. subtilis* colonies were taken with a stereo fluorescence microscope Lumar.V12 (Zeiss, Jena, Germany) equipped with the ZEN lite 2011 (blue edition) software. The applied filter set was Lumar 38 for eGFP detection (Zeiss, Jena, Germany). Images were taken at room temperature.

Western blotting

For Western blot analysis, proteins present in 20 µg cell free crude extract were separated by 12.5% SDS PAGE and transferred onto polyvinylidene difluoride membrane (BioRad, Germany) by electroblotting. Anti-*Strep*-tag II, anti-GFP and anti-GudB polyclonal antibodies were diluted 1:1000, 1:10.000 and 1:15.000, respectively, and served as primary antibodies to detect PdxT-Strep, GFP and

GudB proteins (Commichau et al., 2007b) (PromoKine, Germany; MBL, Japan). The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega, Germany) and the CDP-Star detection system (Roche Diagnostics, Switzerland), as described previously (Commichau et al., 2007b).

Isolation of the PdxST enzyme complex from *B. subtilis*

To isolate the *Strep*-tagged PdxST enzyme complex from *B. subtilis*, a first preculture was grown in LB containing 0.5% glucose for 10 h at 37°C. This preculture was used to inoculate 1 l of LB medium containing 0.5% glucose to an OD_{600} of 0.1. This culture was grown at 37°C until the optical density had reached 2.0. The cells were harvested and disrupted using a French press (20.000 p.s.i., 138.000 kPa; Spectronic Instruments, UK) and the enzyme complex was purified using a Streptactin column (IBA, Göttingen, Germany). Aliquots of the different fractions were subjected to SDS PAGE and analysed by silver staining. The purified proteins were identified by mass spectrometry.

Protein identification by mass spectrometry

Silver nitrate-stained gel slices were destained by incubation in 30 mM $K_3[Fe(CN)_6]$, and 100 mM $Na_2S_2O_3$ until colourless and washed three times in water before processing gel slices as previously described (Thiele et al., 2007). Briefly, gel pieces were washed twice with 200 µl of 20 mM NH_4HCO_3, and 50% (v/v) acetonitrile (ACN) for 30 min at 37 °C and dried by adding 200 µl of ACN two times for 15 min. Trypsin solution (10 ng/µl trypsin in 20 mM ammonium bicarbonate) was added until gel pieces stopped swelling, and digestion was allowed to proceed for 16 –18 h at 37 °C. Peptides were extracted from gel pieces by incubation in an ultrasonic bath for 30 min in 40 µl of 0.1% (v/v) acetic acid followed by a second extraction with 40 µl of 50% ACN in 0.05% acetic acid. The supernatants containing peptides were collected, combined, ACN-depleted by evaporation, and transferred to microvials for mass spectrometric analysis. Peptides were separated by a non-linear water-ACN gradient in 0.1% acetic acid on an nanoAcquity UPLC reversed-phase column (BEH130, C18, 100 µm x 100 mm, Waters Corporation, Milford, MA) with a nano-UPLC system (Waters) coupled on line with a LTQ (linear trap quadrupole) Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) operated in data-dependent MS/MS mode. Proteins were identified by searching all MS/MS spectra against a *B. subtilis* protein database [(4254 entries; extracted from SubtiList using SEQUEST version 2.7 rel. 11 (Sorcerer built 4.04, Sage-N Research Inc., Milpitas, CA)] (Tab. S 13.7). Initial mass tolerance for peptide identification on MS and MS/MS peaks were 10 ppm and 1 Da, respectively. Up to two missing tryptic cleavages were allowed. Methionine oxidation (+15.99492 Da) and propionamide modification on cysteine (+71.037109 Da) were set as variable modifications. Protein identification results were evaluated by determination of probability for peptide and protein assignments provided by PeptideProphet and ProteinProphet (Institute for Systems Biology, Seattle, WA, USA) incorporated in

the Scaffold software package release 4.3.2 (Proteome Software, Portland, OR, USA). Proteins were identified by at least two peptides with minimal peptide scores of XCorr=2.2 at z=2 and XCorr=2.5 at z=3 and a peptide probability >95% reflecting protein probability of >95%. Sequence coverage of the isolated PdxS and PdxT-Strep enzymes was 82% and 87%, respectively.

Determination of PdxST enzymatic activity

The activity of the *Strep*-tagged PdxST enzyme complex purified from *B. subtilis* was analyzed as previously described (Raschle et al., 2005). Briefly, reactions were carried out in 50 mM Tris-HCl, pH 8.0, at 37 °C containing 1.44 µM of the isolated PdxST-*Strep* enzyme complex and 0.5 and 1 mM of the substrates ribose 5-phosphate and DL-glyceraldehyde 3-phosphate, respectively. Glutamine at 10 mM served as the nitrogen source. The formation of pyridoxal phosphate was monitored at a wavelength of 414 nm using the Synergy MX multi-well plate reader (BioTek, USA). Samples lacking the purified enzyme complex served as control.

Results and Discussion

Construction and evaluation of a cryptic core promoter

The conserved elements of prokaryotic promoters, i. e. the -35 and -10 regions TTGACA and TATAAT, respectively, bound by sigma factor σ^A are well-studied and can be rationally engineered (Blazeck & Alper, 2013). To obtain an inactive promoter that could be activated by spontaneous mutagenesis, we increased the spacing between the -35 and -10 regions of a synthetic promoter by duplication of a 9 bp sequence, resulting in a perfect 18 bp DR (Fig. 5.2A, see Tab. S 13.3 for the construction). The resulting promoter was designated as P^{CR}. Because the spacer length between the -35 and -10 regions of a promoter may drastically affect its performance, we expected the P^{CR} promoter to be transcriptionally inactive (cryptic). To determine its activity, we fused the P^{CR} promoter to a *lacZ* reporter gene encoding the β-galactosidase. The isogenic P^+ promoter lacking one unit of the 18 bp DR served as the control (Fig. 5.2A). Both plasmids carrying the promoter *lacZ* fusions were introduced into the *amyE* locus of the *B. subtilis* wild-type strain 168, giving strains BP472 (P^{CR}-*lacZ*) and BP429 (P^+-*lacZ*). To evaluate the activities of the promoters in the two strains, we propagated a single colony of each strain on a SP rich medium plate containing the chromogenic substrate X-Gal for the β-galactosidase. As shown in Fig. 5.2B strain BP472 harboring the P^{CR}-*lacZ* fusion formed white colonies, indicating that the cryptic promoter is inactive. By contrast, strain BP429 harboring the P^+-*lacZ* fusion formed dark blue colonies, suggesting that the P^+ promoter lacking one unit of the 18 bp DR is highly active (Fig. 5.2B).

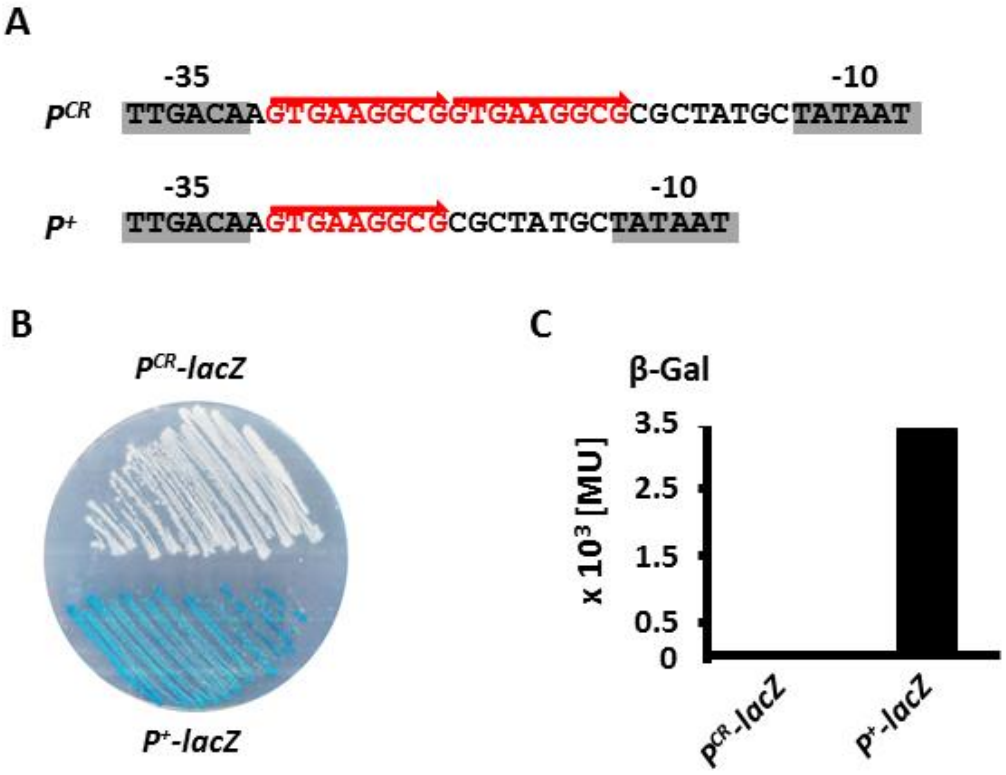

Fig. 5.2 Activities of the cryptic and the active promoters.
(A) Sequences of the cryptic and the active promoters P^{CR} and P^{+}, respectively. **(B)** *In vivo* activities of the cryptic and the active promoters P^{CR} and P^{+}, respectively. Strains BP472 (P^{CR}-*lacZ*) and BP429 (P^{+}-*lacZ*) were propagated on SP agar plates supplemented with 80 µg/ml X-Gal and the plates were incubated for about 48 h at 37°C. **(C)** β-galactosidase assay to quantify the activities of the translational promoter-lacZ fusions. Representative results from three independent experiments are shown. The maximum deviation of the series of representative data did not exceed 10%.

While the P^{CR}-*lacZ* fusion was not expressed in strain BP472 (21 U/mg of protein) the P^{+}-*lacZ* fusion was indeed highly expressed in strain BP429 because a quantitative β-galactosidase assay revealed an enzymatic activity of about 3500 U/mg of protein, which is rather high for translational *lacZ* reporter fusions (Schilling et al., 2007) (Fig. 5.2C). In conclusion, the cryptic P^{CR} promoter seems to be perfectly suited to establish an inducer-free expression system that is based on spontaneous mutational activation of the inactive promoter (excision of one 9 bp repeat unit of the DR) and the enrichment of the mutant bacteria during growth under selection.

Activation of gene expression by selection-driven promoter decryptification

A *B. subtilis* strain lacking the glutamate dehydrogenase (GDH) *rocG* gene encoding the active GDH RocG shows a severe growth defect on rich medium (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The growth defect of the *rocG* mutant strain is relieved by the rapid emergence of suppressor mutants that have activated the cryptic *gudB*^{CR} gene by the precise excision of 9 bp of an 18 bp DR. The DR is present in the ORF of the *gudB*^{CR} gene and renders the encoded GudB^{CR} protein unstable and inactive (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The “decryptified” *gudB* gene encodes the functional GDH GudB that restores glutamate homeostasis of the cell (Gunka et al., 2013). It has also

been shown that the cryptic *gudB*^{CR} gene is stably inherited because a lower GDH activity provides the bacteria with a selective growth advantage when exogenous glutamate is scarce (Gunka et al., 2013; Stannek et al., 2014). Thus, depending on the supply with glutamate (e.g., growth on rich medium or not) either the cryptic *gudB*^{CR} gene is inherited among the bacteria or the gene spontaneously "decryptifies" and the active *gudB* gene is propagated in a cell population.

The observation that the DR in the *gudB*^{CR} gene harbors a mutational hot spot provoked us to design and construct a system for inducer-free activation of gene expression in bacteria. This system is based on the instability of the DR in the *P*^{CR} promoter that, if mutated, drives the expression of the growth-promoting *gudB* gene in the background of a GDH-deficient strain. For this, we fused the cryptic *P*^{CR} promoter to the decryptified *gudB* gene and integrated the construct into the chromosome of the *B. subtilis* strain BP200 (*ΔgudB*), giving strain BP201 (see Tab. S 13.3 and Tab. S 13.1 for the construction of plasmids and strains, respectively). Next we inactivated the second GDH encoding *rocG* gene by transformation using chromosomal DNA of the *rocG*$^{-}$ strain GP747. The resulting strain BP205 only contains the functional GDH-encoding *gudB* gene fused to the mutable *P*^{CR} promoter (Fig. 5.3A). To illustrate the application potential of the expression system, we constructed two additional artificial operons and integrated them into the chromosome of a GDH-deficient strain, giving strains BP207 and BP216 (Fig. 5.3A; Tab. S 13.1). In addition to the *gudB* gene, strain BP207 harbors the *gfp* gene encoding the green fluorescent protein (GFP) to facilitate the identification of mutant bacteria that have mutated the cryptic *P*^{CR} promoter. In strain BP216 the cryptic *P*^{CR} promoter controls the expression of the *gudB* and *gfp* genes, and the native *pdxST* operon encoding the tagged PdxST vitamin B6 synthase complex for purification *via* the Strep-tag protein purification system (Fig. 5.3A). The full sequence of the artificial *P*^{CR} *gudB pdxST-tag gfp* operon is given in Fig. S 13.8. As described above, the expression of the *gudB* gene in the background of a GDH-deficient strain provides the bacteria with a strong selective growth advantage on rich medium. Therefore, it can be expected that once the *P*^{CR} promoter was spontaneously "decryptified" the selective pressure acting on the bacteria results in the rapid clonal expansion of mutants harboring the active *P*$^{+}$ promoter. Indeed, visual inspection of aging colonies of strains BP205 (*P*^{CR} *gudB*), BP207 (*P*^{CR} *gudB gfp*) and BP216 (*P*^{CR} *gudB pdxST-tag gfp*) revealed that the emerged mutants (white papillae) obviously do not have a growth defect like GDH-deficient bacteria on rich medium (Fig. 5.3B). As expected, no GFP signal was observed when the colony of strain BP205 was analyzed by fluorescence microscopy because this strain does not contain a *gfp* gene. However, the mutants derived from strains BP207 and BP216 showed a GFP signal, suggesting that the *P*^{CR} promoter in the suppressors derived from these strains was indeed decryptified (Fig. 5.3B). Next we isolated suppressor mutants from strains BP205, BP207 and BP216, and designated them as BP206 (*P*$^{+}$ *gudB*), BP208 (*P*$^{+}$ *gudB gfp*) and BP219 (*P*$^{+}$ *gudB pdxST-tag gfp*), respectively. As expected, fluorescence microscopic inspection of the suppressor mutants at the level of single cells revealed that

all suppressors derived from strains BP207 and BP216 showed a homogeneous GFP signal (Fig. 5.3C). Decryptification of the P^{CR} promoters of all isolated suppressor mutants including that of strain BP206 (derived from strain BP205 lacking the *gfp* gene) was verified by DNA sequencing analysis (Fig. S 13.9). In all analyzed suppressor mutants one 9 bp unit of the DR in the P^{CR} promoter was precisely excised.

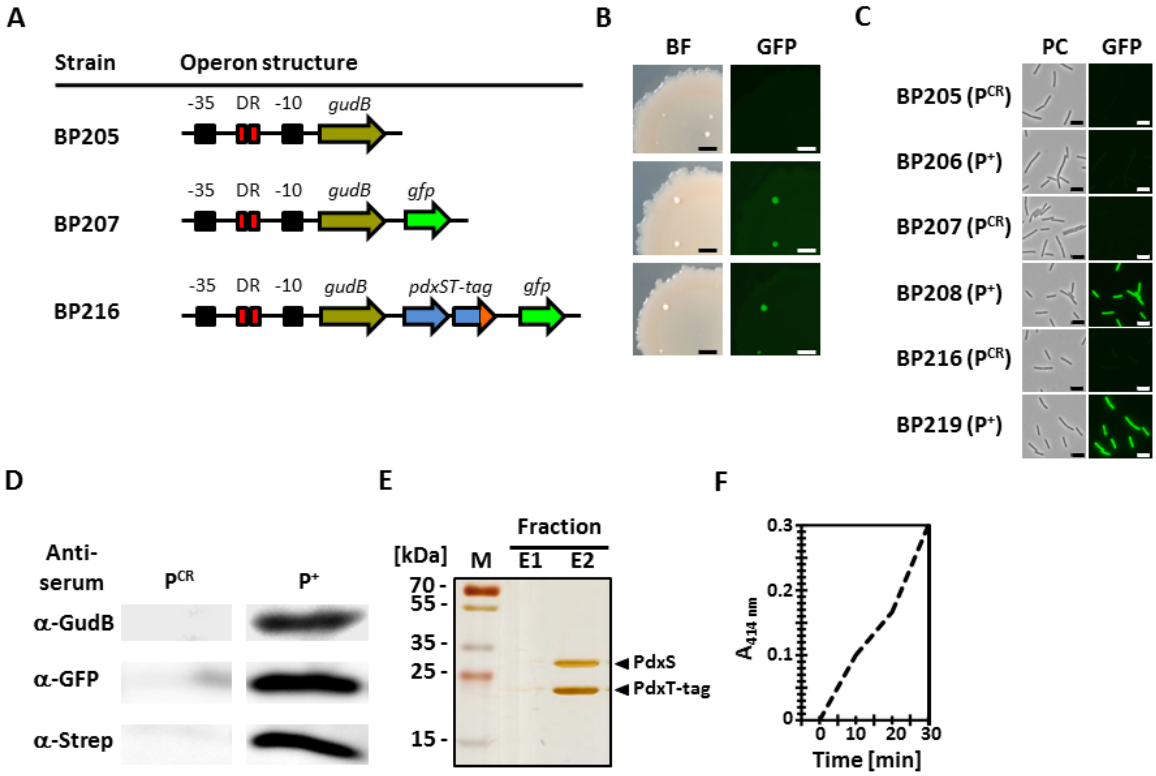

Fig. 5.3 Characterization of the engineered *B. subtilis* strains harboring the artificial operons.
(A) Operon structures of the engineered *B. subtilis* strains having inactivated the *rocG* and the *gudB* genes encoding the native glutamate dehydrogenases RocG and GudB, respectively. **(B)** The emergence of suppressor mutants (white papillae) in aging colonies of the *B. subtilis* strains that are shown in **(A)** was visualized by bright field (BF) microscopy. The suppressor mutants have decryptified the P^{CR} promoters. Suppressor mutants derived from strains BP207 and BP216 harboring the *gfp* gene were identified by monitoring the GFP fluorescence signal. Exposure time, 1.5 s; scale bar, 1 mm. **(C)** Single-cell characterization of strains harboring the cryptic and the spontaneously activated promoters P^{CR} and P^{+}, respectively, by phase contrast (PC) and fluorescence microscopy. Exposure time, 3 s; scale bar, 5 µm. **(D)** Western blot analysis to confirm the synthesis of GudB, PdxT-Strep and GFP in strain BP219 (*P^{+} gudB pdxST-tag gfp*). Cell free crude extracts (20 µl) were separated by SDS PAGE and the proteins were identified using GudB, GFP and Strep-tag antisera. The parent strain BP216 (*P^{CR} gudB pdxST-tag gfp*) harboring the cryptic P^{CR} promoter served as the negative control. **(E)** Purification of the Strep-tagged PdxST vitamin B6 synthase complex from the *B. subtilis* strain BP219 (*P^{+} gudB pdxST-tag gfp*) by Strep-tag Streptactin affinity chromatography. The proteins in 20 µl of the elution fractions E1 and E2 were separated by SDS PAGE and visualized by silver staining. **(F)** Enzyme assay to demonstrate the activity of the purified of the Strep-tagged PdxST vitamin B6 synthase complex shown in **(E).** A representative measurement from three independent experiments using a concentration of 40 µM of the PdxST enzyme complex is shown.

To verify that the suppressor mutant strain BP219 (*P^{+} gudB pdxST-tag gfp*) produces the GDH GudB, the PdxT-Strep subunit of the vitamin B6 PdxST enzyme complex and GFP, we performed Western blot analyses (Fig. 5.3D). The parent strain BP216 (*P^{CR} gudB pdxST-tag gfp*) served as the negative control. While the parent strain BP216 did not produce the three proteins, the mutant strain BP219 harboring the decryptified P^{+} promoter synthesized the GudB and PdxT-Strep proteins as well as GFP (Fig. 5.3D). Next, we tested whether the functional vitamin B6 synthase PdxST-Strep can be

isolated from cell free crude extract of strain BP219 (*P*$^{+}$ *gudB pdxST-tag gfp*). For this, we performed a Strep-tag protein purification experiment and evaluated the elution fractions by SDS PAGE (Fig. 5.3E). In the elution fraction 2 (E2) two proteins were identified with molecular masses of 21 kDa and 32 kDa that correspond to the PdxT-Strep (Sample 2) and PdxS (Sample 1) subunits of the vitamin B6 synthase complex, respectively. Mass spectrometric analysis revealed that the two isolated proteins were indeed the PdxT-Strep and PdxS proteins from *B. subtilis* (Fig. 5.3E and Tab. S 13.7). Finally, we performed an enzyme assay to test whether the isolated enzyme complex was capable of forming vitamin B6. As shown in Fig. 5.3F the isolated enzyme complex was active and formed significant amounts of vitamin B6. In conclusion, the system we present here is suitable for inducer-free activation of gene expression and for the production of proteins, as illustrated by the isolation of a functional enzyme complex from *B. subtilis*.

There are several applications for the inducer-free expression system in synthetic biology because the enrichment of mutant bacteria producing a protein of interest can be achieved with any gene that provides the cells with a selective growth advantage. Moreover, for wastewater treatment one could envision constructing strains harboring multiple cryptic operons encoding metabolic pathways that, if expressed degrade anthropogenic substances. In contrast to conventional expression systems the system presented here does not require a transcription activator or repressor because the decryptified σ^{A}-dependent *P*$^{+}$ promoter is constitutively transcribed by the RNA polymerase. It is safe to assume that the expression system is not restricted to bacteria because DRs are also mutational hotspots in eukaryotic organisms (Vinces et al., 2009).

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (http://www.dfg.de; CO 1139/1-1), the Fonds der Chemischen Industrie (http://www.vci.de/ fonds), the Göttingen Centre for Molecular Biology (GZMB), and the Max-Buchner-Forschungsstiftung (http://www.dechema.de/mbf.html; MBFSt-Kennziffer 3381). We are grateful to Jonathan Rosenberg for critical reading of the manuscript.

6 Identification of GabP as the third proline transporter in *Bacillus subtilis*

The results described in this chapter were published in:

ZAPRASIS, A., HOFFMANN, T., **STANNEK, L.**, GUNKA, K., COMMICHAU, F.M. & BREMER, E. (2014) The γ-aminobutyrate permease GabP serves as the third proline transporter of *Bacillus subtilis*. *Journal of Bacteriology*, 196, 515–526.
doi: 10.1128/JB.01128-13

Author´s contribution:

The study was designed by EB and AZ and interpreted by AZ, FMC and EB. Analyses of suppressor mutants including measurements of cellular proline pools, enzyme activity assays and transport assays were performed by AZ and TH. Evaluation of genome sequencing was done by LS and KG. The paper was written by AZ, FMC and EB.

Identification of GabP as the third proline transporter
in *Bacillus subtilis*

Abstract

PutP and OpuE serve as proline transporters when this imino acid is used by *Bacillus subtilis* as a nutrient or as an osmostress protectant, respectively. The simultaneous inactivation of the PutP and OpuE systems still allows the utilization of proline as a nutrient. This growth phenotype pointed to the presence of a third proline transport system in *B. subtilis*. We took advantage of the sensitivity of a *putP opuE* double mutant to the toxic proline analog 3,4-dehydro-DL-proline (DHP) to identify this additional proline uptake system. DHP-resistant mutants were selected and found to be defective in the use of proline as a nutrient. Whole-genome resequencing of one of these strains provided the lead that the inactivation of the γ-aminobutyrate (GABA) transporter GabP was responsible for these phenotypes. DNA sequencing of the *gabP* gene in 14 additionally analyzed DHP-resistant strains confirmed this finding. Consistently, each of the DHP-resistant mutants was defective not only in the use of proline as a nutrient but also in the use of GABA as a nitrogen source. The same phenotype resulted from the targeted deletion of the *gabP* gene in a *putP opuE* mutant strain. Hence, the GabP carrier not only serves as an uptake system for GABA but also functions as the third proline transporter of *B. subtilis*. Uptake studies with radiolabeled GABA and proline confirmed this conclusion and provided information on the kinetic parameters of the GabP carrier for both of these substrates.

7 Factors that mediate and prevent degradation of GudB^{CR} in *Bacillus subtilis*

The results described in this chapter were published in:

STANNEK, L., GUNKA, K., CARE, R.A., GERTH, U. & COMMICHAU, F.M. (2015) Factors that mediate and prevent degradation of the inactive and unstable GudB protein in *Bacillus subtilis*. *Frontiers in Microbiology*, 5, 758.
doi: 10.3389/fmicb.2014.00758

Author´s contribution:

LS evaluated GFP-GudB^{CR}/GFP-GudB protein levels by fluorescence microscopy (Fig. 7.2A). Overexpression plasmids for complementation studies were constructed by LS. Complementation analyses, including plate reader experiments and Western blots, were performed by LS (Fig. 7.3A and Fig. 7.3B). LS constructed plasmids and strains to obtain the quadruple *gfp-gudB^{CR}* mutant. With this mutant and the newly constructed *gfp-gudB^{CR} ΔmcsB,* LS analyzed the role of phosphorylation on the GFP-GudB^{CR} stability (Fig. 7.5).

Factors that mediate and prevent degradation of GudB^{CR}
in *Bacillus subtilis*

Abstract

The Gram-positive model bacterium *Bacillus subtilis* contains two glutamate dehydrogenase-encoding genes, *rocG* and *gudB*. While the *rocG* gene encodes the functional GDH, the *gudB* gene is cryptic (*gudB^{CR}*) in the laboratory strain 168 due to a perfect 18 bp-long direct repeat that renders the GudB enzyme inactive and unstable. Although constitutively expressed the GudB^{CR} protein can hardly be detected in *B. subtilis* as it is rapidly degraded within stationary growth phase. Its high instability qualifies GudB^{CR} as a model substrate for studying protein turnover in *B. subtilis*. Recently, we have developed a visual screen to monitor the GudB^{CR} stability in the cell using a GFP-GudB^{CR} fusion. Using fluorescent microscopy we found that the GFP protein is simultaneously degraded together with GudB^{CR}. This allows us to analyze the stability of GudB^{CR} in living cells. By combining the visual screen with a transposon mutagenesis approach we looked for mutants that show an increased fluorescence signal compared to the wild type indicating a stabilized GFP-GudB^{CR} fusion. We observed, that disruption of the arginine kinase encoding gene *mcsB* upon transposon insertion leads to increased amounts of the GFP-GudB^{CR} fusion in this mutant. Deletion of the cognate arginine phosphatase YwlE in contrast results in reduced levels of the GFP-GudB^{CR} fusion. Recently, it was shown that the kinase McsB is involved in phosphorylation of GudB^{CR} on arginine residues. Here we show that selected arginine-lysine point mutations of GudB^{CR} exhibit no influence on degradation. The activity of McsB and YwlE, however, are crucial for the activation and inhibition, respectively, of a proteolytic machinery that efficiently degrades the unstable GudB^{CR} protein in *B. subtilis*.

Introduction

Posttranslational modifications of proteins allow bacteria to control several important cellular processes. Phosphorylation is such a posttranslational modification event that can severely affect the function of a protein, which is targeted by a specific kinase (Pawson & Scott, 2005; Jers et al., 2008; Kobir et al., 2011). In bacteria, phosphorylation of enzymes and of enzyme regulators is important for the re-direction of fluxes through central metabolic pathways (LaPorte & Koshland, 1983.; Cozzone & El-Mansi, 2005; Niebisch et al., 2006). Moreover, posttranslational modification of RNA- and DNA-binding transcription factors by phosphorylation may result in induction or repression of gene expression (Bird et al., 1993; Stülke et al., 1997; Jung et al., 2012; Mascher, 2014).

In the past years, several studies revealed that beside serine, threonine, histidine, and cysteine also amino acids like tyrosine and arginine are phosphorylated in bacteria (Meins et al., 1993; Hoch, 2000; Deutscher & Saier, 2005; Macek et al., 2007; Kobir et al., 2011) For instance, the activity of the UDP-glucose dehydrogenase in the Gram-positive model bacterium *Bacillus subtilis* is controlled by reversible phosphorylation of a tyrosine residue (Mijakovic et al., 2004). Phosphorylation of tyrosine residues has also been shown to be important for controlling the activity of DNA-binding proteins (Mijakovic et al., 2006; Derouiche et al., 2013). Recently, phosphoproteomic studies revealed that phosphorylation of arginine residues is an emerging posttranslational modification, which is implicated in general stress response in *B. subtilis* (Elsholz et al., 2012; Trentini et al., 2014; Schmidt et al., 2014). The kinase responsible for arginine phosphorylation in *B. subtilis* was shown to be McsB (Fuhrmann et al., 2009). Under normal growth conditions McsB is bound and inhibited by the ClpC ATPase subunit of the ClpCP protease complex and/or the activator of McsB kinase activity, McsA. At the same time, the DNA-binding transcription factor CtsR represses the genes of the CtsR-regulon (Derré et al., 1999a). In contrast, if the bacteria encounter heat stress, ClpC preferentially interacts with misfolded proteins and releases McsB, which finally targets CtsR for degradation (Kirstein et al., 2005). Inactivation of CtsR results in upregulation of genes that encode proteins of a central protein quality network. The proteins of this network include chaperones, proteases and adaptor proteins that improve the recognition of substrates by proteases (Elsholz et al., 2010a; Battesti & Gottesman, 2013). Recent findings indicate that the detachment of CtsR from the DNA provoked by heat seems to be mediated by an intrinsic protein domain that senses heat rather than by McsB-dependent phosphorylation of arginine residues (Elsholz et al., 2010b). By contrast, upon oxidative stress, McsA does not longer bind to and inhibit McsB, which subsequently removes CtsR from the DNA (Elsholz et al., 2011b). Thus, the way of how the DNA-binding activity of CtsR is controlled by oxidative stress and by heat is strikingly different.

In recent global phosphoproteomic studies using a *B. subtilis ywlE* mutant strain lacking the cognate phosphatase YwlE of the kinase McsB, several arginine phosphorylation sites were detected (Elsholz et al., 2012; Trentini et al., 2014; Schmidt et al., 2014). Two phosphorylatable arginine residues

in the ClpC protein were shown to be important for McsB-dependent activation of the ATPase subunit of the ClpCP protease complex (Elsholz et al., 2012). In the same study it has been shown that the arginine kinase McsB and the cognate phosphatase YwlE may influence the expression of different global regulons. However, the impact of arginine phosphorylation on the physiology of *B. subtilis* is not yet fully understood. Analyses of the dynamic changes in the arginine phosphoproteome in response to heat and oxidative stress revealed that only a minor fraction of the phosphorylation sites were differentially modified (Schmidt et al., 2014).

We are interested in the regulation of glutamate metabolism in *B. subtilis*. In addition to *de novo* synthesis of the important amino group donor glutamate, the bacteria may use glutamate as a source of carbon and nitrogen (for a recent review see Gunka & Commichau, 2012). Utilization of glutamate requires expression of the *rocG* and *gudB* genes encoding the catabolically active glutamate dehydrogenases (GDHs) RocG and GudB, respectively (Belitsky & Sonenshein, 1998; Gunka et al., 2013). Some isolates of *B. subtilis* like the "wild" ancestor strain NCIB3610 indeed synthesize two active GDHs allowing the bacteria to use glutamate as the single source of carbon and nitrogen (Zeigler et al., 2008; unpublished results). In the domesticated *B. subtilis* strain 168 only the *rocG* gene encodes a functional GDH (Belitsky & Sonenshein, 1998; Zeigler et al., 2008). In this strain, the *gudB^{CR}* gene is cryptic (CR) due to a perfect 18 bp-long direct repeat (DR). This occurs in the part of the gene encoding the active center of the enzyme (Belitsky & Sonenshein, 1998). The GudB^{CR} is enzymatically inactive and also subject to rapid proteolytic degradation, especially when the bacteria starve for nutrients, which is the case when bacteria enter stationary phase (Gerth et al., 2008; Gunka et al., 2012, 2013). Although ClpP was shown to slightly affect GudB^{CR} stability (Gerth et al., 2008), other factors that are involved in the recognition and degradation of the protein are unknown. Interestingly, McsB was shown to phosphorylate the inactive GudB^{CR} protein on four arginine residues (Elsholz et al., 2012). It is tempting to speculate that this phosphorylation serves as a label that directs the inactive GudB^{CR} protein to the proteolytic machinery (see below).

In the present study, we apply a visual screen that is based on a GFP-GudB^{CR} fusion to monitor the GudB^{CR} stability *in vivo*. By applying microscopical and biochemical techniques, we found that GFP and GudB^{CR} are simultaneously degraded. Thus, the visual screen is suitable to analyze the cellular amount of GudB^{CR}. To identify novel factors that are involved in GudB^{CR} degradation, we combined the visual screen with a transposon mutagenesis approach. Afterward we looked for mutants that show an increased fluorescence, indicating increased amounts of the GFP-GudB^{CR} fusion. Among the transposants we found one insertion in the *mcsB* gene encoding the arginine kinase McsB. Moreover, inactivation of the cognate phosphatase YwlE resulted in a decreased fluorescence of a strain synthesizing the GFP-GudB^{CR} fusion. The possible mechanisms of how the activity of the kinase McsB and the cognate phosphatase YwlE affect the amount of the GudB^{CR} protein are discussed.

Materials and methods

Chemicals, media, and DNA manipulation

The oligonucleotides were purchased from Sigma-Aldrich (Germany) and are listed in Tab. S 13.4. *B. subtilis* chromosomal DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Germany). Plasmids were isolated from *E. coli* using the Nucleospin Extract Kit (Macherey and Nagel, Germany). PCR products were purified using the PCR Purification Kit (Qiagen, Germany). Phusion DNA polymerase, restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (Germany) and used according to the manufacturer's instructions. Other chemicals and media were purchased from Sigma-Aldrich (Germany), Carl Roth (Karlsruhe, Germany) and Becton Dickinson (Heidelberg, Germany). Sequencing of DNA was performed by the SeqLab Sequence Laboratories (Göttingen, Germany).

Bacterial strains, growth conditions, and construction of mutant strains

B. subtilis strains (Tab. S 13.1) were grown in LB and SP medium, respectively. LB and SP plates were prepared by the addition of 17 g agar/l (Roth, Germany) to LB and SP (8 g nutrient broth/l, 1 mM $MgSO_4$, 13 mM KCl, supplemented after sterilization with 2.5 µM ammonium ferric citrate, 500 µM $CaCl_2$, and 10 µM $MnCl_2$), respectively. When required, media were supplemented with antibiotics at the following concentrations: ampicillin (100 µg/ml), kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), lincomycin/erythromycin (25/2 µg/ml), tetracyclin (12.5 µg/ml) and spectinomycin (150 µg/ml). *B. subtilis* was transformed with plasmid and chromosomal DNA according to a previously described two-step protocol (Kunst & Rapoport, 1995).

Construction of plasmids

The plasmids for complementation of the *ywlE* and *mcsB* mutations in *B. subtilis* were constructed as follows. The *ywlE* and *mcsB* genes were amplified by PCR from chromosomal DNA using the oligonucleotide pairs LS92/LS93 and LS97/LS98, respectively (Tab. S 13.4). The PCR products were digested with the enzymes BamHI and PstI and ligated to the plasmid pBQ200 that was cut with the same enzymes. The plasmids harbouring the *ywlE* and *mcsB* genes and their native ribosome-binding sites were designated pBP183 and pBP186, respectively. Expression of the genes is driven by the constitutively active P_{degQ} promoter (Martin-Verstraete et al., 1994). The quadruple *gfp-gudB^{CR}* mutant (designated as *gfp-gudB^{CR}*-mut), encoding the GudB^{CR} protein in which the arginine residues 56, 83, 421, and 423 were replaced by lysine, was constructed by the Multiple-mutation reaction (MMR; Hames et al., 2005). The mutated *gudB^{CR}* allele was amplified with the oligonucleotide pair KG188/LS96 and the mutagenic oligonucleotides LS94, LS95 and LS96 using plasmid pBP4 as a template. The MMR product was digested with the enzymes MfeI and BamHI, and ligated to the plasmid pAC5 that was cut

with the enzymes EcoRI and BamHI. The resulting plasmid was designated as pBP184. This plasmid was used to amplify the promoter-less quadruple *gudB^{CR}* mutant allele by PCR using the oligonucleotide pair KG181/LS96. The *gfp* gene containing the ribosome-binding site of the *B. subtilis gapA* gene was amplified by PCR from plasmid pBP8 using the oligonucleotide pair KG180/KG190. The *gfp* and *gudB^{CR}* genes were fused by PCR using the external oligonucleotides KG190 and LS96, the PCR product was digested with BamHI and EcoRI, and ligated to the plasmid pBP7 that was cut with the same enzymes. The resulting plasmid pBP187 contains the native *gudB* promoter and integrates in single copy into the *amyE* locus. Replacement of the arginine codons in the *gfp-gudB^{CR}* gene was confirmed by DNA sequencing. All cloning procedures were performed with the *E. coli* strain DH5α (Sambrook et al., 1989).

Transposon mutagenesis

For transposon mutagenesis of the *B. subtilis* strain BP25, we used the mini-Tn*10* delivery vector pIC333 (Steinmetz & Richter, 1994) as described previously (Chauvaux et al., 1998). The transposants were grown on SP agar plates for 48 h at 42°C and the intensity of the GFP signal was evaluated by stereo fluorescence microscopy. For the determination of the site of mini-Tn*10* insertion, we made use of the fact that the integrated DNA fragment does not contain any EcoRI restriction sites. The chromosomal DNA of the mutants was digested with EcoRI and re-ligated. The ligation mixture was used to transform *E. coli* DH5α (Sambrook et al., 1989). For all mutants that were further analyzed, we obtained plasmids conferring spectinomycin resistance (Tab. S 13.3). The insertion sites of the mini-Tn*10* transposon were determined by DNA sequencing of the plasmids using the oligonucleotides pIC333_seq up and pIC333_seq down.

Western blotting

For Western blot analyses, proteins present in 20 – 50 µg of cell free crude extracts were separated by 12.5% SDS PAGE and transferred onto polyvinylidene difluoride membrane (BioRad, Germany) by semi-dry electroblotting. Anti-GFP (PromoKine, Germany; MBL, Japan), anti-YwlE, anti-McsB and anti-GapA polyclonal antibodies were diluted 1:10.000, 1:1000, 1:5.000 and 1:30.000, respectively, and served as primary antibodies. The antibodies were visualized using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega, Germany) and the CDP-Star detection system (Roche Diagnostics, Switzerland) as described previously (Commichau et al., 2007a).

Fluorescence microscopy

For fluorescence microscopy, cells were grown in SP medium to optical densities as indicated, and analysed on agarose microscopy slides. Fluorescence images were obtained with an Axioskop 40 FL fluorescence microscope, equipped with digital camera AxioCam MRm and AxioVision Rel (version

4.8) software for image processing (Carl Zeiss, Göttingen, Germany) and Neofluar series objective at x 100 primary magnification. The applied filter set was eGFP HC-Filterset (band-pass [BP] 472/30, FT 495, and long-pass [LP] 520/35; AHF Analysentechnik, Tübingen, Germany) for GFP detection. Pictures of *B. subtilis* colonies were taken with a stereo fluorescence microscope Lumar.V12 (Zeiss, Jena, Germany) equipped with the ZEN lite 2011 (blue edition) software. The applied filter set was Lumar 38 for eGFP detection (Zeiss, Jena, Germany). Images were taken at room temperature and an exposure time of 1 s.

Monitoring GFP-GudB^{CR} levels in growing cultures

Cellular amounts of the GFP-GudB^{CR} fusion protein were determined by monitoring the fluorescence (excitation 489/9.0 nm, emission 509/9.0 nm) in a growing bacterial culture using the Synergy MX II multimode microplate reader (BioTek). For this purpose, 4 ml LB medium were inoculated with the precultures to an OD_{600} of 0.1. The cultures, that had an approximate OD_{600} of 1.0, were used to inoculate a 96 well plate (Corning, Sigma) containing 180 µl medium per well. To avoid evaporation, the outermost wells were filled with 200 µl sterile water. The plates were incubated for a maximum of 10 h at 37°C and fast shaking speed. OD_{600} was measured every 10 min throughout the experiment. Background fluorescence of the parental strains was subtracted from the raw fluorescence of all *gfp* fusion strains at the same OD_{600}. The cellular amounts of the GFP-GudB^{CR} fusion protein correspond to the fluorescence divided by the OD_{600} at each time point.

Results

A stable screening system for identifying factors involved in GudB^{CR} degradation

The fact that also the GFP-GudB^{CR} protein is degraded (Gunka et al., 2012, 2013) qualifies it as a substrate to uncover the proteolytic machinery. Before identifying factors that contribute to GudB^{CR} degradation, we constructed the *rocG* plus strain BP25 that is genetically stable (Gunka et al., 2012) and synthesizes the active GDH RocG as well as the inactive GFP-GudB^{CR} fusion. To test if the GFP-GudB^{CR} fusion protein is degraded in this strain, we compared the fluorescence signal of cells to those of strain BP26 harbouring the active *gfp-gudB* fusion. As shown in Fig. 7.1A, while the bacteria with the active GFP-GudB fusion were strongly fluorescent, the fluorescence signal of bacteria synthesizing the inactive GFP-GudB^{CR} protein was reduced. Thus, the inactive GFP-GudB^{CR} fusion is also degraded in the new strain background. We also tested whether the two strains can be distinguished from each other by monitoring the fluorescence emitted by colonies that were grown on rich medium agar plates. For this purpose, the strains BP25 (*gfp-gudB^{CR}*) and BP26 (*gfp-gudB*) were grown in liquid medium, mixed in a 1:1 ratio and appropriate dilutions were propagated on SP plates to allow growth of individual colonies. By visual inspection of the plates using a stereo fluorescence microscope we found several

colonies that were grown close to each other and showed different fluorescence signals (Fig. 7.1B). We then re-streaked some of the colonies showing different fluorescence signals on agar plates to obtain individual colonies. Next, we performed colony PCRs and confirmed that the higher and lower fluorescence signals were due to the presence of the *gfp-gudB* and *gfp-gudB^{CR}* alleles, respectively. In conclusion, the visual screen seems to be suitable to look for mutants, lacking factors that enhance or decrease proteolytic degradation of GFP-GudB^{CR}.

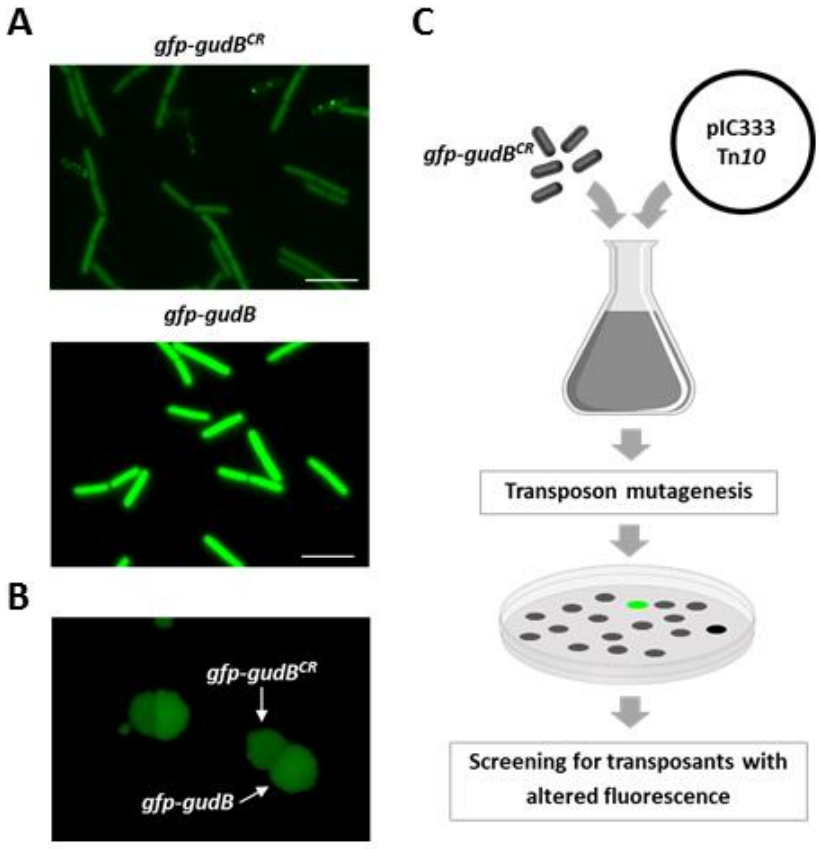

Fig. 7.1 Fluorescence of strains BP25 (*gfp-gudB^{CR}*) and BP26 (*gfp-gudB*) synthesizing the GFP-GudB^{CR} and GFP-GudB proteins, respectively, at the single cell (A) and at the colony level (B); transposon mutagenesis to identify factors involved in GFP-GudB^{CR} degradation (C).
For single cell analysis the bacteria were grown in SP medium. Exposure time, 5 s; scale bar, 5 µm. To monitor fluorescence of colonies the strains were grown in SP medium, mixed and appropriate dilutions were propagated on SP plates, which were incubated for 24 h at 37°C. Exposure time, 1 s.

Identification of MscB contributing to GudB^{CR} degradation

To identify factors that are involved in degradation or stabilization of GudB^{CR}, we performed a transposon mutagenesis with strain BP25 (*gfp-gudB^{CR}*) using the mini-Tn*10* delivery vector pIC333 (Steinmetz & Richter, 1994). Afterward, we screened for mutants that show an altered fluorescence signal using a stereo fluorescence microscope (Fig. 7.1C). Appropriate dilutions of the transposants were propagated on SP plates that were incubated for 48 h at 42°C. By visual inspection of about 8000 transposants we could identify one mutant that showed no fluorescence signal, whereas a second mutant showed an increase in fluorescence intensity. While the first mutant had obviously lost the

ability to synthesize GFP because the transposon was inserted into the *gfp* gene, the mutant showing increased fluorescence had integrated the transposon at position 580 into the arginine kinase encoding *mcsB* gene (Fuhrmann et al., 2009). This transposon mutant was designated as BP69.

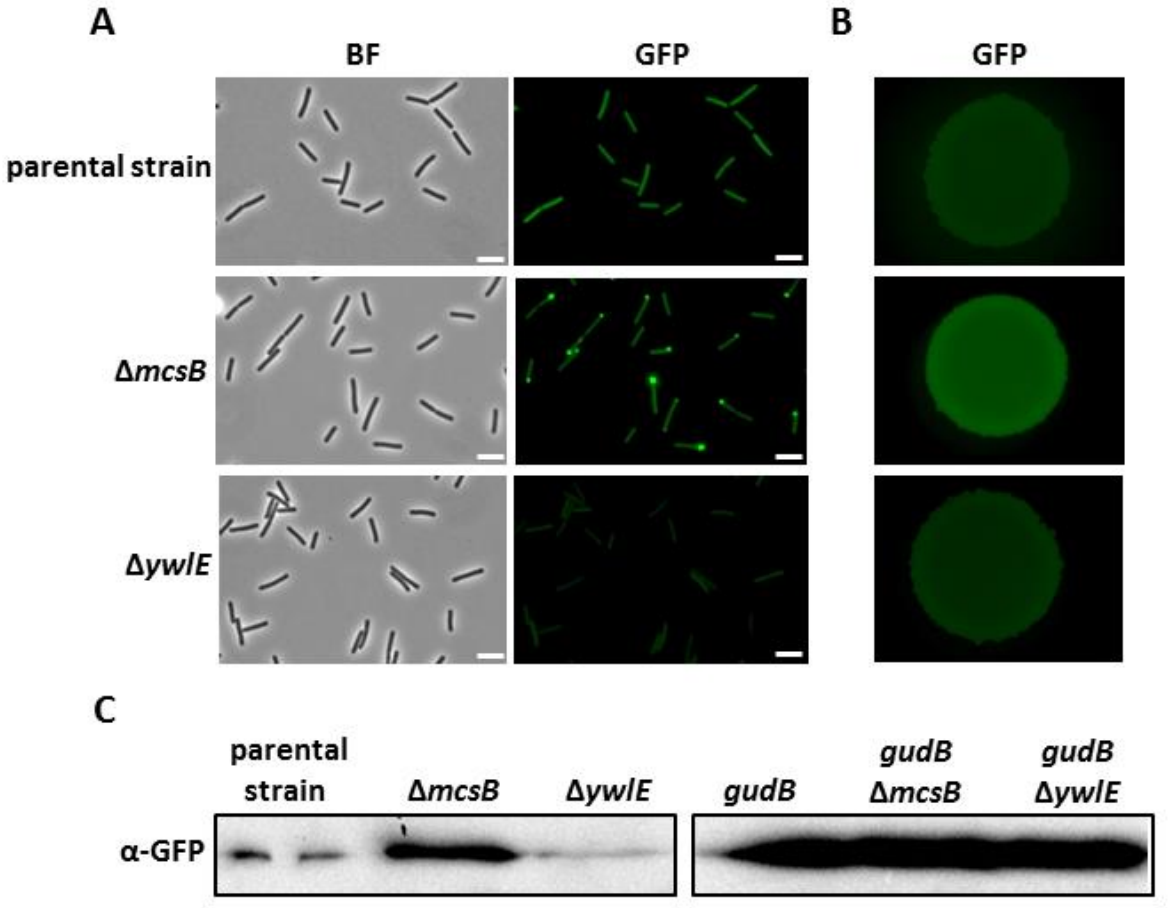

Fig. 7.2 Evaluation of the GFP-GudB^{CR}/GFP-GudB levels by fluorescence microscopy and Western blotting.
For fluorescence microscopic analyses of single cells **(A)** the parental strain B25 (*gfp-gudB^{CR}*) and the strains BP69 (*gfp-gudB^{CR} mcsB*) and BP74 (*gfp-gudB^{CR} ywlE*) were grown in SP medium. For bright field and fluorescence microscopy the exposure times were 150 ms and 5 s, respectively. For the evaluation of the GFP-GudB^{CR} level by stereo fluorescence microscopy **(B)** the bacteria were grown in SP medium until stationary growth phase and 10 µl of cell suspensions with an approximate OD_{600} of 1 were dropped on a SP plate. The plate was incubated for 24 h at 37°C. Exposure time, 1 s; scale bar, 5 µm. For Western blot analysis **(C)** the strains B25 (*gfp-gudB^{CR}*), BP69 (*gfp-gudB^{CR} mcsB*) and BP74 (*gfp-gudB^{CR} ywlE*) as well as the isogenic strains BP26 (*gfp-gudB*), BP311 (*gfp-gudB mcsB*) and BP75 (*gfp-gudB ywlE*) expressing the active *gfp-gudB* fusion were grown in SP medium and 30 µg of the cell free crude extracts were loaded onto a 12.5 % SDS PAGE. The fusion proteins were detected using GFP polyclonal antibodies.

A re-evaluation of the fluorescence signal of single cells and of a colony of the *mcsB* transposon mutant revealed that the cellular amount of the GFP-GudB^{CR} fusion was increased when compared to that of the parent strain BP25 (Fig. 7.2A and Fig. 7.2B). The lack of McsB also resulted in the formation of large aggregates of the GFP-GudB^{CR} fusion protein at the cell poles (Fig. 7.2A), an observation that can be made when aggregation prone proteins are synthesized in bacteria (Rokney et al., 2009; Villar-Pique et al., 2012). In conclusion, using transposon mutagenesis in combination with a visual screen, we identified the arginine kinase McsB being a novel factor that contributes to GudB^{CR} degradation.

McsB and YwlE are involved in GudB^{CR} stability

To underpin the role of arginine phosphorylation in the degradation of the GudB^{CR} protein, we inactivated the *ywlE* gene in the strain BP25 (*gfp-gudB^{CR}*). In case the arginine phosphatase YwlE counteracts the function of its cognate kinase McsB, we expected to observe that single cells as well as colonies of the *ywlE* mutant BP74 would show a reduced fluorescence. This was indeed the case for single cells of the *ywlE* mutant strain in comparison to cells of the *mcsB* mutant and parent strains BP69 and BP25, respectively (Fig. 7.2A). Although less pronounced, fluorescence of the *ywlE* mutant was also reduced at the level of single colonies (Fig. 7.2B). However, a quantification of the fluorescence of the GFP-GudB^{CR} fusion protein monitored in growing cultures in the *ywlE* mutant strain clearly demonstrates that the phosphatase YwlE affects GudB^{CR} stability (see below, Fig. 7.3A).

Next, we confirmed that the kinase McsB and the phosphatase YwlE affect the cellular levels of the GFP-GudB^{CR} fusion protein. For this purpose, we cultivated the parent strain BP25 as well as the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively, in SP medium until stationary phase (the OD_{600} was around 3.0) and analyzed the amounts of the GFP-GudB^{CR} fusion protein by Western blotting using GFP-specific antibodies. As shown in Fig. 7.2C, in strain BP69 lacking McsB the cellular amount of the GFP-GudB^{CR} fusion protein was strongly increased. By contrast, the inactivation of the *ywlE* gene resulted in a decrease of GFP-GudB^{CR} levels. In conclusion, the semi-quantitative Western Blot analyses are in perfect agreement with the fluorescence microscopical studies.

McsE and YwlE do not influence the cellular levels of the active GudB protein

Subsequently, we wanted to answer the question of whether McsB and YwlE do also influence the cellular amounts of the enzymatically active GFP-GudB fusion protein lacking the duplication of three amino acids in the active centre of the enzyme. For this purpose, we cultivated the parent strain BP26 (*gfp-gudB*) synthesizing the active GFP-GudB fusion and the isogenic *mcsB* and *ywlE* mutant strains BP311 and BP75 (Tab. S 13.1), respectively, in SP medium until stationary phase (OD_{600} of about 3.0). Afterward, we quantified the amount of the GFP-GudB protein by Western blotting using antibodies specific for GFP. As shown in Fig. 7.2C, irrespective of the absence of either McsB or YwlE all strains synthesized similar amounts of the active GFP-GudB fusion protein. In conclusion, only the cellular amount of the inactive GFP-GudB^{CR} but not that of the active GFP-GudB fusion protein is significantly affected by McsB.

Complementation of the *mcsB* and *ywlE* mutations

For complementation studies of the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively, we constructed the plasmids pBP186 (*mcsB*) and pBP183 (*ywlE*). Both plasmids are derivatives of the non-integrative overexpression plasmid pBQ200 and gene expression is driven by the constitutively active P_{degQ} promoter (Martin-Verstraete et al., 1994). The plasmids pBP186 and pBP183 were

introduced into the corresponding mutant strains by transformation. Next, we compared the cellular amounts of the GFP-GudB^{CR} fusion protein in the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively, with those of the isogenic complementation strains by monitoring the fluorescence, which reflects the cellular amounts of the GFP-GudB^{CR} fusion protein during growth of the bacteria. The parent strain BP25 (*gfp-gudB^{CR}*) served as a control.

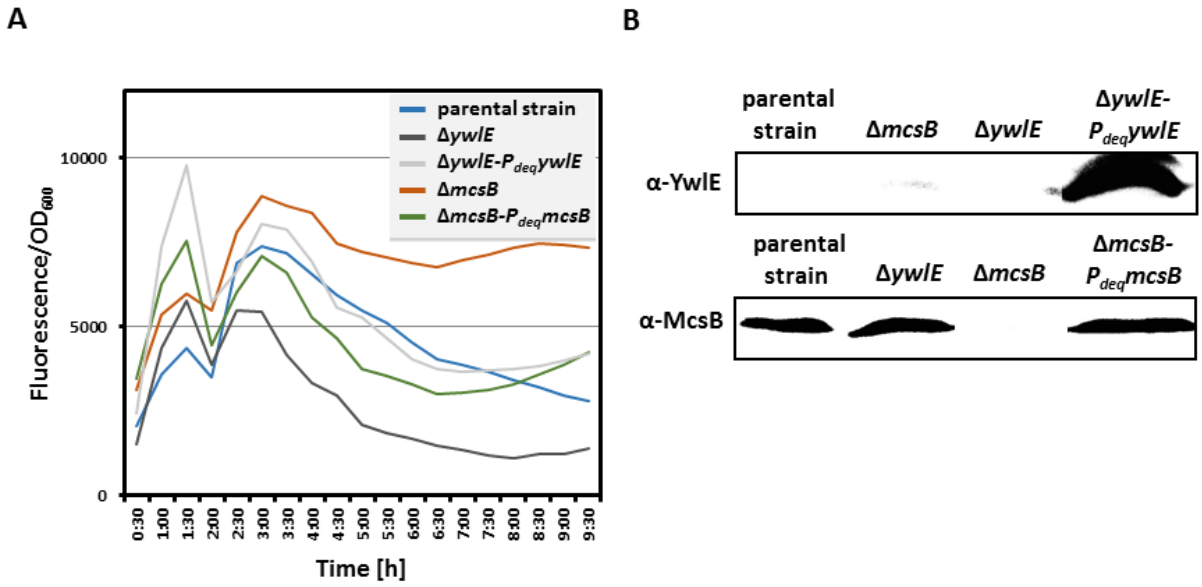

Fig. 7.3 Complementation of the *mcsB* and *ywlE* mutations.
To verify the complementation of the *mcsB* and the *ywlE* mutations *in vivo* **(A)**, the strains BP69 (*gfp-gudB^{CR} mcsB*) and BP74 (*gfp-gudB^{CR} ywlE*), and the isogenic strains BP69-pBP186 and BP74-pBP183 expressing *mcsB* and *ywlE* from the overexpression vector pBQ200 were grown in SP medium and the relative cellular levels of the GFP-GudB^{CR} fusion is reflected by the GFP signal divided by the OD_{600}. The parental strain BP25 (*gfp-gudB^{CR}*) served as a control. All strains entered stationary phase around 6 h of growth. The maximum deviation of the series of representative data shown here was <30%. For the Western blot analysis **(B)** the bacteria were cultivated in SP medium. 40 µg and 50 µg of the cell free crude extracts were loaded onto a 12.5% SDS PAGE for the detection of the YwlE and McsB proteins, respectively, using polyclonal antibodies.

As shown in Fig. 7.3A, the emitted fluorescence of all cultures was similar during exponentially growth. In the stationary phase the fluorescence signal was much higher in the *mcsB* mutant strain BP69 when compared to that of the parent strain BP25. By contrast, inactivation of the *ywlE* resulted in a strong decrease of the fluorescence signal. Overexpression of the *mcsB* and *ywlE* genes in the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively, restored the fluorescence signal in the stationary phase almost to the extent of the parent strain. Western blot experiments using antibodies specific for McsB and YwlE confirmed overexpression of the arginine kinase and the phosphatase from the complementation plasmids in the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively (Fig. 7.3B). In conclusion, the cultivation experiments to detect the cellular levels of the GFP-GudB^{CR} fusion protein are in good agreement with the previous experiments showing that the lack of the McsB and YwlE results in elevated and reduced levels, respectively, of the inactive GDH. Moreover, together with the Western blot experiments the cultivation experiments also revealed that the *mcsB* and *ywlE* mutations can be complemented by expressing the *mcsB* and *ywlE* genes from plasmids.

McsB seems to act independently of ClpC and ClpP on GudB^{CR} degradation

The *mcsB* gene lies immediately upstream of the *clpC* gene in the *ctsR mcsA mcsB clpC* operon. Since the *mcsB* mutation can be complemented, it can be ruled out that enhanced cellular levels of the GFP-GudB^{CR} fusion are a consequence of a polar effect of the transposon insertion into the *mcsB* gene leading to a reduced *clpC* expression and a lower proteolytic activity. However, the lower proteolytic activity in the *mcsB* mutant strain might be due to the missing of McsB-dependent activation of the ClpC-ClpP protease complex. To exclude this possibility, we compared the cellular amounts of the GFP-GudB^{CR} fusion in the background of the *clpC* and *clpP* mutant strains BP98 and BP99, respectively, to that of the parent strain BP25 (*gfp-gudB^{CR}*). For this purpose, we grew the bacteria in SP medium and collected samples from exponential and stationary phases and performed Western blot analyses (Fig. 7.4).

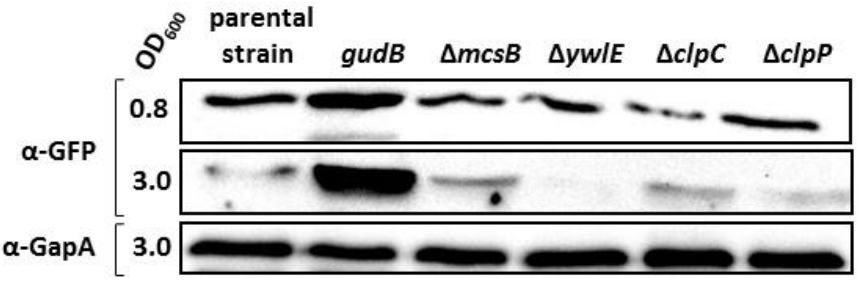

Fig. 7.4 McsB acts independently of ClpC and ClpP.
For the Western blot analysis the parental strain BP25 (*gfp-gudB^{CR}*) and the strains BP26 (*gfp-gudB*), BP69 (*gfp-gudB^{CR} mcsB*), BP74 (*gfp-gudB^{CR} ywlE*), BP98 (*gfp-gudB^{CR} clpC*) and BP99 (*gfp-gudB^{CR} clpP*) were cultivated in SP medium until the indicated optical densities (OD_{600}). 30 µg of the cell free crude extracts were loaded onto a 12.5% SDS PAGE for the detection of the GFP and GapA proteins using polyclonal antibodies.

The strain BP26 (*gfp-gudB*) as well as the *mcsB* and *ywlE* mutant strains BP69 and BP74, respectively, served as controls. As expected, in contrast to the inactive GFP-GudB^{CR} fusion protein the active GFP-GudB variant was more abundant during exponential and stationary phase. Moreover, as observed in the previous experiments, the GFP-GudB^{CR} levels were increased and decreased in the *mcsB* and *ywlE* mutants, respectively (see also Fig. 7.2C). Finally, the GFP-GudB^{CR} levels in the *clpC* and *clpP* mutant strains BP98 and BP99, respectively, were similar to that of the parent strain BP25 (*gfp-gudB^{CR}*). Using GapA and GFP antibodies, we show that only the GFP-GudB^{CR} fusion but not GapA was degraded in stationary growth phase samples. Thus, McsB is involved in GudB^{CR} degradation in a rather ClpP and ClpC-independent manner.

Replacement of phosphorylation sites does not affect McsB-dependent GudB^{CR} degradation

In a recent phosphoproteome analysis it has been shown that the inactive GudB^{CR} protein is phosphorylated on the arginine residues at positions 56, 83, 421 and 423 (Elsholz et al., 2012). To evaluate whether phosphorylation of these sites is important for the degradation of the GFP-GudB^{CR} protein, we replaced the arginine by the structurally similar amino acid lysine and monitored the

amount of the GudB^{CR} variant *in vivo*. For this purpose the parent strain BP25 (*gfp-gudB^{CR}*), the *mcsB* mutant strain BP69 (*mcsB gfp-gudB^{CR}*), the quadruple GFP-GudB^{CR} mutant strain BP230 (*gfp-gudB^{CR}-mut* (R56K R83K R421K R423K)), and the isogenic *mcsB* mutant strain BP231 (*mcsB gfp-gudB^{CR}-mut* (R56K R83K R421K R423K)) were cultivated in SP medium. Simultaneously, the cellular levels of the fusion proteins were determined by monitoring the fluorescence during bacterial growth. As shown in Fig. 7.5, the fluorescence measurements revealed that the cellular levels of the fusion proteins in strains BP25 (*gfp-gudB^{CR}*) and BP230 (*gfp-gudB^{CR}*-mut (R56K R83K R421K R423K)) was much lower in comparison to those of the isogenic *mcsB* mutant strains BP69 (*mcsB gfp-gudB^{CR}*), and BP231 (*mcsB gfp-gudB^{CR}*-mut (R56K R83K R421K R423K)). In conclusion, these observations suggest that phosphorylation of the arginine residues 56, 83, 421 and 423 sites is rather not important for the degradation of the inactive GudB^{CR} protein.

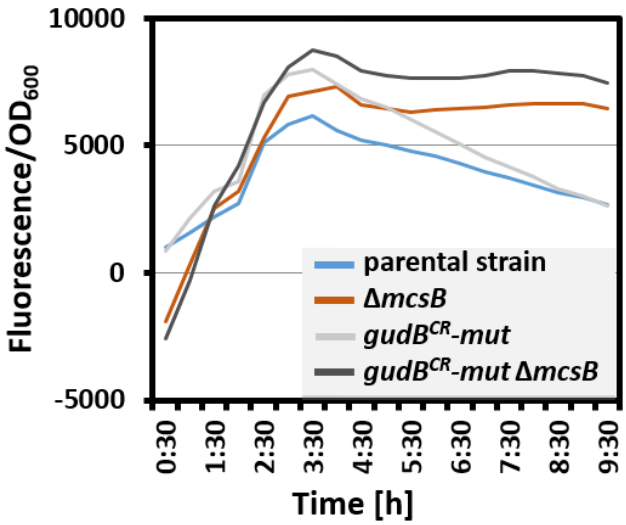

Fig. 7.5 Impact of McsB on the cellular levels of the GFP-GudB^{CR} and GFP-GudB^{CR}(R56K, R83K, R421K, R423K) proteins. The strains BP25 (*gfp-gudB^{CR}*), BP69 (*gfp-gudB^{CR} mcsB*), BP230 (*gfp-gudB^{CR}-mut*), and BP231 (*gfp-gudB^{CR}-mut mcsB*) were cultivated in SP medium and the relative cellular levels of the GFP-GudB^{CR} fusion is reflected by the GFP signal divided by the OD_{600}.The parental strain BP25 (*gfp-gudB^{CR}*) served as a control. All strains entered stationary phase around 6h of growth.The maximum deviation of the series of representative data shown here was <30%.

Discussion

In the present study, we found that the inactivation of the *mcsB* arginine kinase gene resulted in stabilization of the inactive GDH GudB^{CR} during stationary growth phase of *B. subtilis*. Thus, beside its role in controlling the degradation of the DNA-binding transcription factor CtsR (Elsholz et al., 2010b) and delocalization of proteins involved in the development of transformability of *B. subtilis* (Hahn et al., 2009), McsB activity also mediates degradation of GudB^{CR}. Moreover, we found that the arginine phosphatase YwlE counteracts the function of McsB and prevents degradation of GudB^{CR}.

There are several possibilities how McsB and YwlE might stimulate and prevent GudB^{CR} degradation, respectively. As it has been reported previously for the proteolytic degradation of CtsR

(Elsholz et al., 2012), McsB-dependent activation of the ATPase subunit ClpC of the ClpCP protease complex by phosphorylation of two specific arginine residues could also be crucial for GudB^{CR} degradation. However, according to our Western blot analysis ClpP and ClpC appear apparently not involved in GudB^{CR} degradation. Recent global phosphoproteomic studies have revealed that in the absence of YwlE several proteins, among them the GudB^{CR} protein are phosphorylated on arginine residues (Elsholz et al., 2012; Trentini et al., 2014; Schmidt et al., 2014). These studies prompted us to address the question of whether the phosphorylation of GudB^{CR} by McsB could serve as a label for proteolysis. However, although the cellular levels of the GudB^{CR} quadruple mutant, in which the arginine residues 56, 83, 421 and 423 were replaced by lysine residues, were slightly increased, the protein was still degraded in a McsB-dependent manner when the bacteria entered stationary phase (see Fig. 7.5). Thus, the degradation of GudB^{CR} seems to be rather indirectly influenced by McsB. Finally, an unknown proteolytic machinery that remains to be identified might be responsible for the degradation of the misfolded and inactive GDH GudB^{CR}. On one hand the activity of the proteolytic machinery might be controlled by McsB-dependent phosphorylation of an unknown adaptor protein that specifically recognizes GudB^{CR} and directs the protein to the protease for degradation. On the other hand McsB could be important for the activation of one of the AAA+ proteases or other unknown proteases that remain to be identified. One could also envision that McsB acts itself as the adaptor that mediates proteolysis of the GudB^{CR} protein. The interaction between McsB and GudB^{CR} could result in coincidental phosphorylation of the GDH. This could also be the case for the other arginine phosphorylations of the *B. subtilis* proteome (Elsholz et al., 2012).

As described above it is interesting to note that only the domesticated *B. subtilis* strains 160, 166 and 168, of which the latter one is used worldwide in basic research and in industry, harbour the *gudB^{CR}* gene that is enzymatically inactive and unstable (Zeigler et al., 2008). It has been suggested that the *gudB^{CR}* allele appeared as a consequence of X-ray mutagenesis and subsequent adaptation for rapid growth of the bacteria in minimal medium lacking the amino group donor glutamate (Burkholder & Giles, 1947). This hypothesis is supported by the observation that a strain that synthesizes in addition to RocG also the enzymatically active GDH GudB is rapidly outcompeted by the laboratory strain 168 (*rocG gudB^{CR}*) when exogenous glutamate is not available (Gunka et al., 2013; Stannek et al., 2014). Obviously, the presence of both, RocG and GudB is disadvantageous for the bacteria because the catabolic GDHs degrade the endogenously produced glutamate, which is needed in anabolism. Thus, under laboratory growth conditions a permanent selective pressure must act on the bacteria, which prevents the accumulation of mutants that have spontaneously mutated the cryptic *gudB^{CR}* gene and synthesize in addition to RocG the functional GDH GudB. Moreover, the selective pressure acting on the *B. subtilis* strain 168 might be an explanation for the observation that the cryptic *gudB^{CR}* gene is stably inherited since the bacterium has been domesticated. Recently, it has been shown that bacteria

rapidly loose genes and reduce their genome sizes when adapted to specialized environments. This might also be observed in the laboratory by experimental evolution of bacterial cell populations (Lee & Marx, 2012; Koskiniemi et al., 2012). Therefore, it is somewhat surprising that *B. subtilis* affords to waste energy by permanently synthesizing an inactive enzyme that is subject to rapid degradation. However, under certain growth conditions it must be advantageous for *B. subtilis* to harbour the cryptic *gudB^{CR}* gene that, when activated by spontaneous mutagenesis (Gunka et al., 2012), encodes a functional GDH. Indeed, a derivative of the *B. subtilis* 168 expressing *rocG* and *gudB* can use glutamate as a single source of carbon and nitrogen (Gunka et al., 2013). Thus, under very specific nutritional conditions bacteria that are endowed with high-level GDH activity have a strong selective growth advantage.

In the future it will be interesting to identify additional factors that are involved in the rapid degradation of the enzymatically inactive GDH GudB^{CR}. This goal might be achieved by monitoring the cellular amounts of the GFP-GudB^{CR} fusion protein in a mutant collection that have inactivated all non-essential genes by targeted gene deletion or by a next time saturated transposon mutagenesis. The identification of novel factors that are involved in GFP-GudB^{CR} proteolysis might be facilitated by monitoring growth and fluorescence over time because the fusion protein seems to be preferentially degraded in stationary phase. Moreover, it will be interesting to address the question whether arginine phosphorylation influences the physiological functions of other proteins in *B. subtilis*.

Acknowledgments

This work was supported by the grant CO 1139/1-1 of the Deutsche Forschungsgemeinschaft (http://www.dfg.de), the Fonds der Chemischen Industrie (http://www.vci.de/fonds), the Göttingen Centre for Molecular Biology (GZMB), and the Max-Buchner-Forschungsstiftung (http://www.dechema.de/mbf.html; MBFSt-Kennziffer 3381) to F. M. C.. Work of K. G. in the authors' lab was supported by the grant SFB860. Sabine Lentes is acknowledged for expert technical support. We are grateful to Miriam Dormeyer for critical reading of the manuscript and for helpful discussions.

8 Synergistic control of glutamate biosynthesis by glutamate dehydrogenases and glutamate

The results described in this chapter were published in:

STANNEK, L., THIELE, M.J.; ISCHEBECK, T.; GUNKA, K.; HAMMER, E., VÖLKER, U. & COMMICHAU, F.M. (2015) Evidence for synergistic control of glutamate biosynthesis by glutamate dehydrogenases and glutamate in *Bacillus subtilis*. *Environmental Microbiology,* 17, 3379-3390.
doi: 10.1111/1462-2920.12813

Author´s contribution:

The study was designed and interpreted by LS and FMC. LS constructed plasmids and strains and performed most of the experiments including Western blotting, *in vivo*-crosslinking experiments, bacterial two-hybrid analyses, ß-galactosidase assays and sample preparation for GC-MS analyses. TI performed GC-MS analyses. KG and MT contributed to the study by constructing several plasmids and strains. EH and UV identified proteins by mass spectrometry and evaluated the results. LS and FMC wrote the paper.

Abstract

In the Gram-positive bacterium *Bacillus subtilis*, glutamate is synthesized by the glutamine synthetase and the glutamate synthase (GOGAT). During growth with carbon sources that exert carbon catabolite repression, the *rocG* glutamate dehydrogenase (GDH) gene is repressed and the transcription factor GltC activates the expression of the GOGAT encoding *gltAB* genes. In the presence of amino acids of the glutamate family, the GDH RocG is synthesized and the enzyme prevents GltC from binding to DNA. The dual control of glutamate biosynthesis allows the efficient utilization of the available nutrients. Here we provide genetic and biochemical evidence that, like RocG, also the paralogous GDH GudB can inhibit the transcription factor GltC, thereby controlling glutamate biosynthesis. Contradictory previous observations show that high-level of GDH activity does not result in permanent inhibition of GltC. By controlling the intracellular levels of glutamate through feeding with exogenous arginine, we observed that the GDH-dependent control of GltC and thus expression of the *gltAB* genes inversely correlates with the glutamate pool. These results suggest that the *B. subtilis* GDHs RocG and GudB in fact act as glutamate sensors. In conclusion, the GDH-mediated control of glutamate biosynthesis seems to depend on the intracellular glutamate concentration.

9 Identification of the proteolytic machinery involved in GudB^{CR} degradation

Introduction

The *B. subtilis* laboratory strain synthesizes two GDH´s - the active RocG and GudB^{CR} (Belitsky & Sonenshein, 1998). The latter is enzymatically inactive as it is probably misfolded due to a perfect direct repeat of 18 bp present in the region encoding the active center of the protein. Nonetheless, the inactive enzyme is constitutively transcribed and subject to rapid degradation (Gunka et al., 2012). Until know, the proteolytic machinery degrading the inactive GudB^{CR} enzyme was not identified. Recently, it was discovered, that the degradation of GudB^{CR} is influenced by the presence of the arginine kinase McsB and the cognate phosphatase YwlE (Stannek et al., 2015a). The stability of GudB^{CR} was analyzed by measuring the fluorescence intensity of the GFP-GudB^{CR} fusion protein expressed in different strain backgrounds. In a *mcsB* deletion strain GudB^{CR} is more stable compared to the wild-type strain. In contrast, deletion of *ywlE* results in degradation quicker than in wild-type cells. In comparison, no effect on the stability of the active GudB^{+} enzyme was detectable regarding the presence or absence of the arginine kinase or phosphatase (Stannek et al., 2015a). McsB is known for its activity as an adapter protein of the ClpCP protease. The ATPase ClpC is phosphorylated on two arginine residues by McsB rendering ClpC active (Elsholz et al., 2012). Adapter protein function of McsB is proved for the ClpCP-dependent degradation of the DNA-binding transcription factor CtsR (Elsholz et al., 2010b). Clear evidence for the involvement of Clp-proteases in the degradation of GudB^{CR} is not provided so far. A slight influence of ClpP on the stability of GudB^{CR} was observed during glucose-limited stationary phase (Gerth et al., 2008). This observation could not be confirmed in Western Blot studies analyzing the GudB^{CR} protein stability in *clpC* and *clpP* deletion strains (Stannek et al., 2015a).

Phosphoproteome analyses delivered hints for phosphorylation of the GudB^{CR} enzyme on four different arginine residues in a McsB-dependent manner (Elsholz et al., 2012). Phosphoylation of the GudB^{CR} enzyme on arginine residues being a signal for degradation of the protein was already excluded. Nonetheless, McsB and YwlE strongly affect the stability of GudB^{CR} (Stannek et al., 2015a). However, these observations do not allow to conclude that the GDH´s stability is influenced due to a direct interaction between GudB^{CR} and McsB or YwlE. Instead, McsB and YwlE activities could also regulate other processes which have an effect on GudB^{CR} stability. Bacterial adenylate cyclase two-hybrid (B2H) analyses were applied to detect primary protein-protein interactions between GudB^{CR}/GudB^{+} and proteins of the proteolytic machinery in *B. subtilis* (Karimova et al., 1998).

Results and Discussion

B2H analyses were used to address the possibility of direct protein-protein interactions between both the inactive and active GudB enzyme and McsB and YwlE. In order to identify the protease degrading the inactive enzyme GudB^{CR}, B2H were performed as well. In parallel, interactions between the active GudB^{+} enzyme and the Clp-proteases were conducted. The genes encoding the inactive and active GDH, the arginine kinase McsB, the cognate arginine phosphatase YwlE and the genes encoding the domains of the ClpCP and ClpXP protease were cloned into the plasmids of the B2H system to elucidate interactions. B2H experiments were performed according to Stannek et al., 2015b.

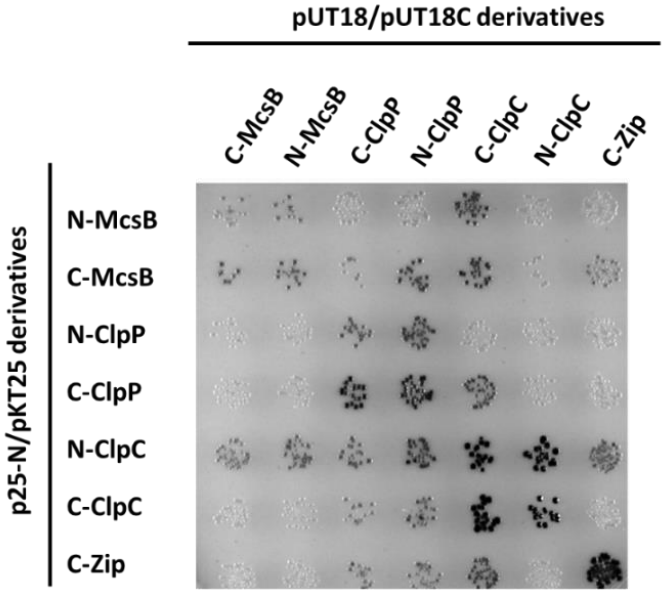

Fig. 9.1 B2H analysis to study the interactions among the arginine kinase McsB and the ClpCP protease subunits. The *mcsB*, *clpP,* and *clpC* genes were cloned in the plasmids pUT18, pUT18C, p25-N, and pKT25. Plasmids pUT18 and pUT18C allow the expression of the proteins fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid p25-N and pKT25 allow the expression of the proteins fused to the N or the C terminus of the T25 domain of the adenylate cyclase. The *E. coli* transformants were incubated for 48 h at 28 °C.

Self-interaction was observed for all the proteins analyzed, indicating the formation of dimers or larger oligomers. Self-interaction was expected for the hexamer-forming GDH´s, for the ClpC and ClpX ATPases forming hexameric rings as well as for the ClpP protease known for its heptameric rings (see Fig. 9.1, Fig. 9.4; Wang et al., 1997; Striebel et al., 2009; Gunka et al., 2010). Furthermore, the interaction of the adaptor protein McsB and the ATPase ClpC was proved (Elsholz et al., 2011a). An interaction between McsB and the proteolytic subunit ClpP was detected, too (Fig. 9.1). As expected an interaction between ATPases ClpC and ClpX and the proteolytic subunit ClpP was observed (Fig. 9.3 and Fig. 9.4).

The stability of the GudB^{CR} protein is influenced by the presence of the arginine kinase McsB (Stannek et al., 2015a). It was speculated that McsB is the adaptor protein mediating GudB^{CR} proteolysis by Clp-proteases (Stannek et al., 2015a). The B2H analyses did not support the theory of

McsB to be an adaptor protein for GudB^{CR}. GudB^{CR} showed no interaction with the arginine kinase McsB or the arginine phosphatase YwlE. This suggests that McsB and YwlE affect other proteins which regulate the stability of the inactive GudB^{CR} enzyme. The stable enzyme GudB^{+} was found to interact weakly with McsB but not with YwlE. Additionally, the results display interactions between the inactive GudB^{CR} enzyme and the ATPase ClpC. These observations indicate that the ClpCP protease is involved in the degradation of GudB^{CR} (Gerth et al., 2008). Meanwhile additional experiments using an improved protein extraction method and novel antibodies against the GDH GudB revealed a ClpCP and McsB-dependent proteolysis of GudB^{CR}, too (Gerth et al., unpublished data). Interestingly, also the active GDH GudB^{+} interacts with the ClpC ATPase. GudB^{+} furthermore interacts with the ClpP proteolytic subunit (Fig. 9.3). A faint interaction between GudB^{+} and the protease ClpXP was also observable (Fig. 9.4). This indicates, that both the active and the inactive GudB enzyme are degraded in a Clp-dependent manner.

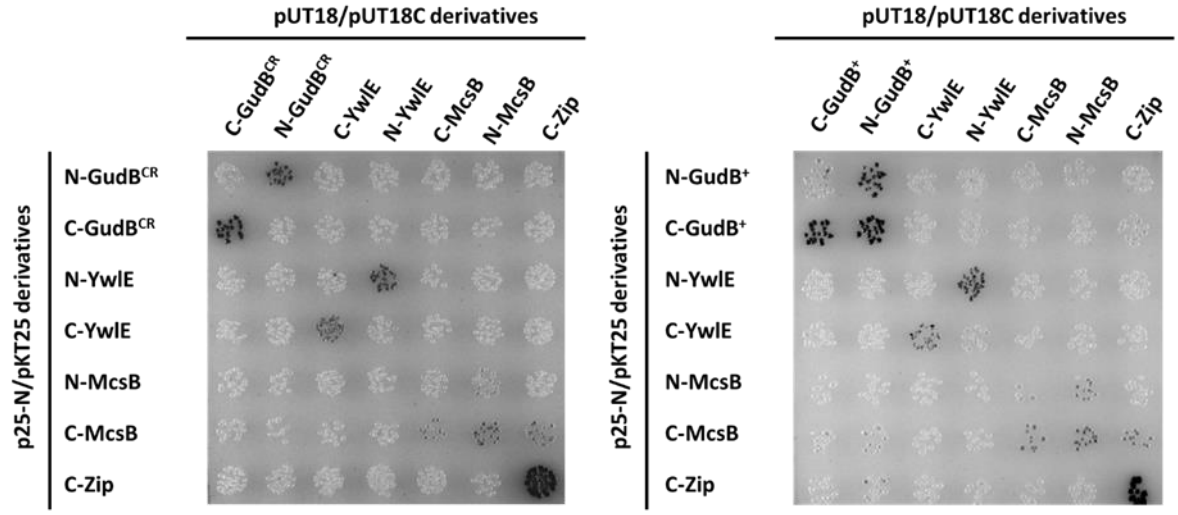

Fig. 9.2 B2H analysis to study the interactions among the glutamate dehydrogenase GudB^{CR}/GudB^{+} and the arginine kinase McsB and the cognate phosphatase YwlE.
The *gudB^{CR}*, *gudB^{+}*, *ywlE* and *mcsB* genes were cloned in the plasmids pUT18, pUT18C, p25-N, and pKT25. Plasmids pUT18 and pUT18C allow the expression of the proteins fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid p25-N and pKT25 allow the expression of the proteins fused to the N or the C terminus of the T25 domain of the adenylate cyclase. The *E. coli* transformants were incubated for 48 h at 28 °C.

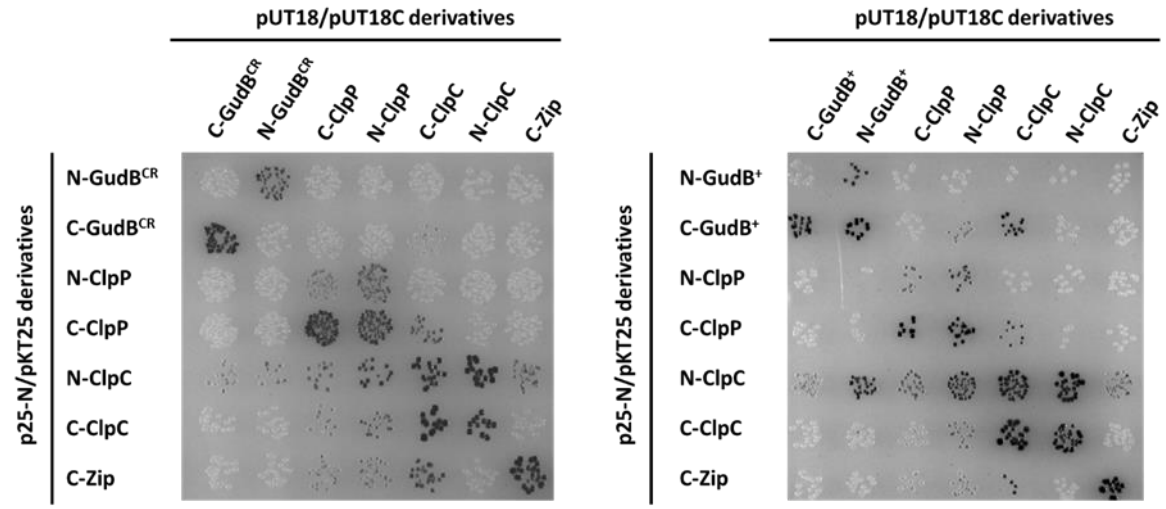

Fig. 9.3 B2H analysis to study the interactions among the glutamate dehydrogenase GudB^{CR}/GudB^{+} and the ClpCP protease subunits.
The *gudB^{CR}*, *gudB^{+}, clpP* and *clpC* genes were cloned in the plasmids pUT18, pUT18C, p25-N, and pKT25. Plasmids pUT18 and pUT18C allow the expression of the proteins fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid p25-N and pKT25 allow the expression of the proteins fused to the N or the C terminus of the T25 domain of the adenylate cyclase. The *E. coli* transformants were incubated for 48 h at 28 °C.

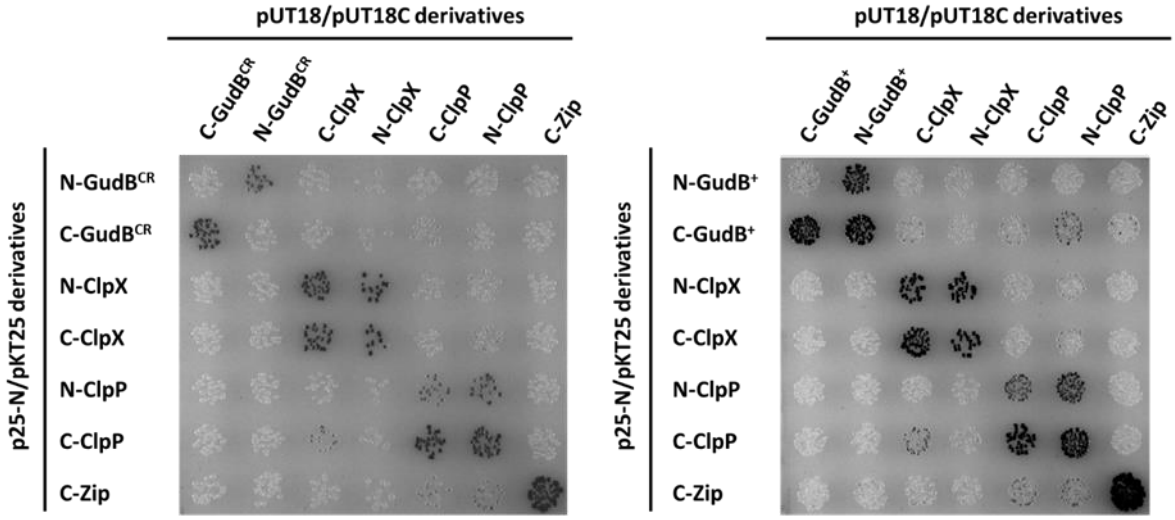

Fig. 9.4 B2H analysis to study the interactions among the glutamate dehydrogenase GudBCR/GudB+ and the ClpXP protease subunits.
The *gudB^{CR}*, *gudB^{+}, clpX* and *clpP* genes were cloned in the plasmids pUT18, pUT18C, p25-N, and pKT25. Plasmids pUT18 and pUT18C allow the expression of the proteins fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid p25-N and pKT25 allow the expression of the proteins fused to the N or the C terminus of the T25 domain of the adenylate cyclase. The *E. coli* transformants were incubated for 48 h at 28 °C.

10 The rate of transcription influences the stability of the direct repeat in the *gudB*^{CR} allele

Introduction

Glutamate is one of the key metabolites within the cell of any living organism. Being the most important amino group donor for nitrogen containing compounds, glutamate is present in high intracellular amounts (Whatmore et al., 1990; Bennett et al., 2009). To maintain glutamate homeostasis, *Bacillus subtilis* has developed a sophisticated regulatory system (Gunka & Commichau, 2012). Remarkable in the regulation of the glutamate metabolism is the involvement of trigger enzymes – bifunctional enzymes active in metabolism and in the control of gene expression (Commichau & Stülke, 2008). Genes involved in glutamate synthesis and degradation are regulated by transcription factors which themselves are subject to regulation through trigger enzymes, depending on the metabolic status of the cell. If the regulation is disrupted, the formation of suppressor mutants balancing the glutamate metabolism is observed, not only in *B. subtilis* but also in enterobacteria (Belitsky & Sonenshein, 1998; Yan, 2007; Commichau et al., 2008).

In *B. subtilis*, glutamate is exclusively synthesized by the glutamate synthase GOGAT. Glutamate is formed from 2-oxoglutarate and glutamine. Thus, the synthesis connects the carbon with the nitrogen metabolism. By the degradation of glutamate, *B. subtilis* is also able to use glutamate as an additional carbon and nitrogen source. This reaction is performed by the strictly catabolic glutamate dehydrogenase (GDH). The GDH is not only involved in glutamate degradation, but depending on the glutamate concentration within the cell also regulates the activity of GltC, the transcriptional activator of the *gltAB* genes encoding the glutamate synthase GOGAT (Commichau et al., 2007a; Stannek et al., 2015b).

Interestingly, in the genome of the laboratory *B. subtilis* strain 168, two glutamate dehydrogenases are encoded, GudB^{CR} and RocG (Belitsky & Sonenshein, 1998). While the *rocG* gene is only transcribed under certain growth conditions, e.g. in medium containing high amounts of arginine, the *gudB*^{CR} gene is constitutively transcribed (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The GudB^{CR} protein, however, is enzymatically inactive due to a mutation acquired during domestication (Burkholder & Giles, 1947; Zeigler et al., 2008). A perfect direct repeat with a repeat unit of 9 bp is present in the region encoding the active center of the protein. However, one part of the perfect direct repeat of the inactive pseudogene *gudB*^{CR} is rapidly excised in a *rocG* deletion strain plated on complex medium (Belitsky & Sonenshein, 1998). The resulting *gudB*$^{+}$ gene encodes an active GudB^{+} protein, fully able to take over the glutamate degrading and GltC regulating functions of RocG (Stannek et al., 2015b).

Direct repeat sequences are found throughout the genomes of many organisms. Sequences containing direct repeats are prone to mutations (Kovtun & McMurray, 2008). While these hypermutable loci are regarded as engines of bacterial adaptation, mutation of direct repeat sequences in the human genome can cause severe illnesses (for a review see Zhou et al., 2014a). Therefore, it is interesting to identify possible mechanisms and proteins involved in the expansion and contraction of direct repeats.

Regarding the high mutagenesis rate of the direct repeat present in the *gudB*^{CR} gene, the transcription repair coupling factor Mfd was found to be important (Gunka et al., 2012). Mfd is involved in the displacement of RNA polymerases stalled at DNA lesions during the transcription process. Mfd not only releases the RNA polymerase together with the transcript but additionally recruits the nucleotide excision repair machinery (Ayora et al., 1996). Mfd is highly conserved and homologs are found in many organisms, including the gram negative model organism *Escherichia coli* (Roberts & Park, 2004). In *B. subtilis*, a *mfd* deficient strain exhibits a much lower mutation frequency compared to the wild-type strain in stationary phase (Ross et al., 2006). As Mfd couples DNA repair with transcription, the role of transcription on the mutation frequency of the inactive *gudB*^{CR} gene was analyzed in this study. It was investigated whether the mutation rate of the direct repeat in the *gudB*^{CR} gene correlates with the level of transcription. Therefore, the *gudB*^{CR} gene was placed under the control of the promoters with different strength and the mutation rate was analyzed by the appearance of suppressor mutants.

Experimental procedures

Construction of plasmids

The plasmids for the promoter-*lacZ* fusions were constructed as follows. To construct the P_{alf2}-*lacZ* fusion the primer pairs LS23 and LS24 containing digested ends of the BamHI and EcoRI restriction sites were hybridized and ligated to the plasmid pAC6 cut with BamHI and EcoRI. For the P_{alf4}-*lacZ* fusion the primer pairs LS27 and LS28 were used. The plasmids harboring the P_{alf2}-*lacZ* fusion and the P_{alf4}-*lacZ* fusion were designated as pBP162 and pBP164, respectively. The plasmids containing the promoter-*gudB*^{CR} fusions were constructed as described below. For the P_{alf2}-*gudB*^{CR} fusion the *gudB*^{CR} gene was amplified using the primer pair LS29 (containing the promoter region of P_{alf2} and the shine dalgarno) and LS31. The PCR products were digested with BamHI and EcoRI and ligated into the plasmid pAC5 which was digested with the same enzymes. The plasmid was designated as pBP166. Plasmid pBP167 harboring the P_{alf4}-*gudB*^{CR} fusion was constructed like pBP166, except that the insert was amplified with LS30 (containing the promoter region of P_{alf4} and the shine dalgarno) and LS31.

ß-galactosidase assay

To analyze the strength of the newly constructed promoters, the *lacZ* expression was quantitatively determined. The respective *B. subtilis* strains were grown at 37° C and 220 rpm in 12 ml SP medium and harvested at an optical density OD_{600} of 0.5-0.8. The ß-galatactosidase activity was determined with cell extracts obtained by lysozyme treatment using o-nitrophenyl galactopyranoside as substrate as described previously (Kunst & Rapoport, 1995). One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of *o*-nitrophenol per min at 28° C.

Analysis of mutation frequencies

To determine the mutation frequency of strains harboring promoter-*gudB*^{CR} fusions of different strengths, single colonies of the strains were grown overnight in 4 ml minimal medium containing glucose at 28°C and 220 rpm until they reached an OD_{600} of 1.5-2.5. Afterwards, the cultures were adjusted to OD_{600} 1.0 with 0.9% NaCl and 10 µl were spottet or plated on complex medium. The plates were incubated at 37° C for several days and the appearing suppressor mutants were documented using the Stereo Microscope Lumar.V12 (Zeiss, Jana) equipped with the ZEN lite 2011 (blue edition) software and the Molecular Imager®Gel Doc™XR+Systems.

Results and Discussion

To analyze if the mutation frequency of the *gudB*^{CR} gene correlates with the expression level of the gene, the following system was developed (see Fig. 10.1). In a previous study, the strength of the native *gudB*^{CR} promoter P_{gudB} was already determined to be rather high (Gunka et al., 2012). Working with an artificial promoter system allowed to construct a perfect artificial promoter (P^+) stronger than the P_{gudB} promoter (Dormeyer et al., 2015). The perfect -10 and -35 region are 17 nucleotides apart from each other, the optimal spacing distance for the housekeeping sigma factor of the RNA polymerase. A translational promoter-*lacZ* fusion was constructed first to evaluate the promoter strength via ß-galatactosidase activity measurements. Based on the promoter P^+, promoters with altered -10 and - 35 regions were constructed. The P_{alf2} promoter has an altered -35 region and the P_{alf4} promoter has an altered -35 and -10 region. The promoter-*lacZ* fusions were integrated into the *amyE* locus of *B. subtilis* strain 168. The resulting strains were designated as BP191 (P_{alf2}-*lacZ*) and BP193 (P_{alf4}-*lacZ*). Together with BP429 (P^+-*lacZ*) and GP1101 (P_{gudB}-*lacZ*) the strains were grown in SP medium and cells were harvested during exponential growth phase. To elucidate the strength of the different promoters compared to the native *gudB* promoter, a ß-galactosidase activity assay was performed. The ß-galactosidase assay revealed a higher activity of the P^+ promoter compared to the native *gudB* promoter P_{gudB} as previously observed (Thiele, 2013). The A→T replacement in the -35 region of the P_{alf2} promoter reduced the promoter strength by almost 70 %, the T→C replacement in the -10 region of the P_{alf4} promoter resulted in an 80 % lower expression of the *lacZ* fusion.

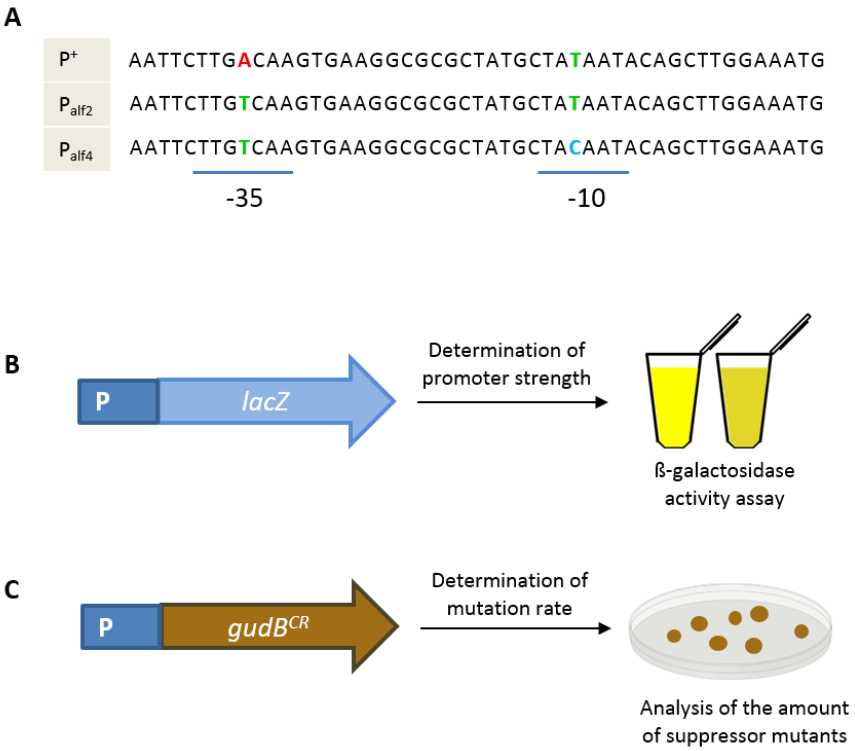

Fig. 10.1 Schematic illustration of experiments performed to analyze the influence of transcription on mutation rate
(A) The *P*$^{+}$ promoter is characterized by perfect -35 and -10 regions. The promoters P_{alf2} and P_{alf4} have non-perfect -35 or/and -10 regions. **(B)** The promoter strength was analyzed by ß-galacatosidase assays performed with *B. subtilis* strains harboring the promotor-*lacZ* fusions. **(C)** The *gudB*^{CR} gene was put under the control of the different promoters. In adequate strain backgrounds the effect of the transcription rate on the mutagenesis process of the direct repeat in the *gudB*^{CR} gene was analyzed via the amount of appearing suppressor mutants.

Exhibiting a different strength than the native *gudB*^{CR} promoter, the *P*$^{+}$, P_{alf2} and P_{alf4} promoters were suitable to analyze if the mutation rate of the *gudB*^{CR} direct repeat is influenced by the transcription rate of the gene. Thus, plasmids were constructed with *gudB*^{CR} fused to the artificial promoters. The *gudB*^{CR} deficient strain BP442 was transformed with plasmids pBP166 (P_{alf2}-*gudB*^{CR}) and pBP167 (P_{alf4}-*gudB*^{CR}), resulting in strains BP196 and BP194, respectively. Plasmids pBP301 (*P*$^{+}$-*gudB*^{CR}) and pBP303 (*P*$^{-}$-*gudB*^{CR}) were introduced into the *gudB*^{CR} deficient strain GP1160, resulting in strains BP401 and BP411, respectively (Thiele, 2013). Next, the respective strains were transformed with the chromosomal DNA of the transposon mutant GP747 to inactivate the *rocG* gene. The resulting strains were BP197 (P_{alf2}-*gudB*^{CR} *ΔrocG*) and BP195 (P_{alf4}-*gudB*^{CR} *ΔrocG*). Together with strain GP1163 (P_{gudB}-*gudB*^{CR} *ΔrocG*) harboring the native *gudB* promoter and strains BP405 (*P*$^{+}$-*gudB*^{CR} *ΔrocG*) and BP412 (*P*$^{-}$-*gudB*^{CR} *ΔrocG*) the strains harboring the weaker promoters were subsequently cultivated in minimal medium and plated on complex medium. As negative control served strain BP412 harboring a promoter-less *gudB*^{CR} gene in the *amyE* locus. Over several days the appearance of suppressor mutants was analyzed.

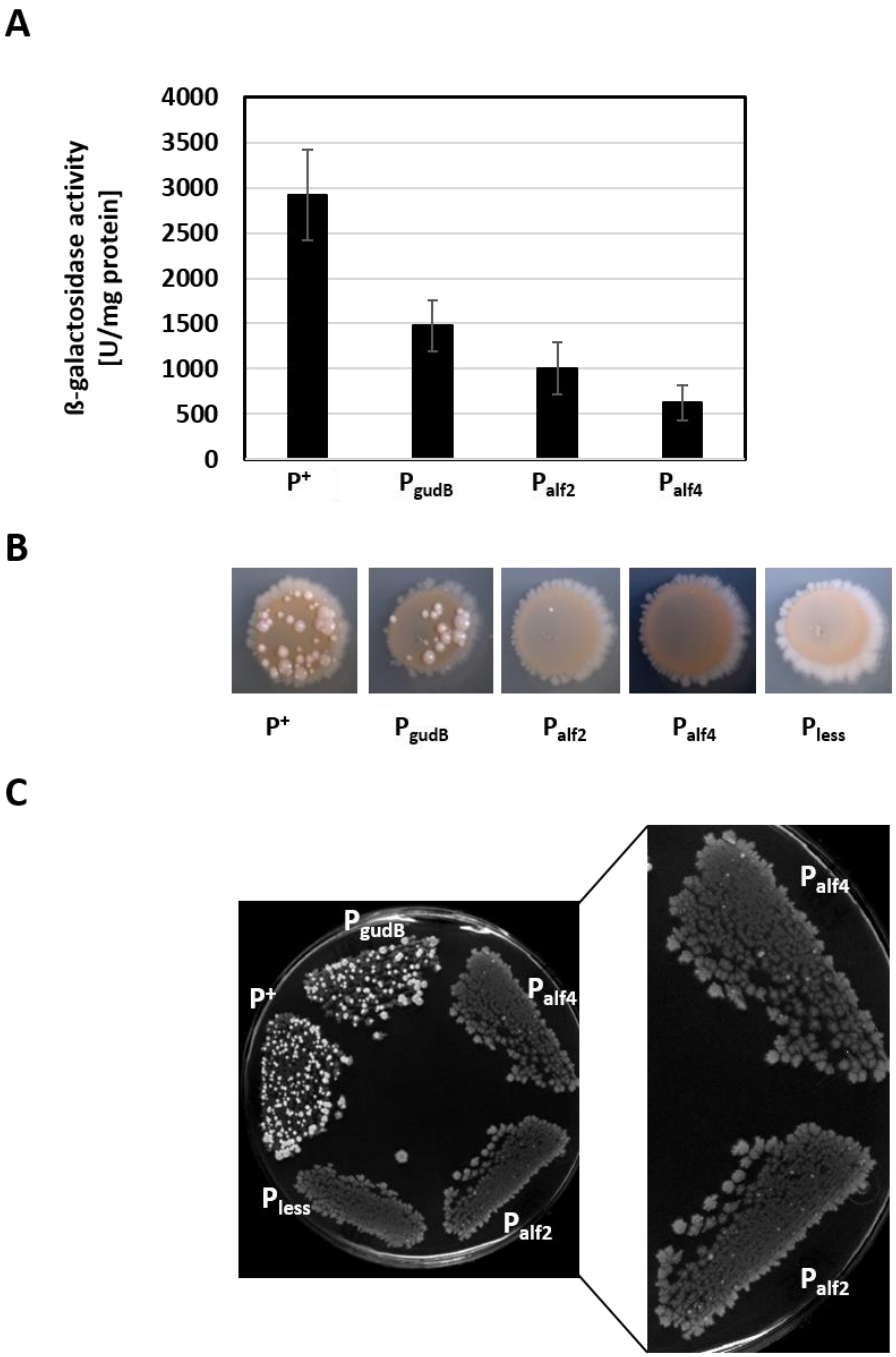

Fig. 10.2 The effect of transcription rate on *gudB*^{CR} mutagenesis
(A) The expression levels of the *lacZ* gene of strains encoding *P*$^{+}$*-lacZ* (BP429), P_{gudB}*-lacZ* (GP1101), P_{alf2}*-lacZ* (BP191), and P_{alf4}*-lacZ* (BP193) were determined via ß-galactosidase activity assays. ß-galactosidase activities are given as units per mg^{-1} of protein. Experiments were carried out threefold. Results are presented as means ±SD. **(B)** Formation of suppressor mutants of *B. subtilis* strains BP405 (*P*$^{+}$*-gudB*^{CR} *ΔrocG*), GP1163 (P_{gudB}*-gudB*^{CR} *ΔrocG*), BP197 (P_{alf2}*-gudB*^{CR} *ΔrocG*), BP195 (P_{alf4}*-gudB*^{CR} *ΔrocG*), and BP412 (*P*$^{-}$*-gudB*^{CR} *ΔrocG*) 6 days post incubation (dpi) on SP medium. Cultures of the strains were spotted on the agar plate. **(C)** Formation of suppressor mutants of *B. subtilis* strains BP405 (*P*$^{+}$*-gudB*^{CR} *ΔrocG*), GP1163 (P_{gudB}*-gudB*^{CR} *ΔrocG*), BP197 (P_{alf2}*-gudB*^{CR} *ΔrocG*), BP195 (P_{alf4}*-gudB*^{CR} *ΔrocG*), and BP412 (*P*$^{-}$*-gudB*^{CR} *ΔrocG*) 21 dpi on SP medium. Cultures of the strains were streaked out on the agar plate.

As shown in Fig.10.2, the highest number of suppressor mutants was observed for strain BP405, harboring the strongest promoter *gudB*^{CR} gene fusion (*P*$^{+}$*-gudB*^{CR}). Strain GP1163, representing native conditions, exhibited – compared to strain BP405 – a lower, but still a high mutation frequency, as observed earlier (Gunka et al., 2012). Corresponding to the weaker strength of the promoter strains

BP195 and BP197, harboring the P_{alf4}- and P_{alf2}-*gudB*^{CR} gene fusions, respectively, the lowest number of suppressor mutants appeared on complex medium. As expected, the strain with the non-transcribed *gudB*^{CR} gene, BP412, formed no suppressor mutants. However, the suppressor mutants of strains BP195 and BP197, harboring the weaker promoters, appeared late. During our studies, a slightly different outcome of the experiment was observed with respect to the plating method used (data not shown here). Apparently, it makes a difference if the bacteria are spotted on the agar plates or the cells are streaked out on a bigger surface. In general, the appearance of suppressor mutants can be better analyzed using a streak-out method compared to the drop assay. Here, a lower concentration of cells with respect to the size of area in which the cells are plated is present on the agar plate. Probably, the cells grow better on less-densely colonized medium and are viable for a longer time allowing the mutation of the *gudB*^{CR} gene to occur also under a lower transcription rate. The lower than expected mutation frequency of the strains harboring the weaker transcribed promoter-*lacZ* fusions could indicate that the concentration of cells used in this assay is still too high for the suppressor mutation to occur. For the future, it will be interesting to establish a suitable protocol for the analysis of the mutation frequency of the direct repeat in the *gudB*^{CR} gene. Such a protocol would be a valuable tool to identify further factors involved in the mutation process of the direct repeat.

However, the study showed that the spontaneous mutation frequency of the *gudB*^{CR} gene's direct repeat tends to correlate with the transcription level. Besides the fact that the mutation frequency decreases in a *mfd* deletion strain, this observation additionally supports the hypothesis of GudB^{+} suppressor mutants appearing due to a transcription associated mutagenesis mechanism (Gunka et al., 2012). In several model systems it has been shown already that transcription promotes repeat instability (Lin et al., 2009). For instance, an effect of transcription rate on mutation rate was observed in yeast (Kim et al., 2007). Regardless of whether transcription or DNA replication contribute to the repeat instability, models propose that exposed single-stranded DNA forms aberrant secondary structures like hairpins or slipped-strand DNA duplexes (Levinson & Gutman, 1987; Bierne et al., 1997; Viguera et al., 2001). If a slipped DNA structure is formed during transcription of the *gudB*^{CR} gene, the RNA polymerase is probably not able to proceed. As a consequence, Mfd removes the stalled RNA polymerase from the template strand and recruits other factors involved in the repair mechanism (Ayora et al., 1996). There is evidence that also RNase H ribonucleases are relevant (Thiele, 2013). RNase H enzymes are known to remove R-loops, RNA:DNA structures formed due to slipped DNA, for instance (Wimberly et al., 2013; Skourti-Stathaki & Proudfoot, 2014). Further factors still need to be identified in future studies.

11 Discussion

11.1 Mutate or be moribund – suppressor mutants overcome glutamate imbalance

A complex control system of the glutamate metabolism in *B. subtilis* enables the bacterium to adapt to changing conditions of nitrogen and carbon supply in the environment. As glutamate is the major amino group donor for nitrogen containing compounds, transcription of genes and protein activities being part of the glutamate metabolism are highly regulated (see introduction and Gunka & Commichau, 2012). In the past years, ongoing research revealed so far unknown regulatory processes within the nitrogen and glutamate metabolism in *B. subtilis* (Murray et al., 2013; Schumacher et al., 2015). Still, there are gaps of knowledge on how *B. subtilis* achieves such a fine-tuned homeostasis of glutamate metabolism. In this study, new aspects regarding the regulation of the glutamate metabolism in *B. subtilis* were obtained.

The vital importance of keeping the glutamate metabolism in balance is reflected by the appearance of suppressor mutants in strains suffering from glutamate imbalance. Examples for the occurrence of suppressor mutations under both conditions – the apparently toxic accumulation of glutamate and glutamate shortage – were observed in *B. subtilis* (Belitsky & Sonenshein, 1998; Commichau et al., 2008; Flórez et al., 2011). Depending on the reason for glutamate imbalance, the acquired mutations had different consequences. On the one hand, genes involved in glutamate metabolism were inactivated, e.g. the *gltAB* genes. On the other hand, suppressor mutations were detected enabling the use of new metabolic pathways for glutamate degradation. Astonishing, however, is the activation of an actually inactive GDH by a mutagenesis process. The *B. subtilis* laboratory strain 168 synthesizes two GDHs – the active RocG and the inactive GudB^{CR}. Probably the most studied suppressor mutation in the *B. subtilis* strain 168 is the activation of the inactive *gudB^{CR}* gene to *gudB^{+}* in a *rocG* deletion strain grown on complex medium (Belitsky & Sonenshein, 1998; Gunka et al., 2012, 2013). The glutamate-degrading activity of the GudB^{+} protein suppresses the growth defect of a *rocG* deletion strain on complex medium.

In this work, the flexibility of the glutamate metabolism and the urgency of glutamate homeostasis was demonstrated once again (see chapter 3). Growth of GudB^{+} cells in medium lacking exogenous glutamate resulted in the accumulation of cells with an inactivated *gudB* gene due to intragenic mutations (Gunka et al., 2013). Through inactivating the constantly active GDH GudB^{+} a futile cycle of glutamate synthesis and degradation under conditions of low glutamate levels is prevented under glutamate limiting conditions.

11.1.1 The relevance of suppressor mutants for research

The above described examples not only demonstrate that suppressor mutants are often required to be able to live, but that the analysis of suppressor mutants also helps researchers to understand life. In bacterial populations which are impaired in growth and maladapted to their environment, appearance of suppressor mutations can be observed regularly. As bacteria have a short generation time, mutations can evolve and accumulate quickly, manifesting in phenotypic changes. Not only researchers in the field of glutamate metabolism work with suppressor mutants. Also other research groups harness the appearance of suppressor mutants to uncover unknown functions of proteins.

As already stated, proline serves not only as carbon, nitrogen and energy source (Moses et al. 2012), but it is additionally an osmostress protectant for *B. subtilis* (Brill et al., 2011; Zaprasis et al., 2013). Proline can either be taken up by the osmotically inducible transporter OpuE or the substrate-inducible transporter PutP (von Blohn et al., 1997; Spiegelhalter & Bremer, 1998; Moses et al., 2012). Moses et al. delivered first hints for the presence of a third proline uptake systems, as a *putP opuE* double mutant was still sensitive to 3,4-dehydro-DL-proline (DHP) (Moses et al., 2012). DHP is a proline analog which causes protein-misfolding upon incorporation. By whole-genome sequencing of suppressor mutants of a *putP opuE* deletion strain resistant against DHP, GabP –normally active in the uptake for the nonproteinogenic amino acid ϒ-aminobutyrate (GABA) – was identified as the third proline transporter (see chapter 6, Zaprasis et al., 2014). This study also emphasizes the enormous value of whole-genome sequencing to identify mutations.

However, analyzing suppressor mutants is also of interest for industrial research. Especially the aforementioned example of the development of a new metabolic pathway to degrade glutamate via a suppressor mutation in AnsR underlines an interesting aspect for analyzing suppressor mutants (Flórez et al., 2011). Spontaneous mutations can confer a growth advantage to bacteria, maybe by novel alternative pathways or altered enzyme activities. Discovering and exploiting these alternatives is of special interest for industrial purposes. Interesting examples can be found in studies performed already 20 years ago. Often overexpression of recombinant proteins can have toxic effects for the host bacterium. Two suppressor mutant strains of *E. coli* strain BL21 (DE3), often used for overexpression of proteins, were isolated allowing high-level synthesis of recombinant proteins using the T7 RNA polymerase expression system (Miroux & Walker, 1996). In these mutant strains the toxic effect was alleviated due to a mutation in the promoter of the T7 RNA polymerase (Wagner et al., 2008).

Another example taking benefit from the appearance of suppressor mutants is described by an inducer-free activation system used to control gene expression (chapter 4, Dormeyer et al., 2015). A tight regulation of gene expression of industrial relevant proteins is useful for production strains. In the past years, the possibility of achieving such a control via promoter engineering was set out to use

(Brautaset et al., 2009; Zhou et al., 2014b; Vogl et al., 2014). Combining the knowledge about the mutagenesis of the direct repeat present in the inactive *gudB*^{CR} gene in a *rocG* deletion strain under high glutamate concentrations and the selection-driven accumulation of the strain harboring the active GudB^{+} enzyme an industrially attractive system for activating gene expression was developed (Gunka et al., 2012, 2013; Dormeyer et al., 2015). The system is based on the mutational activation of a cryptic promoter harboring a perfect direct repeat in the binding region of the RNA polymerase sigma factor. Cells which have mutated the promoter, to which a growth conferring gene is fused, proliferate rapidly under selective conditions. Inducer-free expression of a protein of industrial interest can thus be achieved by fusing the respective gene to the synthetic promoter that is activated by spontaneous mutagenesis (Dormeyer et al., 2015).

11.2 Diverge or disappear – paralogous genes in bacteria

The two GDH encoding genes in *B. subtilis* are paralogs. Organisms can obtain paralogous genes either by gene duplication or by horizontal gene transfer. The parental wild-type strain of the *B. subtilis* laboratory strain 168 synthesizes two functional GDHs, RocG and GudB^{+} (Zeigler et al., 2008). The inactivation of the *gudB*$^{+}$ gene occurred during domestication (Burkholder & Giles, 1947). It is assumed that the *rocG* and *gudB* genes emerged through gene duplication. Gene duplications represent an important evolutionary force because the genomic plasticity with respect to adaptation increases (Zhang, 2003; Magadum et al., 2013). Without gene duplication there propably would not be today´s microbial diversity. Already in 1970, Ohno predicted that upon gene duplication one of the genes – being redundant – can aquire mutations (Ohno, 1970). However, different outcomes are possible as described in the following (see Tab. 11.1).

Tab. 11.1 Evolutionary fate of duplicated genes
Gene duplication can have different evolutionary fates according to Zhang et al., 2003

1. Pseudogenization	Either of the duplicated genes becomes inactive
2. Conservation of gene function	Duplicated genes maintain function of original gene
3. Subfunctionalization	Duplicated genes adopt some functions of original gene
4. Neofunctionalization	Either of the duplicated genes acquires new functions through mutation

The first is a process called pseudogenization and results in functional redundancy of genes. The expression of two identical genes is usually not advantegous for the cell and expression of redundant genes is energetically costly (Wagner, 2005). Mutations are aquired in one of the paralogous gene copies. These mutations are for instance present in the promoter region or as nonsense mutations in the coding region. Thus, pseudogenization results either in nonexpressed or nonfunctional genes (Zhang, 2003). Duplicated genes are often eliminated from the genome again. Second, in some cases

the gene function of duplicated genes is conserved if an increased protein amount is beneficial for the cell. Duplicated genes with the same function are either retained in the genome by gene conversion or strong selection against mutations within the genes. However, the maintenance of two identical duplicated genes is rather unusual. Instead, as a third fate of duplicated genes, subfunctionalization might occur. During this process each of the daughter genes takes over part of the parental gene´s function. Furthermore, the daughter genes can even improve in performing the function of the original gene. The forth possible outcome of gene duplication is the most important process with respect to evolution. Neofunctionalization is the process of acquiring new functions by mutation. Beneficial mutations result in gene fixation in the genome and the organism being better adapted to the environment (Zhang, 2003).

11.2.1 Examples of paralogs in different organisms

As mentioned above, it is assumed that gene duplication or also the acquirement of paralogs by horizontal gene transfer contribute immensely to evolution allowing novel protein functions to develop (Zhang, 2003; Magadum et al., 2013). Support for this statement was delivered especially with the given technical possibility to sequence and analyze whole genomes. Indeed, throughout all three domains of life, many genes arose by gene duplication. In *Mycoplasmae pneumonia*, 44 % of the genes seem to be generated by gene duplication, in *Saccharomyces cerevisiae* one third of the genome comprises originally duplicated genes (Himmelreich et al., 1996; Rubin et al., 2000). The first genome analysis of *B. subtilis* revealed that paralogs constitute 47 % of the genes present in the genome (see Fig. 11.1). The generation of the paralogs found in *B. subtilis* is ambiguous. Interestingly, some repetitive DNA sequences were detected in genome regions which were identified as or are putative prophages. Generally, it was observed that many paralogs are engaged in the transport of molecules into and out of the cells or in the regulation of transcription (Zeigler et al., 2008).

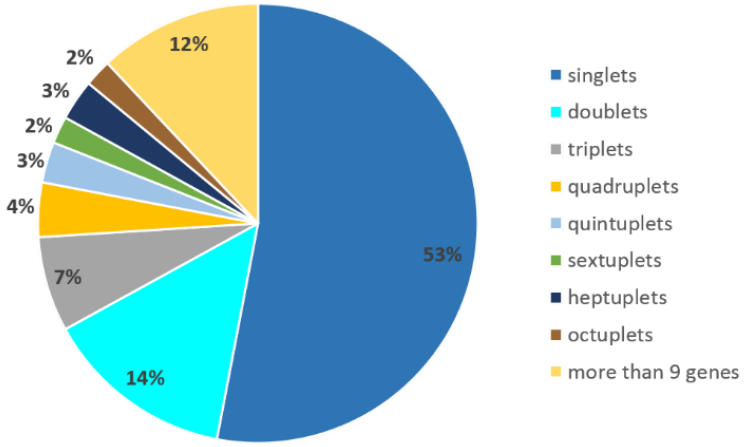

Fig. 11.1 Gene paralogs present in the genome of *B. subtilis*
The distribution of *B. subtilis* gene paralogs in the genome was determined on the basis of the complete genome sequence using Smith and Waterman algorithm. Image adapted from Kunst et al., 1997.

The following examples demonstrate to which level the acquirement of paralogous genes contributes to evolution in diverse organisms (Zhang, 2003; Magadum et al., 2013). A comparison of the function of the *B. subtilis* GDHs GudB^{+} and RocG to the given examples reveals that we encounter a special situation regarding the control of the glutamate metabolism in *B. subtilis*.

In *Candida albicans* four copies of the Lys regulator are present in the genome which resulted from successive gene duplication. Over time, the Lys regulator paralogs acquired as many differences that each Lys paralog controls distinct groups of target genes (Perez et al., 2014).

Another example of subfunctionalization of proteins after gene duplication is represented by RNase J enzymes. In Firmicutes, at least two copies of the gene are present in the genome. *Bacillus thuringiensis*, for instance, features four gene copies of the RNase J. In *B. subtilis* two endoribonucleases J1 and J2 were identified (Even et al., 2005). The paralogs share 50 % sequence identity. Although the proteins are similar in sequence, only the RNase J1 is characterized by essentiality (Kobayashi et al., 2003). Interestingly, the *B. subtilis* RNase J1 was the first bacterial enzyme with dual 5´-to-3´ exoribonucleolytic/endoribonucleolytic activity (Mathy et al., 2007). The 5´-to-3´ exoribonucleolytic activity of *B. subtilis* RNase J2 is due to a weaker substrate affinity and lower k_{cat} value extremely poor compared to RNase J1. The endoribonucleolytic activity in contrast is similar between the two enzymes. This indicates that the original RNase J protein was both endo- and exoribonucleolytically active. It is suggested that the subfunctionalization process upon gene duplication resulted in a loss of RNase J2 5´-to-3´ exonuclease activity. However, as RNase J1 retains endonucleolytic activity, the subfunctionalization process in this example was not completed (Mathy et al., 2010).

Recently, the co-existance of the paralogous *phoN* genes in *Pseudomonas putida* was explained. Although the *phoN1* gene was acquired by horizontal gene transfer, it shows a 46 % sequence identity with the endogenous *phoN2* gene. Both genes confer resistance of *P. putida* against an inhibitor of the GS. However, *phoN1* is a phosphinothricin-acetyl transferase and *phoN2* acts on methionine sulfoximine, both of which are glutamine synthetase inhibitors (Páez-Espino et al., 2015). Phosphoinothricin and methionine sulfoximine are synthetically produced herbicides used in agriculture. As the analyzed *P. putida* strain was isolated before these herbicides were used in agriculture, adaptation of *P. putida* to man-created pressure was ruled out. Instead, PhoN1 and PhoN2 are believed to play a role in quorum sensing among soil bacteria. For instance, *Streptomyces* naturally produce phosphoinothricin (Schwartz et al., 2005).

If duplicated genes fulfill the same function under different environmental conditions they are called eco-paralogs (Sanchez-Perez et al., 2008). An example for eco-paralogs is found in the extremely halophilic bacterium *Salinibacter ruber* which encodes two GDHs. While one GDH displays highest activities in the absence of salt, the other requires high salt concentrations for activity. Both GDHs in

S. ruber are active in the reductive amination of 2-OG. However, one of the GDHs additionally showed activity in the oxidative deamination of glutamate (Bonete et al., 2003). Hence, being active under different environmental conditions, the GDHs of this bacterium are eco-paralogs. Regarding the difference in expression of the *B. subtilis* GDHs RocG and GudB$^+$, these paralogs can in a certain way be classified to be eco-paralogs as well (see introduction and 11.2.2).

11.2.2 GudB$^+$ and RocG – the paralogous GDHs of *B. subtilis*

Compared to the examples of paralogs described above, the paralogs GudB$^+$ and RocG of *B. subtilis* have one special characteristic: Until now, no functional difference was detected between the paralogs (see also 11.2.3). RocG and GudB$^+$ have an amino acid sequence identity of 74 %. Interestingly, the main difference between RocG and GudB$^+$ is neither found in the enzymatic reaction they perform nor in their regulatory function in metabolism. Instead, the different promoter of *rocG* and *gudB*$^+$ results in a diverged regulation of the genes. Changes in the regulation of the respective gene expression acquired after gene duplication is another criterion why paralogs are retained in the genome (Gu et al., 2005). As duplicated genes are placed into a new genomic surrounding, it is also highly likely that their expression is regulated differently. A new system regulating the expression of one of the paralogs can furthermore avoid deleterious protein doses (Soskine & Tawfik, 2010). This is certainly true for the GDH paralogs of *B. subtilis*. While the transcription of the *rocG* gene is highly regulated, *gudB*$^+$*/gudB*^{CR} is constitutively transcribed (Belitsky et al., 2004; Gunka et al., 2012). As described in 2.4.3 the expression of *rocG* is induced by arginine or ornithine. Under these conditions the transcription factor RocR is activated which – together with AhrC – positively regulates the expression of the *rocG* gene (Belitsky & Sonenshein, 1999). Furthermore, RocR activates transcription of the *rocABC* operon and *rocDEF* operon (Calogero et al., 1994; Gardan et al., 1995; Ould Ali et al., 2003). Like RocG the proteins encoded in the *rocABC* and *rocDEF* operon are required for the utilization of arginine.

The glutamate degrading enzymatic activity of the active GudB$^+$ enzyme (6.7 U/mg of protein) is higher than for RocG (3.9 U/mg of protein). Furthermore, the k_m value for glutamate is higher for GudB$^+$ (17.9 mM) compared to RocG (2.9 mM) (Gunka et al., 2010). This makes perfectly sense, as the constantly active GudB$^+$ will only degrade glutamate effectively if high glutamate concentrations are present in the cell. In contrast, under low glutamate concentrations, glutamate degradation by GudB$^+$ is prevented due to the low substrate affinity of the enzyme for glutamate. In contrast, RocG is especially needed when high amounts of amino acids belonging to the glutamate family are present in the environment (Calogero et al., 1994; Gardan et al., 1995, 1997).

As stated above, the laboratory *B. subtilis* strain 168 and its siblings 160 and 166 are exceptional in encoding an inactive GDH. The parental Marburg strain and non-domesticated strains like the wild

NCIB 3610 strain encode a functional GDH GudB$^+$ and RocG (Zeigler et al., 2008). Earlier, it was suggested that RocG is the major GDH of *B. subtilis*, whereas GudB$^+$ was regarded to be the minor GDH. Taken together, the observations now demand a different hypothesis. The active GudB$^+$ enzyme constitutes the major GDH of *B. subtilis*. GudB$^+$ fulfills criteria needed for being the only GDH before gene duplication occurred. First, it is constitutively expressed and degrades glutamate also in the absence of glutamate precursors in the environment (Gunka et al., 2012). Therefore, if glutamate is present in high amounts, it can be used as an additional carbon source and accumulation of toxic glutamate concentrations within the cell is prevented (Kimura et al., 2004). In case RocG had been the original GDH in *B. subtilis*, the bacterium would not have been able to gain energy from glutamate degradation in the absence of arginine or other glutamate precursors. Second, the low affinity of the active GudB$^+$ enzyme for glutamate prevents degradation under glutamate limiting conditions (Gunka et al., 2010). Thus, harboring only the GDH GudB$^+$ *B. subtilis* would have been sufficiently adapted to changing environmental conditions.

11.2.3 GudB$^+$ as a paralog completely compensates loss of RocG

As described above, data about the catabolic activities of the paralogs RocG and GudB$^+$ already exist. Clearly, both proteins are capable of degrading glutamate. Several studies were performed, demonstrating that a *rocG* deletion strain synthesizing the active GudB$^+$ protein exhibits the same phenotype as the parental *B. subtilis* laboratory strain (Belitsky & Sonenshein, 1998; Commichau et al., 2008; Gunka et al., 2012). These observations led to the hypothesis, that the active GudB$^+$ enzyme not only degrades glutamate, but also regulates the activity of the transcriptional activator GltC. However, it has never before been confirmed experimentally that GudB$^+$ is a trigger enzyme like its paralog RocG.

In this work, trigger enzyme activities of the GudB$^+$ enzyme were demonstrated for the first time (see chapter 8, (Stannek et al., 2015b)). Similar to RocG, GudB$^+$ was shown to interact with GltC in co-purification experiments. Furthermore, evidence was delivered that the GltC activity is regulated in the presence of GudB$^+$ as single GDH, too. This implicates that GudB$^+$ and RocG are indeed paralogs exhibiting the same enzymatic and regulatory functions. For different strain backgrounds the following conclusions can be drawn: As expected, the GudB$^+$ suppressor mutants can completely compensate the loss of *rocG* in the laboratory strain 168. In the wild Marburg parental strain, GltC is probably regulated by direct protein-protein interactions between either GudB$^+$ or RocG, depending on the growth condition. Thus, as GudB$^+$ alone is able to regulate the endogenous glutamate synthesis, the hypothesis of GudB$^+$ to be the original GDH in *B. subtilis* before gene duplication is supported as well.

Trials to pinpoint the exact mechanism of GltC inhibition upon GDH interaction were unsuccessful so far. Purification of RocG-GltC complexes in order to determine the interaction surface between the GDHs and GltC were not possible so far (data not shown). Prevailing models describing

the mechanisms of divergence via gene duplication state that new functions are more likely to be carried by the duplicated gene rather than by the original gene (Soskine & Tawfik, 2010). If RocG evolved mutations after gene duplication to become stably integrated into the genome, also the interaction surface required for GltC binding might have changed compared to the GudB$^+$ protein. As a consequence, the subunits of the RocG/GltC might only be loosely associated and the complex too unstable to be purified. Thus, with the hypothesis of GudB$^+$ being the major GDH in *B. subtilis*, further experiments using the active GudB$^+$ enzyme as regulator of GltC might lead to success. Interaction between the original *B. subtilis* GDH and the transcriptional activator GltC possibly exhibits stronger binding. This could have been especially important for an ancestral *B. subtilis* strain encoding only GudB$^+$.

11.2.4 Glutamate and the GDHs synergistically control GltC activity

Until now, there still have been some discrepancies in the regulation of the GltC activity by the GDHs. Under some conditions, although high amounts of GDH are present within the cells GltC, the transcriptional regulator of the *gltAB* genes, is not fully inhibited by RocG or GudB$^+$ (Stannek et al., 2015b). The consequence is a futile cycle. While the GDHs degrade glutamate, GOGAT at the same time produces glutamate. Comparable observations were already made earlier in a similar genetic context. Contrary to expectations, high-level GltC activity resulting in *gltAB* expression is detectable in a strain overproducing the negative regulator RocG (Belitsky & Sonenshein, 1998, 2004; Gunka et al., 2010). An explanation for this observation was missing so far.

Heretofore, it was speculated that RocG and GudB$^+$ require a co-factor to fully regulate the GltC activity (Gunka et al., 2010). Support for this hypothesis was delivered by the observation that expression of the *gltAB* genes was repressed as soon as arginine was present in the medium. With these findings a Roc pathway-derived metabolite, including arginine, ornithine, proline or glutamate, was regarded to be a likely candidate for a co-factor. Now, the synergistic control of the GltC activity by the GDHs and glutamate was finally verified (see chapter 8, Stannek et al., 2015b). Given the fact that glutamate is the metabolite directly catabolized by RocG and GudB$^+$, it makes physiologically sense that the inhibitory effect of the GDHs on GltC is dependent on the glutamate level itself. Thus, not the product of the reaction regulates the activity of the enzyme, as in the feed-back inhibition of the GS by glutamine (Fisher, 1999; Murray et al., 2013). Instead, the inhibitory effect of the GDHs on GltC is regulated by glutamate, the substrate the GDHs catabolize (Stannek et al., 2015b).

Although the metabolome analyses were performed in a strain synthesizing constant levels of RocG, it can be assumed that also GudB$^+$ regulates the GltC activity in a glutamate-dependent manner. This regulatory circuit might be particularly important for the wild Marburg strain expressing the functional GudB$^+$ enzyme (Zeigler et al., 2008). As already discussed, the active GudB$^+$ enzyme is

constitutively expressed (Gunka et al., 2012). Without another level of regulation GltC would be inhibited to a certain degree by the active GudB$^+$ enzyme under any condition. Thus, with the additional layer of regulation, also in a strain synthesizing only the constitutively expressed GDH GudB$^+$ the glutamate homeostasis is ensured.

Still, the exact mechanism of how high glutamate levels contribute to GltC inhibition by the GDHs has to be resolved. A model can be predicted by including the results of studies analyzing RocG mutant proteins effected in their ability to control GltC activity. In a strain harboring the wild-type RocG protein, glutamate has to be present in sufficient amounts for inactivation of GltC activity by RocG (Stannek et al., 2015b). In contrast, in strains encoding so-called superrepressor RocG proteins the GltC activity is low in arginine or glutamate containing medium. In medium without glutamate or arginine but with glucose, GltC is still active. Superrepressors are characterized by a lower affinity for glutamate and a weaker catabolic activity (Gunka et al., 2010). These observations allow the following hypothesis: Glutamate regulates the affinity of RocG for GltC, probably through a conformational change of the GDH enabling binding of GltC. Only if enough glutamate is present within the cell, enough GDHs are in the conformational state allowing inhibition of GltC (Stannek et al., 2015b). This idea is supported by the observed regulation of GltC in the strain harboring the superrepressor RocG. Due to the weaker catabolic activity, the RocG mutant proteins are upon binding of glutamate probably longer in the conformational state allowing GltC binding and inhibition. Thus, also under lower glutamate concentrations within the cell GltC will be inhibited by a superrepressor protein RocG. A second reason for the high inhibition of GltC in medium containing glutamate and glucose can be that the superrepressors are already in a conformation allowing them to interact with GltC also under low glutamate levels. In order to corroborate this assumption, a crystal structure of the superrepressor RocG proteins is required to visualize a changed conformation compared to WT-RocG. A further possibility to elucidate which role glutamate still plays for the superrepressors and its GltC binding activity is the following experiment. A strain harboring the Strep-GltC fusion protein and a constitutively expressed superrepressor RocG variant could be analyzed for its GltC binding capacity under changing glutamate concentrations in a low concentration range. The GltC activity of this strain grown under different glutamate levels should then correlate to the binding capacity of the superrepressor RocG protein detected in the co-purification experiment. Probably, a much lower amount of glutamate is required by the strains harboring superrepressor RocG proteins to inhibit GltC than by the wild-type strains.

However, the synergistic control of glutamate biosynthesis by GDHs and glutamate does not thoroughly explain how a strain overproducing an active GDH can grow with the low amounts of internal glutamate determined in the metabolome analyses (Stannek et al., 2015b). Grown in minimal medium containing low amounts of arginine, the GOGAT is highly expressed. Without further

regulation, RocG would constantly degrade glutamate resulting in a futile cycle. The same would apply for a strain synthesizing the active GDH GudB$^+$ grown on minimal medium containing glucose. This provokes the suspicion of the existence of a second regulatory variable involved in the regulation of RocG activity and/or GltC binding. In the presence of the preferred carbon source glucose and under comparatively low levels of glutamate, this factor seems to inhibit RocG or GudB$^+$ (see Fig. 11.2). Conceivable is an inhibition of the GDH through a metabolite, co-factor or protein produced or involved in the degradation of glucose. Hypothetically, the ratio of $NADH_2/NAD^+$ influences the GDH activity. $NADH_2$ produced during glycolysis might change the $NADH_2/NAD^+$ ratio to a level resulting in inhibition of the GDH. NADH-dependent inhibition of the GDH has already been observed for the catabolic activity of the *Streptomyces clavuligerus* GDH. Similarly, the reductive amination reaction of *S. clavuligerus* is inhibited by NAD$^+$ (Miñambres et al., 2000).

The development of an adequate screening system can help to identify such a co-factor. The same strain used for the metabolome analyses can be subject to transposon mutagenesis and plated on minimal medium containing glucose and moderate levels of glutamate (see chapter 8, Stannek et al., 2015b). Cells in which the unknown co-factor inhibits RocG in its binding activity to GltC should form blue colonies on the plate. In contrast, cells in which the negative effector of RocG/GltC binding is missing, should form light blue colonies. Together with glutamate as co-factor representing the nitrogen status of the cell, the finding of a second co-factor signaling the carbon status of the cell would complete the picture of the complex regulatory mechanism of the glutamate metabolism further.

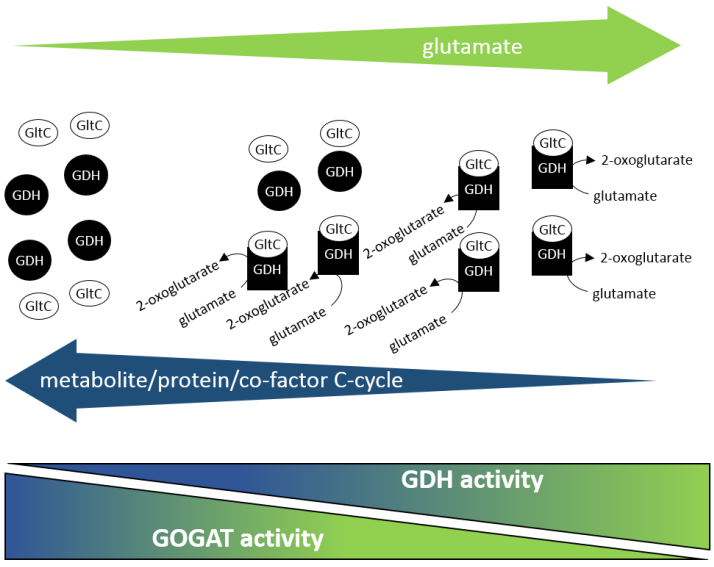

Fig. 11.2 Regulation of enzymatic and regulatory activity of the GDH
Under low intracellular glutamate concentrations the transcriptional activator GltC is not inhibited by the GDH, resulting in expression of the GOGAT. Thus, glutamate can be synthesized. An unknown second factor negatively regulates the enzymatic activity of the GDH under low glutamate concentrations. With increasing glutamate concentrations, the GDH becomes active and indirectly represses *gltAB* expression by binding GltC (adapted from Stannek et al., 2015b).

The GDHs of *B. subtilis*, as stated in chapter 2.3, are strictly catabolic enzymes in contrast to *E. coli* and other Bacilli as *Bacillus megaterium* (Hemmilä & Mäntsälä, 1978; Belitsky & Sonenshein, 1998; Reitzer, 2003; Gunka et al., 2010). Taking up the approach of isolating monofunctional *rocG* mutant alleles, it would be an interesting trial to isolate *B. subtilis* GDH enzymes capable of performing not only the catabolic reaction but also the anabolic reaction. This can be achieved by a screening system aiming at the detection of anabolically active GDHs. Therefore, a glutamate auxotrophic *gtlAB* mutant can be transformed with plasmids from a mutant library harboring mutagenized *rocG* genes (Gunka et al., 2010) and plated on minimal medium containing succinate as only carbon source. Colonies able to grow should harbor a RocG enzyme active in glutamate synthesis as no exogenous glutamate is present for uptake. Analyzing growth of *B. subtilis* strains harboring an anabolically active GDH could give hints about the reason for encoding a catabolically active GDH.

11.3 Glutamate availability and GDH activity determine fitness of *B. subtilis*

The parental wild-type strain of the *B. subtilis* laboratory strain 168 synthesizes two functional GDHs, RocG and GudB^{+} (Zeigler et al., 2008). It was speculated that the mutation in the *gudB^{CR}* gene of the *B. subtilis* laboratory strain 168 emerged and became fixed in the genome during growth on minimal medium lacking glutamate as nitrogen source (Burkholder & Giles, 1947). In principle, the acquirement of the perfect direct repeat in the original *gudB^{+}* gene is a pseudogenization which occurred after the gene duplication (see chapter 11.2 and Zhang, 2003). Until now, the reason for the stable inheritance of the *gudB^{CR}* gene in the strain 168 under laboratory conditions was not known. However, it has been suggested that *B. subtilis* cells encoding only the highly regulated r*ocG* gene might have a growth advantage under certain conditions compared to cells encoding the constitutively transcribed *gudB^{+}* in addition. In this work, intraspecies-competition experiments of bacterial strains encoding *rocG* and *gudB^{CR}* or *rocG* and *gudB^{+}* revealed that the fitness of the cells is determined by the GDH activity and the glutamate concentration available (see chapter 3 and 4; Gunka et al., 2013; Stannek et al., 2014). The strong growth advantage observed in the laboratory strain 168 in medium without exogenous glutamate most likely results from lower glutamate-degrading enzymatic activity of the strain. This implies, that in strain 168 under glutamate limited conditions, the endogenously formed glutamate can be used for important anabolic reactions, e.g. the synthesis of other amino acids. In a strain synthesizing the active GudB^{+}, it can be assumed that the endogenously synthesized glutamate is used for carbon metabolism. Therefore, with the pseudogenization of the *gudB* gene the *B. subtilis* strain 168 became perfectively adapted to the laboratory conditions. Concordantly, it was demonstrated that mutants accumulate which have inactivated the only active GDH when cells synthesizing the constantly expressed GudB^{+} enzyme are grown in medium lacking glutamate (Gunka et al., 2013). These experiments demonstrate evolution in a fast-mode. Selective conditions allow

faster growth of fitter mutants, explaining the stable inheritance of the *gudB*^{CR} in the laboratory strain 168.

11.3.1 Birds of a feather flock together- Heteromultimer formation of GudB^{+} and RocG

Recently, formation of a RNase J1 and J2 heteromeric complex, which is likely to be the principle form of the enzymes in *B. subtilis*, was shown *in vivo* and *in vitro*. Interestingly, the RNase J1/J2 complex has altered endonucleolytic cleavage site preference and activities compared to the individual enzymes displaying similar activities and specificities (Mathy et al., 2010). In the Trypanosomatids, protozoan parasites, a unique regulatory strategy by heteromultimer formation by paralogs is available. Meanwhile two paralogous gene pairs were found encoding for S-adenosylmethionine decarboxylase and deoxyhypusine synthase, both of them are involved in polyamine metabolism. One of each paralog is enzymatically inactive. Heteromultimer formation between the inactive/dead paralog and the active counterpart exhibiting low enzymatic activity results in 1200- or 3000-fold increase of activity of the S-adenosylmethionine decarboxylase and deoxyhypusine synthase, respectively (Willert et al., 2007; Nguyen et al., 2013).

Transferring the formation of heteromultimers on the GDHs of *B. subtilis*, several implications are conceivable depending on the interaction between the inactive GDH GudB^{CR}, its active variant GudB^{+} and the active GDH RocG. As discussed earlier, besides the active GDH RocG, the inactive highly unstable protein GudB^{CR} is constitutively expressed (Gunka et al., 2012). Formation of a GudB^{CR}/RocG heteromultimeric complex in the *B. subtilis* laboratory strain 168 can have different consequences. First, heterocomplex formation of RocG and GudB^{CR} would probably result in a nonfunctional GDH hexamer due to the misfolded GudB^{CR} enzyme. Second, as the inactive GudB^{CR} variant is rapidly degraded, interaction of RocG with GudB^{CR} might lead to degradation of RocG along with GudB^{CR}. Similar to the different activity of the RNase J1/J2 complex, a GudB^{+}/RocG hexamer could exhibit an altered efficiency as well (Mathy et al., 2010). Heretofore, no data demonstrating heteromultimer formation of the paralogous GDHs encoded in *B. subtilis* is available. Using bacterial-two hybrid analyses this question was addressed in the present work (see chapter 8, Stannek et al., 2015b). The bacterial-two hybrid analyses revealed an interaction between the active GDHs RocG and GudB^{+}. Subsequent *in vivo* co-purification experiments corroborated these finding (Stannek et al., 2015b). Hence, both experiments showed for the first time heterohexamer formation of the active GDHs RocG and GudB^{+}, which are encoded in the wild *B. subtilis* Marburg strains, for example (Zeigler et al., 2008; Stannek et al., 2015b). Presumably, not only the homohexamers but also the heterohexameric complex regulates the DNA-binding activity of the transcriptional activator GltC. Whether the specific activity of a GudB^{+}/RocG hexameric GDH differs from the homohexameric proteins as shown for RNase J1/J2 remains to be elucidated in further experiments (Mathy et al., 2010). In case a different activity

is detectable, it will be interesting to analyze, if homohexamer or heterohexamer formation is prefered in a *B. subtilis* strain synthesizing the two active GDH paralogs.

While the bacterial-two hybrid analyses revealed an interaction between the inactive and the active GudB enzyme, no interaction between GudB^{CR} and RocG was detectable in the analyzed extend (Stannek et al., 2015b). This observation is especially of importance for the laboratory *B. subtilis* strain 168. It is conceivable that the inactive and highly unstable GudB^{CR} protein does not interfere with the activity of the only active GHD RocG *in vivo*.

11.3.2 Proteolysis of the inactive GDH GudB^{CR}

Though the presence of the inactive and probably misfolded GDH GudB^{CR} does apparently not result in unwanted degradation of the active GDH RocG in the *B. subtilis* laboratory strain (see chapter 8, Stannek et al., 2015b), the accumulation of the aberrant folded and non-functional GudB^{CR} protein might interfere with other cellular processes. Indeed, misfolding and aggregation of proteins threatens the cell´s viability. To ensure proteostasis, the maintenance of proteome homeostasis, a variety of proteolytic machineries removes misfolded or aggregated proteins (Hartl et al., 2011). Up to now, the GudB^{CR} degrading protease could not clearly be identified. Only ClpP, the proteolytic subunit of the Clp-proteases, is known to slightly influence the GudB^{CR} stability in cells of stationary growth phase under glucose starvation (Gerth et al., 2008). This observation delivered first hints for a ClpCP-dependent proteolysis of GudB^{CR}. The ClpCP protease belongs to the Clp proteases of *B. subtilis*. The Clp proteases are composed of the ClpP proteolytic subunit forming a complex with either of the AAA+ ATPases ClpX, ClpC or ClpE (Derré et al., 1999b; Krüger et al., 2000).

In this work, searching for factors involved in the degradation of GudB^{CR} a GFP-based system was used. For the first time two proteins, the arginine kinase McsB and the respective arginine phosphatase YwlE, were found to influence the stability of GudB^{CR}. The active GudB^{+} enzyme, in contrast, is unaffected. In a *mcsB* deletion strain GudB^{CR} is more stable, whereas deletion of the arginine phosphatase YwlE decreased the stability of GudB^{CR} (Stannek et al., 2015a). This observation allowed to set up three different hypotheses providing an explanation for McsB-dependent stabilization of GudB^{CR}. First, as GudB^{CR} is one of the proteins found to be phosphorylated on arginine residues, phosphorylation alone might be a signal targeting GudB^{CR} for degradation (Elsholz et al., 2012). Second, as an adaptor protein for the ClpCP protease, McsB could target GudB^{CR} for ClpCP-dependent degradation. A well-studied example of McsB-dependent protein degradation is the heat-shock induced proteolysis of CtsR by the ClpCP protease (Kirstein et al., 2005). The heat-shock repressor CtsR prevents transcription of the *clpC* operon, encoding inter alia the genes *ctsR*, *mcsB* and *clpC* (Derré et al., 1999a). Third, McsB might indirectly influence the stability of GudB^{CR} via a general activation of the ClpCP protease. Latest experiments discovered that two arginine residues of the ClpC

ATPase have to be phosphorylated by the arginine kinase McsB for McsB-dependent ClpC activity (Elsholz et al., 2012).

Phosphorylation of the four arginine residues found in phosphoproteomic studies could rapidly be excluded to influence the stability of GudB^{CR} (Elsholz et al., 2012; Stannek et al., 2015a). A GudB^{CR} variant with replaced arginine phosphorylation sites is still degraded in an McsB-dependent manner (Stannek et al., 2015a). On the one hand, a potential impact on the physiology of *B. subtilis* due to arginine phosphorylation, as shown for other proteins, is questionable regarding GudB^{CR} (Elsholz et al., 2012; Schmidt et al., 2014). On the other hand, these observations underline the involvement of McsB in the degradation of GudB^{CR}. Recent studies also denied the requirement of CtsR phosphorylation by McsB on arginine residues for degradation (Elsholz et al., 2010b). Contradictory to previous studies, the study additionally detected that McsB does not regulate CtsR activity *per se* (Fuhrmann et al., 2009). Instead activated auto-phosphorylated McsB serves as an adaptor protein for the ClpCP protease and captures non-functional CtsR proteins which lost their DNA-binding capacity during heat stress (Elsholz et al., 2010b). However, no protein-protein interaction between McsB and GudB^{CR} was detected in B2H analyses (see chapter 9). Thus, the role of McsB as adaptor protein for GudB^{CR} proteolysis can be excluded. As no McsB-GudB^{CR} interaction was present, a possible physiological relevance of the phosphorylated arginine residues of GudB^{CR} is doubtful as well (Elsholz et al., 2012). Furthermore, no interaction between arginine phosphatase YwlE and GudB^{CR} was detectable. This observation indicates that the proteolytic process of GudB^{CR} is not regulated through a protein-protein interaction between GudB^{CR} and McsB or YwlE.

Consistent with the previously observed effect of ClpP on the GudB^{CR} stability, GudB^{CR} and the ClpC ATPase interacted in the B2H analyses (see chapter 9; Gerth et al., 2008). However, also GudB^{+}, the active variant of the enzyme, interacted with ClpC and to a weaker intensity with ClpP. Hence, both enzymes seem to be degraded in a ClpCP-dependent manner. Taken together, the observations favor the last hypothesis (see Fig. 11.3). McsB in its autophosphorylated form activates the ClpCP protease by phosphorylating two arginine residues of the ClpC ATPase (Elsholz et al., 2012). YwlE dephosphorylates ClpC resulting in inactivation of the ATPase. Assuming that indeed phosphorylated ClpC is required for the GudB^{CR} proteolysis, deletion of *mcsB* impairs GudB^{CR} proteolysis. This would explain the increase of GFP-GudB^{CR} stability in a *mcsB* deletion strain. Contrariwise, GudB^{CR} is proteolyzed more rapidly in an *ywlE* mutant, because the ClpCP protease is kept in its active state. Moreover, it is known that YwlE dephosphorylates active McsB. The resultant inactivation of McsB in turn prevents McsB-dependent ClpC activation (Kirstein et al., 2005; Elsholz et al., 2011a).

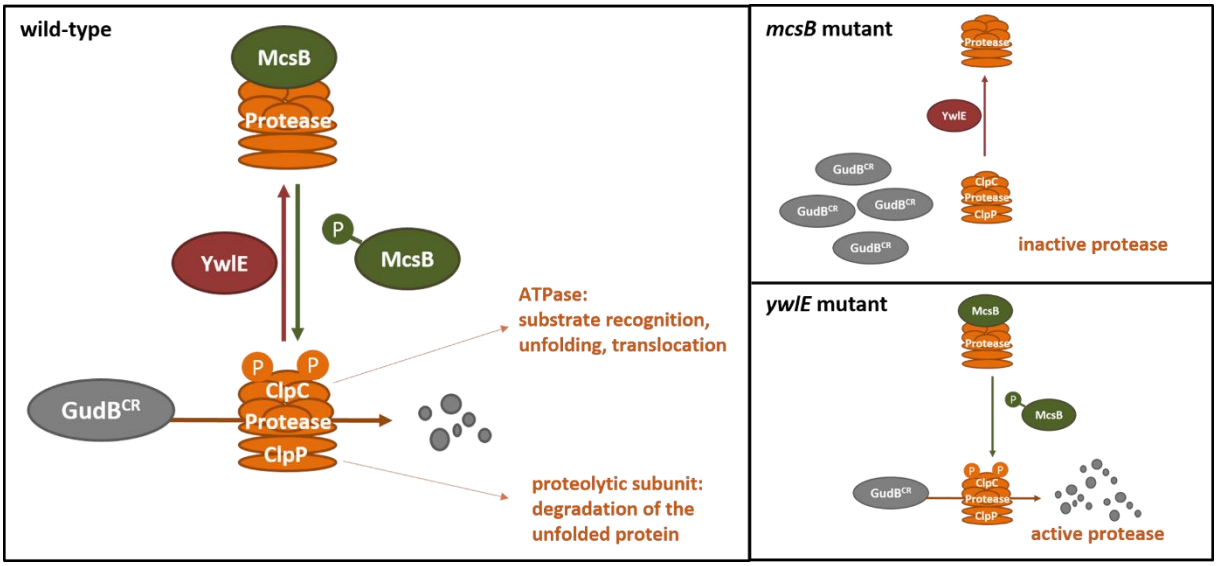

Fig. 11.3 Proteolysis of GudB^{CR} by the ClpCP proteases in a McsB and YwlE dependent manner
In wild-type *B. subtilis* cells the auto-phosphorylated arginine kinase McsB activates the ClpC ATPase by phosphorylating two arginine residues. Subsequently, GudB^{CR} is unfolded by ClpC and degraded by ClpP forming the proteolytic subunit of the protease. The arginine phosphatase YwlE dephosphorylates ClpC, resulting in inactivation of ClpC activity. In a *mcsB* mutant, ClpC cannot be activated by McsB. Thus, the GDH is more stable. In contrast, in a *ywlE* mutant GudB^{CR} is degraded more rapidly as ClpC can be activated by McsB, but not inactivated through dephosphorylation.

Especially under conditions of nutrient starvation during entry into stationary phase GudB^{CR} was shown to be rapidly degraded (Gerth et al., 2008; Gunka et al., 2012; Stannek et al., 2015a). In *B. subtilis*, ClpP is the major peptidase responsible for the degradation of proteins accumulating under stress or starvation conditions. Hence, the hypothesized ClpCP-dependent degradation of GudB^{CR} fits perfectly to already existing data. Additionally, ClpP fulfills essential function in intracellular protein quality control (Kock et al., 2004). Thus, the ClpCP protease would be suitable for the degradation of the inactive GudB^{CR} GDH.

Still, it is striking that no adaptor protein has been identified yet. It is possible that no adaptor protein is required for the GudB^{CR} degradation. Assumably the presence of the perfect direct repeat present in the region encoding the active center of the protein results in misfolding of GudB^{CR} (Belitsky & Sonenshein, 1998; Gunka et al., 2012). Often, upon protein misfolding, hydrophobic residues normally buried inside the protein´s core become exposed on the surface. Maybe these hydrophobic residues are directly recognized and the misfolded protein is captured by the ATPase ClpC. Upon translocation, the unfolded protein is degraded inside the proteolytic cavity. Such mechanisms are already well-studied in eukaryotes, for instance (Fredrickson et al., 2011). However, this would only be an appropriate explanation for the proteolysis of the inactive GudB^{CR} protein, not for the correctly folded GudB^{+}. Thus, it is still interesting for further studies to analyze if other ClpC adaptor proteins, for instance MecA, are involved in the GudB^{CR}/GudB^{+} proteolysis. In the first case, the adaptor protein MecA is well-known for its involvement in competence development of *B. subtilis*. However, MecA is generally required for ClpC oligomer assembly and for the degradation of misfolded or aggregated

proteins (Schlothauer et al., 2003; Kirstein et al., 2006). Finally, a further function of McsB and YwlE in the proteolysis of GudB^{CR} cannot be ruled out and may be detected in the future.

11.4 Model for the mutagenesis process of the direct repeat

Although the physiological relevance for the activation of the inactive *gudB^{CR}* gene is well-understood, the mechanism behind the mutagenesis process is ambiguous. A mutation frequency of 10^{-4} of the direct repeat in the *gudB^{CR}* gene under selective growth conditions is the highest mutation frequency observed for *B. subtilis* so far (Gunka et al., 2012). The high mutation frequency is in line with the observation from numerous other studies demonstrating that direct repeat sequences are mutational hotspots in the genome of many organisms. As shown for the direct repeat in the *gudB^{CR}* gene in *B. subtilis*, the hypermutability of direct repeat sequences can be regarded to promote evolvability of bacteria (Bichara et al., 2006; Zhou et al., 2014a).

Earlier, the mutation frequency decline factor Mfd was already shown to be important for the high mutation rate of the *gudB^{CR}* gene observed in a *rocG* deletion strain grown on complex medium (Gunka et al., 2012). Additionally, it was observed that the direct repeat of *gudB^{CR}* is more unstable in transcribed regions compared to untranscribed regions (Thiele, 2013; Dormeyer et al., 2015). As Mfd removes stalled RNA polymerases from the template strand during transcripton, the process of transcription itself was regarded as a factor influencing the mutation rate of the direct repeat. Here, indeed a tendency of correlation between the transcription rate of the *gudB^{CR}* gene and the mutation frequency of the direct repeat was detected (see chapter 10). Besides Mfd, also the two paralogous RNaseH enzymes RNase HI and RNase HI as well as the translesion polymerase PolY1 have previously been found to be involved in the direct repeat mutagenesis process (Thiele, 2013).

Currently, the DNA strand slippage is a proposed model for the underlying mechanism of direct repeat instabilities (Streisinger et al., 1966; Bierne et al., 1997; Zhou et al., 2014a). Interpreting the roles of the idenfied factors involved in the mutagenesis process of the direct repeat in *gudB^{CR}*, we propose the following model. In case the strand slippage model also accounts for the direct repeat in *gudB^{CR}*, a consequence might be the formation of a hairpin structure. During transcription, the RNA polymerase probably stalls due to the hairpin structure. In consequence, the nascent RNA forms RNA:DNA heterocomplexes, so called R-loops (Skourti-Stathaki & Proudfoot, 2014). Subsequently, Mfd displaces the stalled RNA-polymerase from the template strand and RNase H enzymes cleave the RNA from the RNA:DNA hybrids. Until now, the factor excising preferentially the first part of the direct repeat is still unknown. However, it can be assumed that the translesion polymerase PolY1 fills the gap between the strands. Indeed, in a very recent study, first evidence was delivered that Mfd and PolY1 function in the same pathway. Both proteins are required for the increased mutation rate of the genes encoded on the lagging strand (Million-Weaver et al., 2015).

11.5 Perspectives

In this work, the physiological relevance of the inactive and active GDHs encoded in the *B. subtilis* genome was revealed. Furthermore, glutamate being the co-factor required for the GDH to inhibit GltC was positively proven. Still, there are open questions regarding the exact control mechanism of glutamate biosynthesis. For a better understanding of how the GDH-GltC interaction results in inhibition of *gltAB* expression, co-crystallization experiments would be of great value. Moreover, the determination of the interaction surface between the GDH and GltC is an interesting project for the future.

Obviously, a synergistic control of glutamate and the GDHs on GltC activity ensures the synthesis of the major amino group donor once required. To avoid a futile cycle of glutamate synthesis and degradation, the presence of another unknown factor controlling the activity of the catabolic GDHs is supposed. Identification of this factor using an appropriate screening system is a worthwhile approach. Once identified, it would be interesting to study the interplay of glutamate and the respective yet unknown factor on the GDH-GltC interaction in more detail. *In vitro* experiments, for instance isothermal titration calorimetry, with functional GDH and GltC enzymes, could deliver useful insights into the regulatory mechanisms. Therefore, it is required to purify a functional GDH able to regulate GltC also under *in vitro* conditions. Additionally, electrophoretic mobility shift assays performed with GltC, the GDHs and the two cofactors required for regulation could dissect how GltC-binding to the *gltAB* promotor region is affected under different conditions.

12 Literature

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13 Appendix

13.1 *Bacillus subtilis* strains

Tab. S 13.1 *B. subtilis* strains.

Name	Genotype	Construction	Author
168	*trpC2*	laboratory collection	
BP9	*trpC2 amyE::(gltA-lacZ aphA3) ΔrocG::cat gudB^{CR} -gfp spc*	pBP1→GP754	Gunka et al., 2013
BP10	*trpC2 amyE::(gltA-lacZ aphA3) ΔrocG::cat gudB^{+}-gfp spc*	pBP1→GP801	Gunka et al., 2013
BP22	*trpC2 ΔgudB^{CR}::aphA3 rocG::Tn10 spc amyE::(gfp-gudB^{CR} cat)*	pBP8→ GP1161	Gunka et al., 2013
BP23	*trpC2 ΔgudB^{CR}::aphA3 rocG::Tn10 spc amyE::(gfp-gudB^{+} cat)*	pBP9→ GP1161	Gunka et al., 2013
BP25	*trpC2 ΔgudB^{CR}::aphA3 amyE::(gfp-gudB^{CR} cat)*	pBP8 → GP1160	Stannek et al., 2015a
BP26	*trpC2 ΔgudB^{CR}::aphA3 amyE::(gfp-gudB^{+} cat)*	pBP9 → GP1160	Stannek et al., 2015a
BP31	*trpC2 gudB^{CR} rocG::Tn10 spc amyE::(gudB$^{CR}{}_{SacI}$ gfp cat)*	pBP11→ GP747	Gunka et al., 2013
BP40	*trpC2 amyE::(yfp cat)*	pBP26→168	Gunka et al., 2013
BP41	*trpC2 amyE::(cfp cat)*	pBP27→168	Gunka et al., 2013
BP42	*trpC2 gudB^{+} amyE::(gltA-lacZ aphA3) ΔrocG::cat*	The same as GP801	Gunka et al., 2013
BP44	*trpC2 gudB^{+} (T896G) amyE::(gltA-lacZ aphA3) ΔrocG::cat*	Derived from GP801	Gunka et al., 2013
BP46	*trpC2 gudB^{+} (Δ673-738) amyE::(gltA-lacZ aphA3) ΔrocG::cat*	Derived from GP801	Gunka et al., 2013
BP47	*trpC2 gudB^{+} (C1222^{ins} T1223^{ins}) amyE::(gltA-lacZ aphA3) ΔrocG::cat*	Derived from GP801	Gunka et al., 2013
BP48	*trpC2 gudB^{+} (Δ766) amyE::(gltA-lacZ aphA3) ΔrocG::cat*	Derived from GP801	Gunka et al., 2013
BP52	*trpC2 gudB^{+} amyE::(cfp cat)*	BP41→GP804	Gunka et al., 2013
BP69	*trpC2 ΔgudB^{CR}::aphA3 mcsB::Tn10 spc amyE::(gfp-gudB^{CR} cat)*	pIC333 → BP25	Stannek et al., 2015a
BP74	*trpC2 ΔgudB^{CR}::aphA3 ywlE::tet amyE::(gfp-gudB^{CR} cat)*	GP1459 → BP25	Stannek et al., 2015a
BP75	*trpC2 ΔgudB^{CR}::aphA3 ywlE::tet amyE::(gfp-gudB^{+} cat)*	GP1459 → BP26	Stannek et al., 2015a
BP98	*trpC2 ΔgudB^{CR}::aphA3 amyE::(gfp-gudB^{CR} cat) clpC::spc*	*clpC::spc* → BP25	Stannek et al., 2015a
BP99	*trpC2 ΔgudB^{CR}::aphA3 amyE::(gfp-gudB^{CR} cat) clpP::tet*	*clpP::tet* → BP25	Stannek et al., 2015a
BP156	*trpC2 gudB^{+} amyE::(yfp cat)*	pBP26→ GP804	Gunka et al., 2013

Name	Genotype	Construction	Author
BP191	*trpC2 amyE::(P_{alf2}-lacZ cat)*	pBP162→168	This study
BP193	*trpC2 amyE::(P_{alf4}-lacZ cat)*	PBP164→168	This study
BP194	*trpC2 amyE::(P_{alf4}-gudB^{CR} cat)*	pBP167→ BP442	This study
BP195	*trpC2 amyE::(P_{alf4}-gudB^{CR} cat) rocG::Tn10 spc*	GP747 →BP194	This study
BP196	*trpC2 amyE::(P_{alf2}-gudB^{CR} cat)*	pBP166→ BP442	This study
BP197	*trpC2 amyE::(P_{alf2}-gudB^{CR} cat) rocG::Tn10 spc*	GP747 →BP196	This study
BP200	*trpC2 ΔgudB^{CR}::cat*	GP27 → 168	Dormeyer et al., 2015
BP201	*trpC2 ΔgudB^{CR}::cat lacA::(P^{CR} gudB^{+} aphA3)*	pBP168 → BP200	Dormeyer et al., 2015
BP202	*trpC2 ΔgudB^{CR}::cat lacA::(P^{CR} gudB^{+} gfp aphA3)*	pBP169 → BP200	Dormeyer et al., 2015
BP205	*trpC2 ΔgudB^{CR}::cat rocG::Tn10 spc lacA:: (P^{CR} gudB^{+} aphA3)*	GP747 → BP201	Dormeyer et al., 2015
BP206	*trpC2 ΔgudB^{CR}::cat rocG::Tn10 spc lacA::(P^{+} gudB aphA3)*	BP205 spontaneous on SP	Dormeyer et al., 2015
BP207	*trpC2 ΔgudB^{CR}::cat rocG::Tn10 spc lacA::(P^{CR} gudB^{+} gfp aphA3)*	GP747 → BP202	Dormeyer et al., 2015
BP208	*trpC2 ΔgudB^{CR}::cat rocG::Tn10 spc lacA::(P^{+} gudB^{+} gfp aphA3)*	BP207 spontaneous on SP	Dormeyer et al., 2015
BP213	*trpC2 ΔgudB^{CR}::cat lacA::(P^{CR} gudB^{+} pdxST gfp aphA3)*	pBP172 → BP200	Dormeyer et al., 2015
BP216	*trpC2 ΔgudB^{CR}::cat lacA::(P^{CR} gudB^{+} pdxST gfp aphA3) rocG::Tn10 spc*	GP747 → BP213	Dormeyer et al., 2015
BP219	*trpC2 ΔgudB^{CR}::cat lacA::(P^{+} gudB^{+} pdxST gfp aphA3) rocG::Tn10 spc*	BP216 spontaneous on SP	Dormeyer et al., 2015
BP220	*trpC2 ΔgudB::cat rocG::Tn10 spc amyE::(gltA-lacZ aphA3) gltAB::tet*	GP807→ GP28	Stannek et al., 2015b
BP230	*trpC2 ΔgudB^{CR}::aphA3 amyE::(gfp-gudB^{CR} R56K, R83K, R421K, R423K cat)*	pBP187 → GP1160	Stannek et al., 2015a
BP231	*trpC2 ΔgudB^{CR}::aphA3 mcsB::Tn10 spc amyE::(gfp-gudB^{CR} R56K, R83K, R421K, R423K cat)*	BP69 → BP230	Stannek et al., 2015a
BP311	*trpC2 ΔgudB^{CR}::aphA3 mcsB::Tn10 spc amyE::(gfp-gudB^{+} cat)*	pBP45 → BP26	Stannek et al., 2015a
BP401	*trpC2 ΔgudB^{CR}::kan amyE::P^{+}-gudB^{CR} cat*	pBP301→ GP1160	Thiele, 2013
BP405	*trpC2 ΔgudB^{CR}::kan ΔrocG::spec amyE::P^{+}-gudB^{CR} cat*	GP747→ BP401	Thiele,2013
BP411	*trpC2 ΔgudB^{CR}::kan amyE::P^{-}-gudB^{CR} cat*	pBP303→ GP1160	Thiele, 2013
BP412	*trpC2 ΔgudB^{CR}::kan ΔrocG::spec amyE::P^{-}gudB^{CR} cat*	GP747→ BP411	Thiele, 2013

Name	Genotype	Construction	Author
BP429	*trpC2 amyE ::(P⁺–lacZ cat)*	pBP300 →168	Dormeyer et al., 2015
BP442	*trpC2 ΔgudB^{CR}::aphA3*	LFH→168	Thiele, 2013
BP472	*trpC2 amyE ::(P^{CR}–lacZ cat)*	pBP311→168	Dormeyer et al., 2015
GP27	*trpC2 ΔgudB^{CR}::cat amyE::(gltA-lacZ aphA3)*		Commichau et al., 2007b
GP28	*trpC2 ΔgudB^{CR}::cat rocG::Tn10 spc amyE::(gltA-lacZ aphA3)*		Commichau et al., 2007b
GP32	*trpC2 rocG::Tn10 spc amyE::(gltA-lacZ aphA3) gudB⁺*		Commichau et al., 2007b
GP342	*trpC2 amyE::(gltA-lacZ aphA3)*		Wacker et al., 2003
GP747	*trpC2 rocG::Tn10 spc*		Commichau et al., 2007b
GP753	*trpC2 rocG::Tn10 spc gudB⁺*		Gunka et al., 2012
GP754	*trpC2 amyE::(gltA-lacZ aphA3) ΔrocG::cat*		Commichau et al., 2007a
GP801	*trpC2 gudB⁺ amyE::(gltA-lacZ aphA3) ΔrocG::cat*		Commichau et al., 2008
GP804	*trpC2 gudB⁺ amyE::(gltA-lacZ aphA3)*		Commichau et al., 2008
GP807	*trpC2 ΔgltAB::tet*	LFH→ 168	Stannek et al., 2015b
GP1101	*trpC2 amyE::(P_{gudB}-lacZ)*		Gunka et al., 2012
GP1160	*trpC2 ΔgudB^{CR}::aphA3*		Gunka et al., 2012
GP1161	*trpC2 ΔgudB^{CR}::aphA3 rocG::Tn10 spc*		Gunka et al., 2012
GP1163	*trpC2 ΔgudB^{CR}::aphA3 rocG::Tn10 spc amyE::(gudB^{CR} cat)*		Gunka et al., 2012
GP1165	*trpC2 ΔgudB^{CR}::aphA3 rocG::Tn10 spc amyE::(gudB⁺ cat)*	GP1163 spontaneous on SP	This study
GP1459	*trpC2 ΔywlE::tet*	BDO01 → 168	Stannek et al., 2015a

13.2 *Escherichia coli* strains

Tab. S 13.2 *E. coli* strains.

***E. coli* strain**	**Genotype**	**Author**
DH5a	*recA1 endA1 gyrA96 thi hsdR17r_K- m_K+relA1 supE44 Φ80ΔlacZΔM15 Δ(lacZYAargF)U169*	Sambrook et al., 1989
XL1-blue	*λ⁻ endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB⁺ lacIq Δ(lacZ)M15] hsdR17(r_K- m_K+)*	Stratagene

BTH101	*F⁻ cya-99 araD139 galE15 galK16 rpsL1 (Str^R) hsdR2 mcrA1 mcrB1*	Karimova et al., 1998

13.3 Plasmids

Tab. S 13.3 Plasmids.

Plasmid	Vector	Construction	Author
pAC5		Integration of DNA into the *B. subtilis amyE* locus	Martin-Verstraete et al., 1992
pAC6		Integration of DNA into the *B. subtilis amyE* locus	Stülke et al., 1997
pBP1	pGP1870 (BamHI/SalI)	*gudB^{CR}* (BamHI/SalI) KG125/KG126	Gunka et al., 2013
pBP4	pAC5 (EcoRI/BamHI)	*P$_{gudB}$^{CR} –gudB^{CR}* (EcoRI/BamHI) KG184/KG185	Stannek et al., 2015a
pBP7	pAC5 (EcoRI/BamHI)	*P$_{gudB}$* (MfeI/BamHI) KG188/KG189	Gunka et al., 2013
pBP8	pBP7 (EcoRI/BamHI)	*gfp-gudB^{CR}* (EcoRI/BamHI) KG190/KG180 (*gfp*), KG181/KG92 (*gudB^{CR}*)	Gunka et al., 2013
pBP9	pBP7 (EcoRI/BamHI)	*gfp-gudB^{+}* (EcoRI/BamHI) KG190/KG180 (*gfp*), KG181/KG92 (*gudB*)	Gunka et al., 2013
pBP11	pAC5 (EcoRI/BamHI)	*gudB^{CR}$_{SacI}$-gfp* (EcoRI/BamHI) ST1/KG198/KG197	Gunka et al., 2013
pBP26	pBP7 (EcoRI/BamHI)	*yfp* (MfeI/BglII) KG201/KG208	Gunka et al., 2013
pBP27	pBP7 (EcoRI/BamHI)	*cfp* (MfeI/BglII) KG199/KG206	Gunka et al., 2013
pBP45		transposon plasmid *mcsB*	Stannek et al., 2015a
pBP106	pGP882 (BglII/SmaI)	*aphA3* (BglII/SmaI) KG47/FC200	Dormeyer et al. 2015
pBP162	pac6 (EcoRI/BamHI)	*P$_{alf2}$* (EcoRI/BamHI) LS23/LS24	This study
pBP164	pac6 (EcoRI/BamHI)	*P$_{alf4}$* (EcoRI/BamHI) LS27/LS28	This study
pBP166	pac5 (EcoRI/BamHI)	*P$_{alf2}$-gudB^{CR}* (BamHI/EcoRI) LS29/LS31	This study
pBP167	pac5 (EcoRI/BamHI)	*P$_{alf4}$-gudB^{CR}* (BamHI/EcoRI) LS30/LS31	This study
pBP168	pBP106 (PstI/EcoRI)	*P^{CR} gudB^{+}* (MfeI/PstI) LS32/LS33	Dormeyer et al. 2015

Plasmid	Vector	Construction	Author
pBP169	pBP168 (XbaI/EcoRI)	*gfp$_{mono}$* (XbaI/EcoRI) LS34/LS35	Dormeyer et al. 2015
pBP172	pBP169 (BglII/SalI)	*pdxST* (BglII/SalI) LS36/LS37	Dormeyer et al. 2015
pBP173	pUT18 (XbaI/EcoRI)	*gudB^{CR}* (XbaI/EcoRI) LS42/LS47	Stannek et al., 2015b
pBP174	pUT18C (XbaI/EcoRI)	*gudB^{CR}* (XbaI/EcoRI) LS42/LS46	Stannek et al., 2015b
pBP175	pkT25 (XbaI/EcoRI)	*gudB^{CR}* (XbaI/EcoRI) LS42/LS46	Stannek et al., 2015b
pBP176	pUT18 (XbaI/EcoRI)	*gudB^{+}* (XbaI/EcoRI) LS42/LS47	Stannek et al., 2015b
pBP177	pUT18C (XbaI/EcoRI)	*gudB^{+}* (XbaI/EcoRI) LS42/LS46	Stannek et al., 2015b
pBP178	pkT25 (XbaI/EcoRI)	*gudB^{+}* (XbaI/EcoRI) LS42/LS46	Stannek et al., 2015b
pBP179	pGP380	Strep-*gudB^{+}* (BamHI/PstI) LS64/KG136	Stannek et al., 2015b
pBP183	pBQ200 (BamHI/PstI)	*ywlE* (BamHI/PstI) LS92/LS93	Stannek et al., 2015a
pBP184	pAC5 (EcoRI/BamHI)	*gudB^{CR}* (R56K R83K R421K R423K) (EcoRI/BamHI) KG188/LS94/LS95/LS96	Stannek et al., 2015a
pBP186	pBQ200 (BamHI/PstI)	*mcsB* (BamHI/PstI) LS97/LS98	Stannek et al., 2015a
pBP187	pBP7 (EcoRI/BamHI)	*gfp-gudB^{CR}* (R56K R83K R421K R423K) (EcoRI/BamHI) KG181/LS96/KG180/KG190	Stannek et al., 2015a
pBP188	p25N (XbaI/EcoRI)	*gudB^{CR}* (XbaI/EcoRI) LS42/LS47	This study
pBP189	p25N (XbaI/EcoRI)	*gudB^{+}* (XbaI/EcoRI) LS42/LS47	This study
pBP190	pUT18 (XbaI/EcoRI)	*mcsB* (KpnI/XbaI) LS99/LS100	This study
pBP191	pUT18C (XbaI/EcoRI)	*mcsB* (KpnI/XbaI) LS99/LS100	This study
pBP192	pkT25 (XbaI/EcoRI)	*mcsB* (KpnI/XbaI) LS99/LS100	This study
pBP193	p25N (XbaI/EcoRI)	*mcsB* (KpnI/XbaI) LS99/LS100	This study
pBP194	pUT18 (XbaI/EcoRI)	*ywlE* (KpnI/XbaI) LS101/LS102	This study
pBP195	pUT18C (XbaI/EcoRI)	*ywlE* (KpnI/XbaI) LS101/LS102	This study
pBP196	pkT25 (XbaI/EcoRI)	*ywlE* (KpnI/XbaI) LS101/LS102	This study

Plasmid	Vector	Construction	Author
pBP197	p25N (XbaI/EcoRI)	*ywlE* (KpnI/XbaI) LS101/LS102	This study
pBP198	pUT18 (XbaI/EcoRI)	*clpP* (KpnI/XbaI) LS103/LS104	This study
pBP199	pUT18C (XbaI/EcoRI)	*clpP* (KpnI/XbaI) LS103/LS104	This study
pBP200	pkT25 (XbaI/EcoRI)	*clpP* (KpnI/XbaI) LS103/LS104	This study
pBP201	p25N (XbaI/EcoRI)	*clpP* (KpnI/XbaI) LS103/LS104	This study
pBP202	pUT18 (XbaI/EcoRI)	*clpC* (KpnI/XbaI) LS105/LS106	This study
pBP203	pUT18C (XbaI/EcoRI)	*clpC* (KpnI/XbaI) LS105/LS106	This study
pBP204	pkT25 (XbaI/EcoRI)	*clpC* (KpnI/XbaI) LS105/LS106	This study
pBP205	p25N (XbaI/EcoRI)	*clpC* (KpnI/XbaI) LS105/LS106	This study
pBP206	pUT18 (XbaI/EcoRI)	*clpX* (KpnI/XbaI) LS109/LS110	This study
pBP207	pUT18C (XbaI/EcoRI)	*clpX* (KpnI/XbaI) LS109/LS110	This study
pBP208	pkT25 (XbaI/EcoRI)	*clpX* (KpnI/XbaI) LS109/LS110	This study
pBP209	p25N (XbaI/EcoRI)	*clpX* (KpnI/XbaI) LS109/LS110	This study
pBP300	pAC6 (EcoRI/BamHI)	P^{+} (EcoRI/BamHI) ST5/ST6	Dormeyer et al. 2015
pBP301	pBP300 (BamHI)	$gudB^{CR}$ (BamHI) KG134/KG92	Thiele, 2013
pBP302	pBP300 (BamHI)	$gudB^{+}$ (BamHI) KG134/KG92	Dormeyer et al. 2015
pBP303	pAC6 (EcoRI/BamHI)	$gudB^{CR}$ (EcoRI/BamHI) KG210/KG92	Thiele,2013
pBP311	pAC6 (EcoRI/BamHI)	P^{CR} (EcoRI/BamHI) ST7/ST8	Dormeyer et al. 2015
pBQ200	pHT315	allows overexpression proteins in *B. subtilis*	Martin-Verstraete et al., 1994
pDG1514		template for the amplification of the *tet* gene	Guérout-Fleury et al., 1995
pDG780		template for the amplification of the *aphA3* gene	Guérout-Fleury et al., 1995
pGP380		allows overexpression of N-terminal Strep-tag fusion proteins in *B. subtilis*	Herzberg et al., 2007

Plasmid	Vector	Construction	Author
pGP529	pBQ200	overexpression of *rocG*	Commichau et al., 2008
pGP651	pAC5 (EcoRI/BamHI)	P_{gudB} (EcoRI/BamHI) ST1/ST2	Gunka et al., 2012
pGP878	pUT18 (KpnI/XbaI)	*rocG* (KpnI/XbaI) KG36/KG37	Stannek et al., 2015b
pGP879	pUT18C (KpnI/XbaI)	*rocG* (KpnI/XbaI) KG36/KG37	Stannek et al., 2015b
pGP880	p25N (KpnI/XbaI)	*rocG* (KpnI/XbaI) KG36/KG37	Stannek et al., 2015b
pGP881	pKT25 (KpnI/XbaI)	*rocG* (KpnI/XbaI) KG36/KG37	Stannek et al., 2015b
pGP882		integration of DNA into the *B. subtilis lacA* locus	Diethmaier et al., 2011
pGP916	pBQ200	overexpression of *gltC*	Commichau et al., 2007a
pGP1870		C terminal GFP fusions from the native locus	Rothe et al., 2013
pIC333		transposon mutagenesis	Steinmetz and Richter, 1994
p25-N		P_{lac}-mcs-*cyaA aphA3*	Claessen et al., 2008
pKT25		P_{lac}-*cyaA*-mcs *kan*	Karimova et al., 1998
pKT25::zip		P_{lac}-*cyaA-zip kan*	Karimova et al., 1998
pUT18		P_{lac}-mcs-*cyaA bla*	Karimova et al., 1998
pUT18c		P_{lac}-*cyaA*-mcs *bla*	Karimova et al., 1998
pUT18c::zip		P_{lac}-*cyaA-zip bla*	Karimova et al., 1998
pCFPbglS		template for the amplification of the *cfp* gene	Bisicchia et al., 2010
pSG1154	pJS2	template for the amplification of the *gfp* gene	Lewis & Marston, 1999
pYFPbglS		template for the amplification of the *yfp* gene	Bisicchia et al., 2010

13.4 Oligonucleotides

Tab. S 13.4 Oligonucleotides.

Name	Sequence 3'-5'	Gene/Purpose
FC146	CGATGCGTTCGCGATCCAGGC	sequencing of pUT18 constructs

Name	Sequence 3'-5'	Gene/Purpose
FC147	CCAGCCTGATGCGATTGCTGCAT	sequencing of p25-N constructs
FC148	GTCACCCGGATTGCGGCGG	sequencing of pUT18C constructs
FC149	GCTGGCTTAACTATGCGGCATCAGA	sequencing of pUT18C constructs
FC150	GATTCGGTGACCGATTACCTGGC	sequencing of pKT25 constructs
FC151	CGCCAGGGTTTTCCCAGTCACG	sequencing of pKT25 constructs
FC159	GCGGTCAAAGAAGATACATCTGATTTTCCTC	direct repeat analysis *dnaA*_fwd
FC160	CGCTACTGCGAGGGAAGCAGC	direct repeat analysis *dnaA*_rev
FC161	GGAAGCGGCGAGGTTGTTGATC	direct repeat analysis *tcyA*_fwd
FC162	GCCAAGCGTCAATGCCGGCAG	direct repeat analysis *tcyA*_rev
FC163	CAATGAATTAGCTATCGGAAAAGAAGGCC	direct repeat analysis *yrvM*_fwd
FC164	GGCCGACGCCTGACCGCG	direct repeat analysis *yrvM*_rev
FC165	GGCGATTGGAGGAGACATCATG	direct repeat analysis *aroH*_fwd
FC166	CTTCTTCAGTATCCCGTTCAACTGTAG	direct repeat analysis *aroH*_rev
FC167	GGCTGCGACCATTTGCAGGGC	direct repeat analysis *ykoW*_fwd
FC168	CGGCGCTGTTATTGCGACGGC	direct repeat analysis *ykoW*_rev
FC169	GTTTGCGGTCCTTTACTTCTTGCTG	direct repeat analysis *yrbF*_fwd
FC170	CGTGCAATCCCCCGATTGTCACTAC	direct repeat analysis *yrbF*_rev
FC171	GAAGATCCATGTGTCACCGTCTGC	direct repeat analysis *yisV*_fwd
FC172	GAAGGTAGGACGGCTTCTCCGTC	direct repeat analysis *yisV*_rev
FC173	GAGAAGGAGGAGGAACAGCAAGAG	direct repeat analysis *yjzB*_fwd
FC174	CATAATTGAACCAATTTGGTCCATAATATGCATG	direct repeat analysis *yjzB*_rev
FC175	GGACAGCTCGGGGCTTGGCG	direct repeat analysis *spoIIAA*_fwd

Name	Sequence 3'-5'	Gene/Purpose
FC176	GCTTCACCGCAGGAGAGATAGCG	direct repeat analysis *spoIIAA*_rev
FC177	GTAGATGAAGAAGTCACAGTACAAACACCG	direct repeat analysis *greA*_fwd
FC178	CACCCACATTGTGGACGAGGTGTTTC	direct repeat analysis *greA*_rev
FC179	GGTCTGATCGATGGGATGTCACGC	direct repeat analysis *amyE*_fwd
FC180	CCGTCTGCCCCGTCATTCAATGC	direct repeat analysis *amyE*_rev
FC181	GTCGATGTTCAGACGCTCAGCTTCAG	direct repeat analysis *putP*_fwd
FC182	CTCCCAAGCCAGAACCAACGCG	direct repeat analysis *putP*_rev
FC183	GATCAGCTGCCTGACATCGCAACG	direct repeat analysis *yhgE*_fwd
FC184	CTCCCTCAGTTAATCTGTCAATCGCATC	direct repeat analysis *yhgE*_rev
FC185	CGGAGCTGCTGAAGAGCAAAGCG	direct repeat analysis *rplL*_fwd
FC186	CTTTAGCTTCTTTCAAGCCAAGACCAGTG	direct repeat analysis *rplL*_rev
FC187	CAGCGGCGGTGAAGAAGAATGGC	direct repeat analysis *slrR*_fwd
FC188	GAGTCTGTTCGTAAAAGTGAACAATTCTTCC	direct repeat analysis *slrR*_rev
FC200	AAAAGATCTGAATTCGGATCCTCTAGAAGCTTGTCGACTGCAGGATAAACCCAGCGAACCATTTG	*aphA3* [BglII, EcoRI, BamHI, XbaI, HindIII, SalI, PstI]
JG44a	CACAAGCGGTTTGCCTGTAAT	sequencing *gudB*_rev
kan fwd	CAGCGAACCATTTGAGGTGATAGG	*aphA3*_fwd LFH PCR
kan rev	CGATACAAATTCCTCGTAGGCGCTCGG	*aphA3*_rev LFH PCR
kan-check fwd	CATCCGCAACTGTCCATACTCTG	sequencing of down_fragment LFH
kan-check rev	CTGCCTCCTCATCCTCTTCATCC	sequencing of up_fragment LFH
KG36	AAATCTAGAGATGTCAGCAAAGCAAGTCTCGAAAGATGAAG	*rocG*_fwd B2H [XbaI]
KG37	TTTGGTACCCGGACCCATCCGCGGAAACGCGAT	*rocG*_rev B2H [KpnI]
KG47	TTTCCCGGGATCGATACAAATTCCTCGTAGGC	kanamycin cassette_rev [SmaI]
KG92	TTTGGATCCTCATTATATCCAGCCTCTAAAACGCG	*gudB*_rev [BamHI]
KG100	GCAGCAATAACACCGGCAATAA	*gudB*_rev downstream fragment LFH PCR
KG101	CCTATCACCTCAAATGGTTCGCTGGCTGGATATAAGTTGATGATTTGCAT	*gudB*_fwd downstream fragment LHF PCR

Name	Sequence 3'-5'	Gene/Purpose
KG103	GCCATAATCCGGAGATTCATG	*gudB*_fwd upstream fragment LFH PCR
KG104	CGATTTCCGCTGCGATATGC	*gudB*_rev down-fragment sequencing LFH PCR
KG105	GGTTGATGATATCAGGATGGAG	*gudB*_fwd up-fragment sequencing LFH PCR
KG118	GCGGCTAAGAAGAGAGGCATCGATA	sequencing *gudB*_fwd
KG125	AAAGGATCCGCAAAAGTTGTCGGCATCTCAGATGC	*gudB*_fwd with 3x Flag [BamHI]
KG126	TTTGTCGACTATCCAGCCTCTAAAACGCGAAGCTTC	*gudB*_rev w/o stop 3x Flag [SalI]
KG134	AAAGGATCCCTAGGAGGTTAACTCAAATGGCAGC	*gudB*_fwd with SD [BamHI]
KG136	TTTCTGCAGTCATTATATCCAGCCTCTAAAACGCGAAGCTT	*gudB*_rev + 2x stop [PstI]
KG166	GCGGGATACGTTTTCACC	direct repeat analysis *gudB*_fwd
KG167	CACCGCCATATGGAAGATC	direct repeat analysis *gudB*_rev
KG180	TTTGTATAGTTCATCCATGCCATGTGTAATC	*gfp*_rev w/o stop
KG181	ACATGGCATGGATGAACTATACAAAATGGCAGCCGATCGAAACACCG	*gudB*_fwd (overhang *gfp*)
KG184	AAAGAATTCTCATTATATCCAGCCTCTAAAACGCG	*gudB*_rev [EcoRI]
KG185	TTTGGATCCCATTCAGCTTTCAGAAAGCTTACAGCGAATC	promoter region *gudB*_fwd [BamHI]
KG188	AAACAATTGCATTCAGCTTTCAGAAAGCTTACAGCGAATC	promoter region *gudB*_fwd [MfeI]
KG189	TTTAGATCTGGATCCCCGGGAATTCGAATCTTCTGTTTCTCACATGCTCCCTTTC	promoter region *gudB*_rev [EcoRI, SmaI, BamHI, BglII]
KG190	AAAGAATTCAAAGGAGGAAACAATCATGAGTAAAGGAGAAGAACTTTTCACT	*gfp* + SD(*gapA*)_fwd [EcoRI]
KG196	GGCTGATCGCTCTGACAT	*gudB*_rev
KG197	TTTGGATCCTTATCATTTGTATAGTTCATCCATGCCATGTGTAATC	*gfp*_rev + 2x stop codon [BamHI]
KG198	[P]-GTCGTTTAGAGAGCTCGAGCGTCTGAGCAG	mutagenesis *gudB* for introduction of SacI_fwd
KG199	AAACAATTGAAAGGAGGAAACAATCATGGTTTCAAAAGGCGAAGAACTGTTTACG	*cfp*_fwd for N-terminal fusion + SD(*gapA*) [MfeI]

Name	Sequence 3'-5'	Gene/Purpose
KG201	AAACAATTGAAAGGAGGAAACAATCATGGATTCAATAGAAAAGGTAAGCGAATTTGC	*yfp*_fwd for N-terminals fusion + SD(*gapA*) [MfeI]
KG206	TTTAGATCTTCATTACTTATAAAGTTCGTCCATGCCAAGTGTAATG	*cfp*_rev for C-terminal fusion + 2x stop codon [BglII]
KG208	TTTAGATCTTCATTACTTGTACAGCTCGTCCATGCCGA	*yfp*_rev for C-terminal fusion + 2x stop codon [BglII]
LS23	AATTCTTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATG	P_{alf2}_fwd [EcoRI/BamHI]
LS24	GATCCATTTCCAAGCTGTATTATAGCATAGCGCGCCTTCACTTGACAAG	P_{alf2}_rev [EcoRI/BamHI]
LS27	AATTCTTGTCAAGTGAAGGCGCGCTATGCTACAATACAGCTTGGAAATG	P_{alf4}_fwd [EcoRI/BamHI]
LS28	GATCCATTTCCAAGCTGTATTGTAGCATAGCGCGCCTTCACTTGACAAG	P_{alf4}_rev [EcoRI/BamHI]
LS29	AAAGAATTCTTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATGGATCTCTAGGAGGTTAACTCAAATGGCAGC	P_{alf2} with SD+start of *gudB*_fwd [EcoRI]
LS30	AAAGAATTCTTGTCAAGTGAAGGCGCGCTATGCTACAATACAGCTTGGAAATGGATCTCTAGGAGGTTAACTCAAATGGCAGC	P_{alf4} with SD+start of *gudB*_fwd [EcoRI]
LS31	AAAGGATCCGGCATGATTGTTTCCTCCTTTTCATTATATCCAGCCTCTAAAACGCGAAGCTTC	*gudB*_rev + SD *lacZ* [BamHI]
LS32	AAACTGCAGTTGACAAGTGAAGGCGGTGAAGGC	P^{CR}_fwd (pBP302) [PstI]
LS33	TTTCAATTGGAATTCTCTAGATTATATCCAGCCTCTAAAACGCGAAGCTTCA	*gudB*_rev with stop [MfeI, EcoRI, XbaI]
LS34	AAATCTAGAGTCGACCTCGAGAGATCTAAGGAGGAAACAATCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG	*gfp*_fwd with SD(*gapA*) [XbaI, SalI, XhoI, BglII]
LS35	TTTGAATTCTTATTTGTATAGTTCATCCATGCCATGTGTAATCC	*gfp*_rev [EcoRI]
LS36	AAAGTCGACATTAGGGGGACCAAGAAATGGCTCAAAC	*pdxS*_fwd with nat. SD [SalI]
LS37	TTTAGATCTTTATTTTTCGAACTGCGGGTGGCTCCATACAAGTGCCTTTTGCTTATATTCCTCAACC	*pdxT*_rev with Strep-tag [BglII]

Name	Sequence 3'-5'	Gene/Purpose
LS42	AAATCTAGAGATGGCAGCCGATCGAAACACCGG	*gudB*_fwd B2H [XbaI]
LS46	TTTGAATTCTTATATCCAGCCTCTAAAACGCGAA GCTTCA	*gudB*_rev B2H with stop [EcoRI]
LS47	TTTGAATTCGATATCCAGCCTCTAAAACGCGAAGCTTCA	*gudB*_rev B2H w/o stop [EcoRI]
LS48	AAATGACGCCTGATATCCCTATCACAACG	DNA sequencing *lacA*
LS61	ATGTCCGATACGCGCTTTTGCCATTAC	DNA sequencing *pdxST*_rev intern
LS62	AATTGCCGGTTCAGATAATCCTCATTTAGG	DNA sequencing *pdxST*_fwd intern
LS63	ATCAGACAACCCTGCTAAATTTGCGAAAGC	DNA sequencing *pdxST*_fwd intern II
LS64	AAAGGATCCATGGCAGCCGATCGAAACACCGG	*gudB*_fwd [BamHI]
LS92	AAAGGATCCAATAGAGAAAAATAAGGGGTGACTGACATGGATATTA	*ywlE* + SD _fwd [BamHI]
LS93	TTTCTGCAGTTATCTACGGTCTTTTTTCAGCTGTTTTGCCAG	*ywlE*_rev + stop [PstI]
LS94	[P]-TAACGGTAAAAATACCTGTTAAGATGGACGAC GGTTCAGTAAAG	mutagenesis *gudB*_fwd (R56K)
LS95	[P]-AACGAAAGGCGGGATAAAGTTTCACCCGAACGTAACA	mutagenesis *gudB*_fwd (R83K)
LS96	TTTGGATCCTTATATCCAGCCCTTAAACTTCGAAGCTT CAGCCATTTTG	mutagenesis *gudB*_rev (R421K, R423K)
LS97	AAAGGATCCGTACAGATAGTGAGGAGGAACAGGAGTAA	*mcsB* + SD _fwd [BamHI]
LS98	TTTCTGCAGTCATATCGATTCATCCTCCTGTCTTTTCCC	*mcsB*_rev + stop [PstI]
LS99	AAATCTAGAGATGTCGCTAAAGCATTTTATTCAGGACGCAC	*mcsB*_fwd B2H [XbaI]
LS100	TTTGGTACCCGTATCGATTCATCCTCCTGTCTTTTCCCATTC	*mcsB*_rev B2H [KpnI]
LS101	AAATCTAGAGATGGATATTATTTTTGTCTGTACTGGAAATACGTGC	*ywlE*_fwd B2H [XbaI]
LS102	TTTGGTACCCGTCTACGGTCTTTTTTCAGCTGTTTTGCCAG	*ywlE*_rev B2H [KpnI]
LS103	AAATCTAGAGATGAATTTAATACCTACAGTCATTGAACAAACGAACC	*clpP*_fwd B2H [XbaI]
LS104	TTTGGTACCCGCTTTTTGTCTTCTGTGTGAGTCAAAATTTTGTCAATC	*clpP*_rev B2H [KpnI]
LS105	AAATCTAGAGATGATGTTTGGAAGATTTACAGAACGAGCTCAAAAAG	*clpC*_fwd B2H [XbaI]

Name	Sequence 3'-5'	Gene/Purpose
LS106	TTTGGTACCCGATTCGTTTTAGCAGTCGTTTTTACGACAAATTCG	*clpC*_rev B2H [KpnI]
LS107	TGTCGCCGTCGTAGCGGAACTG	sequencing of pUT18 constructs_rev
LS108	GGATGCCGGGAGCAGACAAGC	sequencing of pUT18C constructs_rev
LS109	AAATCTAGAGATGTTTAAATTTAACGAGGAAAAAGGACAATTAAAATGCTCG	*clpX*_fwd B2H [XbaI]
LS110	TTTGGTACCCGTGCAGATGTTTTATCTTGGCTTACCTCAGTG	*clpX*_fwd B2H [KpnI]
LS111	AGCCGATTGGGCGTTTAGGATCTTTCAAT	DNA sequencing *clpC_rev* intern
M13_puc_fwd	GTAAAACGACGGCCAGTG	sequencing of pUC derivatives_fwd
M13_puc_rev	GGAAACAGCTATGACCATG	sequencing of pUC derivatives_rev
MT13	CCGAGCGCCTACGAGGAATTTGTATCGGAATTGATATATGATGTCCGCGCTTTCATCAAG	*gudB*_rev upstream fragment LFH PCR including promoter
pac5 fwd	GCGTAGCGAAAAATCCTTTTC	sequencing of pAC constructs_fwd
pac5 rev	CTGCAAGCGATAAGTTGG	sequencing of pAC constructs_rev
pIC333_seq down	TTTGCATGCTTCAAAGCCTGTCGGAATTGG	sequencing transposon plasmids
pIC333_seq up	AAGAGCGCCCAATACGCAAACCGCC	sequencing transposon plasmids
pKT25 rev	GCAAGGCGATTAAGTTGGGTAACGC	DNA sequencing B2H pKT25_rev
ST1	AAAGAATTCCATTCAGCTTTCAGAAAGCTTACAGCGAATC	promotor region *gudB*_fwd [EcoRI]
ST5	AATTCTTGACAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATG	P^{+}_fwd [EcoRI/BamHI]
ST6	GATCCATTTCCAAGCTGTATTATAGCATAGCGCGCCTTCACTTGTCAAG	P^{+}_rev [EcoRI/BamHI]
ST7	AATTCTTGACAAGTGAAGGCGGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATG	P^{CR}_fwd [EcoRI/BamHI]
ST8	GATCCATTTCCAAGCTGTATTATAGCATAGCGCGCCTTCACCGCCTTCACTTGTCAAG	P^{CR}_rev [EcoRI/BamHI]

13.5 Supplementary Material Chapter 3 – Selection-driven accumulation of *gudB*$^{+}$ suppressor mutants in *Bacillus subtilis*

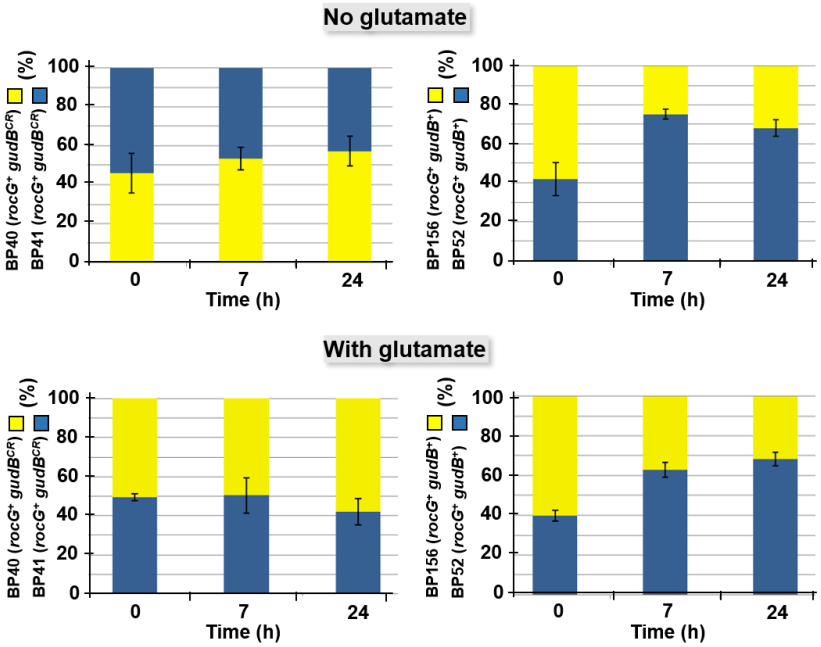

Fig. S 13.1 Effect of the yfp and cfp fluorophore genes on growth of *B. subtilis*.
Mixed populations of strains BP40 (*rocG*$^{+}$ *gudB*^{CR} *amyE::yfp*) and BP41 (*rocG*$^{+}$ *gudB*^{CR} *amyE::cfp*) or BP52 (*rocG*$^{+}$ *gudB*$^{+}$ *amyE::cfp*) and BP156 (*rocG*$^{+}$ *gudB*$^{+}$ *amyE::yfp*) were grown for up to 24 h in C minimal medium supplemented with glucose and ammonium (C-Glc), and C-Glc minimal medium supplemented with glutamate. Prior to co-cultivation (0 h), and after 7 h and 24 h of growth dilutions of cells were plated on complex medium. The surviving cells that emerged after 12 h of incubation were identified by fluorescence microscopy and counted. The bars represent standard deviations for at least four independently repeated experiments.

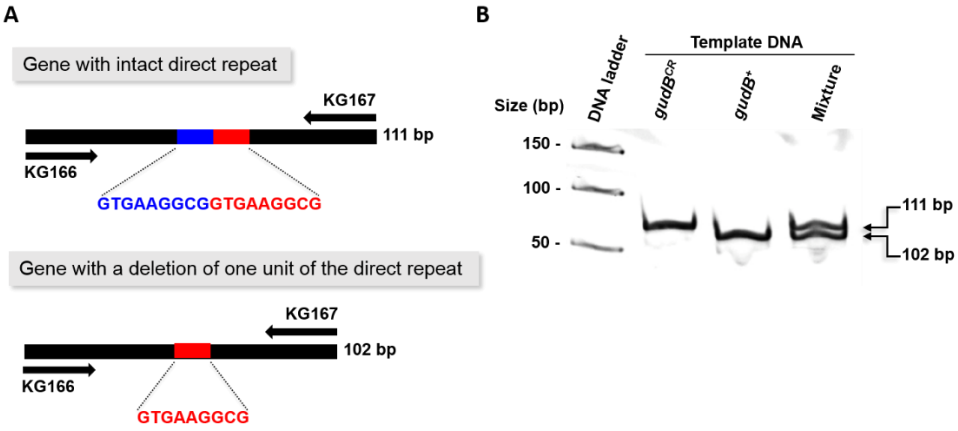

Fig. S 13.2 Analysis of DR integrity in cell population.
(A) Schematic illustration of the colony PCR to detect deletion of the 9 bp-long single repeat unit of the *gudB*^{CR} DR (see Materials and Methods). KG166 and KG167 are forward and reverse oligonucleotides, respectively, that hybridise close to the tandem repeat of the *gudB*^{CR} gene. **(B)** Evaluation of the method to analyse the state of *gudB* in a population of cells. The DNA molecules were generated by colony PCR using template DNA from *B. subtilis* strains GP342 (*gudB*^{CR}) and GP801 (*gudB*$^{+}$). The 1:1 mixture of co-cultivated strains GP342 and GP801 was analysed by colony PCR to detect the presence of the *gudB*^{CR} and *gudB* alleles in a population of cells. The 50 bp Gene Ruler (Thermo scientific, #SM0373) served as DNA ladder.

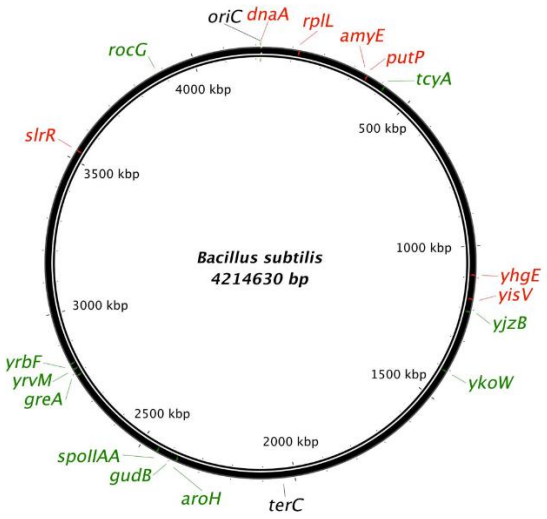

Fig. S 13.3 Location and direction of 16 genes with 9 bp-long DRs on the *B. subtilis* chromosome.
Genes that are highlighted in red and green are encoded on the plus and minus strand, respectively. The circular map of the *B. subtilis* chromosome was generated using the open source BLAST Ring Image Generator software 0.95 (http://sourceforge.net/projects/brig/). The genes were positioned according to the Subtilist database (http://genolist.pasteur.fr/SubtiList/).

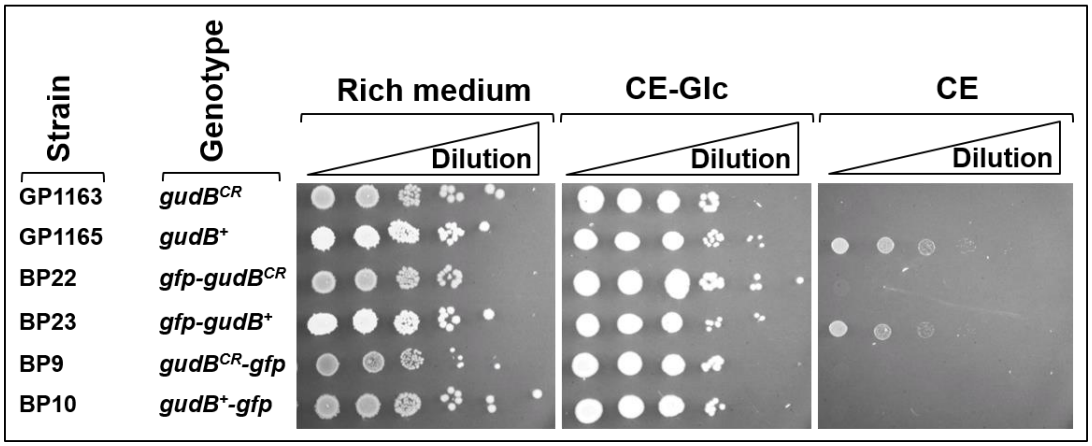

Fig. S 13.4 *In vivo* activities of GudB^{CR} and GudB variants fused to GFP.
5 µl were plated from serial dilutions (from 10^{-1} till 10^{-6}) of cell suspensions of the control strains GP1163 (*rocG*$^+$ *gudB*^{CR}) and GP1165 (*rocG*$^+$ *gudB*$^+$), and the strains BP22, BP23, BP9 and BP10 expressing the *gfp-gudB*^{CR}, *gfp-gudB*$^+$, *gudB*^{CR}*-gfp* and *gudB*$^+$*-gfp* fusions, respectively. The dilutions were spotted on SP medium agar plates (rich medium), and C minimal medium supplemented either with glucose and glutamate (CE-Glc medium) or with glutamate and ammonium (CE medium). The plates were incubated for 48 h at 37°C.

```
241 - ggc ggg ata cgt ttt cac ccg aac gta aca
271 - gaa aaa gag gtg aag gcg gtg aag gcg ctt
301 - tca att tgg atg agt tta aaa tgc ggc ata
331 - att gat ctt cca tat ggc ggt ggt aaa ggc
361 - gga att gtt tgt gat cca agg gat atg tcg
391 - ttt aga gag ctc gag cgt ctg agc aga ggg
421 - tat gtc aga gcg atc agc caa att gtc ggc
```

Fig. S 13.5 Part of the sequence of the gudB$^{CR}_{SacI}$-gfp allele.
The recognition site GAGCTC for the restriction endonuclease *Sac*I that is highlighted in green was generated by modification of the CTG leucine codon at position 402 to the leucine codon CTC (see Tab. S 13.3). The 9 bp DR of the *gudB^{CR}* gene is highlighted in red. Letters highlighted in black and pink indicate the regions where the oligonucleotides KG166 and KG196 hybridise.

Tab. S 13.5 9 bp long tandem repeats present in essential (indicated by a superscript "e") and non-essential genes of *the B. subtilis* chromosome.
Tandem repeats can be either in frame or not in frame. The red letter indicates the nucleotide that renders the direct repeat imperfect. n. a., data not available.

Gene	Protein	Locus tag	Function	DNA sequence	Expression [31]	
					Exp. Phase (120 Min.)	Stat. phase (400 Min.)
gudB^{CR}	GudB^{CR}	BSU22960	Inactive glutamate dehydro-genase	GTG AAG GCG GTG AAG GCG	Inter-mediate	Low
putP	PutP	BSU03220	Proline permease	CTG GCT GTT CTG GCT GTT	Low	Inter-mediate
greA	GreA	BSU27320	Transcription elongation factor	GTG AAA ATT GTG AAA ATT	High	Low
spoIIAA	SpoIIAA	BSU23470	Anti-anti sigma factor	AAG CAA ATT AAG CAA ATT	Inter-mediate	High
ykoW	YkoW	BSU13420	c-di-GMP synthase and phosphodi-esterase	GAA CAA TTC GAA CAA TTC	Inter-mediate	Low
amyE	AmyE	BSU03040	a-amylase	AAT ACA CAA AAT ACA CAA	Low	Inter-mediate
aroH	AroH	BSU22690	Chorismate mutase	ATT CGC GGA ATT CGC GGA	High	Low
yisV	YisV	BSU10880	Putative GntR family transcription factor	GCA CTT CAG GCA CTT CAG	Low	Low
yjzB	YjzB	BSU11320	Unknown	GTT TCT CAG GTT TCT CAG	Low	Inter-mediate

Gene	Protein	Locus tag	Function	DNA sequence	Expression [31]	
					Exp. Phase (120 Min.)	Stat. phase (400 Min.)
yhgE	YhgE	BSU10160	Unknown	GCG CAG GTG GCG CAG GTG	Inter-mediate	Low
yrbF	YrbF	BSU27700	Unknown	CAG CAA AAG CAG CAA AAG	High	Low
yrvM	YrvM	BSU27540	Unknown	GGA GTC GGG GGA GTC GGG	High	Low
slrR	SlrR	BSU34380	Control of SlrA and SinR	GTG CAA GCC GTA CAA GCC	Inter-mediate	High
rplL^{e}	RplL	BSU01050	Ribosomal protein L12	ATC AAA GTT ATC AAA GTT	High	Inter-mediate
tcyA	TcyA	BSU03610	Cysteine transporter binding protein	CTT TCT AAA ATT TCT AAA AAA	High	Low
dnaA^{e}	DnaA	BSU00010	Replication initiation protein	TAT ACT TTT GAT ACT TTT GTC	n. A.	n. A.

Tab. S 13.6 Raw data of the experiment shown in Fig. 3.4.

Time (h)	Experiment	Medium	*gudB*^{CR} colonies (blue)	*gudB*$^{+}$ colonies (white)	*gudB*^{CR} colonies (blue, in %)	*gudB*$^{+}$ colonies (white, in %)
0	1	Preculture	141	0	100	0
0	2	Preculture	73	0	100	0
0	3	Preculture	144	0	100	0
0	4	Preculture	156	0	100	0
7	1	C-Glc	167	0	100	0
7	2	C-Glc	219	0	100	0
7	3	C-Glc	145	0	100	0
7	4	C-Glc	184	0	100	0
7	1	CE-Glc	165	0	100	0
7	2	CE-Glc	129	0	100	0
7	3	CE-Glc	178	0	100	0
7	4	CE-Glc	104	0	100	0
7	1	SP	72	2	97,3	2,7
7	2	SP	91	4	95,8	4,2

Time (h)	Experiment	Medium	*gudB*^{CR} colonies (blue)	*gudB*$^{+}$ colonies (white)	*gudB*^{CR} colonies (blue, in %)	*gudB*$^{+}$ colonies (white, in %)
7	3	SP	72	3	96	4
7	4	SP	48	0	100	0
24	1	C-Glc	173	0	100	0
24	2	C-Glc	127	1	99,2	0,8
24	3	C-Glc	180	0	100	0
24	4	C-Glc	208	1	99,5	0,5
24	1	CE-Glc	111	2	98,2	1,8
24	2	CE-Glc	86	6	93,5	6,5
24	3	CE-Glc	109	3	97,3	2,7
24	4	CE-Glc	121	24	83,4	16,6
24	1	SP	1	107	0,9	99,1
24	2	SP	16	105	13,2	86,8
24	3	SP	4	70	5,4	94,6
24	4	SP	9	171	5	95

13.6 Supplementary Material Chapter 5 - Inducer-free activation of gene expression by selection-driven promoter decryptification in *Bacillus subtilis*

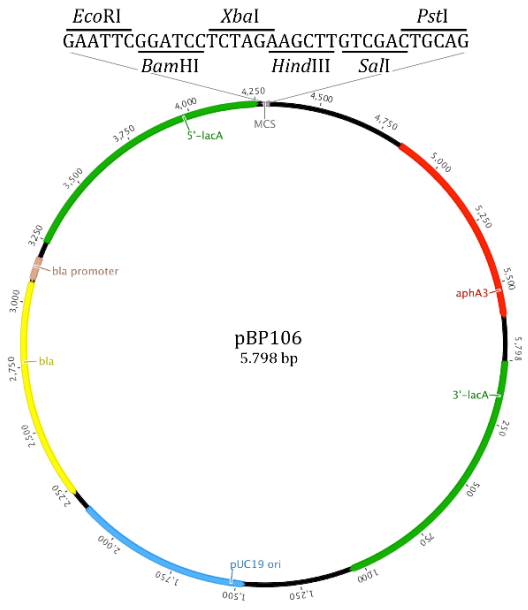

Fig. S 13.6 Map of the plasmid pBP106.
MCS, multiple-cloning site. The pUC19 origin of replication (ori) (Vieira & Messing, 1982) and the *bla* ampicillin resistance gene allow replication and selection, respectively, in *E. coli.*

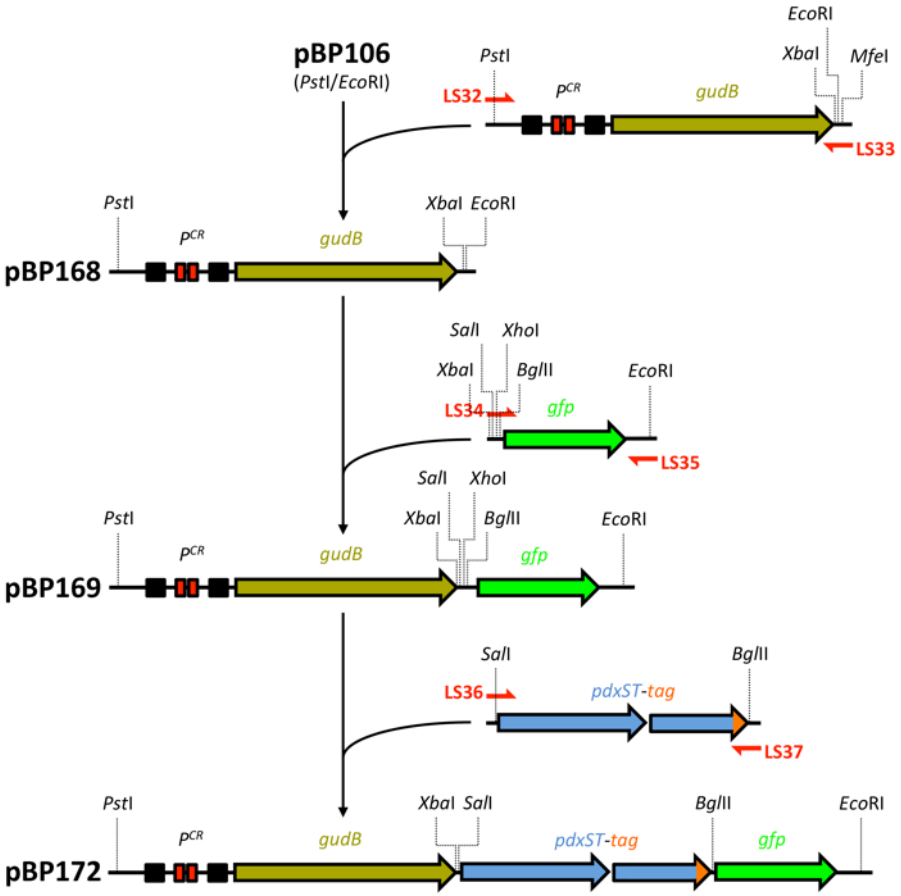

Fig. S 13.7 Construction of the artificial *P^{CR}-gudB-pdxST-Strep-gfp* operon.

CTGCAGTTGACAAGTGAAGGCGGTGAAGGCGCGCTATGCTATAATACAGCTTGGAAATGGATCCCTAGGAGGTTA
ACTCAAATGGCAGCCGATCGAAACACCGGTCATACAGAAGAGGACAAACTTGATGTATTAAAATCAACCCAAACC
GTAATACATAAGGCTCTGGAAAAATTGGGATATCCCGAAGAGGTATACGAATTGTTAAAAGAGCCGATGAGATTA
TTAACGGTAAAAATACCTGTTCGTATGGACGACGGTTCAGTAAAGATTTTCACAGGATATCGTGCGCAGCACAAT
GACTCTGTCGGTCCAACGAAAGGCGGGATACGTTTTCACCCGAACGTAACAGAAAAAGAGGTGAAGGCGCTTTCA
ATTTGGATGAGTTTAAAATGCGGCATAATTGATCTTCCATATGGCGGTGGTAAAGGCGGAATTGTTTGTGATCCA
AGGGATATGTCGTTTAGAGAGCTGGAGCGTCTGAGCAGAGGGTATGTCAGAGCGATCAGCCAAATTGTCGGCCCG
ACAAAAGACGTGCCGGCACCGGATGTATTTACAAACTCACAAATCATGGCTTGGATGATGGATGAGTATTCAAGA
ATTGATGAATTTAATTCGCCTGGATTTATTACAGGCAAACCGCTTGTGCTTGGCGGATCTCACGGGAGAGAATCT
GCGACAGCAAAAGGTGTTACCATCTGTATTAAAGAAGCGGCTAAGAAGAGAGGCATCGATATTAAAGGTGCGCGT
GTCGTTGTCCAAGGCTTCGGAAACGCGGGAAGCTATTTGGCAAAATTTATGCATGATGCGGGGGCAAAAGTTGTC
GGCATCTCAGATGCGTATGGCGGACTTTATGATCCGGAAGGCCTTGATATCGATTATTTACTCGACCGACGCGAC
AGCTTCGGTACCGTAACAAAGCTTTTCAACGATACCATTACCAACCAAGAGCTGCTGGAGCTGGATTGTGATATT
CTCGTTCCTGCTGCGATTGAAAATCAAATTACAGAAGAAAATGCCCATAATATCCGGGCTAAAATTGTCGTTGAA
GCAGCGAACGGACCAACAACGCTTGAAGGAACAAAAATTCTTTCAGACCGGGACATTCTGCTTGTACCAGACGTG
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GAAGAGGTAGAAGAAAAATTAGAAAAAATGATGGTCAAATCATTTAACAATATTTACGAAATGGCTAACAACCGA
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CAGAAATGCAAAAAGGCGGCGTCATCATGGACGTCATCAATGCGGAACAAGCGAAAATCGCTGAAGAAGCTGGAG
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ACCTGTCCACACAATCTAAACTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAA
CAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAAGAATTC

Fig. S 13.8 Full sequence of the artificial *gudB-pdxST-Strep-gfp* operon.
Restriction sites are underlined. The 9 bp-long direct repeat in the synthetic promoter is highlighted in red. Perfect -35 and -10 regions of the promoter are highlighted in black. Ribosome binding sites are highlighted in turquoise. Start and stop codons are shown in green and red letters, respectively. Open reading frames (ORF) are shown in black and bold letters. The *Strep*-tag II coding sequence is highlighted in blue.

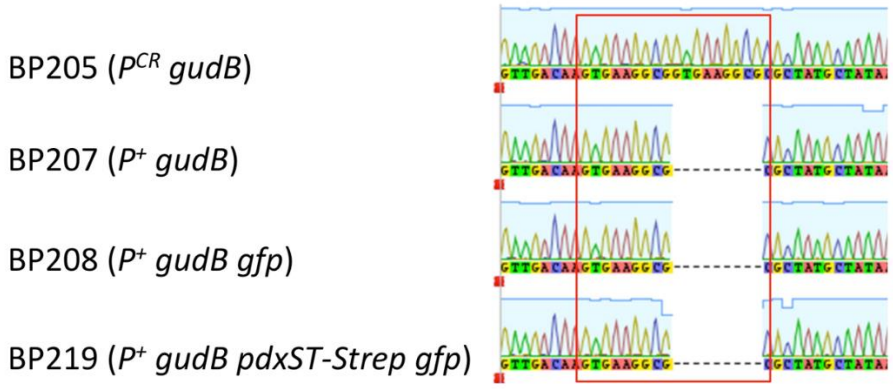

Fig. S 13.9 Sequence analysis of the direct repeat.
Sequence analysis of the direct repeat to confirm the deletion of one part of the 18 bp-long direct repeat (indicated by the red rectangle) in the suppressor mutants BP207, BP208 and BP219 with the "decryptified" synthetic promoter.

Tab. S 13.7 Number of spectral counts as a measure of abundance of the proteins identified by mass spectrometry in the gel slices.

Protein	Molecular weight [kDa]	Sample 1	Sample 2
PdxS	**32**	**1219**	**105**
PdxT	**21**	**19**	**502**
RpsC	24	3	6
PrsA	33	13	-
RpsB	28	8	-
GtaB	33	10	-
YxkC	23	-	12
YvcJ	34	8	-
RpsE	18	-	7
Rok	22	-	4
Tsf	32	3	-
FhuD	34	3	-
Tpi	27	3	-
YxeB	36	2	-
RplF	20	-	2
YceC	22	-	3
PurQ	25	2	-
FbaA	30	3	-
Prs	35	3	-
PyrE	24	-	2
BkdAB	36	2	-
RplA	25	3	-
RpsT	10	-	2
Hbs	10	-	2
LipA	34	2	-

14 CURRICULUM VITAE

PERSONAL DATA

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Nationality	German

EDUCATION

2012-2015	**Ph.D. Thesis** **Department of General Microbiology, Dr. Fabian M. Commichau** Control of glutamate homeostasis in the Gram-positive model organism *Bacillus subtilis*
2009-2011	**Georg-August-University of Göttingen, Germany** **M.Sc. Microbiology and Biochemistry** **Master Thesis** **Department for General Microbiology, PD Dr. Michael Hoppert** Nitrogen assimilation in the marine chemosymbiotic bivalve *Loripes lacteus*
2006-2009	**Georg-August-University of Göttingen, Germany** **B.Sc. Biology** **Bachelor Thesis** **Department for General Microbiology**, **PD Dr. Michael Hoppert** Characterization of microbial biofilms on building stones.
1999-2006	**Gymnasium Corvinianum, Northeim**