Characterization of proteins of the Asp23 protein family in *Bacillus subtilis*

Dissertation

for the award of the degree

“Doctor rerum naturalium”

of the Georg-August-Universität Göttingen

within the doctoral program “Microbiology and Biochemistry”

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

submitted by

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from Uelzen

Göttingen 2016

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I hereby declare that this thesis entitled “Characterization of proteins of the Asp23 protein family in *Bacillus subtilis*” has been written independently and with no other sources and aids than quoted.

Dominik Tödter

Danksagung

Mein erster Dank geht an dieser Stelle an Prof. Dr. Jörg Stülke. Dass du mittlerweile schon zum dritten Mal eine Abschlussarbeit von mir betreut hast, spricht eigentlich für sich. Ich danke dir ganz herzlich für deine Betreuung und Unterstützung, nicht nur während meiner Doktorarbeit, sondern auch schon seit meiner Bachelorarbeit. Danke, dass du mir diesen Abschluss ermöglicht hast!

Dr. Fabian Commichau danke ich nicht nur für die Übernahme des Korreferats, sondern vor allem auch für seine Hilfsbereitschaft und Anregungen während meiner Doktorarbeit. Des Weiteren danke ich Prof. Dr. Ralf Ficner für die Teilnahme an meinem Thesis Committee, sowie Prof. Dr. Stefanie Pöggeler, Prof. Dr. Henning Urlaub und Prof. Dr. Ivo Feußner für die Teilnahme an meiner Prüfungskommission.

Erwähnen möchte ich auch die Hilfe von Achim Dickmanns, der mir bei Fragen zur Proteinaufreinigung oft mit seinem fachlichen Rat zur Seite stand. Außerdem danke ich meinen Bachelor- und Masterstudenten Laura Griese, Lars Zimmerningkat und Anika Klewing für ihre Beiträge zu meiner Arbeit. Besonders Anika war eine große Hilfe und auch nach ihrer Abgabe immer für ein Gespräch offen. Danke dafür! Meinen „Labor-Homies“ Daniel Reuß und Dr. Christopher Zschiedrich danke ich für die wunderbare Atmosphäre im Labor und im Büro! Ihr hattet immer ein offenes Ohr für mich und seid für jeden Spaß zu haben gewesen.

Ein besonderer Dank geht an die beiden guten Seelen des Labors, Dr. Katrin Gunka und Christina Herzberg. Ihr seid mir nicht nur bei wissenschaftlichen Fragen immer eine große Hilfe gewesen, sondern es war auch immer angenehm mit euch über alltägliche Dinge sprechen zu können. Danke für eure Offenheit und Hilfsbereitschaft, die ihr schon seit Jahren allen Studenten und Kollegen entgegenbringt. Haltet das Labor weiterhin so am Laufen! In diesem Zuge danke ich auch Silvia Carrillo-Castellón dafür, dass sie mir durch ihre Arbeit den Laboralltag sehr erleichtert hat.

Weiterhin danke ich auch Jonathan Rosenberg und Jan Gundlach für interessante (Fach)gespräche, manchmal auch bei einem(!) Bier, und für die schöne Zeit außerhalb des Labors. Allen übrigen und ehemaligen Kollegen der AGS und der HIF sei ebenfalls für die Unterstützung und Hilfsbereitschaft, sowie das tolle Arbeitsklima gedankt.

Zu guter Letzt danke ich meinen Freunden, insbesondere den „Uelzener Jungs“, Martin, Jana, Sabine und Ira, meiner Familie und vor allem meinen Eltern dafür, dass sie mich in den letzten Jahren stets motiviert und gefördert haben. Danke für eure Unterstützung und euren Rückhalt!

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List of abbreviations

% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
Amp	ampicillin
APS	ammonium persulfate
ATP	adenosine triphosphate
B.	*Bacillus*
BACTH	Bacterial two-hybrid
BGSC	Bacillus Genetic Stock Center
bp	base pairs
CDP*	disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate
CoA	coenzyme A
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
deion. H_2O	deionized water
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
et al.	*et alia*
FA	formaldehyde
Fig.	figure
fwd	forward
gDNA	genomic DNA
GFP	green fluorescent protein
Glc	glucose
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani (medium)
LFH	long flanking homology
MCR	malonyl-CoA reductase
mRNA	messenger RNA
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Ni^{2+}-NTA	nickel-nitrilotriacetic acid
OD	optical density
P	promoter
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
pH	power of hydrogen
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT	room temperature
rev	reverse
S.	*Staphylococcus*
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SP	sporulation medium
SPINE	Strep-protein interaction experiment
Tab.	table
TEMED	N,N,N´,N´-tetramethylethylendiamine
Tris	tris-(hydroxymethyl)-aminomethane
U	units
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YFP	yellow fluorescent protein

Summary

The acetyl-CoA carboxylase (ACCase) catalyzes the production of malonyl-CoA, the precursor in fatty acid biosynthesis. In almost all organisms, this is a fundamental process since fatty acids are the main components of membrane lipids and serve as precursor for energy supplying pathways. Especially in organisms like the soil bacterium *Bacillus subtilis*, that have to deal with different and quickly changing environmental conditions, the control of fatty acid homeostasis is important. Although many examples of ACCase regulation are found in other organisms, nothing is known about the regulation of the acetyl-CoA carboxylase in *B. subtilis*. The aim of this work was the characterization of the Asp23 protein YqhY. It could be shown that YqhY is able to interact with the AccAD subcomplex or the protease ClpCP. Moreover, cells lacking YqhY acquired suppressor mutations that were often located in the subunits of the acetyl-CoA carboxylase. Further investigations demonstrated that some mutations lead to decreased acetyl-CoA carboxylase activity. Therefore, it was assumed that YqhY regulates the activity of the ACCase by either acting as inhibitor or by promoting the degradation of the subunits. Despite these observations, an inhibitory effect of YqhY on the activity of the ACCase complex could not be proven and unchanged protein amounts of the subunits in the absence of ClpP indicated that they are not subject to proteolysis. However, localization experiments displayed the localization dependency of AccA on YqhY, providing the possibility of YqhY impacting the acetyl-CoA carboxylase activity by delocalization of parts of the complex. It is also conceivable that the absence of YqhY causes the accumulation of malonyl-CoA. This highly active compound could nonspecifically acylate many proteins of different pathways, leading to their inactivity and toxic effects for the cell. Although the precise role of YqhY remains elusive, the results of this work indicate a regulatory function in fatty acid synthesis. They provide possible ways how YqhY could be involved in this pathway and serve as a basis for future investigations.

1 Introduction

1.1 YqhY, an Asp23 protein with a (non-)essential function

The Gram-positive bacterium *Bacillus subtilis* is one of the best studied organisms. The completely sequenced genome and the comprehensive knowledge about cellular processes makes it a perfect model organism for ongoing studies. This and the ability to easily manipulate the genetic structure, are also reasons for the intensive application in industries. Nevertheless, not all essential functions and pathways are totally understood and are in need of further studies. A fundamental aspect in this approach is the determination of the minimal gene set and basic requirements for *B. subtilis* to survive, like it is done in the recent work of Reuß *et al.* (2016). The authors suggested a reduced genome of 523 protein encoding genes and 119 genes coding for RNAs that allows growth at 37°C in complex medium. Among these genes, 245 are currently regarded as essential in *B. subtilis*.

The first essential gene set for *B. subtilis* was established in 2003 (Kobayashi *et al.*). In this study, only single genes were verified. But, the essentiality of a protein depends on certain environmental conditions and some functions can be executed by several proteins. The diadenylate cyclases CdaA, DisA and CdaS or the enoyl-ACP reductases FabI and FabL are examples for that (Mehne *et al.*, 2013; Parsons and Rock, 2013). In other words, the function carried out by these proteins only became essential when all encoding genes are deleted in combination. Gene pairs executing essential functions were named isologous (Thomaides *et al.*, 2007). In this first evaluation of such isologous pairs, the unknown genes *yqhY* and *yloU* were added to the list of essential genes, whereas they were previously regarded as nonessential after single knockouts (Kobayashi *et al.*, 2003). The encoded proteins YqhY and YloU are paralogues, sharing an identity of 32%. They are both highly conserved and highly and constitutively expressed in Gram-positive bacteria (Fig. 1a) (Nicolas *et al.*, 2012). Despite these facts already indicating a vital function of these proteins, single knockouts of *yqhY* and *yloU* were possible. Moreover, a successful double deletion of both genes refuted the theory of being an isologous essential gene pair. However, the importance of at least YqhY still remained. This became apparent by the occurrence of suppressor mutations as a consequence of an *yqhY* deletion (Tödter, 2013).

Thus, the gene pair of *yqhY* and *yloU* is not essential, but it is crucial for growth under certain circumstances.

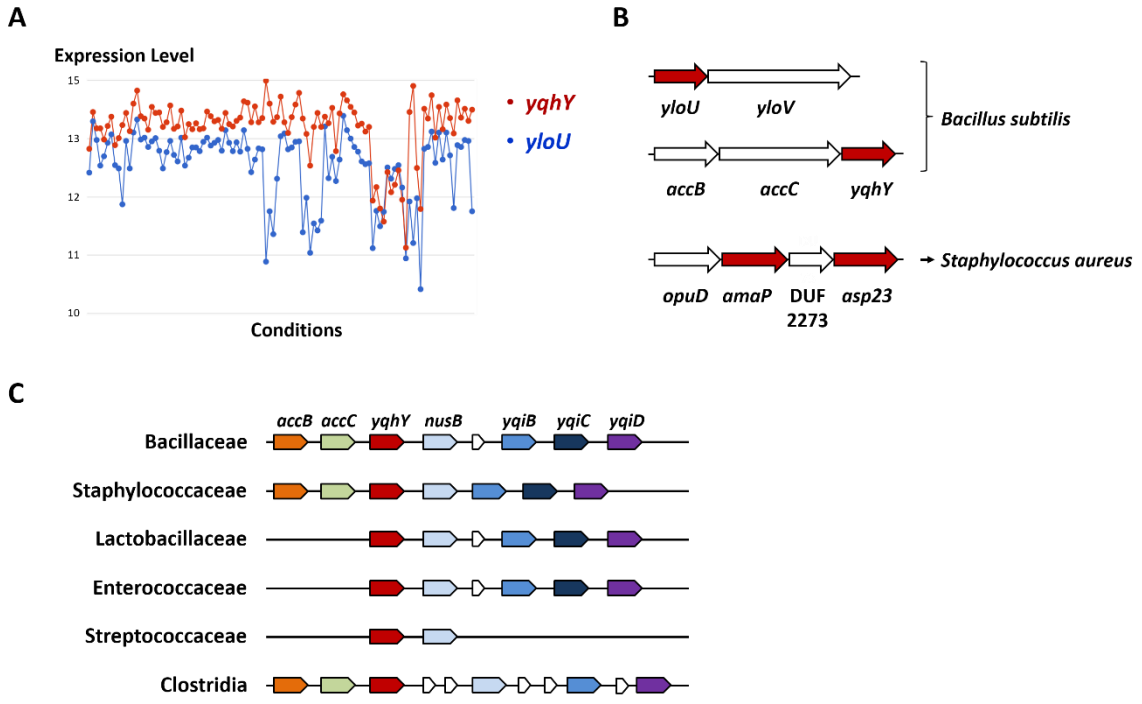

Fig. 1. (A) Expression levels of *yqhY* and *yloU* under different growth conditions (Nicolas *et al.*, 2012; *Subti*Wiki database). The transcription pattern shows constitutive and high expression of *yqhY* and *yloU*. Only under glucose starvation and after phosphate limitation the expression is reduced. **(B) Genetic context of *yloU*, *yqhY* and *asp23*.** Genes encoding Asp23 family members are shown in **red**. The representatives of this family in *B. subtilis* are YloU and YqhY. Their encoding genes a located in two distinct operons with *yloV* (putative dihydroxyacetone/ glyceraldehyde kinase) and *accB* (biotin carboxyl carrier protein) and *accC* (biotin carboxylase subunit), respectively. *asp23* is cotranscribed with genes coding for the osmoprotectant transporter OpuD2, a small transmembrane protein belonging to the DUF2273 family and another transmembrane protein called AmaP (Müller *et al.*, 2014). **(C) Conservation *accBC-yqhY* operon throughout Firmicutes (according to STRING database).** *yqhY* is highly conserved in Firmicutes and often clustered with *accBC*. Downstream of *yqhY* are genes located coding for subunits of exodeoxyribonuclease VII (*yqiB*, *yqiC*) and a geranyltransferase (*yqiD*). Gene names refer to *B. subtilis* 168 wild type genome (NC_000964).

yqhY is located in an operon with *accB* and *accC*, both coding for subunits of the acetyl-CoA carboxylase. This gene cluster is highly conserved throughout Bacillales and YqhY shares high identities with its homologues in several representatives, for example *Staphylococcus aureus* (49%), *Listeria monocytogenes* (54%), *Desmospora sp.* 8437 (34%) and *Paenibacillus vortex* (45%). Outside Bacillales *yqhY* is not embedded in an operon with *accB* and *accC*, but orthologues in *Enterococcus faecalis* (50%), *Lactococcus lactis* (40%) and *Streptococcus pneumoniae* (65%) represent other orders of Bacilli. Even in other classes of Firmicutes, like Clostridia (*Clostridium difficile* (52%)), homologues are present,

showing the broad conservation of *yqhY* in organisms with many different lifestyles. In almost all organisms containing *yqhY*, *nusB* is located downstream coding for a protein involved in transcription termination. Often, a conserved gene cluster follows with genes coding for subunits of exodeoxyribonuclease VII and a geranyltransferase (Fig. 1c). Strikingly, in nearly all Firmicutes the paralogue *yloU* forms an operon with *yloV*, a gene coding for a putative dihydroxyacetone/ glyceraldehyde kinase. This operon is even present in bacteria lacking *yqhY*.

In *B. subtilis*, an interaction of YqhY and YloU could be shown and both proteins were found to be in the cytosolic fraction. YloU is spread equally in the cell, whereas YqhY is localized at the cell poles. This polar localization is dependent on DivIVA, as YqhY shows a dispersed localization in the absence of this membrane anchor (Tödter, 2013). YqhY and YloU belong to the Asp23 protein family. The name giving protein is the alkaline shock protein 23 (Asp23) from *S. aureus* and all members of this family contain a domain of unknown function (DUF322). In most staphylococci Asp23 is one of four DUF322 proteins together with YqhY, YloU and AmaP. It is highly expressed and one of the most abundant proteins in the cytosolic fraction of *S. aureus* (Maass *et al.*, 2011). In contrast to *yqhY* and *yloU*, *asp23* is co-transcribed with three other genes controlled by sigma factor σ^B (Gertz *et al.*, 1999). They encode the osmoprotectant transporter OpuD2, a small transmembrane protein belonging to the DUF2273 family and another transmembrane protein called AmaP (Fig. 1b). AmaP is another representative of the Asp23 family and functions as a membrane anchor for Asp23. The deletion of *amaP*, resulting in a delocalized Asp23 or the absence of Asp23 itself, lead to an increased expression of cell wall stress genes. The transcription of most of these genes is also upregulated in the presence of vancomycin (Müller *et al.*, 2014). This antibiotic disturbs cell wall synthesis in Gram-positive bacteria and is used as one of the last actions against life-threatening infections, often caused by multi-resistant *S. aureus* strains.

Since *S. aureus* and *B. subtilis* are closely related, understanding the function of YqhY and YloU would help to decipher the function of Asp23 and in doing so, support the identification of new drug targets. In addition, the high conservation of proteins containing the DUF322 domain strongly points out the importance in cellular processes. Therefore, it is important to elucidate the physiological role of YqhY and YloU, in order to understand the fundamental mechanisms of the cell.

1.2 Fatty acid metabolism in prokaryotes

Fatty acid metabolism is an essential pathway in most organisms, since the provision of fatty acids as membrane components and precursor for energy supplying pathways is crucial. Moreover, bacterial cells are often exposed to different environmental conditions. In order to adapt to abrupt changes in temperature, osmolarity, pH or salinity, an alteration of the membrane composition is necessary to adjust to those changes (Zhang and Rock, 2008). There are two types of fatty acid synthesis (FAS). Type I is found in eukaryotes and consists of only one protein carrying out all reactions of the pathway. In contrast, the FAS II in bacteria, plants and parasites is composed of several proteins of which each enzyme catalyzes a single reaction of the pathway (White *et al.*, 2005).

The initiation phase of the FAS II in *B. subtilis* (Fig. 2) is started by the conversion of acetyl-CoA to malonyl-CoA performed by the acetyl-CoA carboxylase (ACCase). This enzyme complex is built up by four subunits, AccA, AccB, AccC and AccD (further description below) (Cronan and Waldrop, 2002). Afterwards, the malonate group is transferred to the acyl carrier protein (ACP) by the malonyl transacylase FabD (Zhang and Rock, 2008). The produced malonyl-ACP is then formed to β-ketoacyl-ACP by FabH (Parsons and Rock, 2013). In Gram-negative bacteria FabH condenses acetyl-CoA with malonyl-ACP to produce straight-chain fatty acids. On the other hand, many Gram-positive bacteria like *B. subtilis* and *S. aureus* preferentially condense short-chain acyl-CoA with malonyl-ACP to create branched-chain fatty acids. *B. subtilis* possesses two FabH isozymes (FabHA and FabHB) that enable also the consumption of acetyl-CoA for the production of straight-chain fatty acids (Choi *et al.*, 2000). The differences in substrate specificity of FabH are determined by the hydrophobic binding pocket. Structural analyses revealed that in *Escherichia coli* FabH this pocket is only big enough to harbor acetyl-CoA or propionyl-CoA, whereas in *S. aureus* FabH is able to bind acyl-CoAs with up to five carbon atoms (Qiu *et al.*, 2005).

After the initiating step of FabH, β-ketoacyl-ACP is reduced by FabG in a NADPH dependent manner in the first reaction of the elongation cycle. The resulting product β-hydroxyacyl-ACP is then dehydrated to *trans*-2-enoyl-ACP by FabA and FabZ in *E. coli*. Although both enzymes catalyze the same reaction, only FabA is able to perform the *cis-trans* isomerase reaction needed to synthesize unsaturated fatty acids. Gram-positive

bacteria only possess FabZ and therefore, they mainly produce saturated fatty acids. An exception is *Streptococcus pneumoniae* which is also capable of forming unsaturated fatty acids. This is facilitated by FabM, an enzyme carrying out the same reaction as FabA despite having no similarity to it (Parsons and Rock, 2013). The completing step of elongation is the reduction of *trans*-2-enoyl-ACP to acyl-ACP. This is fulfilled by the widely expressed enzyme FabI in the presence of NADH or NADPH (Heath and Rock, 1995). In *B. subtilis* the same reaction is executed by a second enoyl-ACP reductase (FabL) with a strong preference for NADPH (Heath *et al.*, 2000).

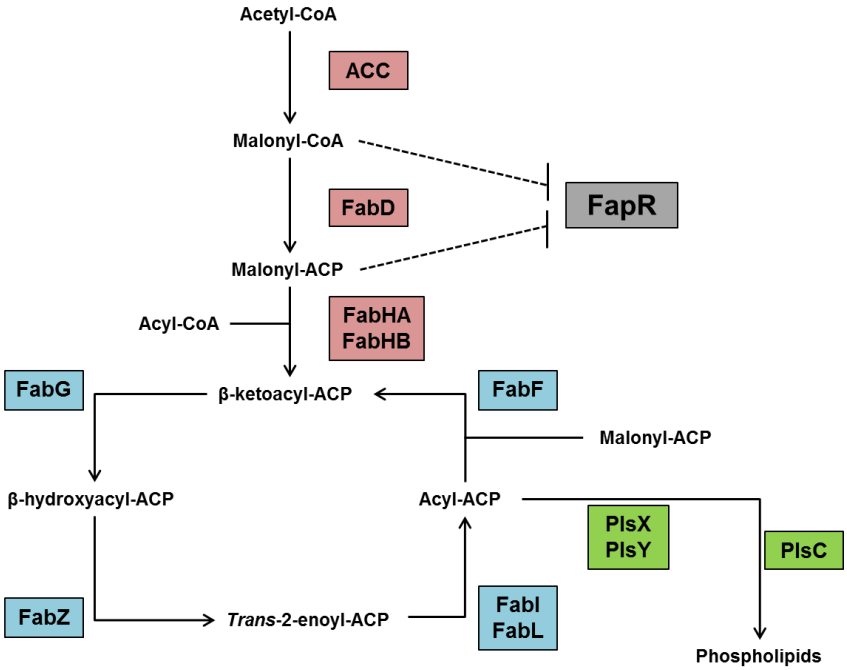

Fig. 2. Schematic overview of the fatty acid synthesis in *B. subtilis* (Klewing, 2015)**.** Initiation cycle (**red**) is started by the production of malonyl-CoA by the acetyl-CoA carboxylase (ACC). The malonate group is transferred to the acyl carrier protein (ACP) by the malonyl transacylase FabD and subsequently the β-ketoacyl-ACP synthase FabH condenses acyl-CoA with malonyl-ACP. The elongation cycle (**blue**) is a repeating process, in which FabG reduces β-ketoacyl-ACP in a first reaction. In the second step, the resulting β-hydroxyacyl-ACP is dehydrated to enoyl-ACP by FabZ. At the end, the enoyl-ACP reductases FabI or FabL complete the elongation. The resulting acyl-ACP is either condensed by FabF for a further round of elongation or it is used for phospholipid synthesis (**green**). PlsX and PlsY acylate glycerol-3-phosphate to 1-acyl-glycerol-3-phosphate, to which another fatty acid is added to the 2-position by PlsC.

There are two possibilities for the further utilization of the newly produced acyl-ACP. Either it is condensed by FabB or FabF for another elongation round or it has reached a sufficient chain length and is used for phospholipid formation. In the latter process, the peripheral membrane protein PlsX converts acyl-ACP to acyl-phosphate. This step is

essential since the following enzyme PlsY is not able to use acyl-ACP or acyl-CoA as substrates. The glycerol-phosphate acyltransferase PlsY connects a fatty acid to the 1-position of glycerol-3-phosphate. In γ-proteobacteria this reaction can also be taken over by PlsB. At the end, the internal membrane protein PlsC acylates the 2-position of 1-acyl-glycerol-3-phosphate (Lu *et al.*, 2006).

An important feature of fatty acids is their composition. Depending on different environmental conditions, like temperature changes, the structure of the membrane is altered by the incorporation of unsaturated fatty acids. At low temperatures an increased membrane fluidity is necessary and as a result the ratio of unsaturated fatty acids in phospholipids is higher. With rising temperatures the cell is in need of an increased membrane rigidity, which bacteria accomplish by decreasing the amount of unsaturated fatty acids. As described above, in Gram-positive bacteria FabH is able to bind long-chain acyl-CoA, derived from exogenous fatty acids. This enables the introduction of *iso* or *anteiso* methyl branches, depending on the precursor (Zhang and Rock, 2008). For example, the availability of isoleucine is decisive for the provision of the branched chain *anteiso* precursor for FabH (Singh *et al.*, 2008). Another mechanism is used in *E. coli*. Here, FabA isomerizes *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP at the 10-carbon stage in the elongation module (Heath and Rock, 1996a). However, a following elongation of the FabA product can only be initiated by FabB, because FabF is not able to condense *cis*-3-decenoyl-ACP (Zhang and Rock, 2008). Since most bacteria do not contain the *fabA*-*fabB* genes, this route is only common in γ-proteobacteria. Other organisms came up with distinct solutions. In *B. subtilis* the desaturase Des inserts *cis* double bounds in fatty acids. The expression of the *des* gene is induced at low temperatures (Weber *et al.*, 2001) and controlled by the two-component regulator DesRK. The membrane fluidity determines the state of the membrane-associated kinase DesK, which activates the transcription of *des* through phosphorylation of DesR (Aguilar *et al.*, 2001).

Another way to control membrane homeostasis is transcriptional regulation. The transcription factor FadR is present in Gram-positive and Gram-negative bacteria. The homolog in *E. coli* is a member of the GntR family and a repressor of the *fad* regulon containing all genes required for transport, activation and degradation of fatty acids. (Dirusso *et al.*, 1992). FadR binding to its operator depends on the presence of acyl-CoAs. The outer membrane protein FadL transports exogenous fatty acids into the cell

(Higashitani *et al.*, 1993) and the acyl-CoA synthase FadD converts them into long-chain acyl-CoAs (Pech-Canul *et al.*, 2011). These in turn bind to FadR which leads to a release of FadR from its DNA binding site and de-repression of the *fad* regulon genes (Dirusso *et al.*, 1992). In addition, FadR also acts as an transcription activator of *fabA* and *fabB* (Henry and Cronan, 1991). Its counterpart is FabR which represses *fabA* and *fabB* expression. Therefore, these two proteins control the properties of the membrane, since FabA and FabB are responsible for unsaturated fatty acid synthesis (Zhang *et al.*, 2002). Although, the *B. subtilis* FadR also represses the genes of the β-oxidation, is does not accommodate any activator function. Furthermore, as a member of the TetR family, FadR in *B. subtilis* contains a distinct structure and the inhibiting long-chain acyl-CoAs are provided by the cytosolic synthetases LcfA and LcfB (Fujita *et al.*, 2007; Parsons and Rock, 2013).

In order to regulate fatty acid biosynthesis the transcription factor FapR controls a regulon containing *acpA, fabD, fabF, fabG, fabHA, fabHB, fabI, fapR, plsC* and *plsX*. This master regulator is highly conserved in *Bacillus*, *Listeria* and *Staphylococcus*, but not discovered in other species (Schujman *et al.*, 2003). FapR is exclusively inhibited by malonyl-CoA. Upon binding of the ligand, the structural arrangement is changed and FapR is released from the DNA. Due to the ability of sensing the levels of malonyl-CoA, FapR is a perfect sensor for the status of fatty acid synthesis and capable to adjust the expression of the elongation cycle genes (Schujman *et al.*, 2008). A disadvantage of this mechanism is the control only by the precursor of the synthesis. In *E. coli* another way of feedback inhibition is evolved by the end product acyl-ACP. One of three targets is the acetyl-CoA carboxylase, which is inhibited by long-chain acyl-ACP (Davis and Cronan, 2001). The other two are FabH and FabI (Heath and Rock, 1996). All three enzymes are responsible for decisive reactions in fatty acid biosynthesis, since they provide the precursor malonyl-CoA or initiate or complete the elongation cycle. All in all, bacteria developed several mechanisms to precisely regulate the production and the composition of fatty acids in order to adapt to different environmental conditions.

1.3 Acetyl-CoA carboxylase

The first committed step in fatty acid synthesis is the formation of the precursor malonyl-CoA, carried out by the acetyl-CoA carboxylase (Fig. 3) (Cronan and Waldrop, 2002). Its catalytic process is divided into two half-reactions. The first one is done by the biotin carboxylase (AccC) and the biotin carboxyl carrier protein (AccB). Both form a subcomplex consisting of two AccC homodimers bound to four AccB (Fig. 4a) (Broussard *et al.*, 2013a). In an ATP dependent reaction, AccC transfers a carboxyl group from bicarbonate to biotin, which is covalently attached to a single lysine residue of AccB. In the second half-reaction, acetyl-CoA is carboxylated by the carboxyltransferase (AccAD) using the carboxyl group from caboxybiotin-AccB (Polyak *et al.*, 2012). This second subcomplex is assembled by AccA and AccD to a $\alpha_2\beta_2$ heterotetramer (Fig. 4b), which forms the multimeric acetyl-CoA carboxylase together with AccB and AccC (Broussard *et al.*, 2013b). It was shown that AccC and the carboxyltransferase both maintain their catalytic functions when they are separated, but only if unbound biotin is used as substrate (Guchhait *et al.*, 1974). In the case of biotin linked to AccB, reflecting the *in vivo* status, the subcomplexes have little to no activity, whereas only the whole ACCase complex exerts full activity (Broussard *et al.*, 2013b).

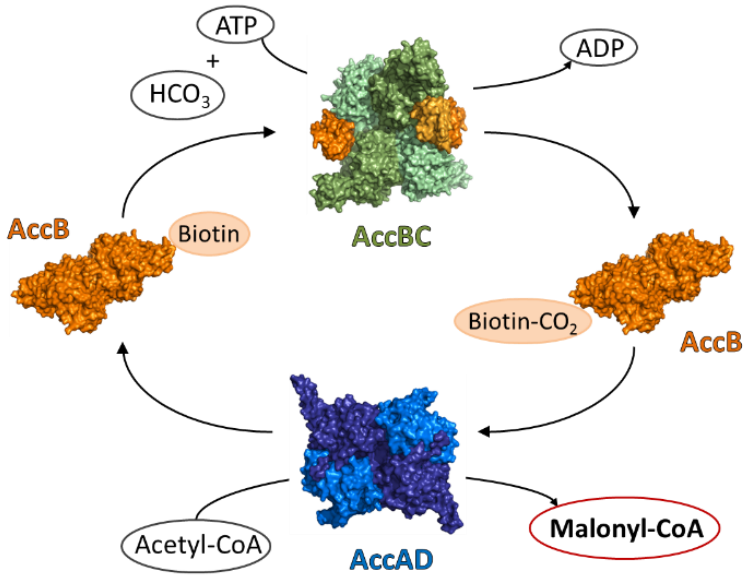

Fig. 3. Formation of malonyl-CoA by the acety-CoA carboxylase. Biotin is covalently bound to the biotin carboxyl carrier protein (AccB) and carboxylated by the biotin carboxylase (AccC) in the presence of ATP and bicarbonate. Following, the carboxyl group is transferred to acetyl-CoA by the carboxyltransferase (AccAD) resulting the precursor for fatty acid biosynthesis, malonyl-CoA. PDB files used here are, 3G8C for AccB, 4HR7 for AccBC and 2F9I for AccAD.

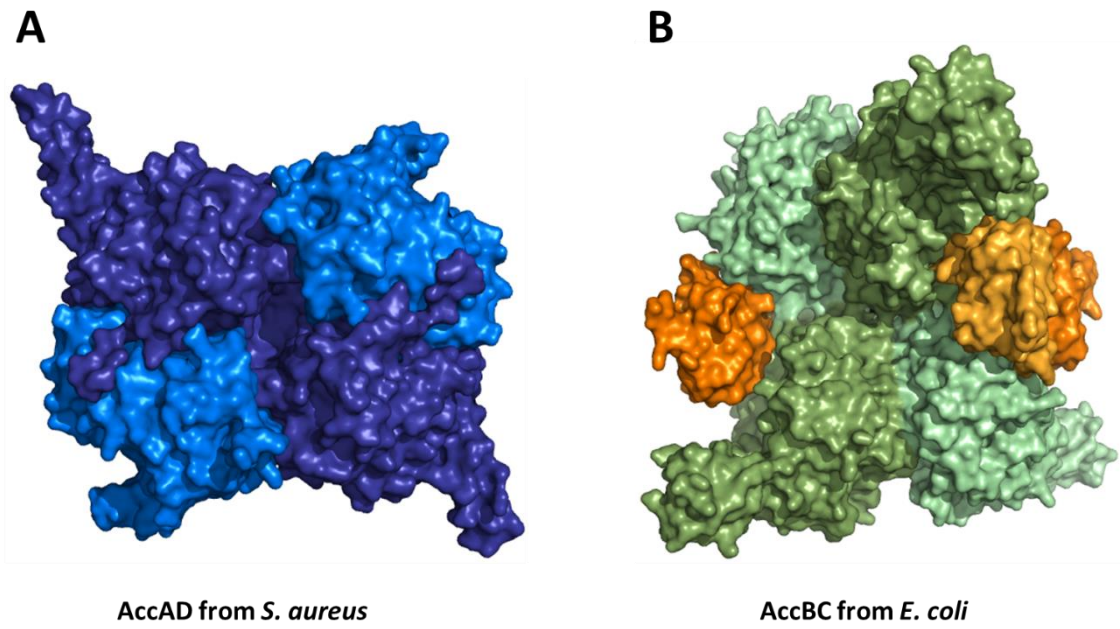

Fig. 4. Acetyl-CoA carboxylase subcomplexes. (**A**) The carboxyltransferase consists of two AccA (deep blue) and two AccD (light blue) subunits forming an $\alpha_2\beta_2$ heterotetramer (Broussard *et al.*, 2013b). (**B**) Complex of the biotin carboxyl carrier protein AccB (orange, gold) and the biotin carboxylase AccC (light green, green). Two AccC homodimers form a tetramer, which is bound to four AccB molecules (Broussard *et al.*, 2013a). PDB files used here are 2F9I for AccAD and 4HR7 for AccBC.

The genes encoding the biotin carboxyl carrier protein and the biotin carboxylase are *accB* and *accC*. These two genes are often found in clusters is Gram-positive as well as in Gram-negative bacteria. This allows coordinated expression which is important, since overexpression of the two subunits results either in disruption of biotin synthesis (Abdel-Hamid and Cronan, 2007) or inhibition of fatty acid synthesis (Karow *et al.*, 1992). The expression of this operon is dependent on the growth rate (Li and Cronan, 1993), which is also the case in *B. subtilis* (Marini *et al.*, 2001). Here, *accB* and *accC* cluster as well, but they are additionally located in an operon with *yqhY*. This supports the idea, that YqhY is involved in the synthesis of fatty acids or its regulation in *B. subtilis*.

The carboxyltransferase encoding genes are *accA* and *accD*, which are located in the same operon and upregulated by Spo0A in *B. subtilis* (Molle *et al.*, 2003). During sporulation Spo0A binds to its binding box upstream of the -35 region and reactivates the malonyl-CoA synthesis (Pedrido *et al.*, 2013).

In addition to the above mentioned inhibition by long-chain acyl-ACP (Davis and Cronan, 2001), there is another way to control ACCase activity in *E. coli*. The PII-like protein GlnB

forms a complex with AccB and AccC and thereby decreases the ACCase activity about 40%. This inhibition was reversed upon 2-oxoglutarate binding to GlnB and by GlnB uridylylation. The dependency on 2-oxoglutarate allows GlnB to measure the nutrient availability and to control the rate of malonyl-CoA production according to the need of fatty acids (Gerhardt *et al.*, 2015). Such a regulatory mechanism by a PII-like protein is not known in *B. subtilis*. Like most Gram-positive bacteria, *B. subtilis* only possess one PII GlnK homolog. Although GlnK is closely related to GlnB and their functions partially overlap (Forchhammer, 2008), the ACCase activity is not influenced by GlnK (Gerhardt *et al.*, 2015).

The high conservation and the essential role in initiation of fatty acid synthesis, make the acetyl-CoA carboxylase an interesting target of inhibition studies and drug therapy. Nevertheless, many aspects about its regulation, especially in *B. subtilis*, remain elusive and need further investigation. A yet unknown role in ACCase regulation in *B. subtilis* could be fulfilled by the unknown protein YqhY, since the knockout leads to mutations affecting the ACCase subunits and the encoding gene is located in an operon with the *accBC* cluster.

1.4 Protein degradation in *Bacillus subtilis*

The degradation of proteins plays a major role in protein homeostasis in all cells. The control of protein quality and removal of misfolded and aggregated proteins is especially important in cells exposed to threatening environments like heat, oxidative stress or salt stress. Moreover, proteolysis is also involved in signaling pathways by controlling the abundance of transcription factors and other regulators. In eukaryotes, ubiquitin is used to label proteins for degradation via the proteasome. Bacteria came up with other ways to get rid of ineffective proteins or control certain pathways by proteolysis (Battesti and Gottesman, 2013). The AAA+ (ATPase associated with diverse cellular functions) proteases are degradation complexes that utilize adaptor proteins or recognition sequences, called degrons, to dispose selected proteins. These complexes contain an ATPase subunit which is built up by Hsp100/Clp proteins. *E. coli* possesses the ATPases ClpA, ClpX and HslU, whereas *B. subtilis* has two additional ATPases in ClpC and ClpE, while lacking ClpA (Kirstein *et al.*, 2009b). The ATPases form a hexameric ring, in which

substrates are unfolded and forwarded through a central axial pore to a chamber in the proteolytic unit (Sauer and Baker, 2011). In case of ClpP, this chamber, containing the active sites, is assembled by two heptameric rings (Wang *et al.*, 1997) and only small peptides can pass the rings to reach the active center in the absence of an ATPase (Lee *et al.*, 2010). In addition to ClpP, the proteolytic unit can also be build up by HslV (also known as ClpQ). An overview of the AAA+ proteases and their adaptor proteins in *B. subtilis* is shown in Fig. 5.

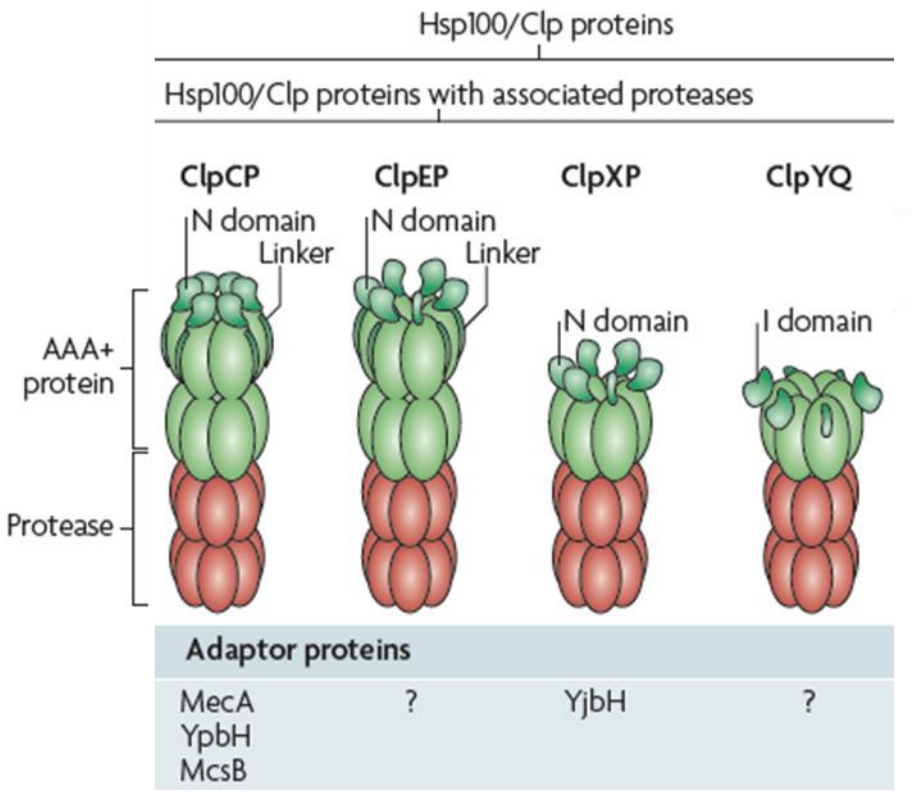

Fig. 5. Composition and adaptor proteins of Hsp100/Clp proteins and AAA+ proteases in *B. subtilis* (adapted from Kirstein *et al.*, 2009b). The proteolytic subunits ClpP and ClpQ are shown in red and the ATPases ClpC, ClpE, ClpX and ClpY are in green. The corresponding adaptor proteins are listed below.

The transcription of ClpC, ClpE, ClpX and ClpP is repressed by CtsR and strongly upregulated during heat stress (see below). Nevertheless, ClpC is also present in non-stressed cells (Molière and Turgay, 2013) and a recent study suggested a model in which ClpC is active independent of any co-factors. It was presumed that post-translationally arginine phosphorylation destabilizes the native structure of proteins and marks them as substrates for ClpCP degradation. Phosphorylated arginine residues are recognized by the N-terminal domain of ClpC which subsequently recruits the target proteins to the proteolytic unit (Trentini *et al.*, 2015). This is in contrast to the previous assumption, that

ClpC activity depends on binding to its adaptor proteins MecA, YpbH and McsB (Kirstein *et al.*, 2006; Kirstein *et al.*, 2007).

Like its paralogue YpbH, MecA tags misfolded and aggregated proteins for ClpCP degradation (Schlothauer *et al.*, 2003). On the other hand, MecA also regulates competence development through binding of ComK. This master regulator activates the transcription of competence genes and protein levels are kept low by ClpCP degradation at exponential growth. During stationary growth, the anti-adaptor ComS is expressed as consequence of a quorum sensing pathway. Subsequently, ComS binds to MecA and replaces ComK. The released ComK induces transcription of its own gene and the genes necessary for competence (Turgay *et al.*, 1998). According to another theory, the degradation of ComK benefits motility gene expression. The activation of the competence genes *comFA* by ComK leads to a read-through transcription of *flgM*. The encoded protein is an anti-sigma factor inhibiting the transcription of the flagellar proteins Hag and FliD. Thus, maintaining low ComK concentrations allows mobility gene expression (Molière and Turgay, 2013).

The third known adaptor protein for ClpC is McsB, responsible for targeting the repressor CtsR (Kirstein *et al.*, 2007). As mentioned before, CtsR prevents expression of the class III heat shock genes, including *ctsR*, *clpC*, *clpE*, *clpP* and *mcsA* and *mcsB* (Derré *et al.*, 1999). The degradation of CtsR by ClpCP occurs after its phosphorylation by McsB, which also bears a kinase activity. The auxiliary protein McsA stimulates the autophosphorylation of arginine residues of McsB, which in turn phosphorylates CtsR (Kirstein *et al.*, 2007; Fuhrmann *et al.*, 2009). This mechanism is antagonized via dephosphorylation of McsB by the arginine phosphatase YwlE (Kirstein *et al.*, 2007). Additionally, ClpC binds to unphosphorylated McsB and this interaction also prevents kinase activity (Elsholz *et al.*, 2011a). Taken together, under normal growth conditions unphosphorylated McsB is connected with ClpC and CtsR represses the transcription of the heat shock genes. A shift to high temperatures provokes a conformational change of CtsR and the regulator is released from the DNA (Elsholz *et al.*, 2010). At the same time, McsB is displaced from ClpC by MecA targeting unfolded proteins (Kirstein *et al.*, 2007). The free McsB is subsequently phosphorylated and marks CtsR for degradation. This means that proteolysis is not needed for activation of heat shock gene transcription, but for removal of inactive CtsR (Molière and Turgay, 2013).

MscB is also involved in oxidative stress response. Exposed to disulfide stress, two conserved cysteine residues of McsA were oxidized, resulting in the separation of the McsAB complex (Elsholz *et al.*, 2011b). Despite the absence of McsA, McsB is still able to bind CtsR and remove it from the operator (Kirstein *et al.*, 2005). In Gram-positive bacteria lacking McsA and McsB, the ATPase ClpE was suggested to take over this function. During disulfide stress, the zinc finger domain of ClpE is oxidized, enabling an interaction between ClpE and CtsR. Again, this finally leads to CtsR inactivation and de-repression of the CtsR regulon (Elsholz *et al.*, 2011b). Apart from that, the role of ClpE is poorly understood. It is hardly detectable under normal growth conditions, but the amount of ClpE is significantly increased for a short time after heat stress, until it is degraded by ClpCP (Gerth *et al.*, 2004). So far, no adaptor protein is known for ClpE (Kirstein *et al.*, 2009b), but ClpEP is proposed to be part of protein quality control in response to high temperatures. In the early phase of heat stress, ClpEP removes inactive CtsR until ClpCP starts to degrade ClpE and finalizes the decay of the remaining CtsR. This points out a backup function for ClpE in heat shock response in *B. subtilis* (Miethke *et al.*, 2006). Moreover, in *Streptococcus mutans*, the ClpEP protease degrades SsrA-tagged proteins (Tao and Biswas, 2015). The *ssrA* gene codes for a so called tmRNA that adds a signal peptide to unfinished proteins. The transcription of the *ssrA* gene is induced by heat stress and high ethanol concentrations (Muto *et al.*, 2000). In case of an incomplete translation, the tmRNA occupies the A-site of a stalled ribosome and transfers alanine to the nascent polypeptide. At the same time, the original mRNA is released and translation is switched to the tmRNA ORF. Afterwards, the residual part of the SsrA-tag (AGKTNSFNQNVALAA) is added and translation terminates at a stop codon in the end. This reaction rather provides a way to release stalled ribosomes than tag specific proteins for degradation (Moore and Sauer, 2007).

In *B. subtilis*, ClpCP and ClpEP lack this ability whereas ClpXP is the only proteolytic unit that degrades SsrA-tagged proteins (Wiegert and Schumann, 2001). A more important role plays ClpXP in the adaptation to oxidative stress. With the help of the adaptor protein YjbH, ClpXP controls the protein levels of Spx (Garg *et al.*, 2009). This transcriptional regulator induces genes involved in thiol homeostasis, cysteine biosynthesis, detoxification and NADPH production (Zuber, 2009). On the other hand, Spx sequesters the C-terminal domain of the α-subunit of the RNA polymerase and prevents the binding

of transcriptional activators like ResD and ComA (Nakano *et al.*, 2003). During normal growth, Spx is constantly degraded by ClpXP (Molière and Turgay, 2013), but it is stabilized under oxidative stress. This is due to oxidation of a zinc-binding domain leading to inactivation of ClpX (Zhang and Zuber, 2007). Another stabilizing factor is YirB, an anti-adaptor protein of YjbH, preventing the interaction of YjbH and Spx and therefore the destruction by ClpXP (Kommineni *et al.*, 2011).

A special Hsp100/Clp ATPase is ClpB in *E. coli*, that does not interact with ClpP (Kirstein *et al.*, 2009b). Instead, ClpB refolds aggregated proteins together with small heat shock proteins and DnaK (Mogk *et al.*, 2003). Homologs of this unfoldase are not present in *B. subtilis*, however, *in vitro* experiments demonstrated disaggregation activity for the ClpC-MecA complex in the absence of ClpP (Schlothauer *et al.*, 2003). These findings implicate that ClpC has a similar function to ClpB in dissolving of aggregated proteins (Molière and Turgay, 2013). LonA and LonB are further exceptions for proteases as both harbor their ATP hydrolysis and proteolysis functions within the same polypeptide chain (Duman and Löwe, 2010). They are thought to be part of sporulation regulation, since *lonB* is particularly transcribed under sporulation conditions and LonA very likely degrades the sigma factor σ^G. Furthermore, σ^H-mediated gene expression is possibly controlled by LonA and LonB at low pH (Molière and Turgay, 2013).

The need of controlled and directed protein degradation is obvious. Bacteria not only face challenges during changing environments, they also have to adjust protein expression and regulate the concentrations of specific proteins at different stages of development. The Gram-negative α-proteobacterium *Caulobacter crescentus* uses an adaptor hierarchy to manage the degradation of key factors during the transition from a swarmer cell stage to a stalked cell stage. For this purpose, the proteolytic complex ClpXP is loaded with several different adaptor proteins depending on the substrates. The first adaptor CpdR promotes the destruction of one class of substrates. Moreover, the priming of ClpXP by CpdR is a prerequisite for binding of the adaptor RcdA that facilitates the degradation of a second class of substrates. The third class of substrates is tethered by PopA which in turn can only bind to RcdA. At the same time, PopA prevents the degradation of RcdA-dependent protease substrates. Thus, at a higher level of the hierarchy adaptors can serve as anti-adaptors for substrates reliant only on the lower levels of the hierarchy (Joshi *et al.*, 2015).

Last but not least, pivotal proteins participating in proteolysis are interesting targets for antibiotic treatment. For example, acyldepsipeptide (ADEP) binds to ClpP and prohibits the complex formation with Clp ATPases, which convey substrate specificity and are needed for the decomposition of large polypeptides. Simultaneously, ADEP binding leads to a conformational change enabling ClpP to nonspecifically degrade unfolded proteins and nascent polypeptides emitted by ribosomes (Kirstein *et al.*, 2009a). The broad range of regulatory functions carried out by proteases as well as the involvement in almost all substantial cellular processes, points out the importance of Clp-mediated protein degradation. Nevertheless, in many of these processes the exact mode of action of the proteases remains unclear and the discovery of new targets and the possible concomitant need of novel adaptor proteins are challenging questions.

1.5 Aim of this work

The aim of this work was the characterization of one representative of the Asp23 protein family in *Bacillus subtilis*, YqhY. Despite all members of this family are both highly conserved and highly expressed in Gram-positive bacteria, almost nothing is known about the functions of Asp23 proteins. Previous studies already pointed out a vital function of YqhY, since the deletion of the encoding gene revealed an impaired phenotype and resulted in the rapid acquisition of suppressor mutations that affect the subunits of the acetyl-CoA carboxylase (Tödter, 2013). This protein complex catalyzes the first committed step in fatty acid biosynthesis, the conversion of acetyl-CoA to malonyl-CoA. The observed genetic link between YqhY and the acetyl-CoA carboxylase suggests an involvement of YqhY in fatty acid synthesis. Studies on the impact of the suppressor mutations were performed and a possible influence of YqhY on the acetyl-CoA carboxylase was investigated, in order to gain further insights for the role of YqhY and Asp23 proteins in general.

2 Materials and Methods

Materials: Chemicals, utilities, equipment, commercial systems, proteins, antibodies, enzymes and oligonucleotides are listed in the appendix.

2.1 Bacterial strains and plasmids

See appendix of this work

2.2 Growth media

Buffers, solutions and media were prepared with deionized water and autoclaved (20 min at 121°C and 2 bar). Thermolabile substances were dissolved and sterilized by filtration. Solutions are related to water, other solvents are indicated. Basic media were supplemented with agar (1.5% w/v) for solidification.

2.2.1 Bacterial growth media and optional additives

IPTG Stock solution: 0.5 M,
final concentration: 0.5 mM in medium

X-Gal Stock solution: 40 mg/ml X-Gal in DMF,
final concentration: 40 µg/ml in medium

5 x C-Salts

20	g	KH_2PO_4
80	g	$K_2HPO_4 \times 3\ H_2O$
16.5	g	$(NH_4)2SO_4$

to 1 l deion. H_2O

III' salts

0.232	g	$MnSO_4 \times 4\ H_2O$
12.3	g	$MgSO_4 \times 7\ H_2O$

to 1 l deion. H_2O

1x C minimal medium	20	ml	5x C salts
	1	ml	Tryptophan (5 mg/ml)
	1	ml	Ferric ammonium citrate (2.2 mg/ml)
	1	ml	III`salts
	to 100 ml deion. H_2O		

1 x CSE medium	20	ml	5 x C-salts
	1	ml	Tryptophan (5 mg/ml)
	1	ml	Ferric ammonium citrate (2.2 mg/ml)
	1	ml	III`salts
	2	ml	Potassium glutamate (40%)
	2	ml	Sodium succinate (30%)
	to 100 ml deion. H_2O		

10x MN medium	136	g	K_2HPO_4 x 3 H_2O
	60	g	KH_2PO_4
	10	g	Sodium citrate x 2 H_2O
	to 1 l deion. H_2O		

MNGE medium		877	µl	10 x MN medium
		1	ml	Glucose (20%)
		50	µl	Potassium glutamate (40%)
		50	µl	Ferric ammonium citrate (2.2 mg/ml)
		100	µl	Tryptophan (5 mg/ml)
		30	µl	$MgSO_4$ x 7H_2O (1 M)
	+/-	100	µl	CAA (10%)
	to 10 ml deion. H_2O			

LB-medium	10	g	Trypton
	5	g	Yeast extract
	10	g	NaCl
	to 1 l deion. H_2O		

SP-Medium	8	g	Nutrient Broth
	0.25	g	$MgSO_4$ x 7 H_2O
	1	g	KCl
	to 1 l deion. H_2O		
	autoclave, after cooling down addition of:		
	1	ml	$CaCl_2$ (0.5 M)
	1	ml	$MnCl_2$ (10 mM)
	2	ml	Ferric ammonium citrate (2.2 mg/ml)

2.2.2 Antibiotics

Antibiotics were prepared as 1000-fold concentrated stock solutions. Ampicillin, spectinomycin, lincomycin and kanamycin were dissolved in deionized water, chloramphenicol, erythromycin and tetracycline in 70% ethanol. All solutions were sterile filtrated and stored at -20°C. Autoclaved medium was chilled down to approximately 50°C, and antibiotics were added to their final concentration. For light sensitive additives such as tetracycline, incubation occurred in the dark.

Selection concentration for *B. subtilis*

Chloramphenicol	5 µg/ml
Erythromycin	2 µg/ml[1]
Kanamycin	5 µg/ml
Lincomycin	25 µg/ml[1]
Spectinomycin	150 µg/ml
Tetracycline	12.5 µg/ml

[1]For selection on *ermC* a mixture of erythromycin and lincomycin was used in their respective concentrations, see above.

Selection concentration for *E. coli*	
Ampicillin	100 µg/ml
Spectinomycin	100 µg/ml
Kanamycin	50 µg/ml

2.3 Methods

2.3.1 General methods

Some general methods used in this work that are described in the literature are listed in Tab. 1.

Tab. 1. General methods

Method	**Reference**
Absorption measurement	Sambrook *et al.*, 1989
Ethidiumbromide staining	Sambrook *et al.*, 1989
Precipitation of nucleic acids	Sambrook *et al.*, 1989
Gel electrophoresis of DNA	Sambrook *et al.*, 1989
Plasmid preparation from *E. coli*	Sambrook *et al.*, 1989
Ligation of DNA fragments	Sambrook *et al.*, 1989
Determination of protein amounts	Bradford, 1976
Gel electrophoresis of proteins (denaturating)	Laemmli, 1970
Sequencing according to the chain termination method	Sanger *et al.*, 1977

2.3.2 Cultivation of bacteria

Unless otherwise stated, *E. coli* was grown in LB medium at 37°C and 200 rpm in tubes and flasks. *B. subtilis* was grown in LB medium, CSE-Glc and MNGE medium at 37°C or 28°C in tubes and Erlenmeyer flasks. Fresh colonies from plates were used for inoculation. Furthermore, overnight liquid cultures were used. Growth was measured at a wavelength of 600 nm. For the calculation of the generation time the optical density of cultures in the logarithmic growth phase was used (Commichau *et al.*, 2015).

Storage of bacteria

E. *coli* was kept on LB medium agar plates up to four weeks at 4°C. For long-term storage DMSO cultures were established. SP agar plates and tubes were used for the long-term storage of *B. subtilis*. For the storage of bacteria in DMSO cultures were prepared with 900 µl of a fresh overnight culture mixed with 100 µl of DMSO (100%). Stocks were frozen and stored at -80°C (Commichau *et al.*, 2015).

2.3.3 Transformation of *Escherichia coli*

Preparation of competent cells in SOB medium

A single colony of *E. coli* DH5α or XL1 Blue was used to inoculate an overnight culture in 4 ml LB medium. This culture was used to inoculate 50 ml of LB medium. The culture was incubated at 37°C and 200 rpm in baffled flasks for 8 h.

6 ml of this culture were used for inoculation of 250 ml SOB-medium and incubated over night at 18°C. After reaching an OD_{600} of 0.5-0.9 the culture was cooled down by incubation for 10 min on ice. The cells were harvested by centrifugation (10 min; 4000 rpm; 4°C) and resuspended in 80 ml of ice-cold TB-buffer. After centrifugation (10 min; 4000 rpm; 4°C), the cells were resuspended in 20 ml of ice-cold TB buffer. DMSO (7% final concentration) was added and aliquots of 200 µl were frozen in liquid nitrogen and stored at -70°C (Commichau *et al.*, 2015).

SOB medium	20	g	Trypton
	5	g	Yeast extract
	0.58	g	NaCl
	0.186	g	KCl
	to 1 l deion. H_2O, autoclave		
	10	mM	$MgCl_2$
	10	mM	$MgSO_4$
TB buffer (pH 6.7)	3.46	g	PIPES
	18.64	g	KCl
	to 1 l deion. H_2O, autoclave		
	2.2	g	$CaCl_2 \times H_2O$
	18.64	g	KCl
	55	ml	$MnCl_2$ (1M)

Preparation of competent cells with $CaCl_2$

A single colony of *E. coli* DH5α or XL1 Blue was used to inoculate an overnight culture in 4 ml LB medium. The next day, 50 ml of LB medium were inoculated to an OD_{600} of 0.05 with the overnight culture and incubated at 37°C and 200 rpm. After reaching an OD_{600} of 0.3-0.5, the cells were harvested by centrifugation (10 min; 5000 rpm; 4°C) and resuspended in 5 ml of ice-cold $CaCl_2$. The cells were kept on ice for 30 min and afterwards centrifuged again (10 min; 4000 rpm; 4°C). The pellet was resuspended in ice-cold $CaCl_2$ and subsequently used for transformation (Commichau *et al.*, 2015).

Transformation of competent *Escherichia coli* cells

100 µl of the competent cells were mixed with DNA and kept on ice for 30 min. The heat shock was performed at 42°C for 60-90 s. Afterwards, the samples were incubated for 5 min on ice. After addition of 400 µl LB medium, the samples were incubated for additional 60 min at 37°C at 200 rpm. 100 µl and the concentrated rest were plated on LB selection plates (Commichau *et al.*, 2015).

2.3.4 Transformation of *Bacillus subtilis*

Preparation of competent cells

10 ml of MNGE medium containing 1% CAA were inoculated with an overnight culture of *B. subtilis* to an OD_{600} of 0.1. This culture was grown at 37°C with agitation until an OD_{600} of 1-1.3 was reached. Then, the culture was diluted with 10 ml MNGE medium without CAA and incubated again for one hour. After this incubation step, 400 µl of competent cells were incubated with DNA for 30 min at 37°C with shaking. Afterwards, 100 µl of expression mix were added and the samples were incubated at 37°C for one hour. 100 µl and the concentrated rest of the cell suspensions were spread onto SP containing the appropriate antibiotics for selection (Commichau *et al.*, 2015).

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

2.3.5 Preparation and detection of DNA

Preparation of plasmid DNA from *E. coli*

Plasmid DNA was prepared from *E. coli* carrying the desired plasmid. For high copy vectors an overnight culture of 4 ml with cells carrying the desired plasmid was harvested. For low copy vectors 20 ml of an overnight culture were harvested. The plasmid DNA was isolated using the NucleoSpin® Plasmid Kit (Macherey-Nagel) according to the manufacturer's instructions. Deionized water was used for elution of the DNA from the columns. All steps were performed at room temperature (Commichau *et al.*, 2015).

Isolation of genomic DNA of *B. subtilis*

B. subtilis cultures were grown overnight in 4 ml LB medium and harvested the next day. The genomic DNA was isolated using the peqGOLD Bacterial DNA Kit (PEQLAB) following the manufacturer's instructions. Deionized water was used for elution of the DNA from the columns (Commichau *et al.*, 2015).

Purification of DNA

After PCR, restriction and dephosphorylation DNA fragments were purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. Deionionized water was used for elution of the DNA from the columns (Commichau *et al.*, 2015).

Agarose gel electrophoresis

For analytical and preparative separation of DNA fragments, agarose gels containing Midori Green stain and 1% (w/v) agarose in TAE buffer were prepared. The DNA samples were mixed with 5x DNA loading dye and loaded onto the gel. The gel was run at a voltage of 80–140 V until the separation of the DNA was sufficient. Afterwards, the DNA was detected and documented by Gel Doc™ XR+ (Bio-Rad). For the estimation of the size of the DNA fragments λ-DNA marker was used (Commichau *et al.*, 2015).

50x TAE buffer (pH 8)	242	g	Tris base
	57.1	ml	Acetic acid (100%)
	100	ml	0.5 M EDTA
	Add to 1 l deion. H_2O		

5x DNA loading dye	5	ml	Glycerol (100%)
	4.5	ml	deion. H_2O
	200	µl	50x TAE buffer
	0.01	g	Bromphenol blue
	0.01	g	Xylene cyanol

DNA extraction from agarose gel

For the isolation of DNA fragments from preparative gels, the bands were visualized at a wavelength of λ_{365}. The bands were cut out and transferred into an Eppendorf tube. The purification of the DNA was performed with the peqGOLD Gel Extraction Kit (PEQLAB) according to the manufacturer's instructions (Commichau *et al.*, 2015).

Sequencing of DNA

Sequencing was done based on the chain termination method (Sanger) with fluorescence labeled dideoxynucleotides. The sequencing reactions were conducted by SeqLab (Göttingen, Germany) and the Laboratorium für Genomanalyse (G_2L) of the Georg-August-University Göttingen.

Restriction of DNA

The restriction of DNA with endonucleases was performed with buffers recommended by the manufacturer. Reaction buffers, concentration of enzymes and DNA as well as incubation temperature and incubation time were chosen according to the manufacturer's instructions. (Commichau *et al.*, 2015).

Dephosphorylation of DNA

To avoid re-circularization of a previously digested DNA vector, the 5' phosphate groups of the linearized vector were removed prior to the ligation reaction. The dephosphorylation of the 5`-prime end of DNA fragments was performed with the FastAP alkaline phosphatase (ThermoFisher) with buffers supplied by the manufacturer. Therfore, 1 µl FastAP (1U/µl) were added to the restriction mix and incubated at 37°C for 10 min (Commichau *et al.*, 2015).

Ligation of DNA

DNA fragments were ligated using T4-DNA ligase (ThermoFisher) with buffers supplied by the manufacturer. The ligation reaction contained 10 - 200 ng of vector DNA and an excess of the DNA fragment (insert to vector molar ratio of 3:1). The reaction was started after addition of 5 U T4-DNA ligase in a final volume of 20 µl. The ligation occurred for at least 1 h at RT or overnight at 16°C (Commichau *et al.*, 2015).

Polymerase chain reaction (PCR)

DNA was amplified by PCR with specific oligonucleotide primers. For each DNA fragment of interest, primers were designed. For cloning of DNA fragments into plasmid vectors, recognition sequences for specific restriction endonucleases were added via primers at both ends of the amplified DNA fragments. Oligonucleotides were purchased from Sigma-

Aldrich (Munich, Germany). All oligonucleotides used in this work are listed in the appendix.

The PCR reactions were performed in a total volume of 50 µl and for amplification *Phusion*™ polymerase was used. For check PCR the *Taq* polymerase (own purification) was used.

Reaction conditions for *Phusion* polymerase (50µl):

10	µl	HF reaction buffer (5x)
1	µl	dNTPs (12.5 µmol/ml)
1	µl	template DNA (2-10 ng/ µl)
1	µl	DNA polymerase
1	µl	forward primer (5 µmol/ µl)
1	µl	reverse primer (5 µmol/ µl)
35	µl	deion. H_2O

The sample was mixed and placed in a thermocycler. After an initial DNA denaturation step, a cycle consisting of three steps including denaturation, primer annealing and primer elongation was performed for 30 times. The reaction was terminated after a final elongation step by cooling down to 15°C. Time and temperature for denaturation, time of annealing and elongation temperature remained constant for each reaction. The annealing temperature (Tm) was dependent on the length of the oligonucleotide and its G+C content. (Commichau *et al.*, 2015).

Standard program for the *Phusion*™ polymerase

denaturation	95°C	5 min	
denaturation	95°C	20 s	30 x
annealing	Tm -5 [°C]	30 s	30 x
elongation	72°C	30 s per 1 kb	30 x
elongation	72°C	10 min	
break	15°C	∞	

Reaction conditions for *Taq* polymerase (25µl):

2.5	µl	*Taq* reaction buffer (10x)
1	µl	dNTPs (12.5 µmol/ml)
1	µl	template DNA (2-10 ng/ µl)
1	µl	DNA polymerase
1	µl	forward primer (5 µmol/ µl)
1	µl	reverse primer (5 µmol/ µl)
12.5	µl	deion. H_2O

Standard program for the *Taq* polymerase

denaturation	95°C	5 min	
denaturation	95°C	30 s	30 x
annealing	Tm -5 [°C]	45 s	30 x
elongation	72°C	60 s per 1 kb	30 x
elongation	72°C	10 min	
break	15°C	∞	

Long flanking homology PCR (LFH-PCR)

Deletion of a gene in *B. subtilis* was performed with the long flanking homology PCR (LFH-PCR) technique. For this purpose, genes that mediate resistance against chloramphenicol, erythromycin, kanamycin and spectinomycin were amplified from the plasmids pDG646, pDG780, pDG1726 and pGEM-cat, respectively (Guérout-Fleury *et al.*, 1995). DNA fragments of about 1 kb flanking the target gene at its 5' and 3' ends were amplified. The 3' end of the upstream fragment as well as the 5' end of the downstream fragment extended into the target gene in a way that all expression signals of genes up- and downstream of the gene remained intact. The joining of the two fragments to the resistance cassette was performed in a second PCR. Joining was allowed by complementary sequences of 25 bp that were attached to the single fragments by the respective primers. Thus, the 3` end of the upstream fragment was linked with the 5` end of the resistance cassette and the 3`end of the resistance with the 5`end of the downstream fragment. For the LFH joining reaction, the up- and downstream fragments

and the resistance cassette were used in equal molar ratios. The fused fragment was amplified by PCR using the forward primer of the upstream fragment and the reverse primer of the downstream fragment. *B. subtilis* was transformed with the purified PCR products and transformants were selected on plates. Clones were examined by check PCR for the integrity of the resistance cassette. The DNA sequence of the flanking regions was verified by sequencing (Commichau *et al.*, 2015).

Reaction mix (50µl):

10	µl	HF reaction buffer (5x)
2	µl	dNTPs (12.5 µmol/ml)
1	µl	*Phusion*™ polymerase
1	µl	upstream fragment
1	µl	downstream fragment
1	µl	resistance cassette

to 50 µl deion. H_2O

After a pre-incubation step, the primer were added to the mix

5 µl	forward primer (5 µmol/ µl)
5 µl	reverse primer (5 µmol/ µl)

LFH standard program

	denaturation	95°C	5 min	
	denaturation	95°C	30 s	10 x
1st step	annealing	Tm -5 [°C]	30 s	10 x
	elongation	72°C	30 s per 1 kb	10 x
	break	15°C	∞	

Addition of primer

LFH standard program

	denaturation	95°C	30 s	20 x
	annealing	Tm -5 [°C]	30 s	20 x
2nd step	elongation	72°C	30 s per 1 kb	20 x
	elongation	72°C	10 min	
	break	15°C	∞	

2.3.6 Preparation and analysis of proteins

Overexpression of proteins in *E. coli*

An overnight culture of *E. coli,* carrying the relevant plasmid, was used to inoculate a new culture in LB medium to an OD_{600} of 0.1. This culture was grown at 37°C (200 rpm) until they had reached an OD_{600} of 0.6-0.8. At this point, the expression of recombinant proteins was induced by the addition of isopropyl-β-D-thio-galactopyranoside (IPTG, final concentration: 1 mM). After 3 h the culture was harvested by centrifugation (15 min; 8000 rpm; 4°C). The pellet was resuspended in buffer W or in ZAP buffer and centrifuged again. After removing the supernatant the cells were stored at -20°C (Commichau *et al.*, 2015).

Cell disruption with the French® press

The cell pellet was resuspended in buffer W or in ZAP buffer and filled in the precooled bomb. The disruption took place with a pressure of 18000 PSI and was performed at least three times (Commichau *et al.*, 2015).

Purification of His_6-tagged proteins

For protein purification, the frozen pellets were resuspended in cold ZAP buffer containing 10 mM imidazole and subsequently disrupted by the French® pressure cell as described above. Cell debris and other insoluble material were removed by ultracentrifugation (1 h; 35000 rpm; 4°C). The supernatant was loaded onto a 2 ml bed volume of Ni^{2+}-NTA resin (Qiagen) in a Poly-Prep Chromatography Column (Biorad). The Ni^{2+}-NTA resin was pre-equilibrated with 20 ml ZAP buffer containing 10 mM imidazole. After washing with 10-20 ml of ZAP buffer containing 10 mM imidazole the His_6-tagged proteins were eluted. The elution was performed with ZAP buffer containing an increasing concentration of

imidazole (for example 50 mM, 100 mM, 200 mM, and 500 mM). To analyze the purification success, samples of each fraction were loaded onto a polyacrylamide gel and after electrophoresis stained with Coomassie brilliant blue. The relevant fractions were combined and dialyzed overnight. The protein concentration was determined using the Bradford assay (Commichau *et al.*, 2015).

ZAP buffer (pH 7.5)	50	mM	Tris base
	20	mM	NaCl
	1	mM	EDTA
	to 1 l deion. H_2O		

Purification of Strep-tagged proteins

For protein purification, the frozen pellets were resuspended in cold buffer W and subsequently disrupted by the French® pressure cell as described above. Cell debris and other insoluble material were removed by ultracentrifugation (1 h; 35000 rpm; 4°C). The supernatant was loaded onto 1 ml Strep-Tactin Sepharose (IBA) in a Poly-Prep Chromatography Column (Biorad). Pre-equilibration of the matrix was performed with 10-20 ml of buffer W. After washing 4-6 times with 1.5 ml buffer W, the bound proteins were eluted with buffer E in 4 fractions (1x 500 µl, 3x 1 ml). The fractions were analyzed by SDS-PAGE (Commichau *et al.*, 2015).

Buffer W (pH 8)	100	mM	Tris base
	150	mM	NaCl
	Add to 1 l deion. H_2O		

Buffer E (pH 8)	100	mM	Tris base
	150	mM	NaCl
	2.5	mM	D-desthiobiotin
or	10	mM	Biotin
	Add to 1 l deion. H_2O		

Dialysis of proteins

The elution fractions were dialyzed against the desired buffer, which was in excess of about 1000 fold, in a dialysis tube overnight. (Commichau *et al.*, 2015).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE as described by Laemmli (1970). Protein samples were mixed with 5x SDS loading dye and denatured by boiling at 95°C for 30 min. The polyacrylamide concentration of the gels was chosen according to the expected proteins sizes varying from 8 to 15% (v/v). Samples were loaded onto the prepared gel. Electrophoresis was performed at 100-180 V until the bromphenol blue had reached the lower end of the gel. During electrophoresis, proteins were first focused in the stacking gel and subsequently separated by their molecular mass in the running gel. The self-constructed protein marker *Page King* and the purchased protein marker PageRuler Plus (ThermoFisher) were used as size standards (Commichau *et al.*, 2015).

5x SDS loading dye	1.4	ml	Tris-HCl pH 6.8 (1.5 M)
	5	ml	Glycerol (100 %)
	0.5	g	SDS
	1.6	ml	β-Mercaptoethanol
	0.02	g	Bromphenol blue
		ml	Bromphenol blue
	to 10 ml deion. H_2O		

5% Stacking gel	1.3	ml	Acrylamide-Bisacrylamide (30%)
	1	ml	Tris-HCl pH 6.8 (1.5 M)
	5.5	ml	deion. H_2O
	80	µl	SDS (10%)
	80	µl	APS (10%)
	8	µl	TEMED
8% Running gel	4	ml	Acrylamide-Bisacrylamide (30%)
	3.8	ml	Tris-HCl pH 8.8 (1 M)
	6.9	ml	deion. H_2O
	150	µl	SDS (10%)
	150	µl	APS (10%)
	9	µl	TEMED
12% Running gel	6	ml	Acrylamide-Bisacrylamide (30%)
	3.8	ml	Tris-HCl pH 8.8 (1 M)
	4.9	ml	deion. H_2O
	150	µl	SDS (10%)
	150	µl	APS (10%)
	6	µl	TEMED
15% Running gel	7.5	ml	Acrylamide-Bisacrylamide (30%)
	3.8	ml	Tris-HCl pH 8.8 (1 M)
	3.4	ml	deion. H_2O
	150	µl	SDS (10%)
	150	µl	APS (10%)
	6	µl	TEMED

Coomassie staining of polyacrylamide gels

Protein gels were stained with Coomassie Brilliant Blue. For this purpose, the gels were incubated in staining solution for 30 min while shaking. Afterwards, the gels were transferred in water and heated in the microwave to remove the staining. This process

was repeated until an optimal contrast between protein bands and background was reached (Commichau *et al.*, 2015).

Staining solution	0.5	%	Coomassie Brillant Blue R250
	10	%	Acetic acid
	45	%	Methanol
	44.5	%	deion. H_2O

Silver staining of polyacrylamide gels

The silver staining of the protein bands was performed as described by (Nesterenko *et al.*, 1994). For staining, the polyacrylamide gels were incubated with the following solutions with shaking (Commichau *et al.*, 2015).

Step	Reagent	Duration
Fixing	Fixing solution	1 to 24 h
Washing	Ethanol 50 %	3 x 20 min
Reduction	Thiosulfate solution	1 min
Washing	deion. H_2O	3 x 20 s
Staining	Impregnating	15-25 min
Washing	deion. H_2O	2 x 20 s
Developing	Developer	until sufficiently stained
Washing	deion. H_2O	2 x 20 s
Stopping	Stop solution	5 min

Fixing solution	50	ml	Methanol (100%)
	12	ml	Acetic acid (100%)
	100	µl	Formaldehyde (37%)
	to 100 ml deion. H_2O		
Thiosulfate solution	20	mg	$Na_2S_2O_3$ x 5 H_2O
	to 100 ml deion. H_2O		
Impregnator	0.2	g	$AgNO_3$
	37	µl	Formaldehyde (37%)
	to 100 ml deion. H_2O		
Developer	6	g	$NaCO_3$
	2	ml	Thiosulfate solution
	50	µl	Formaldehyde (37%)
	to 100 ml deion. H_2O		
Stop solution	1.86	g	EDTA
	to 100 ml deion. H_2O		

Strep-protein interaction experiment (SPINE) for the analysis of protein-protein interactions

The Strep-protein interaction experiment (SPINE) was performed according to Herzberg *et al.*, 2007. This experiment was used to identify potential interaction partners of *B. subtilis* proteins *in vivo*. Therefore, the gene of interest was cloned into the expression vectors pGP380, pGP382, pGP1389 or pGP1460. A preculture of *B. subtilis* carrying the appropriate plasmid was grown for 8 h at 37°C in LB medium. This culture was used to inoculate 50 ml CSE medium containing 0.5% glucose and was grown overnight at 37°C. The next day, one liter of the same medium was then inoculated with the overnight culture to an OD_{600} of 0.1. When this culture had reached an OD_{600} of 1.0, 500 ml were supplemented with paraformaldehyde (4% in PBS) to a final concentration of 0.6%. This culture was and incubated for additional 15-20 min. Both types of culture were harvested

by centrifugation (15 min; 5,000 rpm; 4°C). The cells were washed in buffer W and centrifuged again. The pellets were stored at -20°C. For the preparation of the crude extract, the cell pellet was resuspended in buffer W and the cells were disrupted by the French® pressure cell as described above. Cell debris and other insoluble material were removed by ultracentrifugation (1 h; 35000 rpm; 4°C) and the supernatant was used for protein purification via Strep-Tactin Sepharose (IBA). The protein fractions were finally separated by SDS-PAGE (Commichau *et al.*, 2015).

10x PBS (pH 6.5)	80	g	NaCl
	2	g	KCl
	26.8	g	Na2HPO4 × 7 H2O
	2.4	g	KH2PO4
	to 1 l deion. H_2O		

Western blot analysis

The desired amount of cell free crude extract was previously separated by SDS PAGE. The transfer of the proteins on a PVDF membrane was performed using a semi dry blotting machine. Activation of the PVDF membrane occurred by short incubation (30 s) in 100% methanol. After blotting for 1-2 h with a current of 80 mA, the membrane was incubated in blotto for 1-3 h or overnight. Ensuing, the primary antibody (α-FLAG, 1:10000 in blotto) was added and incubation for 2-3 h or overnight followed. The membrane was then washed three times for 30 min with blotto, the secondary antibody (anti-rabbit, 1:100000 in blotto) was added and after 30 min the membrane was washed again three times for 20 min with blotto. At last the membrane was rinsed in deionized water and subsequently incubated in buffer III for 5 min. Detection of the proteins was enabled by the substrate CDP* (1:100 in buffer III). Chemiluminescence signals were detected by ChemoCam system (Intas).

Transfer buffer	15.1	g	Tris
	72.1	g	Glycine
	750	ml	Methanol (100%)
	to 5 l deion. H_2O		
Blotto	100	ml	TBS (10x)
	25	g	Skim milk powder
	1	ml	Tween 20
	to 1 l deion. H_2O		
Buffer III (pH 9.5)	0.1	M	Tris
	0.1	M	NaCl
	to 1 l deion. H_2O		
TBS (pH 7.6)	60	g	Tris
	90	g	NaCl
	to 1 l deion. H_2O		

Confirmation of AccB biotinylation

The AccB protein was purified with Strep-Tactin Sepharose as described above. After that, *in vitro* biotinylation was carried out in a 10 ml reaction mix:

1	mg	AccB
10	mg	Crude extract of BirA expressing cells
1	mM	Biotin
10	mM	ATP
1	Tablet	cOmplete Mini EDTAfree protease inhibitor
50	mM	Tris
100	mM	KCl

The reaction was incubated for 1 h at 37°C and afterwards at 4°C overnight with slightly agitation. The next day, AccB was purified again using the ÄKTAprime system and a heparin column. The successful biotinylation was confirmed by Western blot analysis using Streptavidin-horseradish peroxidase (HPR) (see above). Therefore, the protein fractions were separated by SDS PAGE and transferred on a PVDF membrane. After

blotting, the membrane was incubated in PBS containing 1% BSA for 1-2 h. Then, the Streptavidin-HPR (1:5000 in PBS containing 1% BSA and 0.1% Tween20) was added, followed by incubation for 1-2 h. The membrane was washed three times for 20 min with PBS and subsequently rinsed for 1 min in the developing solution (see below):

2	ml	1M tris pH8.5
100	µl	2.5 mM luminol in DMSO
44	µl	90mM paracumaric acid in DMSO
6.15	µl	H_2O_2
to 20	ml	deion. H_2O

Detection of the proteins was performed by the ChemoCam system (Intas).

2.3.7 Bacterial Adenylate Cyclase-based two-hybrid system (BACTH)

The bacterial two-hybrid system takes advantage of the reconstitution of the catalytic domain of the *Bordetella pertussis* adenylate cyclase (Karimova *et al.*, 1998). To test a potential interaction between two proteins, the T18 and T25 domains of the adenylate cyclase were fused to the N-terminal domain and to the C-terminal domain of the protein of interest respectively. For this purpose, each gene was cloned into two high copy vectors (pUT18, pUT18C) and two low copy vectors (p25-N, pKT25) using the *E. coli* strain XL1-blue. The co-transformation was carried out in the *E. coli* strain BTH101 and as positive control the plasmids pKT25-Zip and pUT18C-Zip were used. In these two plasmids the T18 and T25 domains are fused to a dimer forming leucine zipper. 30 µl of competent cells were mixed with 5 µg plasmid DNA (2.5 µg of the T18 domain and 2.5 µg of the T25 domain) and incubated for 30 min on ice. After a heat shock for 90 sec at 42°C, 120 µl of LB medium were added to the cells and incubation for 2 h at 30°C followed. At last, 4 µl of the cells were dropped on LB agar plates containing ampicillin, kanamycin, X-Gal and IPTG. Association of the two-hybrid proteins resulted in functional complementation between T25 and T18 fragments and lead to cAMP synthesis. cAMP then triggered the transcriptional activation of the lactose operon. The resulting conversion of X-Gal by the β-galactosidase yielded in the occurrence of blue colonies. Pictures were taken after 24 h and 48 h (Commichau *et al.*, 2015).

2.3.8 Acetyl-CoA carboxylase activity assay

The activity of the acetyl-CoA carboxylase was determined by coupling the conversion of acetyl-CoA to malonyl-CoA to the reaction of the malonyl-CoA reductase (MCR) from *Chloroflexus aurantiacus* (Kroeger *et al.*, 2011). This enzyme uses malonyl-CoA as substrate in a NADPH dependent reaction to produce 3-hydroxypropionate (Hügler *et al.*, 2002). The simultaneous oxidation of NADPH was monitored at 365 nm in a plate reader. The reaction was set up in a 96 well plate containing the ingredients listed below.

0.1	M	KCl
20	mM	$MgCl_2$
10	mM	ATP
0.5	mM	DTT (Dithiothreitol)
10	mM	$NaHCO_3$
1	mM	NADPH
1.6	mM	Acetyl-CoA
5	µg	MCR
100	nM	AccAD
100	nM	AccB
100	nM	AccC
to 150	µl	50 mM Tris/ 100mM KCl buffer (pH 8.5)

The reaction was initiated by the addition of the MCR and incubated at 25°C for 12 h. In case of the positive control without the addition of the ACCase and acetyl-CoA, the initial step was the addition of 0.3 mM malonyl-CoA.

3 Results

3.1 Impact of YqhY on the ACCase

3.1.1 Δ*yqhY* suppressor mutations are located in the acetyl-CoA subunits

Previous experiments showed the non-essentiality of *yqhY* and *yloU* (Tödter, 2013). Nevertheless, the occurrence of suppressor mutants resulting from the knockout of *yqhY* still supports a vital role of YqhY for growth of *B. subtilis*, even if it is not essential. The mutations occurred after approximately five days of growth on SP agar plates. The genomic DNA of several clones was isolated and analyzed by sequencing. In many cases, the mutations were located in the subunits of the acetyl-CoA carboxylase (Fig. 6).

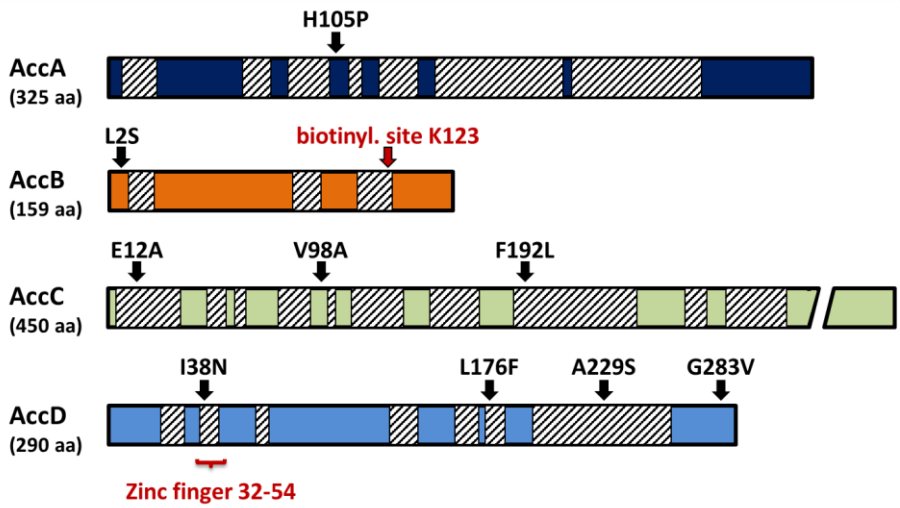

Fig. 6. Mutations in the acetyl-CoA carboxylase subunits. As a result of the deletion of *yqhY*, suppressor mutations were found in the subunits of the acetyl-CoA carboxylase (AccA, AccB, AccC and AccD). All mutations were single substitutions obtained from distinct clones. Many of them were located in highly conserved regions (hatched boxes).

Interestingly, every mutant contains a single substitution and all were found in all four subunits (AccA, AccB, AccC and AccD). Moreover, often highly conserved regions were affected, or the mutations are closely located next to these regions. For example, the substitutions E12A in AccC is close to Y82 and V295. These residues are necessary for biotin attachment in the active site of AccC (Chou *et al.*, 2009). The second mutation F192L is nearby the key side chains K116, K159, E201, H236 and K288 of the ATP binding pocket (Waldrop *et al.*, 2012). The last substitution V98A is not located in a binding region, but it

is also close to a conserved region and therefore maybe involved in complex stability or interaction with AccB. Regarding the substitutions in AccD, L176F and A229S are in close proximity to catalytic sites, which are surrounded by conserved residues. Since both affected residues are also highly conserved, they may be important for ACCase activity. Furthermore, AccD possesses a unique zinc-binding domain that is not only necessary for DNA binding but is probably also important for catalytic activity (Bilder *et al.*, 2006). Thus, the mutation I38N could result in activity changes. Another possibility is an altered DNA binding ability that would also impact the catalytic reaction, because DNA binding inhibits the enzymatic activity of the carboxyltransferase (Benson *et al.*, 2008).

All in all, the obtained substitutions indicate a change in ACCase activity. This could be due to altered substrate affinities, structure changes or decreased abilities to bind other subunits.

3.1.2 The absence of YqhY results in an increased formation of lipophilic clusters

The knockout of *yqhY* showed a severe growth defect resulting in the occurrence of suppressor mutations. Since the majority of these mutations was located in the acetyl-CoA subunits, this indicated an altered fatty acid production. To test this hypothesis, the accumulation of lipophilic components was visualized using the stain FM4-64. The different strains were grown in LB medium and samples were taken at exponential and stationary growth phase. In a first approach, the strains Bs168, GP1765 (Δ*yqhY*), GP2322 (Δ*yqhY* AccD A229S) and GP2323 (Δ*yqhY* AccD I38N) were compared (Fig. 7). Only cells taken at the exponential growth phase revealed a significant effect, whereas cells taken at the stationary growth phase showed no phenotypic alteration compared to the wild type. The absence of YqhY lead to lipophilic accumulations at the cell poles and the cell membrane. This hints at an increased fatty acid synthesis in these areas of the cell. On the other hand, the clustering is revoked after suppressor mutations occurred in AccD. Although, the effect of mutations in the other subunits were not investigated, these observations indicate that the increased cluster formation is abolished in the suppressor mutants.

Moreover, the abundance of AccDA and YqhY was investigated. For this purpose, *yqhY* and the operon of *accDA* were cloned separately into pBQ200. This vector allows

overexpression of target genes in *B. subtilis*. Again, samples were taken at exponential and stationary growth phase and stained with FM4-64. In both growth phases, neither the overexpression of *yqhY* nor *accDA* resulted in a significant phenotype. The cells showed a lipophilic pattern similar to the wild type.

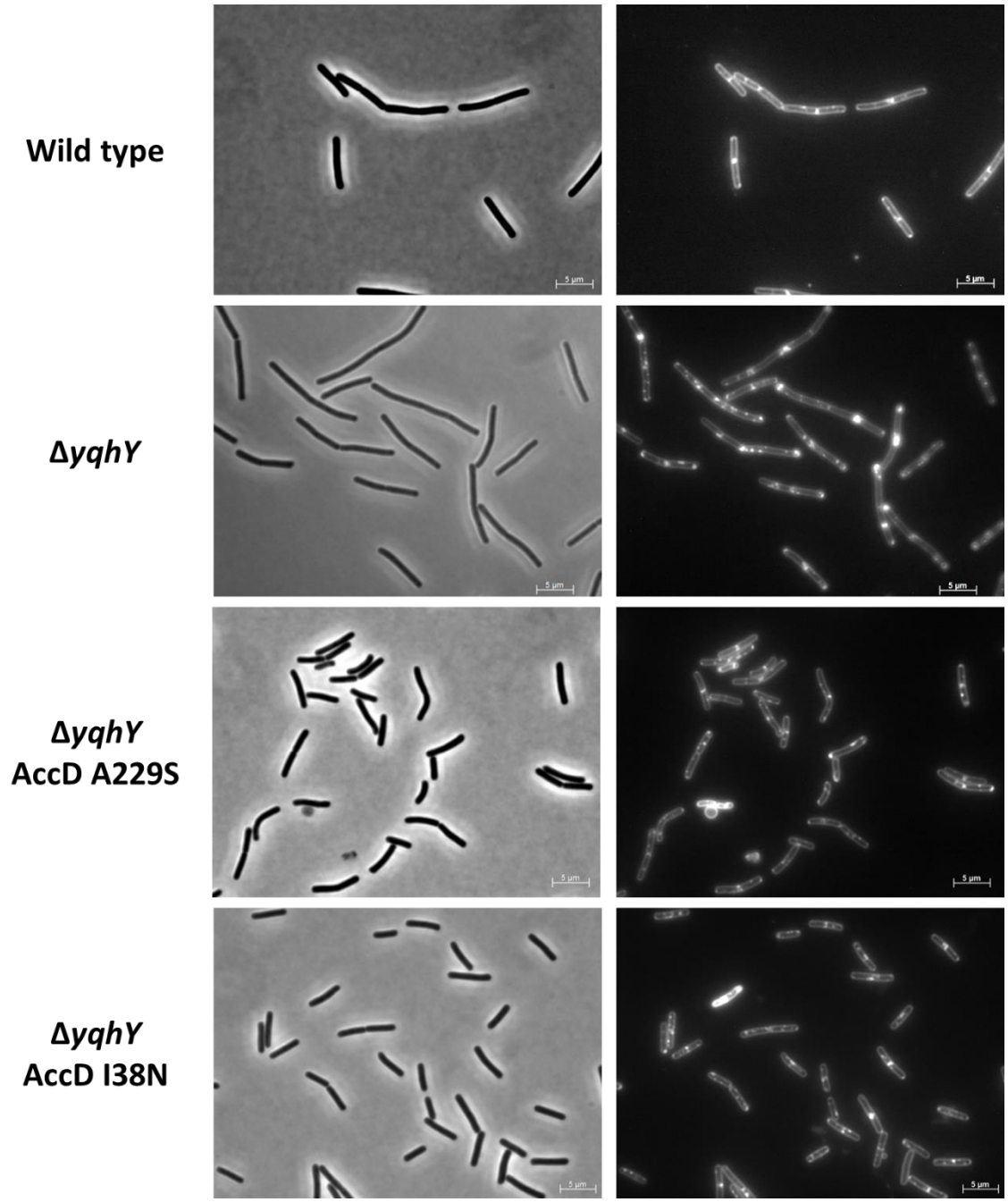

Fig. 7. Staining of lipophilic regions in the absence of YqhY. Samples were taken at exponential growth phase and treated with FM4-64. After the knockout of *yqhY*, lipophilic clusters were visible at the cell poles and near to the cell membrane. This cluster formation was abolished after the appearance of mutations in AccD.

3.1.3 YqhY impacts the localization of AccA

The suppressor mutations in the acetyl-CoA carboxylase subunits and the cell staining with FM4-64 suggest a possible role of YqhY in fatty acid synthesis. A possible function could be YqhY acting as a localization factor. Therefore, it is important to know the localization of YqhY and the subunits of the ACCase. To do this, *accA*, *accB* and *accC* were each cloned into the vector pBP43 to implement a C-terminal GFP-fusion to the encoding proteins. The expression was kept under the control of the respective natural promoter. A GFP-fusion to AccD using the same plasmid was not possible without deleting the essential downstream gene *accA*. But due to the strong complex formation of AccA and AccD, the localization of AccA also represents the localization of AccD. The cells were grown in LB medium and samples were taken at an OD_{600} of 1.5.

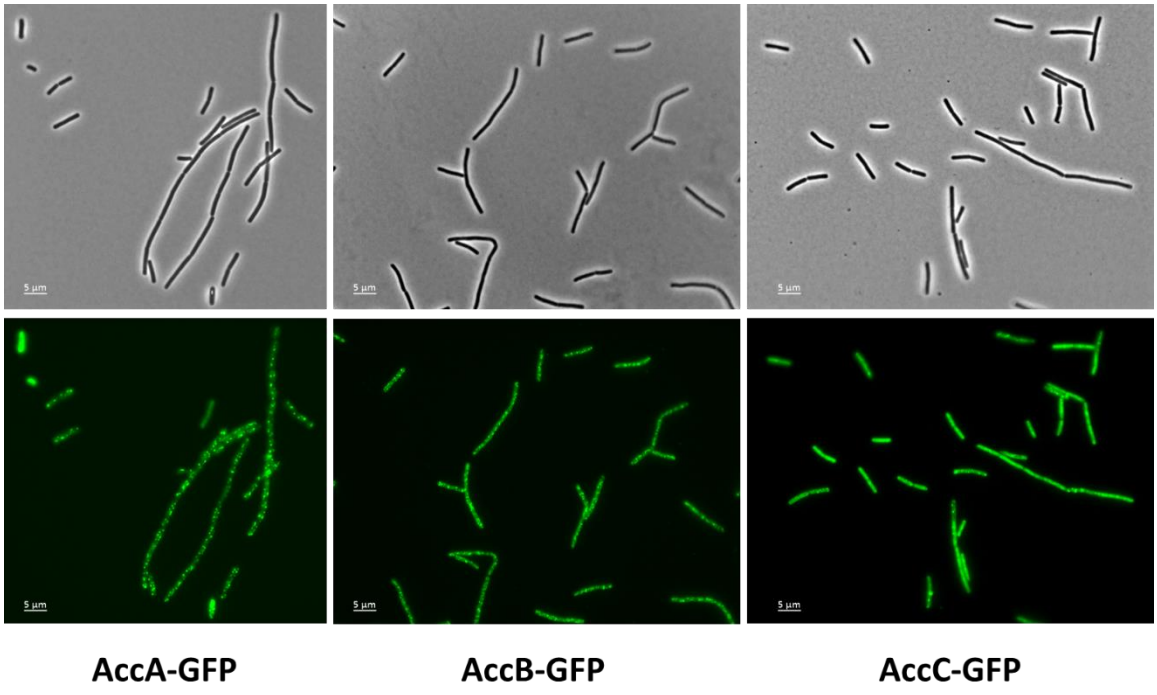

Fig. 8. Localization of AccA, AccB and AccC. The proteins were fused to a monomeric GFP at the C-terminus and visualized via fluorescence microscopy. All subunits were equally distributed in spots over the cell.

As shown in Fig. 8, all tested subunits where scattered equally in spots over the cell. This was in contrast to the cell pole localization of YqhY (Tödter, 2013). However, in the absence of YqhY the equal distribution of AccA changed towards a polar localization. The localization of the other subunits AccB and AccC was not affected in a Δ*yqhY* background.

In addition, the localization of AccA was analyzed in two suppressor mutant strains carrying mutations in AccD (GP1469) and AccA (GP1470). In both strains AccA again accumulates at the cell poles, which means that the occurrence of the suppressor mutations did not restore the phenotype to a wild type pattern (Fig. 9).

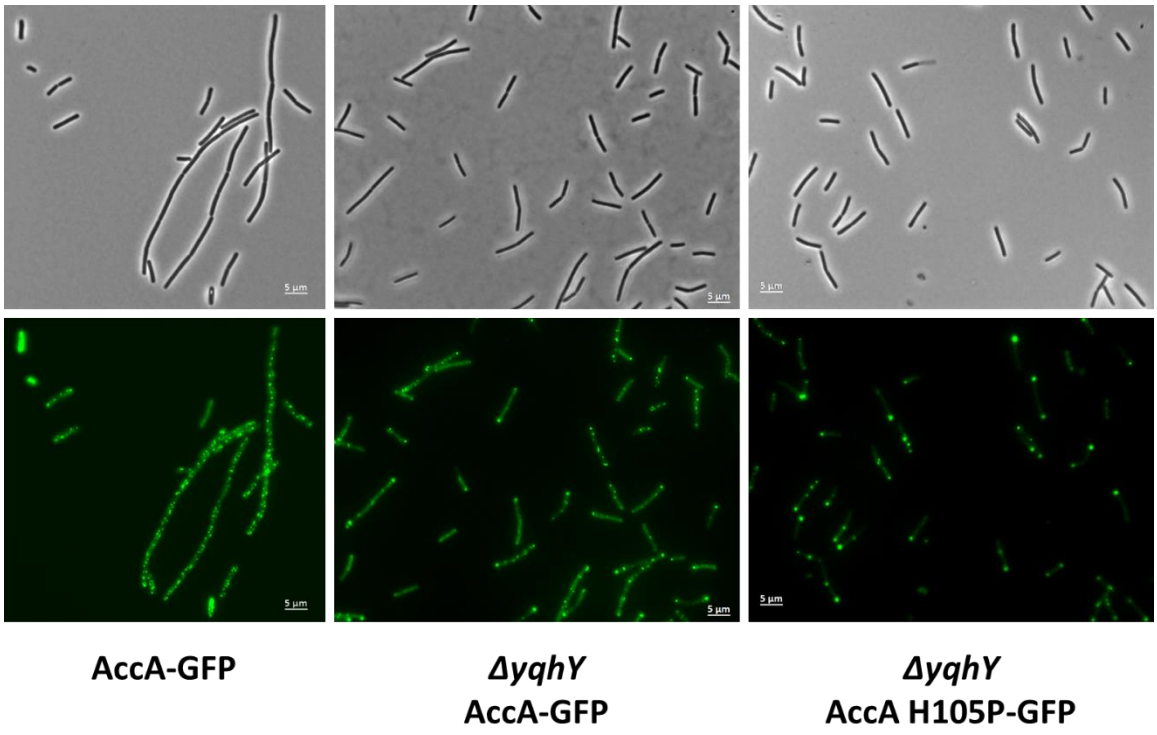

Fig. 9. Localization of AccA in Δ*yqhY* background. AccA was fused to a monomeric GFP at the C-terminus and visualized via fluorescence microscopy. In the wild type AccA was equally distributed in spots over the cell. In contrast to this, in the absence of YqhY the localization pattern changed towards a cell pole localization. This effect also remained after the occurrence of suppressor mutations as it is shown here for a mutation in AccA.

3.1.4 Investigation of an interaction of YqhY with the acetyl-CoA complex

Having a look at the genetic background of *yqhY* already reveals a connection to acetyl-CoA carboxylase. The gene forms an operon with *accB* and *accC*, which encode for the carboxyltransferase. Moreover, the impact on the localization of AccA and the increased formation of lipophilic clusters support the idea that YqhY directly interacts with at least one subunit of the ACCase complex.

3.1.4.1 YqhY interacts with AccC *ex vivo*

At first, the possible interaction of YqhY and the subunits was evaluated in a bacterial two-hybrid assay. In doing so, *yqhY* as well as *accA*, *accB*, *accC* and *accD* were cloned into the bacterial two-hybrid vectors and in this way, the proteins were fused to the T18 and T25 domains. After two days, YqhY, AccA, AccB and AccC showed the already known self-interactions. Besides, the complex formations of the carboxyltransferase (AccDA) and the AccBC complex were visible. Concerning YqhY, there was only an interaction with AccC (Fig. 10).

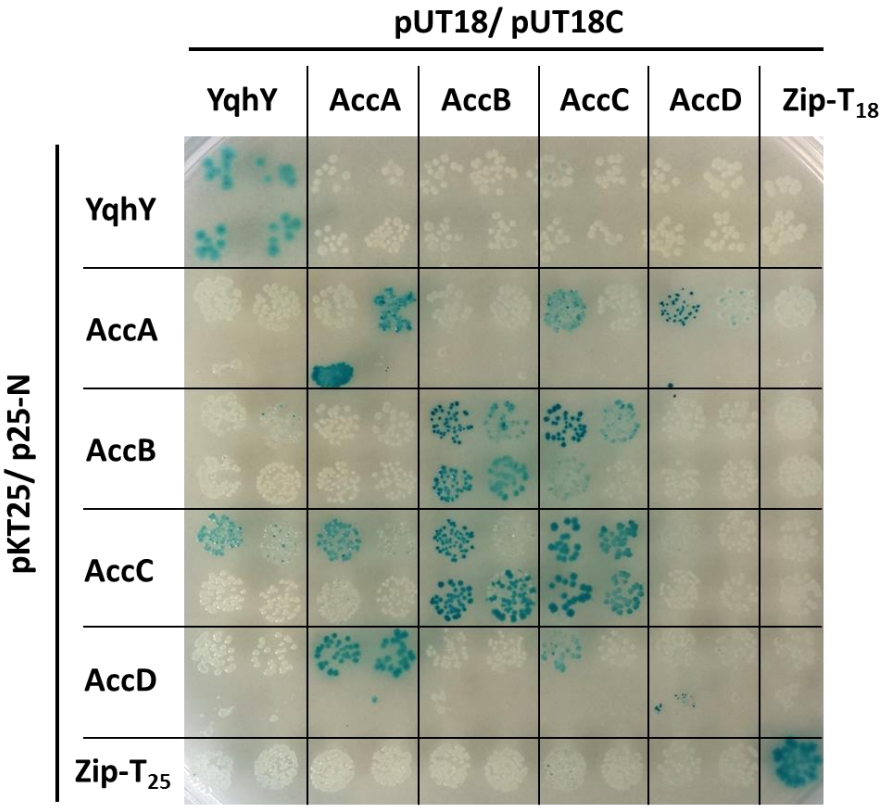

Fig. 10. Bacterial two-hybrid analysis of interactions between YqhY and the ACCase subunits. The respective genes were fused to the domains of the adenylate cyclase and incubated for two days. Interactions, indicated by blue colonies, were observable among the subunits themselves and between YqhY and AccC.

3.1.4.2 YqhY binds AccA and AccD only as a complex

In order to confirm the interaction between YqhY and AccC and examine again the non-interaction with the other subunits, a SPINE was performed. In this approach, YqhY functioned as a bait protein and was fused to a C-terminal Strep-tag. This was carried out by cloning the gene in the vector pGP1460, which integrates into the *lacA* locus and allows

constitutive expression of *yqhY*. The pray proteins, AccA, AccD and CshA, were fused to a C-terminal FLAG tag. *accA* and *cshA* were each cloned into pGP1331 to keep the expression under the control of the natural promoter. The same vector could not be used for a FLAG tag fusion to AccD, because *accA* would be deleted as a consequence. Therefore, *accD* was overexpressed in the vector pGP1370. After the transformation of Bs168 with the plasmids, three different strains were derived. Each of those carried a Strep-tag fusion to YqhY and FLAG tag fusion to AccA, AccD or CshA. These strains were grown in CSE medium containing 0.5% glucose and treated with paraformaldehyde during growth to crosslink adjacent proteins. After the SPINE the proteins were detected via Western blot. The results are shown in Fig. 11.

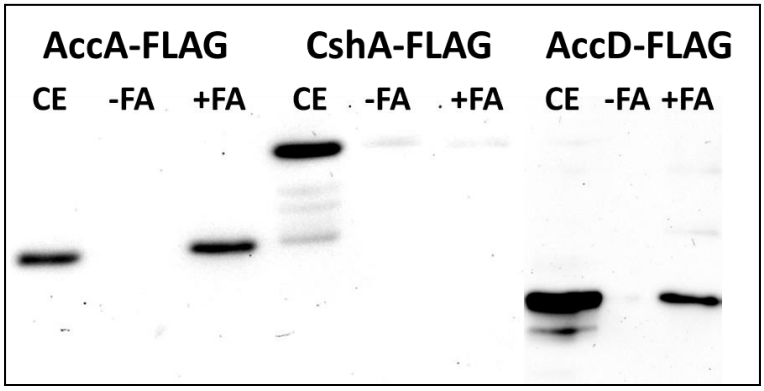

Fig. 11. Proof of interaction between YqhY and the carboxyltransferase via SPINE. Strains expressing YqhY-Strep were grown in CSE medium containing 0.5% glucose, adjacent proteins were crosslinked by the addition of paraformaldehyde (FA). The pray proteins (AccA, CshA and AccD) were fused to a FLAG tag and detected by Western blot analysis. YqhY was purified using a StrepTactin column and was co-eluted with AccA and AccD. CshA functioned as a negative control.

AccA and AccD could be observed in the elution fractions, suggesting an interaction with YqhY. As both proteins form a strong complex, YqhY is expected to bind the AccDA complex instead of interacting with only one of these proteins. This assumption is reinforced by the non-interaction of YqhY and AccA and AccD in the bacterial two-hybrid screen, since only interactions of two proteins can be examined. Nevertheless, the co-purification of AccD has to be evaluated carefully. The protein amount in the crude extract is significantly higher due to the overexpression of *accD*. But in the elution fraction only a little amount of AccD is detectable. In addition to this, a small amount of AccD-FLAG was also detectable in the absence of YqhY-Strep (data not shown), which means that AccD

can already be purified via the biotinylated AccB. Apart from this, AccA could not be purified in the absence of YqhY-Strep (data not shown), confirming that YqhY undoubtedly interacts with AccA.

The same approach was carried out to test the *in vivo* interaction of YqhY and AccB and AccC. This time, the latter proteins were fused to a Strep-tag with the help of pGP1460. On the other hand, YqhY was fused to a FLAG tag using pGP1331. Against the expectations given by the bacterial two-hybrid results, there was neither an interaction of YqhY with AccB nor AccC (data not shown).

3.1.5 Influence of YqhY on the acetyl-CoA carboxylase activity

The foregoing experiments showed an interaction of YqhY and the subunits of the acetyl-CoA carboxylase, demonstrating again a clear connection of YqhY to the fatty acid synthesis. In addition to this, an increased fatty acid synthesis was indicated by the lipophilic accumulation in the absence of YqhY. Giving these preconditions, there is the possibility of YqhY altering the activity the acetyl-CoA carboxylase by direct binding to the complex. To study this hypothesis, an *in vitro* assay was set up in which the reaction of the acetyl-CoA carboxylase was coupled with the reaction of the malonyl-CoA reductase.

3.1.5.1 Purification of proteins used in the activity assay

The first thing to do was the overexpression and purification of YqhY, the ACCase subunits and the malonyl-CoA reductase (MCR). For this purpose, YqhY was brought into the vector pETM-11, resulting in an N-terminal His-tag fusion. The remaining proteins were fused to a Strep tag. All components were overexpressed in *E. coli*, except AccA and AccD. The overexpression of AccA turned out to be toxic for *E. coli*, so *accA* was cloned into pGP382 for overexpression in *B. subtilis*. AccD could be co-purified with Strep tagged AccA. In a first try, AccB was also expressed without a tag in *B. subtilis*, as it can be purified via Strep-Tactin in its biotinylated form. But the protein amount was too low after the purification. Due to this reason, the plasmid pGP1027 was used for Strep tagged AccB purification. The respective fractions are shown in Fig. 12.

The purification of all desired proteins was successful. In case of AccB, the binding partner AccC was co-eluted as well as the biotin carboxylase from *E. coli*, which was able to bind

to the Strep-Tactin column with the help of the biotinylated $AccB_{Ec}$. These contaminations were checked later on to exclude the presence of factors that distort the outcomes of the activity assay. Finally, the elution fractions of the proteins were dialyzed against 50 mM Tris/ 100 mM KCl buffer containing 10% glycerol and frozen at 20°C until usage.

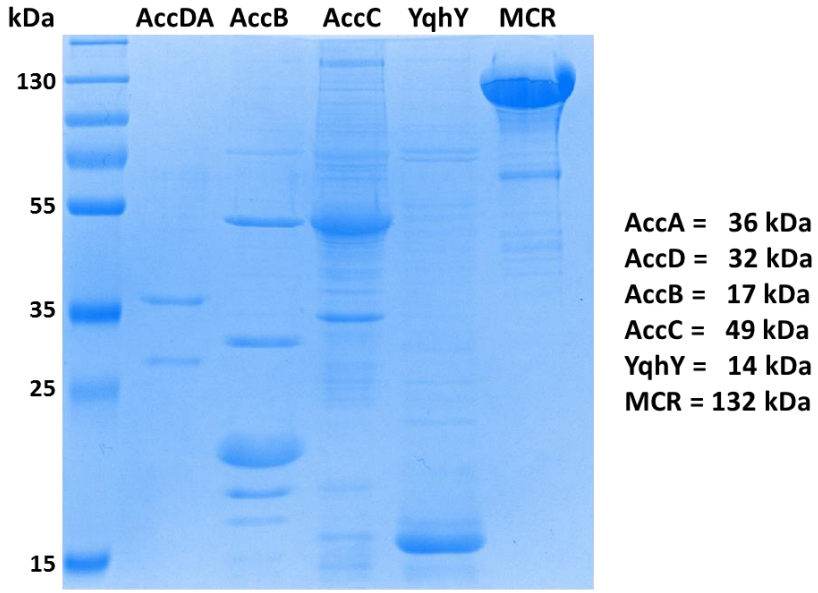

Fig. 12. Purification of the acetyl-CoA subunits, YqhY and malonyl-CoA reductase. The desired proteins were all expressed in *E. coli*, except AccA and AccD (in *B. subtilis*). Afterwards, they were purified on a Strep-Tactin column and Ni^{2+}-NTA column (in case of YqhY), respectively. In the end, the different fractions were separated by SDS-PAGE and visualized by Coomassie staining.

3.1.5.2 Validation of biotinylation of AccB

A crucial condition for the activity of the acetyl-CoA carboxylase is the biotinylation of AccB. To guarantee that this precondition is given, the previously purified AccB was biotinylated *in vitro* and tested in a Western blot using the Streptavidin-horseradish peroxidase (HRP). The biotin adding enzyme of AccB is BirA. This ligase was overexpressed in *E. coli* and subsequently the crude extract of these cells was added to the reaction mix containing AccB, biotin, ATP and protease inhibitor. The following day, this reaction mix was purified with a Heparin column and the obtained elution fractions were separated by SDS-PAGE (Fig. 13a).

The silver staining confirmed the presence of AccB in the elution fractions E1 to E5, together with the co-purified AccB from *E. coli*. To test whether AccB was bound to biotin, the same samples where used for Western blot analysis. In this purpose, biotinylated

proteins were bound to streptavidin coupled with the horseradish peroxidase. This enzyme catalyzes the oxidation of luminol resulting in enhanced light emission. As shown in Fig. 13b, in all fractions other than fraction E5, AccB was detectable, which means that in these samples AccB was biotinylated. Even the purified AccB that was not used for *in vitro* biotinylation was connected to its substrate, demonstrating that an extra biotinylation was unnecessary. Thus, for the following activity assay the pivotal state of AccB was given.

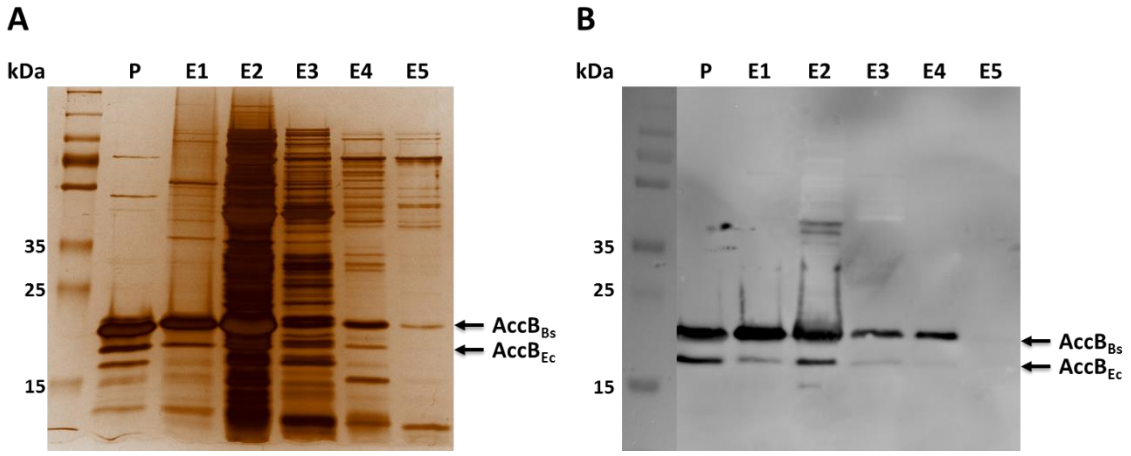

Fig. 13. Confirmation of biotinylation of AccB. The previously purified AccB (**P**) was used for *in vitro* biotinylation and afterwards purified again on a heparin column. The presence of AccB in the elution fractions (**E1-E5**) was confirmed by silver staining (**A**). The same fractions were analyzed for AccB biotinylation by Western blot using streptavidin-HRP (**B**).

3.1.5.3 YqhY does not directly influence the acetyl-CoA carboxylase activity

In order to investigate if the direct interaction of YqhY with the carboxyltransferase implicates a change in ACCase activity, a coupled enzyme assay was set up. In this assay the malonyl-CoA production was followed by NADPH oxidation catalyzed by the malonyl-CoA reductase (MCR). The activity was thereby measured in the presence and absence of YqhY in different amounts. As can be seen in Fig. 12, the addition of the AccB and AccC could also imply the addition of other enzymes that oxidize NADPH or consume the substrates of the ACCase or MCR. Furthermore, YqhY itself could consume one of the ingredients. To exclude both possibilities, the oxidation of NADPH was also monitored in the absence of AccDA on the one hand and only in the presence of YqhY on the other hand (Fig. 14).

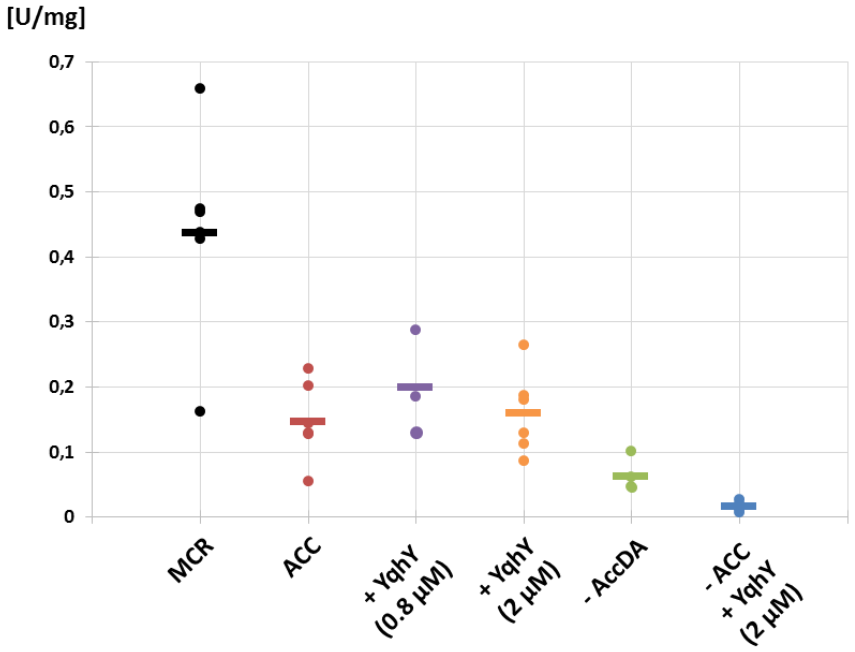

Fig. 14. Impact of YqhY on the ACCase activity. The production of malonyl-CoA by the acetyl-CoA carboxylase (**ACC**) was coupled to the NADPH dependent reaction of the malonyl-CoA reductase (**MCR**). The oxidation of NADPH was monitored in a plate reader. **Dots** represent single measurements and **bars** indicate the mean value of all measurements. Taking outliners into account, there was no significant change in activity by the addition of YqhY.

In all set ups the activity of the MCR was monitored by the oxidation of NADPH. For the positive control, malonyl-CoA was added and therefore the presence of the ACCase was not needed. Compared to this, the MCR activity was significantly lower in the coupled reaction, making obvious that the malonyl-CoA production by the ACCase in general was low. However, a reaction process of the ACCase was noticeable. In the absence of AccA and AccD also an activity was measurable, but since this reaction was always slower than the reaction of the whole complex, the participation of adulterating elements could be excluded. Using a composition without the ACCase and only with YqhY showed no activity at all. So, it was evident that YqhY shows no enzyme activity in producing malonyl-CoA or oxidizing NADPH. The original hypothesis, YqhY alters the activity of the ACCase, could not be confirmed. The addition of YqhY in neither lower nor higher amounts had no meaningful impact on the activity of the ACCase. In some cases the activity was increased, in other cases it turned out to be the opposite. On average, the reaction velocity of the acetyl-CoA carboxylase was equal with or without the YqhY. This made clear that YqhY has

no direct impact on the acetyl-CoA carboxylase activity, although a direct interaction was proven.

3.1.5.4 Suppressor mutations in *yqhY* deletions strains decrease the activity of the acetyl-CoA carboxylase

Despite the fact that YqhY seems to be no activity changing factor, the previous experiments still implied a change in fatty acid production in suppressor mutants. Hence, there was the question if the mutations resulted in a decreased ACCase activity. To further analyze the aftermath of the mutations, the coupled *in vitro* activity assay was used. In this approach, AccD was replaced by mutated versions ($AccD_{I38N}$, $AccD_{A229S}$). At first, the C-terminus of *accA* was amplified and brought into the vector pGP1389. The resulting plasmid pGP1849 was then used for transformation of GP2321 and GP2322, respectively. AccA were thereby fused to a Strep tag. Again, AccA and $AccD_{mut}$ were co-purified and the assay was performed as described above.

The previous set up verified that YqhY has no direct impact on the activity. Contrary to this, the results shown in Fig. 15 demonstrate that some mutations in AccD lead to a lower activity of the ACCase. In case of the $AccD_{I38N}$ mutation, the activity of the acetyl-CoA carboxylase was equal to the wild type activity. On the other hand, the $AccD_{A229S}$ mutation caused a significant decrease in malonyl-CoA production. This example shows, that not all mutations but at least some of them are responsible for a change in activity of the ACCase. This lead to the conclusion that YqhY has no direct impact on the activity, but cells missing this protein have to reduce the activity of the acetyl-CoA carboxylase. This might happen by mutating the subunits to either change the localization or decreasing the activity of the complex.

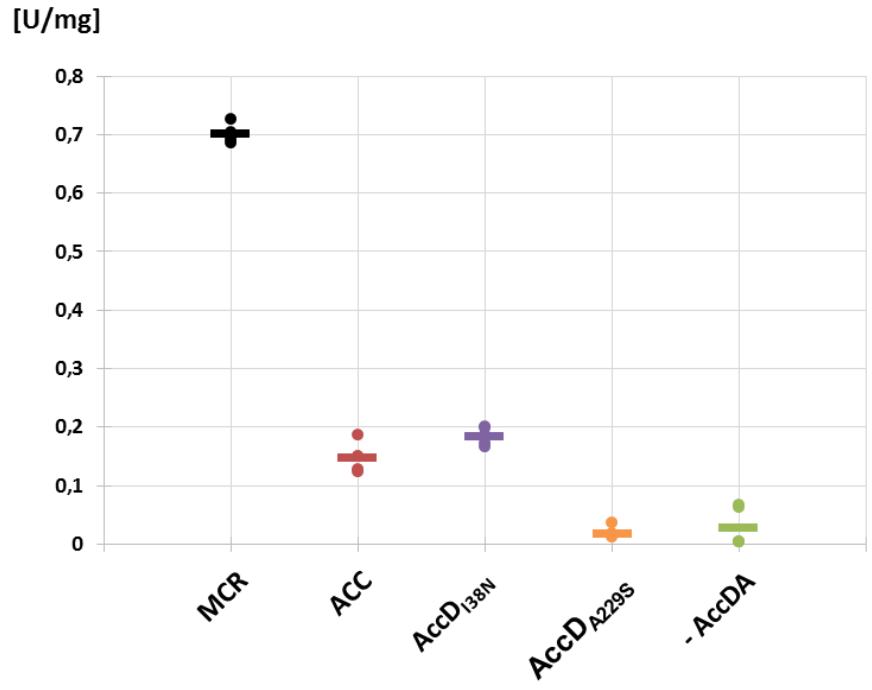

Fig. 15. Effect of suppressor mutations in *yqhY* deletions strains on the ACCase activity. The production of malonyl-CoA by the acetyl-CoA carboxylase (**ACC**) was coupled to the NADPH dependent reaction of the malonyl-CoA reductase (**MCR**). The oxidation of NADPH was monitored in a plate reader. **Dots** represent single measurements and **bars** indicate the mean value of all measurements. Mutations in AccD lead to a reduced activity of the ACCase, shown by the mutation $AccD_{A229S}$. On the other hand, not all mutations located in the subunits have direct influence on the activity as it is demonstrated by the mutation $AccD_{I38N}$.

3.2 The possible role of YqhY in Clp-mediated protein degradation

3.2.1 Δ*yqhY*-suppressor mutants hint to involvement in Clp-mediated proteolysis

Among the suppressor mutants derived after the knockout of *yqhY*, not all carried a mutation in one of the acetyl-CoA carboxylase subunits. Two of those were analyzed by whole genome sequencing revealing a deletion of *ctsR* in one strain and an insertion in *yjbH* in the other strain. Both observations indicated a connection to protein degradation by the Clp protease. YjbH is an adaptor protein for ClpX, promoting the degradation of the transcription regulator Spx via ClpXP. CtsR on the other hand is a transcription repressor of the CtsR regulon that contains genes encoding the proteolytic subunit ClpP, the ATPases ClpC, ClpE and ClpX, as well as the adaptor protein McsB. So, in an organism missing CtsR, these components of the Clp mediated protein degradation are upregulated leading to an increased degradation of substrates of ClpXP, ClpEP and ClpCP.

In order to confirm that the absence of CtsR or YjbH prevents the harming effect appearing after the knockout of *yqhY*, double deletion strains were constructed. In addition to the knockout of *yqhY*, a deletion of *yjbH* or *ctsR* was performed. As a negative control, a double deletion of *yqhY* and *yszB* was carried out. In this strain, *yszB* was replaced by the same resistance marker as *ctsR* and *yjbH*. The expected outgrowth of suppressor mutants could be observed for the *yqhY* single knockout strain and for the Δ*yqhY* Δ*yszB* control strain. In contrast to this, in a Δ*ctsR* and in a Δ*yjbH* background the formation of suppressor mutants was almost not existing (Fig. 16). Therefore, the deletion of *ctsR* or *yjbH* enables the growth of *B. subtilis* in the absence of YqhY without any other adjustments.

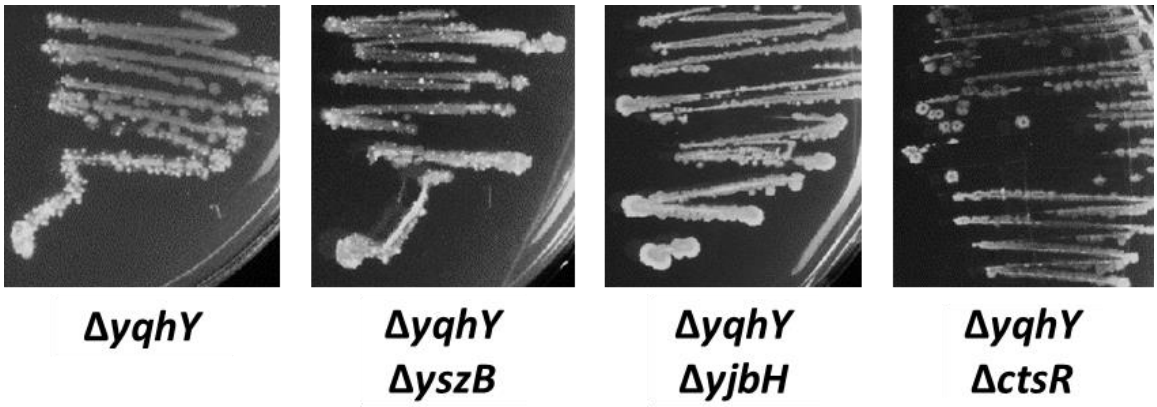

Fig. 16. Occurrence of Δ*yqhY*-suppressor mutants in a Δ*ctsR* and Δ*yjbH* background. The deletion of *yqhY* lead to suppressor mutant formation. This effect is abolished in double knockout strains missing either *yjbH* or *ctsR* in addition to *yqhY*. The double knockout of *yqhY* and *yszB* functioned as a negative control showing the same result as the *yqhY* single deletion.

3.2.2 Interaction of YqhY with Clp proteins

As mentioned above, in the absence of YqhY the degradation of substrates of the Clp machinery is in some cases upregulated, or the fatty acid synthesis is decreased by affecting the acetyl-CoA carboxylase. This lead to the assumption that YqhY might function as an adaptor protein for AccA, AccB, AccC or AccD. In YqhY deletion strains, the production of fatty acids might reach a toxic level, since the amount of the ACCase subunits is too high due to the missing adaptor protein and the concomitant needed degradation. This could be compensated by mutations affecting the functionality of the ACCase or by mutations inducing the degradation of the ACCase complex.

3.2.2.1 YqhY binds Clp ATPases *ex vivo*

To examine the hypothesis of YqhY being an adaptor protein, an interaction of YqhY and the Clp proteins had to be proven. In a first approach, the respective protein interactions were tested in a bacterial two-hybrid assay including YqhY, ClpC, ClpE, ClpP and ClpX. *clpE* was the only gene that has to be brought into the bacterial two-hybrid vectors, for all other genes the necessary constructs were already available. The screen was executed as described above. After two days, the known self-interactions of YqhY, ClpC, ClpE and ClpP were visible (Fig. 17).

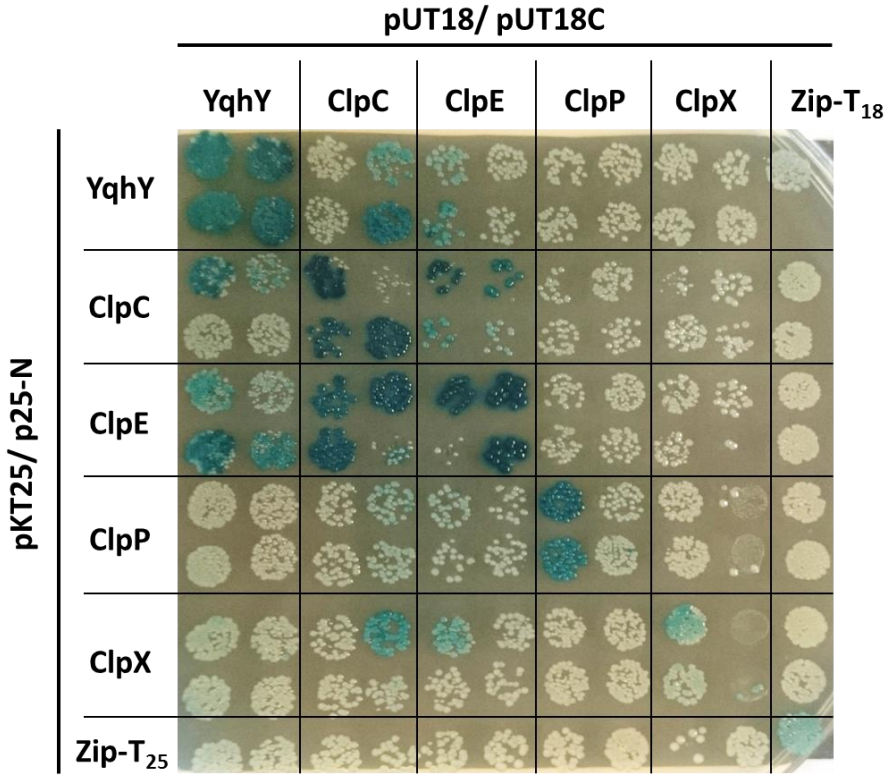

Fig. 17. Bacterial two-hybrid analysis of interactions between YqhY and Clp proteins. The respective genes were fused to the domains of the adenylate cyclase and incubated for two days. Interactions, indicated by blue colonies, were observable among the subunits themselves, between YqhY and ClpE, and YqhY and ClpC.

Regarding ClpX, the missing self-interaction could be observed in repetitions. Moreover, complex formations of ClpC and ClpE were detectable and interactions of YqhY with ClpC and ClpE were shown. A striking missing part is the interaction of ClpP with the ATPases ClpC, ClpE and ClpX. However, interactions of YqhY and ClpC as well as YqhY and ClpE were

identified, supporting the possibility of YqhY acting as an adaptor protein for the Clp degradation complex.

3.2.2.2 YqhY interacts with the ClpCP protease

The obtained interactions of YqhY and ClpC and ClpE in the bacterial two-hybrid screen gave a hint on the involvement in Clp-dependent degradation, but were in need of further evaluation. To confirm these interactions *in vivo*, YqhY, ClpC and ClpP were subject to a SPINE. Again, YqhY was used as bait protein as pointed out in part 3.1.4.2. The strain containing YqhY-Strep was grown in CSE medium containing 0.5% glucose and was treated with paraformaldehyde during growth to crosslink adjacent proteins. This time, the bait proteins ClpC and ClpP were detected by their respective antibodies during Western Blot analysis.

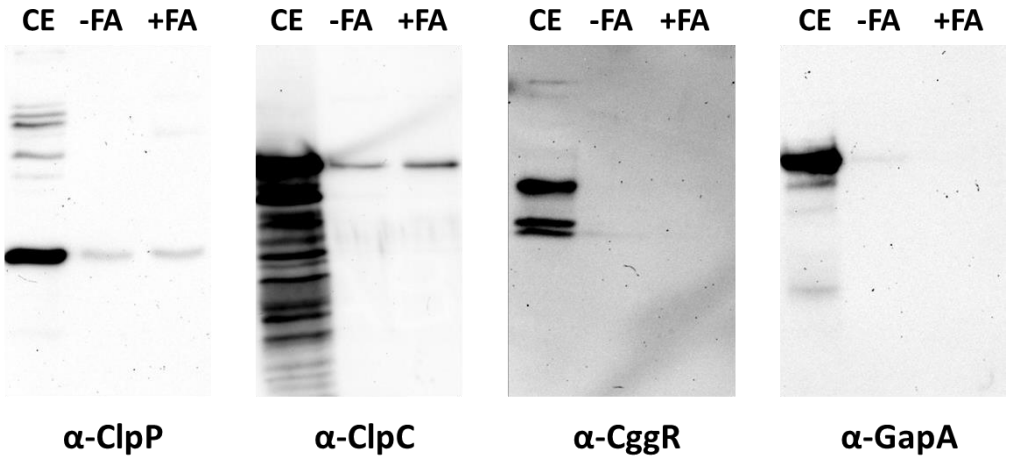

Fig. 18. Proof of interaction between YqhY and the ClpCP complex via SPINE. Strains expressing YqhY-Strep were grown in CSE medium containing 0.5% glucose, adjacent proteins were crosslinked by the addition of paraformaldehyde (**FA**). The pray proteins (ClpC, ClpP, CggR and GapA) were detected by its respective antibody in a Western blot analysis. YqhY was purified using a StrepTactin column and was co-eluted with ClpC and ClpP. CggR and GapA functioned as negative controls.

The detected signals shown in Fig. 18 affirmed the interaction between YqhY and ClpC. Furthermore, an association of YqhY and ClpP was given, indicating a connection to Clp-dependent protein degradation, since ClpP is the main proteolytic unit in this process. Regarding the signal strength of ClpC and ClpP in the elution fractions, only a week or brief interaction could be assumed. Nevertheless, this interactions are given, because

unspecific interactions between YqhY and the two ClpP proteins could be excluded. This became clear by considering the negative controls in which no signal of CggR and GapA was detectable.

A similar approach in which the interaction of YqhY and ClpE should be verified was not successful (data not shown). After the purification of YqhY-Strep, ClpE-FLAG could not be detected in a Western Blot via FLAG tag antibody. On the one hand, this could be due to the fact that there is simply no interaction *in vivo* between these proteins. On the other hand, the expression of *clpE* is basically low and almost only upregulated under heat stress conditions. The short life time and quick degradation by ClpCP or ClpXP (Gerth *et al.*, 2004) make it even more difficult to find the right conditions to detect ClpE. Therefore, an interaction of YqhY and ClpE seems unlikely, but cannot be excluded.

3.2.3 Analyzing the interaction of YqhY with Clp adaptor proteins

The substrate specificity in protein degradation is promoted by adaptor proteins. Normally, only one adaptor protein is necessary to target a certain substrate for decomposition. Recent studies have shown, that an adaptor hierarchy for ClpXP exists in *Caulobacter crescentus*, in which selective protein degradation depends on several adaptor proteins and their degree of assembly (Joshi *et al.*, 2015). Assuming that YqhY is an adaptor protein, the ability of YqhY to bind other adaptor proteins and maybe build up a similar adaptor hierarchy in *B. subtilis* should be investigated. In order to get a first impression, the interactions of YqhY and several adaptor proteins from *B. subtilis* have been checked in a bacterial two hybrid assay (Fig. 19). All relevant genes were cloned into the BACTH vectors and the following screen was carried out as described above.

All tested proteins showed self-interactions. Apart from that, no significant complex formations among the adaptor proteins was noticeable. Only an interaction between YjbH and MecA could be observed. Thus, there were no indications pointing to a network of several adaptor priming the protease complex to confer substrate specificity.

Although, YqhY only interacted with YjbH, this result fits with suppressor mutation located in *yjbH* (s. 3.2.1). If YjbH is sometimes needed to be inactive in the absence of YqhY, a connection of these two proteins is reasonable and an interaction would definitely make sense.

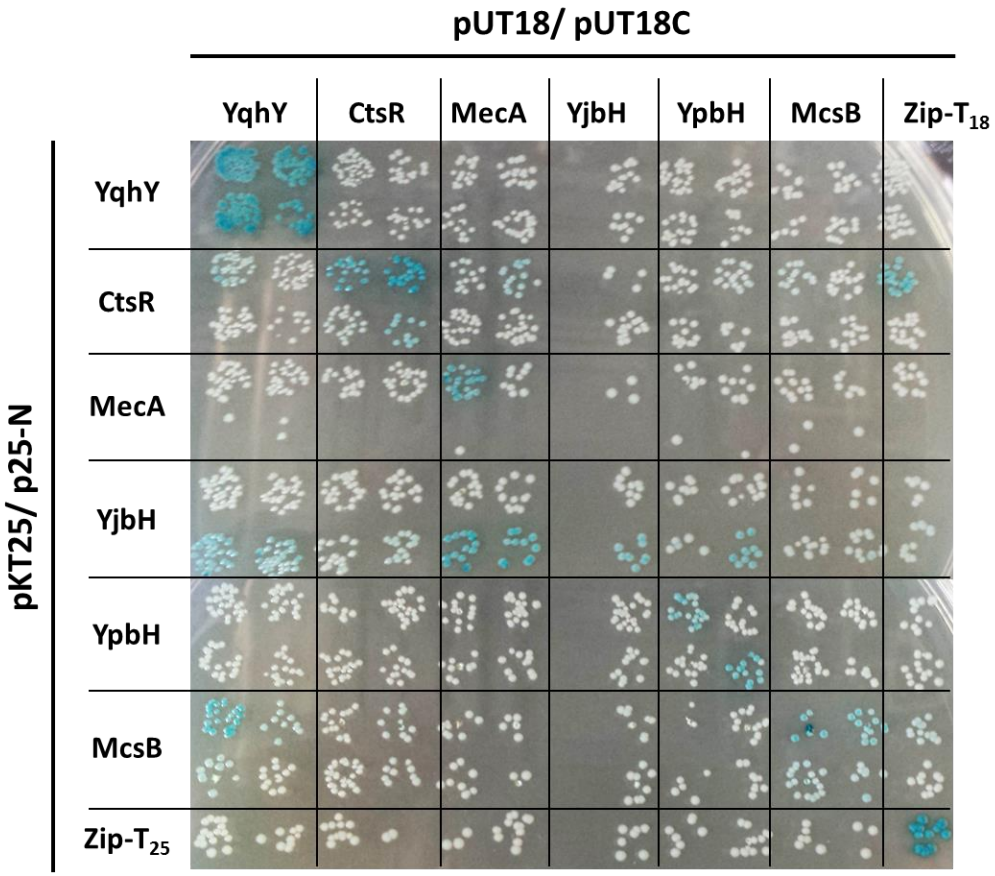

Fig. 19. Bacterial two-hybrid analysis of interactions between YqhY and adaptor proteins. The respective genes were fused to the domains of the adenylate cyclase and incubated for two days. Interactions are indicated by blue colonies. Besides self-interactions of every tested protein, associations between YqhY and YjbH, and YqhY and McsB were observable.

3.2.4 The ACCase subunits are no substrate of ClpCP protease

The observations that YqhY showed interactions with the carboxyltransferase on the one hand and with the ClpCP protease on the other hand, raised the question whether YqhY fulfills the function of an adaptor protein to promote the degradation of AccA and/or AccD. If this assumption is true, the protein amounts of the ACCase subunits have to be decreased in the absence of YqhY or the Clp proteins. To test this hypothesis, the protein amount of AccA, AccB, AccC and AccD was analyzed by Western blot in strains missing YqhY, ClpX or ClpP. The cells were grown in CSE medium containing 0.5% glucose until they reached an OD_{600} of about 1.0 and the proteins were detected by their respective antibodies (Fig. 20).

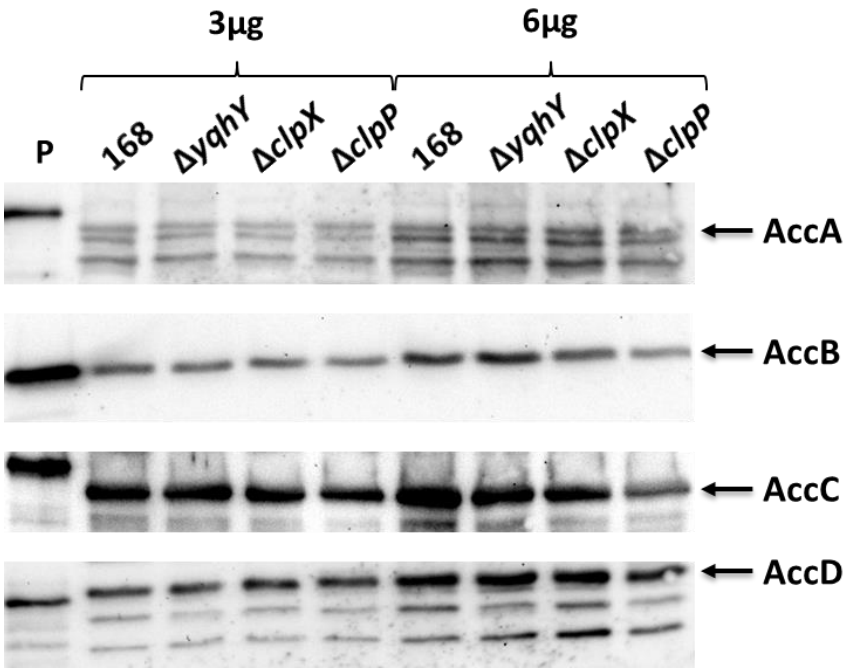

Fig. 20. Protein amount of AccA, AccB, AccC and AccD in *yqhY*, *clpX* and *clpP* deletion strains. The cells were grown in CSE medium containing 0.5% glucose and afterwards, 3 µg and 6 µg of the crude extract were used for Western blot analysis. The target proteins were detected by their respective antibodies. The purified proteins (**P**) functioned as internal marker and the wild type (**168**) was used as a control**.**

In comparison to the wild type, there was no significant change in the protein amount of any subunit in the deletion strains. Although, in case of AccB and AccC the amount seems to be lowered in the absence of ClpX and ClpP, these results could not be repeated. Taking all experimental repetitions into account, a change in expression or an increase in degradation of AccA, AccB, AccC and AccD was not recognizable. Apart from this, a lower amount of the target proteins is improbable since the supposed degrading complex is missing.

Another problem was the unspecific band pattern of the AccA antibody. Therefore, a new approach was carried out to determine the dependency of AccA on ClpX and ClpP. This time, AccA was fused to a FLAG tag keeping the expression under control of the natural promoter. The cells were grown in LB medium and samples were taken at different growth phases. The final detection was again performed via Western Blot using the FLG tag antibody (Fig. 21).

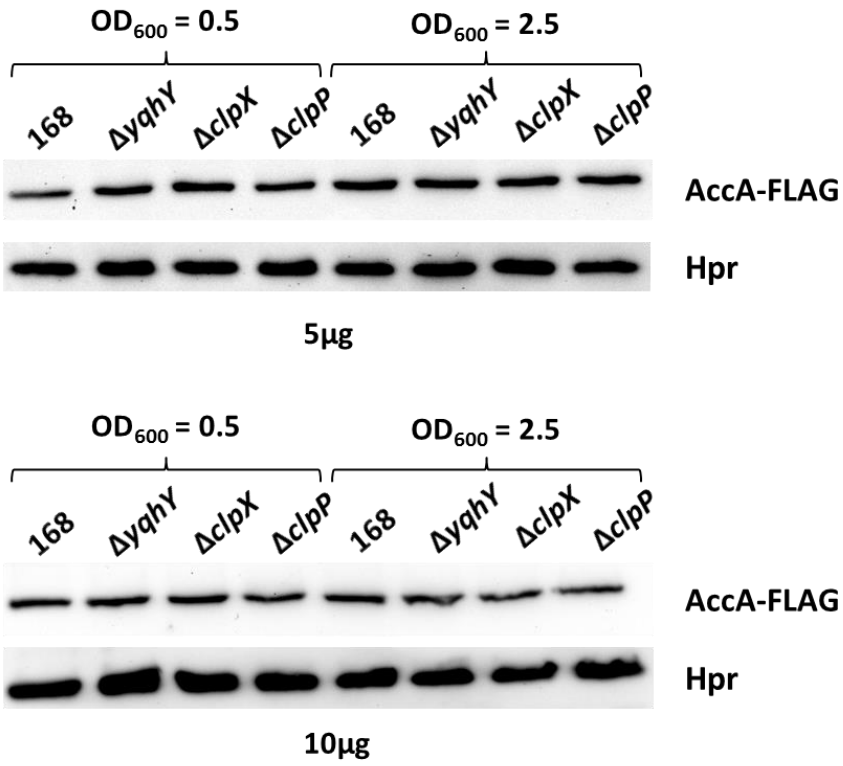

Fig. 21. Protein amount of AccA-FLAG in *yqhY*, *clpX* and *clpP* deletion strains. The cells were grown in LB medium and afterwards, 5 µg and 10 µg of the crude extract were used for Western blot analysis. AccA was detected by the FLAG antibody and the wild type (168) was used as a control. Hpr functioned as a loading control and was detected by its antibody.

The detection of Hpr ensured that for each sample the same amount of protein crude extract (5 µg and 10 µg) was used. However, the deletion of neither *yqhY* nor *clpX* or *clpP* showed any impact on the protein amount of AccA-FLAG independent of the growth phase. All in all, the theory of YqhY being an adaptor protein for the ACCase complex could not be validated. Moreover, the subunits of the acetyl-CoA carboxylase are not substrates of Clp dependent degradation, otherwise the deletion of *clpP* would have resulted in an increased protein amount.

4 Discussion

4.1 The role of YqhY in regulation of acetyl-CoA carboxylase activity

The synthesis of fatty acids is a fundamental process in all organisms. Its first committed step is the production of the precursor malonyl-CoA carried out by the acetyl-CoA carboxylase. This makes this enzyme complex a logical target for regulating the amount of fatty acids. A feedback inhibition by acyl-ACP like in *E. coli* seems reasonable, as the end product regulates the supply of the precursor (Davis and Cronan, 2001). Another possibility is the change of activity triggered by proteins interacting with the ACCase. The PII-like protein GlnB decreases the ACCase activity in *E. coli* through interacting with the biotinylated region of AccB and concomitantly perturbing the transport of biotin to AccAD. This inhibition is dependent on 2-oxoglutarate and nitrogen levels. Low nitrogen conditions facilitate the uridylylation of GlnB, whereas high 2-oxoglutarate amounts favor the interaction of 2-oxoglutarate and GlnB. Both 2-oxoglutarate binding and uridylylation alter the structure of the PII T-loop resulting in decreased binding abilities of GlnB (Gerhardt *et al.*, 2015). It should be noted, that PII-like proteins are only able to inhibit malonyl-CoA production in case of an interaction with both AccB and AccC. This is exemplified by GlnK that only interacts with AccB and has no effect on ACCase activity. Since YqhY only interacted with AccC in a BACTH screen, a similar function to GlnB is unlikely for YqhY. In green algae and land plants, biotin attachment domain-containing (BADC) proteins also act as a negative effector of AccBC. According to a proposed model, AccC binding sites are occupied by BADCs preventing the interaction between AccC and AccB. As a result, the ACCase activity is decreased (Salie *et al.*, 2016).

However, the regulation of the ACCase in *B. subtilis* is poorly understood so far, since neither a feedback inhibition nor the presence of an activity controlling protein is known. The unknown protein YqhY was considered to be such a regulating factor, after *in vivo* experiments showed a direct interaction between YqhY and AccAD. In addition to this, the deletion of *yqhY* caused an accumulation of lipophilic clusters mainly at the cell poles, which was a further indication for a changed fatty acid production. In order to determine if these cluster were formed due to a missing regulatory effect by YqhY, the ACCase activity was measured in dependency of YqhY *in vitro*. The results revealed no significant

impact of YqhY on the ACCase activity, consequently YqhY cannot be regarded as a negative effector.

On the other hand, the purification of a sufficient amount of AccA turned out to be difficult. The overexpression of this protein was toxic for *E. coli* and only a relatively low protein amount could be obtained through overexpression in *B. subtilis*. Since the ACCase complex is believed to dissociate at the low subunit concentrations, high amounts of the subunits are needed to reach a certain threshold for catalytic activity *in vitro* (Davis and Cronan, 2001). For this reason, the low overall activity of the acetyl-CoA carboxylase, measured in the coupled reaction assay, might be explained by an insufficient amount of the subunits added to the reaction mix. Thus, slight changes in activity caused by YqhY could appear to be negligible and are not recognized as regulatory effects. Nonetheless, YqhY has to be somehow involved in regulation of fatty acid production or the synthesis of one of the precursors. In some cases the lack of YqhY is overcome by suppressor mutations resulting in a decreased ACCase activity, which is indicated by the reduced activity of the ACCase caused by the suppressor mutation $AccD_{A229S}$. The effect of repressing the occurrence of suppressor mutants was also given in a Δ*spo0A* background. During growth on sporulation medium, the deletion of *yqhY* has no harmful effect and suppressor mutants were not formed in the absence of Spo0A (data not shown). This is in accordance with the fact that Spo0A activates *accDA* expression under sporulation conditions. Thus, the aftermath of a *yqhY* deletion can be overcome by downregulation of the ACCase, either by the lack of Spo0A or mutations in the subunits.

Another possibility to influence ACCase activity is the correct localization of its subunits. In *Mycobacterium smegmatis*, a homolog of DivIVA, Wag31, interacts with AccA (Xu *et al.*, 2014). Based on this observation and the localization dependency of YqhY on DivIVA, a function as a linker protein between DivIVA and ACCase subunits seemed possible for YqhY. Fluorescence microscopy experiments revealed an equal cytoplasmic distribution of AccA, AccB and AccC in a wild type background, which was in accordance with the results of Meile *et al.*, 2006, but in contrast to the polar localization of YqhY. Due to this contrary localization, YqhY could be considered as an antagonist, preventing the polar accumulation of the acetyl-CoA carboxylase. Indeed, AccA showed a polar localization pattern in the absence of YqhY. Since YqhY is mainly distributed in the whole cell in a Δ*divIVA* background, a deletion of *divIVA* should also display a polar localization of AccA.

In order to examine this theory, the localization of AccA was monitored in a Δ*yqhY* Δ*divIVA* double mutant strain. Against the expectations, AccA was still equally spread over the cell. Thus, an antagonistic role of YqhY against AccA could not be proven, but YqhY nevertheless influences the correct localization of AccA.

A regulatory function for YqhY could not be shown, but the connection of YqhY with ACCase activity cannot be excluded. It is obvious that cells lacking YqhY have most likely an increased malonyl-CoA production possibly resulting in fatty acid overproduction. How this metabolic defect is triggered and why it has a toxic effect for the cell is in need of further investigation.

4.2 Fatty acid uptake in *Bacillus subtilis*

Fatty acid binding proteins (FABPs) are abundantly expressed proteins in eukaryotic cells. These lipid chaperones bind long-chain fatty acids and are involved in fatty acid import and export, as well as in lipid storage (Furuhashi and Hotamisligil, 2008). Since bacteria lack these proteins, they had to evolve other ways to transport and store fatty acids. Many microorganisms store lipids by producing polyhyroxyalkanoates (PHAs) in abundance of carbon sources while lacking other essential nutrients. The synthases of these biopolymers (PhaA, PhaB and PhaC) are expressed in many *Bacillus* species, but *B. subtilis* lacks genes related to PHA biosynthesis (Singh *et al.*, 2009). The missing storage function could be fulfilled by YqhY. In this case, the accumulation of lipophilic clusters in cells lacking YqhY would be due to released fatty acids previously bound to YqhY, instead of an increased fatty acid synthesis.

Another possibility is a role of YqhY in fatty acid uptake. For incorporation of exogenous fatty acids, Gram-positive bacteria need to phosphorylate them in a first step. The resulting acyl-phosphate is either be used by PlsY to initiate phospholipid synthesis or it is converted to acyl-ACP by PlsX. This in turn serves as substrate for PlsC or for fatty acid elongation (Yao and Rock, 2015). The activation of exogenous fatty acids is executed by two proteins, FakA and FakB. The latter one is a fatty acid binding protein and belongs to the DegV protein family, named after its homologue in *B. subtilis*. FakA is responsible for phosphorylation of fatty acids bound to FakB and is homologous to YloV from *B. subtilis*

(Parsons *et al.*, 2014). Since *yloV* is located on an operon with *yloU*, the paralogue of *yqhY*, this is an additional hint for a connection of YqhY to fatty acid homeostasis.

The determination of a role for YqhY in fatty acid uptake is difficult, because YloV and DegV are most likely responsible for fatty acid activation in *B. subtilis*. Although there is no evidence for a protein-mediated fatty acid transporter in bacteria (Parsons *et al.*, 2014), the existence of such a transporter is unlikely and unnecessary at the same time, since fatty acids cross the membrane by spontaneous flipping (Garlid *et al.*, 1996). Broussard *et al.* (2016), identified five conserved residues in FakB2 from *S. aureus* that probably fulfill the same critical functions in all bacterial fatty acid-binding proteins. As YqhY does not contain these residues, a storage or chaperone function of YqhY can also be excluded. Nevertheless, a regulatory effect by binding of YqhY to YloV or DegV is conceivable.

4.3 YqhY, a potential adaptor protein mediating Clp degradation?

It could be shown that YqhY has no direct impact on ACCase activity, but the absence of this protein leads to stress effects, probably caused by excessive amounts of fatty acids. Expression of staphylococcal *asp23* is upregulated upon alkaline stress and is dependent on σ^B. The same sigma factor controls the genes responsible for Clp-mediated protein degradation and their transcription repressor *ctsR* in *B. subtilis*. In this work, sequencing analysis of two Δ*yqhY*-suppressor mutants, in which the ACCase subunits were not affected, revealed the absence of CtsR or the adaptor protein YjbH. In addition, YqhY was shown to interact with AccAD and ClpCP. Thus, YqhY was considered to be part of stress response, possibly functioning as adaptor protein to facilitate the degradation of AccA, AccB, AccC or AccD by Clp proteases. This theory was also supported by the polar localization of YqhY, which was in accordance with the polar cluster formations of Clp and its ATPases. But, not only the Clp proteins co-localize at the cell poles, also the adaptor protein McsB and its substrate CtsR were found in these regions, determining them as important subcellular sites for protein degradation in *B. subtilis* (Kirstein *et al.*, 2008).

Based on the assumption of YqhY being an adaptor protein, the degradation of ACC subunits becomes more unspecific in the absence of YqhY, which leads to a higher amount of the target proteins. In order to compensate this excess and maintain a vital level of ACC

subunits, expression of *clp* genes is upregulated leading to an increased chance of unspecific degradation of ACC. The down-regulation of fatty acid synthesis by degradation of ACCase would restore the wild type situation and seems to be a plausible solution to overcome toxic amounts of fatty acids. To address this theory, the amounts of AccA, AccB, AccC and AccD were checked in the absence of YqhY, ClpX or ClpP through Western blot analysis. In all tested backgrounds the protein level was equal compared to the wild type. Therefore, the ACC subunits are pretty likely no target for Clp degradation, which becomes especially clear by the unchanged protein levels in cells lacking the proteolytic subunit ClpP. Nevertheless, it has to be considered that the experiments took place under normal growth conditions and that heat or oxidative stress induce other factors necessary for substrate targeting. Moreover, there is still the possibility of ClpYQ degrading one of the ACC subunits, since this complex was not part of the investigations and neither an adaptor protein nor any targets for ClpYQ are known.

Taking the bacterial two-hybrid experiments with several Clp adaptor proteins into account, also an adapter hierarchy like in *C. crescentus* was not indicated. The interaction of YqhY and YjbH was relatively weak and besides, MecA and YjbH were the only adaptor proteins interacting with each other. Thus, a network of adaptor proteins activating each other cannot be expected to be present in *B. subtilis*. In order to get further evidence for YqhY being involved in protein degradation, proteomic analysis of a *yqhY* mutant needs to be performed. It is nonetheless possible that the amount of fatty acids is controlled by proteolytic processes. In a comprehensive study, AccA and AccB were proposed as potential targets of Clp-dependent proteolysis. Among the other potential candidates was also YloV (Gerth *et al.*, 2008), previously implicated in fatty acid uptake. In the end, it is difficult to determine new target proteins, because of the immense number of potential substrates and their adaptor proteins.

4.4 Connecting protein degradation and fatty acid synthesis

This work indicates a connection of YqhY with both fatty acid synthesis and protein degradation, but how to combine these distinct processes? The function of YqhY is elusive, but there are possible explanations for how cells are affected by the lack of YqhY. The previous assumption was a downregulation of fatty acid production by mutations in the

ACCase subunits. In accordance to this, another mutant was deficient of CtsR, which results in a higher amount of ClpXP and increased Spx degradation. Since Spx induces *birA* expression (Gaballa *et al.*, 2013) and BirA is essential for biotinylation of AccB, the absence of CtsR probably also results in reduced ACCase activity. In contrast to this, a further mutation resulting in truncated YjbH has the opposite effect, as the protein level of Spx is increased. Due to this conflicting effects, a different connection of fatty acid synthesis and protein degradation has to be found.

Fatty acids are also components of other metabolites, for example surfactin. This lipopeptide contains β-hydroxy fatty acid (Kakinuma *et al.*, 1969) and was shown to be involved in biofilm formation (Zeriouh *et al.*, 2014). Furthermore, it exerts antibacterial activities through destabilization of lipid layers causing the release of aqueous vesicle contents (Carrillo *et al.*, 2003). YqhY could be responsible for surfactin export and its absence leads to a toxic accumulation of surfactin. This can be explained by the key role of Spx that not only activates *birA* transcription but also represses the *srf*-operon encoding surfactin synthases (Zuber, 2004). The suppressor mutations identified in strains lacking YqhY imply three different scenarios to decrease surfactin production. In the first one, a decreased ACCase activity lowers the amount of fatty acids and therefore the availability of precursors for surfactin synthesis. The same effect is achieved in the absence of CtsR, due to a lower amount of BirA as mentioned above. In the last one, the lack of YjbH leads to an increased repression of the *srf* genes by Spx resulting in decreased overall surfactin production. Thus, either an upregulation or downregulation of Spx can have the same effect regarding surfactin production.

Based on the precondition that YqhY is a surfactin exporter, this explains why suppressor mutations hit genes involved in fatty acid synthesis or protein degradation. On the other hand, Asp23 is highly conserved in Firmicutes whereas surfactin is only produced in *Bacillus* species. Therefore, this hypothesis can only be confirmed, if Asp23 proteins are in addition responsible for export of other surfactant-like molecules, for example phenol-soluble modulin (PSM) in *S. aureus* (Periasamy *et al.*, 2012). Moreover, the lack of CtsR leads to upregulation of all Clp proteins resulting in a global response, since proteolysis influences many important cellular processes. That means that a controlled downregulation of surfactin synthesis is not possibly without affecting other pathways.

4.5 Increased malonyl-CoA amounts may lead to unspecific protein acylation

The control of the surfactin synthesis appears to be too complex and the high number of suppressor mutations hitting the acetyl-CoA carboxylase indicate a more obvious problem occurring in the absence of YqhY. The supposed higher ACCase activity increases the amount of malonyl-CoA. Since malonyl-CoA is only a substrate to FabD, this results in a bottleneck effect and accumulation of malonyl-CoA. Due to its similarity to acetyl-CoA (Fig. 22a), an acylation of lysine residues by unbound malonyl-CoA is possible. Lysine acetylation is conserved in organisms from bacteria to human (Kim and Yang, 2011) and was shown to regulate many eukaryotic processes like cell cycle, cell morphology, protein synthesis, mRNA splicing and central metabolism (Kuczyńska-Wiśnik *et al.*, 2016). In prokaryotes little is known about protein acetylation, but it has been proposed to be related to energy metabolism, ribosomal functions and transcription (Zhang *et al.*, 2008; Zhang *et al.*, 2014).

A recent study in *E. coli* demonstrated that acetylation promotes the formation of inclusion bodies and a lower activity of these acetylated proteins. It was further suggested that acetylation of misfolded proteins increases resistance to heat and oxidative stress (Kuczyńska-Wiśnik *et al.*, 2016).

Apart from this, there are several other lysine modifications known, for example lysine malonylation (Peng *et al.*, 2011), succinylation (Zhang *et al.*, 2011) and glutarylation (Tan *et al.*, 2014). All three modifications were thought to have a different impacts on protein functions than acetylation, because they change the charge on lysine. This is concomitant with disruption of ionic interactions with other negatively charged molecules and alterations in protein structure and function (Hirschey and Zhao, 2015). So far, lysine malonylation has been mainly investigated in eukaryotic cells with over thousand proteins identified to be subject of this modification. The most lysine-malonylated substrates were found to be part of ribosomes, valine/ leucine/ isoleucine degradation, proteasome and fatty acid metabolism, including acetyl-CoA carboxylase. In addition, fatty acid oxidation activity was inhibited by lysine malonylation (Colak *et al.*, 2015). The deacetylase SIRT5 is able to remove acyl residues from lysine, since it also possesses desuccinylation, demalonylation (Du *et al.*, 2011) and deglutarylation (Tan *et al.*, 2014) activity. For its

counterpart in *B. subtilis*, SrtN, only deacetylation activity is known, but it was proposed that this enzyme is capable of removing a broad range of acyl groups (Seidel *et al.*, 2016). Nevertheless, substrate malonylation is poorly understood in prokaryotes. In order to get more insight into this field, a recent study investigated lysine malonylation in *E. coli* (Qian *et al.*, 2016). The authors identified 1745 malonylation sites in 594 proteins, of which approximately one third is overlapping with acetylation and succinylation sites (Fig. 22b). As shown in previous studies in eukaryotes, lysine malonylation was highly enriched in protein synthesis and energy metabolism. 68 proteins were related to ribosomes (Fig. 22c) and 19 proteins were associated to citric acid cycle, suggesting that malonylation plays a major role in regulation of substantial processes in bacteria. Moreover, malonylation sites were detected in eleven proteins of fatty acid synthesis, among them FabI, the enzyme catalyzing the last step of fatty acid elongation and malonyl-CoA transacylase FabD which loads the acyl carrier protein with malonyl-CoA prior to fatty acid elongation. Strikingly, the acetyl-CoA carboxylase subunits AccA, AccC and AccD were also subject to malonylation. AccB was not malonylated which can be explained by a protected lysine residue through biotin binding.

These results indicate a regulation of malonyl-CoA production and fatty acid synthesis by malonylation. Assuming that the lack of YqhY leads to higher malonyl-CoA levels, this could result in unspecific protein acylation. Since many important pathways, especially protein synthesis, are affected by malonylation, the cell would have to deal with increasing amounts of misfolded and inactive proteins. Thus, suppressor mutations related to protein degradation and stress response are part of a global response in order to remove protein debris. Suppressor mutations decreasing the ACCase activity would reduce malonyl-CoA concentrations and the overall effect of malonylation. It is also possible that the absence of YqhY leads to delocalization of important proteins, making them targets for unspecific malonylation. These hypotheses give still no answer on the actual function of YqhY, but investigations on protein malonylation could give further hints on its role in *B. subtilis*.

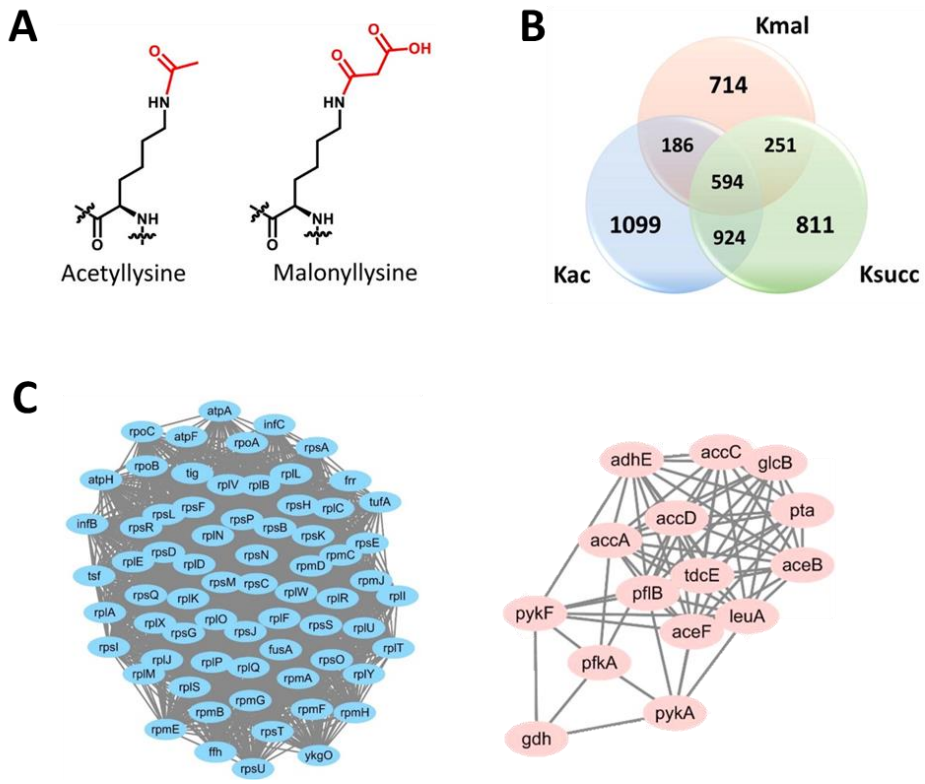

Fig. 22. Lysine malonylation in *E. coli*. (**A**) Comparison of lysine acetylation and malonylation. (**B**) Overlap of lysine acetylation (Kac), succinylation (Ksucc) and malonylation (Kmal) sites. (**C**) Interaction network of lysine malonylated proteins related to ribosome and acetyl-CoA carboxylase (Qian *et al.*, 2016).

4.6 Outlook

A regulation of the acetyl-CoA carboxylase in *B. subtilis* is very likely and there are plenty of examples found in other organisms. The malonylation of ACC subunits is a plausible mechanism to down regulate the enzymatic activity by feedback inhibition through malonyl-CoA. Since malonylation is poorly investigated in prokaryotes and malonylated proteins have not been identified in *B. subtilis* so far, a definition of the malonylome by proteome analysis would give further insights into this mechanism. A changed malonylome in *yqhY* mutant strains would show, if YqhY has an impact on the malonylation of proteins.

Proteomics could also give evidence for YqhY being involved in protein degradation. Without potential target proteins, it is difficult to define YqhY as an adaptor protein, thus, a global protein profile possibly highlights increased protein concentrations in the

absence of YqhY. It should be considered to test interactions with target proteins under heat or oxidative stress, as the proteolytic activity is increased under stress conditions. Regarding a role of YqhY in fatty acid homeostasis, mass spectrometry analysis can provide certainty on the ability to bind fatty acids. Moreover, interaction experiments with YqhY and YloV or DegV could reveal a possible impact on fatty acid uptake and a comparison of surfactin concentrations in wild type cells and *yqhY* mutant strains would either support or decline the hypothesis that YqhY is involved in surfactin export.

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6 Appendix

6.1 Materials

6.1.1 Chemicals

Acrylamide	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Agarose	Peqlab, Erlangen
Ammonium iron (III) citrate	Sigma, Munich
Ammonium Peroxydisulfate	Roth, Karlsruhe
Antibiotics	Sigma, Munich
Bromphenol blue	Serva, Heidelberg
Casein	Sigma, Munich
CDP*	Roche Diagnostics, Mannheim
Coomassie Brilliant Blue, G250	Roth, Karlsruhe
Desthiobiotin	IBA, Göttingen
dNTPs	Roche Diagnostics, Mannheim
Ethidium bromide	Roth, Karlsruhe
D-Fructose-1,6-bisphosphate	Sigma, Munich
D-Glucose	Merck, Darmstadt
Glycerine	Merck, Darmstadt
Immidazole	Sigma, Munich
Isopropyl ß-D-1- thiogalactopyranoside	Peqlap, Erlangen
DL-Malate	Applichem, Darmstadt

ß-Mercaptoethanol	Roth, Karlsruhe
Ni^{2+}-nitrilotriacetic acid superflow	Qiagen, Hilden
Nutrient Broth	Merck, Darmstadt
Pefabloc	Roth, Karlsruhe
Skim milk powder	Oxoid, Heidelberg
Sodium succinate	Fluka, Buchs, Switzerland
Sodium Dodecyl Sulfate	Roth, Karlsruhe
Strep-Tactin Sepharose	IBA, Göttingen
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe
Trypton	Oxoid, Heidelberg
Tween 20	Sigma, München
X-Gal	Peqlab, Erlangen
Yeast extract	Oxoid, Hampshire, U.K.

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

6.1.2 Auxiliary materials

96-Well-plates	Sarstedt, Nürmbrecht
Centrifuge cups	Beckmann, Munich
Cuvettes (microlitre, plastic)	Greiner, Nürtingen
Dialysis tube	Serva, Heidelberg
Eppendorf tubes	Greiner, Nürtingen

Falcon tubes	Sarstedt, Nürmbrecht
Gene amp reaction tubes (PCR)	Perkin Elmer, Weiterstadt
Glas pipette	Brand, Wertheim
Microlitre pipettes (1 µl, 2 µl, 20 µl, 200 µl, 1000 µl, 5 ml)	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen
Pipette tips	Greiner, Nürtingen
Poly-Prep Chromatography columns	Bio-Rad, Munich
Polyvinylidene difluoride (PVDF) membrane	Bio-Rad, Munich

6.1.3 Intruments

ÄKTAprime	GE Healthcare, Freiburg
Autoclave	Zirbus technology, Bad Grund
Biofuge fresco	Heraeus Christ, Osterode
Chemiluminescence system ChemiSmart	Peqlab, Erlangen
Contamination meter	Berthold, Bad Wildbad
Fluorescence microscope Axioskop 40FL + Kamera (AxioCam MRm)	Zeiss, Göttingen
French pressure cell press	SLM Aminco, Lorch
French pressure cell press	Spectonic Unicam, England
Gel electrophoresis apparatus	PeqLab, Erlangen
Ice maschine	Ziegra, Isernhagen

Image eraser	Molecular Dynamics, USA
Heating block Dri Block DB3	Waasetec, Göttingen
Horizontal shaker 3006	GFL, Burgwedel
Hydro tech vacuum pump	Bio-Rad, Munich
LabCycler	SensorQuest, Göttingen
Microplate reader Epoch2	BioTek, Bad Friedrichshall
Microplate reader SynergyMx	BioTek, Bad Friedrichshall
Mini-Protean III System	Bio-Rad, Munich
Nanodrop ND-1000	Thermoscientific, Bonn
Open air shaker Innova 2300	New Brunswick, Neu-Isenburg
Phosphor imagerStorm 860	Molecular Dynamics, USA
pH meter Calimatic	Knick, Berlin
Refrigerated centrifuge	Kendro, Hanau
Scale	Sartorius, Göttingen
Semi Dry Transfer Unit TE70 SemiPhor	Amersham, Freiburg
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer	Amersham, Freiburg
Standard power pack	Bio-Rad, Munich
Thermocycler	Biometra, Göttingen
Ultra centrifuge, Sorvall Ultra Pro 80	Thermoscientific, Bonn

Ultrasonic device	Dr. Hielscher, Teltow
UV Transilluminator 2000	Bio-Rad, Munich
Vortex	Bender & Hobein, Bruchsal
Water-bath incubation system	GFL, Burgwedel
Water desalination plant	Millepore, Schwalbach

6.1.4 Commercial systems

peqGOLD Bacterial DNA Kit	PEQLAB, Erlangen
peqGOLD PCR Purification Kit	PEQLAB, Erlangen
NucleoSpin Plasmid-Kit	Macherey-Nagel, Düren
PageRuler™ Plus Prestained Protein Ladder	ThermoFisher, Waltham, USA
QIAquick PCR Purification Kit	Qiagen, Hilden
Midori Green	Biozym, Hessisch Oldendorf

6.1.5 Antibodies and enzymes

Anti-FLAG®	Sigma-Aldrich, Munich
Anti-Rabbit IgG-AP secondary antibody	Promega, Mannheim
FastAP™	ThermoFisher, Waltham, USA
Lysozym	Merck, Darmstadt
Phusion™ DNA polymerase	Biozym, Hessisch Oldendorf
Restriction nucleases	ThermoFisher, Waltham, USA

T4-DNA ligase	ThermoFisher, Waltham, USA
Taq-DNA polymerase	Roche Diagnostics, Mannheim

6.2 Oligonucleotides

Oligonucleotides were purchased from Sigma Aldrich (Munich, Germany). Underlined are restriction sites.

Name	**Sequenz (5‘-3’)**	**Description**
cat-fwd (kan)	5’CGGCAATAGTTACCCTTATTATCAAG	Amplification of *cat*-cassette from pGem-cat for LFH-PCR
cat-rev (kan)	5’CCAGCGTGGACCGGCGAGGCTAGTTACCC	Amplification of *cat*-cassette from pGem-cat for LFH-PCR
FC146	5’CGATGCGTTCGCGATCCAGGC	Sequencing of pUT18
FC147	5’CCAGCCTGATGCGATTGCTGCAT	Sequencing of p25-N
FC148	5’GTCACCCGGATTGCGGCGG	Sequencing of pUT18C,
FC149	5’GCTGGCTTAACTATGCGGCATCAGA	Sequencing of pUT18C
FC150	5’GATTCGGTGACCGATTACCTGGC	Sequencing of pKT25
FC151	5’CGCCAGGGTTTTCCCAGTCACG	Sequencing of pKT25
FM70	5’CCGGCTCGTATGTTGTGTGGAAT	Sequencing of p25-N
kan-fwd	5’CAGCGAACCATTTGAGGTGATAGG	Amplification of *apha3* from pDG780 for LFH-PCR
kan-rev	5’CGATACAAATTCCTCGTAGGCGCTCGG	Amplification of *apha3* from pDG780 for LFH-PCR
KG64	5’TATCAGGGCCTCGACTACA	Sequencing of pGP882 derivatives, fwd
KG65	5’CGCTGATTAAATACAGCATCGG	Sequencing of pGP882 derivatives, rev
M13 pUC for	5`GTAAAACGACGGCCAGTG	Sequencing of pBS-plasmides and pUC derivatives, fwd

M13 pUC rev	5`GGAAACAGCTATGACCATG	Sequencing of pBS-plasmides and pUC derivatives, rev
ML84	5‘CTAATGGGTGCTTTAGTTGAAGA	Cat check up-fragment (sequencing of up-fragement)
ML85	5‘CTCTATTCAGGAATTGTCAGATAG	Cat check down-fragment (sequencing of down-fragment)
ML107	5’GCTTCATAGAGTAATTCTGTAAAGG	kan check up-fragment (sequencing of up-fragement)
ML108	5’GACATCTAATCTTTTCTGAAGTACATCC	kan check down-fragment (sequencing of down-fragement)
ML109	5’GTCTAGTGTGTTAGACTTTATGAAATC	mls check up-fragment (sequencing of up-fragement)
ML110	5’CTTTAATAATTCATCAACATCTACACC	mls check down-fragment (sequencing of down-fragement)
mls fwd (kan)	5’CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC	LFH-PCR, amplification of *ermC* from pDG647, fwd
mls rev (kan)	5’CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG	LFH-PCR, amplification of *ermC* from pDG647, rev
mls-check fwd	5’CCTTAAAACATGCAGGAATTGACG	LFH-PCR, sequencing of the down-fragment
mls-check rev	5’GTTTTGGTCGTAGAGCACACGG	LFH-PCR, sequencing of the up-fragment
pWH844 fwd	5’TATGAGAGGATCGCATCACCAT	Sequencing of pWH844 constructs
T7-Prom.	5’TAATACGACTCACTATAGGG	Sequencing primer, starts at 5` end of T7 promotor
DT64	AAAGGATCCCCTAGTCCGCAATTTTGGGATGCC	Cloning of *accA* into pGP1331, pBP43, fwd (*Bam*HI)

DT66	TTTGTCGACGTTTACCCCGATATATT GATCTTCAACCG	Cloning of *accA* into pGP1331, pGP382, pBP43, rev (*Sal*I)
DT73	AAAGGATCCCTCACTTATTTAAAGGA GGAAACAATCATGTTAAATATCAAA GAAATCCACGAGCTGATTAAAG	Cloning of *accB* into pGP1460, fwd (*Bam*HI)
DT74	TTTGTCGACCTCCGCTTTTACAAGAA ATAGAGGTTG	Cloning of *accB* into pGP1460, rev (*Sal*I)
DT75	AAAGGATCCCTCACTTATTTAAAGGA GGAAACAATCATGATTAAAAAGCTA TTGATCGCCAACAGAG	Cloning of *accC* into pGP1460, fwd (*Bam*HI)
DT76	TTTGTCGACTGAGCCCATTACATCAT ATGTTTCTAAAAATTTCG	Cloning of *accC* into pBP43, pGP1460, pGP1331, rev (*Sal*I)
DT77	AAATCTAGAAATAATTTTGTTTAACT TTAAGAAGGAGATATAATGAAAGAC AACAGCTTGCTTAAAATGGATCAC	Cloning of *yqhY* into pET-28a, fwd (*Xba*I)
DT78	TTTGCGGCCGCCATTTCTTCGTCGAT TTGGACTTCTTGG	Cloning of *yqhY* into pET-28a, rev (*Not*I)
DT79	AAAGGTACCGGAGAACTTGTACTTC CAAGGAACATTGAATAAATCAATTTT AGTTGCTCAAGAAGCG	Cloning of divIVA C-term. into pGP172, fwd (*Kpn*I)
DT80	TTTGGATCCTTATTCCTTTTCCTCAAA TACAGCGTCGAC	Cloning of divIVA C-term. into pGP172, rev (*Bam*HI)
DT81	AAAGGATCCGAGAACTTGTACTTCC AAGGAATGAAAGACAACAGCTTGCT TAAAATGGATCAC	Cloning of *yqhY* into pwh844, fwd (*Bam*HI)
DT82	TTTCTGCAGTTACATTTCTTCGTCGAT TTGGACTTCTTGG	Cloning of *yqhY* into pwh844, rev (*Pst*I)
DT83	AAAGGATCCCTCACTTATTTAAAGGA GGAAACAATCGTGGCTCCAAGATTA GAATTTGAAAAACCG	Cloning of *accA* into pGP382, fwd (*Bam*HI)

DT84	AAAGGATCCCTCACTTATTTAAAGGA GGAAACAATCTTGTTAAAGGATATA TTCACGAAAAAGAAAAAGTATGC	Cloning of *accD* into pGP382, pGP1370, pBQ200, fwd (*Bam*HI)
DT85	TTTGTCGACATCTTGGAGCCACTCAA TGTCACC	Cloning of *accD* into pGP382, pGP1370, rev (*Sal*I)
DT86	CCTTGTCGTTTCAACACCTTATCTGAT TTC	Sequencing of *accA*D, fwd
DT87	GCTGTTGTGACAGGCAAGGGC	Sequencing of *accA*D, fwd
DT88	CTCCATCCTCCCTATGAAACCTTAC	Sequencing of *accA*D, rev
DT89	AAATCTAGAGGTGGCTCCAAGATTA GAATTTGAAAAACCG	Cloning of *accA* into BACTH, fwd (*Xba*I)
DT90	TTTGGTACCCGGTTTACCCCGATATA TTGATCTTCAACCG	Cloning of *accA* into BACTH, rev (*Kpn*I)
DT91	AAATCTAGAGATGTTAAATATCAAA GAAATCCACGAGCTGATTAAAG	Cloning of *accB* into BACTH, fwd (*Xba*I)
DT92	TTTGGTACCCGCTCCGCTTTTACAAG AAATAGAGGTTGTC	Cloning of *accB* into BACTH, rev (*Kpn*I)
DT93	AAATCTAGAGATGATTAAAAAGCTA TTGATCGCCAACAGAGG	Cloning of *accC* into BACTH, fwd (*Xba*I)
DT94	TTTGGTACCCGTGAGCCCATTACATC ATATGTTTCTAAAAATTTCG	Cloning of *accC* into BACTH, rev (*Kpn*I)
DT95	AAATCTAGAGTTGTTAAAGGATATAT TCACGAAAAAGAAAAAGTATGC	Cloning of *accD* into BACTH, fwd (*Xba*I)
DT96	TTTGGTACCCGATCTTGGAGCCACTC AATGTCACC	Cloning of *accD* into BACTH, rev (*Kpn*I)
DT97	TTTGTCGACATCTTGGAGCCACTCAA TGTCAACTCC	Cloning of *accD* into pGP382, mutation in SD seq. of *accA*, rev (*Sal*I)
DT102	AAAGGATCCCTCACTTATTTAAAGGA GGAAACAATCATGCCATTAACGCCA AATGATATTCACAACAAG	Cloning of *divIVA* into pGP1460, fwd (*Bam*HI)

DT103	TTTCTGCAGTTCCTTTTCCTCAAATACAGCGTCGAC	Cloning of *divIVA* into pGP1460, pGP1331, rev (*Pst*I)
DT104	AAAGGTACCCGGTTCAAGTCATGCAGCAGGC	Cloning of *accB* into pGP1080, pGP1087, fwd (*Kpn*I)
DT105	TTTGGTACCCTCCGCTTTTACAAGAAATAGAGGTTGTC	Cloning of *accB* into pGP1080, pGP1087, rev (*Kpn*I)
DT106	AAAGGATCCGGAGCAAATGGGAGATGCAGCG	Cloning of *accC* into pGP1331, pBP43, fwd (*Bam*HI)
DT107	GGAACTGACCTGATCAAGGAACAAATC	LFH-PCR *yqhY*, *yfp, cat*, (fwd up-fragment, sequencing)
DT108	GATGTTGAATTTGAAGGCTGGGCC	LFH-PCR *yqhY*, *yfp, cat*, (fwd up-fragment)
DT109	CATTTCTTCGTCGATTTGGACTTCTTGG	LFH-PCR *yqhY*, *yfp, cat*, (rev up-fragment)
DT110	CGAGCGCCTACGAGGAATTTGTATCGATGGCTTAACACGAAACCAAGGGG	LFH-PCR *yqhY*, *yfp, cat*, (fwd down-fragment)
DT111	GATCATCTCCAATCAAAATAACCGCCAG	LFH-PCR *yqhY*, *yfp, cat*, (rev down-fragment)
DT112	CTTGAAATTCATTCCCATTGTTTCTGCCG	LFH-PCR *yqhY*, *yfp, cat*, (fwd down-fragment, sequencing)
DT113	CCTGGCGGATGACGGAATTACG	LFH-PCR *yqhY*, *yfp, cat*, (fwd sequencing from *yqhY*)
DT114	GTCCAAATCGACGAAGAAATGATGGTGAGCAAGGGCGAGGAG	Amplification of *yfp,* fwd
DT115	CCTATCACCTCAAATGGTTCGCTGTTACTTGTACAGCTCGTCCATGCCG	Amplification of *yfp,* rev
DT116	GTCCAAATCGACGAAGAAATGATGGTCAGCAAGGGAGAGGAAGATAATATG	Amplification of *mCherry,* fwd

DT117	CCTATCACCTCAAATGGTTCGCTGCT ATTTGTATAATTCGTCCATTCCACCTG TAGAG	Amplification of *mCherry,* rev
DT118	AAACCATGGCGATGAAAGACAACAG CTTGCTTAAAATGGATCAC	Cloning of *yqhY* into pETM-11, fwd (*Nco*I)
DT119	AAAGGATCCATGCCATTAACGCCAA ATGATATTCACAACAAG	Cloning of *divIVA* into pGP1331, fwd (*Bam*HI)
DT120	GCTGTCATATTAGAGTTGAATTCAAA AGTCC	Sequencing of *accB*C, fwd
DT121	AAAGGATCCCCTTACTCTGTCGTGCT TCTTGATGAG	Cloning of *clpC* into pGP1331, fwd (*Bam*HI)
DT122	TTTCTGCAGATTCGTTTTAGCAGTCG TTTTTACGACAAATTCG	Cloning of *clpC* into pGP1331, rev (*Pst*I)
DT123	AAATCTAGAGATGTGGAAAGGACTT ATCCATCAATATAAAGAATTTTTAC	Cloning of *thrC* into pGP888, fwd (*Xba*I)
DT124	TTTGGTACCTCATACACGGGCCGCTC CTTTTAC	Cloning of *thrC* into pGP888, rev (*Kpn*I)
DT125	AAAGGATCCATGAAAGAGGAGACAT TTTATCTTGTCCGTG	Cloning of thrR into pGP380, fwd (*Bam*HI)
DT126	TTTGTCGACTTATGCACCTGAACCTA ATATTTCAACCTTTTC	Cloning of thrR into pGP380, rev (*Sal*I)
DT127	AAATCTAGAGGTGGGACATAATATT TCTGACATCATTGAAC	Cloning of *ctsR* into BACTH, fwd (*Xba*I)
DT128	TTTGGTACCCGTTTTAATTTTAAAGA AGTCAGCATTGCCTTCATC	Cloning of *ctsR* into BACTH, rev (*Kpn*I)
DT129	AAAGGATCCGATGGAAATTGAAAGA ATTAACGAGCATACAGTAAAATT	Cloning of *mecA* into BACTH, fwd (*Bam*HI)
DT130	TTTGGTACCCGTGATGCAAAGTGTTT TTTTATCGTTTCTAGAGC	Cloning of *mecA* into BACTH, rev (*Kpn*I)
DT131	AAATCTAGAGTTGACAAACTATCAG CATGAGCTATACTTCG	Cloning of *yjbH* into BACTH, fwd (*Xba*I)

DT132	TTTGGTACCCGTTTTTCACATGATTG ATATTCATCAGAATAAGTGCTG	Cloning of *yjbH* into BACTH, rev (*Kpn*I)
DT133	AAATCTAGAGATGCGGCTTGAGCGT CTGAATTATAATAAG	Cloning of *ypbH* into BACTH, fwd (*Xba*I)
DT134	TTTGGTACCCGTGAAAAATGAGTTTG TATCGTTTCTACGGCATTAC	Cloning of *ypbH* into BACTH, rev (*Kpn*I)
DT135	AAATCTAGAGATGCGTTGTCAACATT GTCATCAAAACGAG	Cloning of *cplE* into BACTH, fwd (*Xba*I)
DT136	TTTGGTACCCGTTTTGCTCGCACTTT GATTTTATCATCTTCTAAAATC	Cloning of *cplE* into BACTH, rev (*Kpn*I)
DT137	GCGCGACAACACTGAAAGAATATCG	Sequencing of *cplE*
DT138	TTTGTCGACTTAGTTTACCCCGATAT ATTGATCTTCAACCG	Cloning of *accDA* into pBQ200, rev (*Sal*I)
DT139	AAAGAGCTCGAGGAAGCTGGACAAT TAACTGAGAAGGT	Cloning of *clpE* into pGP1331, fwd (*Sac*I)
DT140	TTTGGATCCTTTTGCTCGCACTTTGAT TTTATCATCTTCTAAAATCAC	Cloning of *clpE* into pGP1331, rev (*Bam*HI)

6.3 Plasmids

Name	**Relevant characteristics**	**Primer/ Restriction sites**	**Reference**
pBP43	fusion of the monomeric variant of GFP A206K to the C-terminus of a protein		Cascante-Estepa *et al.*, 2016
pBP190	pUT18-*mcsB*		Stannek, 2015
pBP191	pUT18C-*mcsB*		Stannek, 2015
pBP192	pKT25-*mcsB*		Stannek, 2015

pBP193	p25-N-*mcsB*	Stannek, 2015
pBP198	pUT18-*clpP*	Stannek, 2015
pBP199	pUT18C-*clpP*	Stannek, 2015
pBP200	pKT25-*clpP*	Stannek, 2015
pBP201	p25-N-*clpP*	Stannek, 2015
pBP202	pUT18-*clpC*	Stannek, 2015
pBP203	pUT18C-*clpC*	Stannek, 2015
pBP204	pKT25-*clpC*	Stannek, 2015
pBP205	p25-N-*clpC*	Stannek, 2015
pBP206	pUT18-*clpX*	Stannek, 2015
pBP207	pUT18C-*clpX*	Stannek, 2015
pBP208	pKT25-*clpX*	Stannek, 2015
pBP209	p25-N-*clpX*	Stannek, 2015
pBQ200	allows overexpression of proteins in *B. subtilis*	Martin-Verstraete *et al.*, 1994
pDG646	amplification of *ermC* resistance cassette for LFH PCR Erm^R	Guérout-Fleury *et al.*, 1995
pDG780	amplification of *apha3* resistance cassette for LFH PCR Km^R	Guérout-Fleury *et al.*, 1995
pDG1726	amplification of *aad9* resistance cassette for LFH PCR $Spec^R$	Guérout-Fleury *et al.*, 1995
pGEM-cat	amplification of *cat* resistance cassette for LFH Cat^R	Guérout-Fleury *et al.*, 1995

pGP172	allows expression of proteins carrying a Strep-tag at their N-terminus in *E. coli*	Merzbacher *et al.*, 2004
pGP380	allows overexpression of N-terminal Strep-tag fusion proteins in *B. subtilis*	Herzberg *et al.*, 2007
pGP382	allows overexpression of C-terminal Strep-tag fusion proteins in *B. subtilis*	Herzberg *et al.*, 2007
pGP574	allows overexpression of proteins carrying a Strep-tag at their C-terminus in *E. coli*	Schilling *et al.*, 2006

pGP1027	pGP574	AK12-AK13/ *NdeI*+ *BamH*I	Klewing, 2015
pGP1325	pWH844-*yqhY*	DT25-DT26/ *BamH*I + *Pst*I	Tödter, 2013
pGP1326	pGP1460-*yqhY*	DT27-DT28/ *BamH*I + *Pst*I	Tödter, 2013
pGP1327	pGP1460-*yloU*	DT29-DT30/ *BamH*I + *Pst*I	Tödter, 2013
pGP1328	pGP1331-*yqhY*	DT31-DT32/ *BamH*I + *Pst*I	Tödter, 2013
pGP1329	pGP172-*yqhY*	DT19-DT20/ *BamH*I + *Kpn*I	Tödter, 2013
pGP1331	allows fusion of 3x FLAG tag at the C-terminus of a protein, keeping the expression under control of the natural promotor		Lehnik-Habrink *et al.*, 2010
pGP1333	3’ end of *cshA* with 3x FLAG tag at C-terminus		Lehnik-Habrink *et al.*, 2010
pGP1370	allows fusion of 3x FLAG tag at the C-terminus of a protein		Lehnik-Habrink *et al.*, 2011
pGP1389	allows expression of C-terminal Strep-tag fusion proteins in *B. subtilis*, keeping the expression under control of the natural promotor		Lehnik-Habrink *et al.*, 2011

pGP1460	constitutive expression of C-terminally Strep-tagged proteins in *B. subtilis*; integrates in *lacA*		Mehne *et al.*, 2013
pGP1470	pUT18-*yqhY*	DT45-DT46/ *Xba*I + *Kpn*I	Tödter, 2013
pGP1471	pUT18C-*yqhY*	DT45-DT46/ *Xba*I + *Kpn*I	Tödter, 2013
pGP1472	p25-N-*yqhY*	DT45-DT46/ *Xba*I + *Kpn*I	Tödter, 2013
pGP1473	pKT25-*yqhY*	DT45-DT46/ *Xba*I + *Kpn*I	Tödter, 2013
pGP1482	pBP43-*yqhY*	DT31-DT32/ *BamH*I + *Pst*I	Tödter, 2013
pGP1484	pGP1460-*accB*	DT73-DT74/ *BamH*I + *Sal*I	This study
pGP1485	pGP1460-*accC*	DT75-DT76/ *BamH*I + *Sal*I	This study
pGP1496	pET28a+-*yqhY*	DT77-DT78/ *Xba*I + *Not*I	This study
pGP1497	pGP172-C-terminus of *divIVA*+TEV-site	DT79-DT80/ *Kpn*I + *BamH*I	This study
pGP1498	pWH844-*yqhY*+TEV-site	DT81-DT82/ *BamH*I + *Pst*I	This study
pGP1499	pGP1331-*accA*	DT64-DT66/ *BamH*I + *Sal*I	This study
pGP1723	pGP382-*accA*	DT83-DT66/ *BamH*I + *Sal*I	This study
pGP1724	pGP382-*accD*	DT84-DT85/ *BamH*I + *Sal*I	This study
pGP1725	pGP382-*accD*-G283V	DT84-DT97/ *BamH*I + *Sal*I	This study

pGP1726	pGP1460-*divIVA*	DT102-DT103/ *BamH*I + *Pst*I	This study
pGP1727	pGP1331-*accC*	DT106-DT76/ *BamH*I + *Sal*I	This study
pGP1728	pGP1087-*accB*	DT104-DT105/ *Kpn*I	This study
pGP1729	pGP1871-*yqhY*	DT25-DT28/ *BamH*I + *Pst*I	This study
pGP1730	pBP43-*accA*	DT64-DT66/ *BamH*I + *Sal*I	This study
pGP1731	pBP43-*accC*	DT106-DT76/ *BamH*I + *Sal*I	This study
pGP1732	pGP1080-*accB*	DT104-DT105/ *Kpn*I	This study
pGP1733	pUT18-*accA*	DT89-DT90/ *Xba*I + *Kpn*I	This study
pGP1734	pUT18C-*accA*	DT89-DT90/ *Xba*I + *Kpn*I	This study
pGP1735	p25-N-*accA*	DT89-DT90/ *Xba*I + *Kpn*I	This study
pGP1736	pUT18-*accB*	DT91-DT92/ *Xba*I + *Kpn*I	This study
pGP1737	pUT18C-*accB*	DT91-DT92/ *Xba*I + *Kpn*I	This study
pGP1738	p25-N-*accB*	DT91-DT92/ *Xba*I + *Kpn*I	This study
pGP1739	pKT25-*accB*	DT91-DT92/ *Xba*I + *Kpn*I	This study
pGP1740	pUT18-*accC*	DT93-DT94/ *Xba*I + *Kpn*I	This study

pGP1741	pUT18C-*accC*	DT93-DT94/ *Xba*I + *Kpn*I	This study
pGP1742	p25-N-*accC*	DT93-DT94/ *Xba*I + *Kpn*I	This study
pGP1743	pKT25-*accC*	DT93-DT94/ *Xba*I + *Kpn*I	This study
pGP1744	pUT18-*accD*	DT95-DT96/ *Xba*I + *Kpn*I	This study
pGP1745	pUT18C-*accD*	DT95-DT96/ *Xba*I + *Kpn*I	This study
pGP1746	p25-N-*accD*	DT95-DT96/ *Xba*I + *Kpn*I	This study
pGP1747	pGP1370-*accD*	DT84-DT85/ *BamH*I + *Sal*I	This study
pGP1748	pETM-11-*yqhY*	DT118-DT20/ NcoI + *BamH*I	This study
pGP1749	pGP1331-*divIVA*	DT119-DT103/ *BamH*I + *Pst*I	This study
pGP1750	pGP1331-*clpC*	DT121-DT122/ *BamH*I + *Pst*I	This study
pGP1828	pGP888-*thrC*	DT123-DT124/ *Xba*I + *Kpn*I	This study
pGP1829	pUT18-*ctsR*	DT127-DT128/ *Xba*I + *Kpn*I	This study
pGP1830	pUT18C-*ctsR*	DT127-DT128/ *Xba*I + *Kpn*I	This study
pGP1831	p25-N-*ctsR*	DT127-DT128/ *Xba*I + *Kpn*I	This study
pGP1832	pKT25-*ctsR*	DT127-DT128/ *Xba*I + *Kpn*I	This study

pGP1833	pUT18-*mecA*	DT129-DT130/ *BamH*I + *Kpn*I	This study
pGP1834	pUT18C-*mecA*	DT129-DT130/ *BamH*I + *Kpn*I	This study
pGP1835	p25-N-*mecA*	DT129-DT130/ *BamH*I + *Kpn*I	This study
pGP1836	pKT25-*mecA*	DT129-DT130/ *BamH*I + *Kpn*I	This study
pGP1837	pUT18-*yjbH*	DT131-DT132/ *Xba*I + *Kpn*I	This study
pGP1838	pUT18C-*yjbH*	DT131-DT132/ *Xba*I + *Kpn*I	This study
pGP1839	p25-N-*yjbH*	DT131-DT132/ *Xba*I + *Kpn*I	This study
pGP1840	pKT25-*yjbH*	DT131-DT132/ *Xba*I + *Kpn*I	This study
pGP1841	pUT18-*ypbH*	DT133-DT134/ *Xba*I + *Kpn*I	This study
pGP1842	pUT18C-*ypbH*	DT133-DT134/ *Xba*I + *Kpn*I	This study
pGP1843	p25-N-*ypbH*	DT133-DT134/ *Xba*I + *Kpn*I	This study
pGP1844	pKT25-*ypbH*	DT133-DT134/ *Xba*I + *Kpn*I	This study
pGP1845	pGP172-*accA*-H105P	AK01-AK02/ *Kpn*I + *BamH*I	This study
pGP1846	pGP172-*accD*-L176F	AK07-AK08/ *Kpn*I + *BamH*I	This study
pGP1847	pGP172-*accD*-A229S	AK07-AK08/ *Kpn*I + *BamH*I	This study

pGP1848	pGP172-*accD*-I38N	AK07-AK08/ *Kpn*I + *BamH*I	This study
pGP1849	pGP1389-*accA*	DT64-DT66/ *BamH*I + *Sal*I	This study
pGP1850	pBQ200-*accDA*	DT84-DT138/ *BamH*I + *Sal*I	This study
pGP1870	pUS19-*gfp*		Rothe *et al.*, 2013
pGP1871	pUS19-*yfp*		Rothe *et al.*, 2013
pGP2157	pUT18-*clpE*	DT135-DT136/ *Xba*I + *Kpn*I	This study
pGP2158	pUT18C-*clpE*	DT135-DT136/ *Xba*I + *Kpn*I	This study
pGP2159	p25-N-*clpE*	DT135-DT136/ *Xba*I + *Kpn*I	This study
pGP2160	pKT25-*clpE*	DT135-DT136/ *Xba*I + *Kpn*I	This study
pGP2161	pGP1331-*clpE*	DT139-DT140/ *Sac*I + *BamH*I	This study
pGP2162	pBQ200-*yqhY*	DT27-DT26/ *BamH*I + *Pst*I	This study
pGP2690	pWH844-*birA*	JN335-JN336/ *BamH*I + *Sal*I	J. Gundlach, unpublished
p25-N	P_{lac}-mcs-*cyaA kan*		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
pUT18::zip	P_{lac} -*cyaA*-zip *bla*	Karimova *et al.*, 1998
pWH844	allows expression of proteins carrying a His tag at their N-terminus in *E. coli*	Schirmer *et al.*, 1997

6.4 Strains

***Bacillus subtilis* strains used in this study**

Strain	Genotype	Reference/ Construction
168	*trpC2*	Laboratory collection
4041	*trpC2* Δ*divIVA*::*tet*	Oliva *et al.*, 2010
BKE00830	*trpC2* Δ*ctsR*::*erm*	BGSC
BKE11550	*trpC2* Δ*yjbH*::*erm*	BGSC
BKE27910	*trpC2* Δ*yszB*::*erm*	BGSC
BKG09	*trpC2* Δ*spo0A*::*aphA3*	K. Gunka, UMG
GP1468	*trpC2* Δ*yqhY*::*erm*	Tödter, 2013
GP1469	*trpC2* Δ*yqhY*::*erm* (AccD G283V)	Tödter, 2013
GP1470	*trpC2* Δ*yqhY*::*erm* Δ*yloU*::*cat* (AccA H105P)	Tödter, 2013
GP1473	*trpC2 yqhY-gfp spc,* Δ*divIVA*::*tet*	Tödter, 2013
GP1474	*trpC2 lacA*::(*yqhY*-Strep *aphA3*)	Tödter, 2013
GP1477	*trpC2 lacA*::(*accB*-Strep *aphA3*)	pGP1484 → 168
GP1478	*trpC2 lacA*::(*accC*-Strep *aphA3*)	pGP1485 → 168
GP1479	*trpC2 lacA*::(*accB*-Strep *aphA3*) *yqhY*-3xFLAG *spc*	pGP1328 → GP1477
GP1480	*trpC2 lacA*::(*accC-Strep aphA3*) *yqhY*-3xFLAG *spc*	pGP1328 → GP1478
GP1481	*trpC2 yqhY*-3xFLAG *spc*	pGP1328 → 168

GP1482	*trpC2 lacA*::(*divIVA*-Strep *aphA3*)	pGP1726 → 168
GP1483	*trpC2 accC*-3xFLAG *spc*	pGP1727 → 168
GP1484	*trpC2* P_{spac} *accB*-3xFLAG *erm*	pGP1728 → 168
GP1485	*trpC2 lacA*::(*divIVA*-Strep *aphA3*) *accA*-3xFLAG *spc*	GP1487 gDNA → GP1482
GP1486	*trpC2 lacA*::(*divIVA*-Strep *aphA3*) *accC*-3xFLAG *spc*	pGP1727 → GP1482
GP1487	*trpC2 accA*-3xFLAG *spc*	pGP1499 → 168
GP1488	*trpC2* Δ*yqhY*::*mls* (Suppressor: G283V) *accA*-3xFLAG *spc*	pGP1499 → GP1469
GP1489	*trpC2* Δ*yqhY*::*erm* Δ*yloU*::*cat* (Suppressor: H105P) *accA*-3xFLAG *spc*	pGP1499 → GP1470
GP1490	*trpC2 accA-gfp spc*	pGP1730 → 168
GP1491	*trpC2 accC-gfp spc*	pGP1731 → 168
GP1492	*trpC2 yqhY-yfp cat*	LFH → 168
GP1493	*trpC2* P_{spac} *accB-gfp erm*	pGP1732 → 168
GP1494	*trpC2 lacA*::(*yqhY*-Strep *aphA3*) *accA*-3xFLAG *spc*	GP1487 gDNA → GP1474
GP1495	*trpC2 lacA*::(*yqhY*-Strep *aphA3*) *accC*-3xFLAG *spc*	pGP1727 → GP1474
GP1496	*trpC2 lacA*::(*yqhY*-Strep *aphA3*) *cshA*-3xFLAG *spc*	pGP1333 → GP1474
GP1498	*trpC2 lacA*::(*divIVA*-Strep *aphA3*) *accD*-3xFLAG *erm*	pGP1747 → GP1482
GP1499	*trpC2 lacA*::(*divIVA*-Strep *aphA3*) *cshA*-3xFLAG *spc*	pGP1333 → GP1482
GP1500	*trpC2 lacA*::(*divIVA*-Strep *aphA3*) *yqhY*-3xFLAG *spc*	pGP1328 → GP1482
GP1765	*trpC2* Δ*yqhY*::*cat*	LFH → 168
GP1766	*trpC2 accA-gfp spc* Δ*yqhY*::*erm*	GP1468 gDNA → GP1490
GP1767	*trpC2* P_{spac} *accB-gfp erm* Δ*yqhY*::*cat*	GP1493 gDNA → GP1765

GP1768	*trpC2 accC-gfp spc* Δ*yqhY*::*cat*	GP1491 gDNA → GP1765
GP1769	*trpC2 accA-gfp spc* Δ*divIVA*::*tet*	4041 gDNA → GP1490
GP1770	*trpC2* P_{spac} *accB-gfp erm* Δ*divIVA*::*tet*	4041 gDNA → GP1493
GP1771	*trpC2 accC-gfp spc* Δ*divIVA*::*tet*	4041 gDNA → GP1491
GP1772	*trpC2 accA-gfp spc* Δ*yqhY*::*erm* (Suppressor: G283V)	GP1490 gDNA → GP1469
GP1773	*trpC2 accA-gfp spc* Δ*yqhY*::*erm* Δ*yloU*::*cat* (AccA H105P)	GP1490 gDNA → GP1470
GP1774	*trpC2 accA-gfp spc* Δ*yqhY*::*cat* Δ*divIVA*::*tet*	GP1765 gDNA → GP1769
GP1775	*trpC2 accA-gfp spc* Δ*yqhY*::*erm* Δ*divIVA*::*tet*	GP1766 gDNA → 4041
GP1776	*trpC2 divIVA*-3xFLAG *spc*	pGP1749 → 168
GP1777	*trpC2 lacA*::(*yqhY*-Strep *aphA3*) *divIVA*-3xFLAG *spc*	pGP1749 → GP1474
GP1778	*trpC2 lacA*::(*yqhY*-Strep *aphA3*) *clpC*-3xFLAG *spc*	pGP1750 → GP1474
GP1779	*trpC2* Δ*ctsR*::*erm* Δ*yqhY*::*cat*	BKE00830 gDNA → GP1765
GP1780	*trpC2* Δ*yjbH*::*erm* Δ*yqhY*::*cat*	gDNA BKE11550 → GP1765
GP1781	*trpC2* Δ*yszB*::*erm* Δ*yqhY*::*cat*	gDNA BKE27910 → GP1765
GP1782	*trpC2* Δ*ctsR*::*erm accA*-3xFLAG *spc*	GP1487 gDNA → BKE00830
GP1786	*trpC2* Δ*clpP*::*erm*	GPUG1 gDNA → 168
GP1787	*trpC2* Δ*clpX*::*aphA3*	GPUG2 gDNA → 168
GP1788	*trpC2* Δ*spx*::spc_{200}	GPUG3 gDNA → 168
GP1789	*trpC2* Δ*spx*::spc_{200} Δ*clpX*::*aphA3*	GP1787 gDNA → GP1788
GP1790	*trpC2* Δ*spx*::spc_{200} Δ*clpP*::*erm*	GP1786 gDNA → GP1788
GP1791	*trpC2 accA*-3xFLAG *spc* Δ*yqhY*::*cat*	GP1765 gDNA → GP1487
GP1792	*trpC2 accA*-3xFLAG *spc* Δ*clpX*::*aphA3*	GP1787 gDNA → GP1487
GP1793	*trpC2 accA*-3xFLAG *spc* Δ*clpP*::*erm*	GP1786 gDNA → GP1487

GP1794	*trpC2 accA-gfp spc* Δ*clpX*::*aphA3*	GP1787 gDNA → GP1490
GP1795	*trpC2 accA-gfp spc* Δ*clpP*::*erm*	GP1786 gDNA → GP1490
GP1796	*trpC2 accA*-Strep *spc* Δ*yqhY*::*erm* (Suppressor: AccD A229S)	pGP1849 → GP2322
GP1797	*trpC2 accA*-Strep *spc* Δ*yqhY*::erm (Suppressor: AccD I38N)	pGP1849 → GP2323
GP1798	*trpC2* Δ*yqhY*::*cat* Δ*spo0A*::*aphA3*	BKG09 gDNA → GP1765
GP1799	*trpC2 clpE*-3xFLAG *spc*	pGP2161 → 168
GP1800	*trpC2 lacA*::*(yqhY*-Strep *aphA3) clpE*-3xFLAG *spc*	pGP2161 → GP1474
GP2321	*trpC2* Δ*yqhY*::*erm* (Suppressor: AccD L176F)	GP1468 gDNA → 168
GP2322	*trpC2* Δ*yqhY*::*erm* (Suppressor: AccD A229S)	GP1468 gDNA → 168
GP2323	*trpC2* Δ*yqhY*::*erm* (Suppressor: AccD I38N)	GP1468 gDNA → 168
GPUG1	*trpC2* Δ*clpP*::*erm*	U. Gerth
GPUG2	*trpC2* Δ*clpX*::*aphA3*	U. Gerth
GPUG3	*trpC2* Δ*spx*::*spc*	U. Gerth
QB4913	*trpC2* Δ*clpP*::*spc*	U. Gerth

***Escherichia coli* strains used in this study**

Strain	**Genotype**	**Reference/ Construction**
BL21 (DE3)	*F- lon ompT rBmB hsdS gal* (*cIts857ind1 Sam7 nin5 lacUV5- T7 gene1*)	Sambrook *et al.*, 1989
BTH101	*F- cya-99 araD139 galE15 galK16 rpsL1* (*StrR*) *hsdR2 mcrA1 mcrB1*	Karimova *et al.*, 2005
DH5α	*recA1 endA1 gyrA96 thi hsdR17rK- mK+relA1 supE44 Φ80ΔlacZΔM15* Δ(*lacZYAargF*) *U169*	Sambrook *et al.*, 1989
XL1-Blue	*endA1 gyrA96* (*nalR*) *thi-1 recA1 relA1 lac glnV44 F'*[::*Tn10 proAB+ lacIq* Δ(*lacZ*)*M15*] *hsdR17* (*rK- mK+*)	Karimova *et al.*, 2005

Curriculum vitae

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Education

2000-2007	Lessing-Gymnasium, Uelzen
2007-2011	Study of Biology, Georg-August-University Göttingen, Bachelor thesis in the group of Prof. Dr. Jörg Stülke, Department of General Microbiology at the Georg-August-University Göttingen
2011	Internship at DSM Nutritional Products in Kaiseraugst, Switzerland
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