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ABSTRACT

The ability of a Gram-positive rod-shaped bacterium, *Bacillus subtilis*, to produce and secrete endogenous and heterologous proteins to the environment makes this bacterium an attractive host for production of proteins useful in industry. *B. subtilis* and its close relatives are used to produce more than half of the commercially available enzymes used for the detergent-, food- and beverage industries and for the development of pharmaceuticals. Despite of previous manipulations of the limiting factors that hamper different stages of protein production or secretion, which resulted in a significant improvement of *B. subtilis* as a production host, protein production can still be difficult.

In this thesis stress responses activated under intensive production of homo- and heterologous proteins are identified. A more detailed study of the membrane protein overproduction stress resulted in the characterization of a specific membrane stress response involving a putative membrane protease and in the identification of a novel negative regulator of this response. Also indispensability of the PrsA foldase/chaperone responsible for correct folding of secreted proteins was investigated and it was shown to be crucial for the cell viability due to its indirect involvement in lateral cell wall biosynthesis. Moreover, *B. subtilis* is able to maximize its metabolic efficiency through regulation of carbon metabolism genes by a global regulator, CcpA, which binds to operator sequences, *cre* boxes. This is a relevant aspect of the optimization of cultivation of cells in big fermentations for industry. *cre* boxes were analyzed on a genome-wide scale and differences in their sequences and positions in relation to transcriptional start sites were shown to determine their regulatory efficiencies.

This thesis provides valuable knowledge on (membrane and secreted) protein overproduction and stress-responsive mechanisms, which can be used for further improvement of *B. subtilis* as a production host for industry. Moreover, it offers a better insight in the role of the *cis*-acting *cre*-boxes in determining the strength of carbon catabolism regulation by the global regulator CcpA.

CONTENTS

CHAPTER 1

Introduction and scope of the thesis

Bacillus subtilis as a "cell factory"

Bacillus subtilis is a model rod-shaped Gram-positive bacterium with a GRAS status (Generally Recognized As Safe). The full sequence of the genome of this bacterium was published for the first time in 1997 [1] and resequenced and reannotated recently [2]. The genome was shown to be 4.2 Mb in size and to contain approximately 4200 protein-coding genes. A large proportion of the genome is related to carbon source metabolism, including plant-derived molecules [1]. Around 300 genes are considered indispensable or essential, most of which are involved in metabolism. About half of the essential genes are involved in DNA, RNA metabolism and protein synthesis and the others in cell envelope synthesis, cell shape and division, cell energetics and genes with unknown function [3].

B. subtilis can be naturally found in soil and vegetation [4]. Starvation and different stress conditions are common for this environment. *B. subtilis* has, therefore, developed several survival strategies. Induction of motility and chemotaxis, production of proteases and carbohydrases and antibiotics increase the chance for survival [1]. Moreover, under conditions of nutrient limitations, *B. subtilis* cells can initiate the process of sporulation [5], i.e., formation of endospores that are highly-resistant to starvation and harsh circumstances, and a variety of physical and chemical agents [6]. When the appropriate nutrients are present and the conditions improve, the spores undergo germination followed by outgrowth to vegetative cells [6]. Another strategy of survival is the development of genetic competence allowing uptake of external naked DNA [7]. By recombination, *B. subtilis* can gain new features and adapt to new conditions.

An industrially relevant feature of *B. subtilis* is its ability to secrete proteins to the outside of the cell. "Protein secretion" refers to a process of targeting, docking and translocation of a protein through translocation complexes in the cell membrane. The major pathway for translocation of proteins through the membrane is the Sec machinery, which consists of SecA, the translocation motor, and SecE, SecG and SecY, the integral membrane proteins [8]. The Sec translocon works in an ATP- dependent manner and it recognizes preproteins carrying the Sec-type signal peptide on the N-terminus [8]. Also other components are involved in Sec-dependent protein export: SRP/FtsY and CsaA cytoplasmic chaperons facilitate targeting of the precursors to the translocase in the membrane; type I signal peptidases (SipS-W) and the lipoprotein-specific signal peptidase (Lsp) cleave the preprotein during or shortly after translocation; SppS and TepA are involved in degradation of cleaved signal peptides;

PrsA, BdbBC and/or SpoIIIJ/YqjG are responsible for folding of several secreted proteins; HtrA, HtrB and WprA are involved in the quality control of secretory proteins [8].

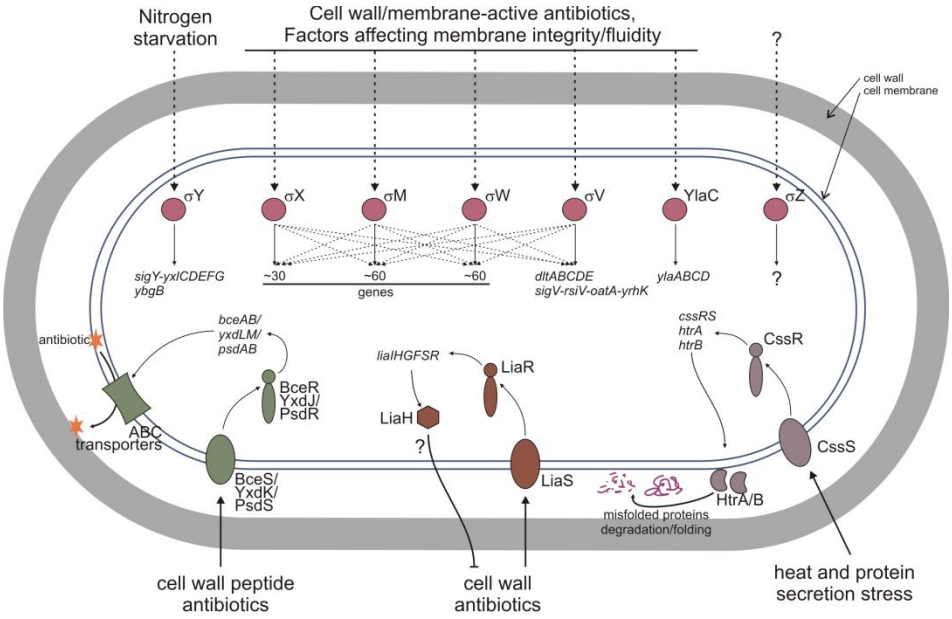

Figure 1. Simplified scheme representing cell envelope stress responses in B. *subtilis*. σY, σX, σM, σW, σV, σZ and YlaC, extracytoplasmic function sigma factors; CssS, LiaS, BceS, YxdK and PsdS, sensor histidine kinases of two-component systems; CssR, LiaR, BceR, YxdJ and PsdR, response regulators of two-component systems; HtrA/B, membrane-anchored chaperone/proteases HtrA and HtrB, LiaH, phage shock protein. BceS/YxdK/PsdS sensor kinases and BceR/YxdJ/PsdR regulators are parts of peptide sensing and detoxification modules (PSD) consisting of two-component systems and ABC transporters. Activation of sigma factors occurs via a cascade of proteolytic degradation of antisigma and anti-antisigma factors. See text for details. This figure was adapted from **[9–11]**.

Proteins containing a highly conserved twin-arginine motif in the N-terminal domain of the signal peptide are exported in their pre-folded state via the twin-arginine translocation (Tat) pathway [12–14] and this process is driven by the proton motive force across the membrane [15–17]. Two distinct translocases with different substrate specificities are present in *B. subtilis*: TatAdCd (involving TatAd and TatCd) facilitating secretion of PhoP, a protein with phosphodiesterase and alkaline phosphatase activity, and TatAyCy (involving TatAy and TatCy) responsible for translocation of YwbN, an iron-dependent peroxidase [18–20]. Both Tat complexes were shown to recognize similar N-terminal signal peptides [21]. Despite of this substrate specificity, the Tat pathway has the potential of secretion of heterologous

proteins as it was shown that an organophosphate-hydrolyzing enzyme, methyl parathion hydrolase (MPH) fused to the twin-arginine signal peptide of TorA from *Escherichia coli* can be secreted in a Tat-dependent way in *B. subtilis* [22].

Proteins lacking a signal peptidase (SPase) cleavage site or lipid-modified proteins or proteins containing transmembrane domains retain at the extracytoplasmic site of the membrane; those with cell wall-binding repeats, stay attached to the cell wall. After proteolysis or due to cell wall turnover, the membrane- and cell wall-attached proteins can be released into the medium [8]. Also proteins lacking signal peptides can leave the cell through cell lysis, holin systems for lytic enzymes of bacteriophages [23], flagellar export for the flagellin (Hag) and two flagellar hook-associated proteins [24–26], or yet unidentified systems. Additional secretion pathways are the pseudopilin export pathway for competence development and ATP-binding cassette (ABC) transporters. Since only few proteins appear to be exported in this manner, they can be regarded as special-purpose pathways [27].

The ability to secrete proteins to the growth medium made *B. subtilis* an enzyme-production "cell factory" competitive to *E. coli*, which is used, in example, for production of human insulin [28]. Proteins produced in *E. coli* usually accumulate within the cell and form aggregates and inclusion bodies. Recovery of the proteins from inclusion bodies can be a problematic process. Moreover, *B. subtilis* is lacking the highly pyrogenic endotoxin lipopolysaccharide (LPS) that is present in the outer membrane of *E. coli* and other Gram-negative bacteria and complicates protein purification, since the end-product must be endotoxin-free [29]. More than half of the commercially available enzymes are produced by *B. subtilis* and its close relatives. The produced proteins are useful enzymes for the detergent, food and beverage industries [30] as for instance alkaline proteases used as washing agent or amylases used for the starch industry [29]. Other successfully produced proteins using *B. subtilis* are proinsulin [31], human epidermal growth factor (hEGF) [32], human interferon α (hIFN-α2b) [33], endocellulase (PuradaxR) [34], endoglucanase [32], and also human papillomavirus type 33 L1 major capsid protein and virus-like particles used for development of a prophylactic vaccine against cervical cancer [35]. Next to industry, *B. subtilis* is used in fundamental research for the production of homologous and heterologous proteins, which then can be isolated and purified for crystallography in order to obtain their crystal structures or for other (*in vitro*) assays. However, when too much protein is produced and transported, cell envelope stress responses are turned on, which may set limits to the production on a big scale.

Cell envelope stress response

Cells of *B. subtilis* and other bacteria respond to chemical and physical stresses affecting the integrity of the cell wall and membrane by activating a cell envelope stress response, which is exerted by two-component regulatory systems (TCS) and extracytoplasmic function (ECF) sigma factors (Fig. 1) [11]. TCSs consist of two proteins, a sensor kinase and a response regulator. In response to specific signal(s), sensor kinases of TCSs autophosphorylate at the conserved histidine residue and transfer the phosphoryl group to the conserved aspartate residue in its cognate regulator, which usually increases the affinity of the regulator for specific DNA sequences and results in expression of the regulated genes [36–38]. ECF sigma factors form a subfamily of eubacterial RNA polymerase sigma factors that are involved in response to extracytoplasmic stimuli and stresses [39]. Their activity is regulated by one or more anti-sigma factors with an extra-cytoplasmic domain and an intracellular domain that prevent the ECF sigma factor from interaction with RNA polymerase [40].

Two-component Systems

High level production of homologous and heterologous proteins, which are translocated through the membrane, as well as heat shock, have been shown to induce a specific stress response in *B. subtilis* through a two-component signal transduction system, CssS-CssR (Fig. 1) [41–43]. CssS is a sensor histidine kinase with two transmembrane domains [44]. Although the direct signal which is perceived by CssS is unknown, the fact that translocation of secreted proteins is required for induction of the CssRS response suggests that the signal originates from some aspect of the secretion apparatus or process, or the accumulation of misfolded proteins [43, 45, 46]. In favor of this hypothesis is recent study showing that the CssS extracellular loop domain functions in the switch between the active and inactive state and in signal perception and/or transduction [45]. The response regulator CssR, stimulated by the histidine kinase, binds to promoter regions and activates expression of its own operon, *cssRS*, leading to amplification of the response, as well as to *htrA* and *htrB*, coding for extracellular chaperone/proteases responsible for refolding or degradation of misfolded proteins within the cell envelope [41, 43, 47]. Both HtrA and HtrB possess transmembrane domains and are probably located at the outer surface of the cell membrane, although truncated forms of HtrA can also accumulate in the growth medium [48]. It has also been suggested that expression of *ykoJ*, *yloA*, *ylxF* and *citM* is also regulated by CssR, although the putative CssR-binding sequence was not found in front of these operons [49].

Next to the response to heat and secreted protein-overproduction stress, CssRS has also been implicated to be involved in cellular response to rhamnolipoid biosurfactants with antimicrobial properties [43]. Moreover, mammalian peptidoglycan recognition proteins (PGRPs), similar to antimicrobial lectins, were shown to bind to the *B. subtilis* cell wall and kill the cells through inducing an exaggerated CssRS stress response [50]. Physiologically, CssRS plays a role in the stationary growth phase, when more proteins are secreted [51].

The CssRS system bears some similarities with the CpxRA system of *E. coli*, which is comprised of the CpxA kinase, the CpxR response regulator and a periplasmic protein CpxP, negatively regulating CpxA activity [52, 53]. CssS and CssR reveal amino acid sequence similarities to CpxA and CpxR, respectively [54]. Both systems respond to cell envelope and secretion stresses [53, 55] and control expression of their own operons as well as genes encoding HtrA-like proteases: *htrA* and *htrB* in *B. subtilis*, and *htrA* (*degP*) in *E. coli* [47, 56–58]. Therefore, the CpxRA system of *E. coli* and CssRS of *B. subtilis* can be considered functional homologues. However, the regulon of CpxR in *E. coli* is bigger than that of CssR in *B. subtilis*. Next to *cpxRA* and *htrA*, it also regulates expression of genes coding for proteins catalyzing peptidyl-prolyl isomerization (i.e., PpiA, PpiD) and disulphide bond formation (i.e., DsbA), phosphatidyl serine decarboxylase (Psd), heat shock membrane-bound zinc metalloprotease (HtpX) and many others [59]. HtpX in *E. coli*, together with a membrane-bound ATP-dependent endopeptidase (FtsH), play a central role in cytoplasmic membrane proteins quality control. This is a mechanism to monitor the state of protein folding and eliminate and/or repair the abnormal membrane proteins accumulating under hazardous environmental changes, which can disturb the membrane structure and function and, eventually, integrity and viability of the cell. FtsH and HtpX contribute to this mechanism by dislocation of misfolded membrane proteins out of the membrane for their degradation and endoproteolytic cleavage of cytoplasmic loops of the substrate proteins, respectively [60]. In *B. subtilis* very little is known how the cells respond to this type of stress. Remarkably, homologs of FtsH and HtpX are present in *B. subtilis*: FtsH and YkrL, respectively. While the role of YkrL had not been studied before, *B. subtilis* FtsH has been shown to be involved in heat and osmotic stress, and sporulation [61–64]. Instead, SigW extracytoplasmic factor and CssRS system were implicated in the membrane quality control in *B. subtilis* [65]. As *B. subtilis* is used as a cell factory also for membrane protein production, it would be desired to gain more insight how the membrane quality process is regulated in this bacterium. This knowledge could be useful in rational design of a better membrane protein producer.

Other two-component systems, namely LiaRS, BceRS, YvcPQ and YxdJK (Fig. 1), were shown to respond to cell wall antibiotics and other factors perturbing the envelope in *B. subtilis* [11]. The LiaRS system, is also called a three-component system as, next to the typical sensor kinase (LiaS) and response regulator (LiaR), it involves a membrane-anchored protein, LiaF, inhibiting LiaS activity under non-stress conditions [66]. It has been shown that LiaR regulates its own operon *liaIHGFSR*, as well as *yhcYZ-yhdA* and *ydhE*, all of which are preceded by a putative LiaR binding site. However, the only *in vivo* relevant target of LiaR seems to be the *lia* operon encoding the three-component system and a phage shock protein-like response protein reminiscent of PspA in *E.coli*, LiaH [10].

The other TCS are parts of the cell wall peptide antibiotics-responsive modules, PSD (peptide sensing and detoxification) which in general consist of a TCS and an ABC transporter: BceRS-AB, YxdJK-LM, and PsdRS-AB (YvcPQ-RS). The BceRS-AB (PSD1) system was implicated in response to cell wall antibiotics such as bacitracin, plectasin, actagardine and mersacidin [9, 67, 68]. The YxdJK-LM system (PSD2) was shown to respond to the human antimicrobial peptide LL-37 [69] and YvcPQ-RS (PSD3) to bacitracin [67].

Extracytoplasmic function (ECF) sigma factors

B. subtilis encodes seven extracytoplasmic function sigma factors: SigM, SigV, SigW, SigX, SigY, SigZ and YlaC (Fig. 1). The *B. subtilis* ECF sigma factors recognize promoters with a similar sequence containing a conserved AAC motif within the -35 region and a CGT motif in the -10 region [40]. Several of these ECFs are induced by cell envelope-active antibiotics and by compounds affecting membrane integrity and/or fluidity [40, 70]. The best characterized ECFs in *B. subtilis* are SigW, SigM and SigX, which control overlapping sets of genes. In many cases, resistance genes are regulated by a single ECF sigma factor. The SigW regulon consists of ~60 genes, including numerous genes encoding membrane-localized proteins, and is activated under membrane stress elicited by cell wall-active antibiotics (e.g., vancomycin), membrane-active antibiotics (e.g., daptomycin), detergents (e.g., Triton X-100), overproduction of membrane proteins, phage infection, alkaline shock, salt stress and antimicrobial peptides [40, 65, 69, 71–76]. It has been shown that SigW responds to compounds that increase membrane fluidity by changing the fatty acid composition [77]. The SigW activity is regulated by a single-pass transmembrane anti-sigma factor RsiW [78–80]. RsiW interacts with SigW keeping it in an inactive state and undergoes stress-induced regulated intramembrane proteolysis (RIP), which leads to degradation of RsiW and in the release of active SigW [81]. The proteins necessary for proteolysis of RsiW are

RasP (YluC) and PrsW membrane-bound proteases, and the ClpXP proteolytic complex [79, 82, 83].

SigM regulates expression of a large number of operons coding for proteins involved in cell wall synthesis and cell division [75]. The SigM response is activated by several stresses like high salinity, heat, ethanol, acid and superoxide stress, and phosphate starvation, as well as by cell-wall active antibiotics such as vancomycin, bacitracin and cationic antimicrobial peptides [67, 69, 78, 84, 85]. In many cases, expression of the genes belonging to the SigM regulon depends also on other ECF sigma factors or other regulators [75]. The SigX regulon consists of operons that have been implicated in peptidoglycan turnover and modulation of the net charge of the cell wall and cell membrane [76, 86]. A *sigX* mutant reveals increased sensitivity to cationic antimicrobial peptides [74]. It was shown that SigM plays a primary and SigX a secondary role in β-lactam resistance [87] and that active SigM or SigX is required for the synthesis of one of the bactericidal antibiotics active against Gram-positive bacteria, sublancin 168 [88].

The knowledge on the other ECF sigma factors (SigV, SigY, SigZ, and YlaC) is still limited. SigY controls expression of its own operon and of at least one other gene of an unknown function, *ybgB* and its activity is not induced by a variety of factors stimulating SigX, SigW and SigM responses [89], but by nitrogen starvation [90]. SigV, YlaC, and also SigM, were shown to be activated by the cell wall-active antibiotics daptomycin and friulimicin B [73]. The SigV factor was also shown to confer resistance to lysozyme by activation of cell wall modification pathways encoded by *dltABCDE* (D-alanylation of teichoic acids) and *oatA* (O-acetylation of peptidoglycan) within the *sigV-rsiV-oatA-yrhK* operon [91, 92]. Its regulon was identified and almost completely overlaps with the genes which are under control of SigM, SigW and SigX [93]. The YlaC factor regulates expression of its own operon (*ylaABCD*) [94] and it was also implicated in oxidative stress resistance [95].

Cellular stress responses

Other cellular quality control systems facilitating the production of high quality proteins by responding to a sudden temperature increase and other stresses are generally called heat shock proteins (Hsps), which, based on their gene regulation, can be divided into classes (regulons). Genes from each class are regulated by a different transcriptional regulator, which can be a sigma factor, a transcriptional repressor or activator. The heat shock genes are expressed constitutively, mostly at low levels, and their expression is

rapidly and transiently induced after a temperature upshift [96]. Most of the heat shock proteins belong either to molecular chaperones, which ensure proper folding or assemblage of proteins [97], or to adenosine triphosphate (ATP)–dependent proteases responsible for degradation of misfolded proteins which are unable to refold to their native conformation [98]. In *B. subtilis*, there are at least six classes of heat shock genes responding to non-native proteins, which arise under the denaturing influence of heat, salt or ethanol (Table 1).

Table 1. Heat shock responses in *B. subtilis*.

Class	Regulon name	Regulator	Inducers	Main regulated genes	Function of major heat shock genes products
I	HrcA	HrcA repressor	Heat	*groES-groEL* *hrcA-grpE-dnaKJ-yqeTUV*	DnaK chaperone machine
II	σB	σB sigma factor	Different stressors	> 150	General stress proteins
III	CtsR	CtsR repressor	Different stressors	*ctsR-mcsA-mcsB-clpC* *clpP* *clpE*	ATP-dependent proteases (ClpCP, ClpEP)
IV	?	?	Heat	*htpG*	Molecular chaperone
V	CssRS	CssS sensor and CssR regulator	Heat, secretory proteins overproduction	*cssRS* *htrA* *htrB*	Membrane-anchored proteases (HtrA, HtrB)
VI	?	?	Different stressors	> 80	Diverse functions

Class I heat shock genes – HrcA regulon

Class I heat shock genes belong to the heptacistronic *dnaK* and bicistronic *groE* operons. The *dnaK* operon consists of the genes *hrcA* coding for the negative regulator of both operons, *groE*, *dnaK,* and *dnaJ* encoding the DnaK chaperone machine, and *yqeT*, *yqeU* and *yqeV*. The *groES* and *groEL* genes, coding for molecular chaperones, form the *groE* operon [99–101]. Both operons are preceded by a SigA (housekeeping sigma factor) promoter and a perfect inverted 9-bp repeat separated by a 9-bp spacer with the DNA sequence TTAGCACTC-N_9-GAGTGCTAA. This inverted repeat, called CIRCE (controlling inverted repeat of chaperone expression), is a *cis*-acting binding site for the negative regulator, HrcA [102–104]. The activity of HrcA is modulated by the GroE chaperonin machinery [105]. The HrcA repressor is present in the cells under both the active and inactive (unable to bind to CIRCE element) form. Under non-stress conditions, the GroE system converts the majority of HrcA molecules into the active form. When the cells experience heat stress, abnormal proteins titrate the GroE

chaperonins, causing an increase of the inactive form of HrcA and intensified transcription of the *dnaK* and *groE* operons [96].

Class II heat shock genes – SigB regulon

The class II heat shock genes are under positive control of the alternative sigma factor, SigB and they encode proteins of different categories: direct protection (proteases, catalases, thioredoxins, arsenate reductases), modulation of SigB activity (antisigma, anti-antisigmafactors, phosphatases), influx and efflux (permeases, antiporters, symporters), metabolism (dehydrogenases, glucosidase, pyruvate oxidase) and turnover (cysteine protease, ribonuclease R) [106]. This regulon responds not only to classical heat shock stress (such as the heat shock itself and ethanol) but also to a range of other stresses like salt, oxidation, desiccation or acid stress, and oxygen, glucose or phosphate starvation. It is therefore considered a general stress response regulon [107]. The 8-gene *sigB* operon is under control of a SigA-dependent promoter, ensuring constitutive expression, and a SigB-dependent promoter, leading to amplification of the response under stress conditions [108, 109]. The SigB activity is modulated in a complex way by antisigma factor RsbW and anti-antisigma factor RsbV [110]. Under physiological conditions, SigB is sequestered by RsbW, which prevents SigB interaction with RNA polymerase and transcription. Additionally, RsbW is responsible for inactivation of RsbV by phosphorylation. If the cells are exposed to a stress condition, one of the phosphatases, RsbP or RsbU, removes the phosphate from RsbV~P. Dephosphorylated RsbV attacks the SigB-RsbW complex, causing release of SigB and activation of the expression of more than 150 genes [96, 111].

Class III heat shock genes – CtsR regulon

Class III heat shock genes consist of six genes organized in three transcriptional units: the tetracistronic *clpC* operon (*ctsR-mcsA-mcsB-clpC*) and the monocistronic *clpP* and *clpE* operons. All operons are preceded by two promoters: *clpE* by two σA-dependent promoters and *clpC* operon and *clpP* by σA- and σB-dependent promoters [112, 113]. The class III heat shock gene repressor, CtsR binds to the operator sequences, i.e., heptanucleotide direct repeats with a highly conserved sequence (A/G)GTCAAANAN(A/G)GTCAAA [114]. At temperatures optimal for growth, the CtsR regulon genes are expressed at a low level and are strongly derepressed after a temperature upshift. The *mcsA* and *mcsB* genes code for modulators of the CtsR activity and *clp* genes for the subunits of ClpCP and ClpEP ATP-dependent proteases involved in degradation of misfolded proteins [115–117]

Class IV, V and VI heat shock proteins

The only class IV heat shock gene, *htpG*, coding for a molecular chaperone, is strongly induced upon a temperature upshift and its expression is under control of a yet unidentified positive transcriptional regulator [118, 119]. The class V consists of the CssR regulon described above and class VI comprises of genes responding to stress but the expression of which is regulated by other mechanism(s) than in classes I-V. Genes falling into this class are, e.g., *ftsH*, *clpX* and *lonA-ysxC* operon coding for ATP-dependent proteases [63, 120, 121], and *ahpC-ahpF*, *nfrA-ywcH* operons encoding alkyl hydroperoxide reductase and NADPH-linked nitro/flavin reductase, respectively [122, 123] and many others [124].

Attempts to improve *B. subtilis* as a "cell factory"

Although *B. subtilis* for a number of reasons is a preferred host for production of homo- and heterologous proteins at a large scale (see the first section of this chapter), cell envelope and cellular stress responses as well as mechanisms inhibiting the secretion pathway may compromise protein production. The limitations can result from, e.g., a low transcription level, inefficient translation, the presence of intracellular proteases, deficiency in chaperon complexes, poor targeting to the translocase in the cell membrane, jamming of the secretion machinery and, after the translocation, from deficiency in signal peptidases, chaperones, foldases, and the presence of extracytoplasmic proteases [27, 29, 125]. Also stress responsive systems, like CssRS or ECF sigma factor SigW may hamper protein production [65].

The rational manipulations of the protein secretion machinery and stress responsive systems resulted in improvement of *B. subtilis* as a protein production host. Deletion of six extracellular proteases (*aprA*, *nprE*, *nprB*, *epr*, *bpf* and *mpr*) lead to improved protein production as shown for TEM β-lactamase [126]. Further enhanced production of intracellular (GroES/EL, DnaK/DnaJ/GrpE) and extracellular (PrsA) molecular chaperons resulted in a better production and subsequent characterization of an antidigoxin and fibrin-specific single-chain antibody fragments [127, 128]. PrsA also facilitated production of recombinant lipoxygenase from *Anabaena sp.*, which has application in bread making and aroma production [129], and the biotechnologically important thermoresistant AmyL α-amylase of *Bacillus licheniformis* and other α-amylases [130–132]. Additional usage of a strong promoter and an efficient signal sequence on a multicopy plasmid resulted in optimization of human interleukin-3 (hIL-

3) production [133]. The contribution of (synthetic) strong and inducible promoters to high level protein production was also shown with other examples [134–137]. Specific overproduction of one of the signal peptidases (responsible for removal of amino-terminal signal peptides from translocated through the membrane proteins in order to release these proteins from the *trans* side of the membrane), SipT, enhanced the secretion of α-amylase, AmyQ [138]. Moreover, it was suggested that the increased net charge of the cell wall as a result of the *dlt* operon interruption, which is involved in the d-alanylation of teichoic acids, had a positive influence of AmyQ and recombinant anthrax protective antigen (rPA) secretion [139, 140]. Specific modifications of signal peptides as shown for two lipolytic enzymes, cutinase from *Fusarium solani pisi* and a cytoplasmatic esterase of metagenomic origin, and secretory protein itself, as shown in case of detoxified *Clostridium perfringens* β-toxin (β-toxoid), resulted in an improved production [141, 142]. Also high-throughput screening for optimal (native or mutated) signal peptides contributed to improvement of the production and secretion of the industrially important secreted protease, subtilisin BPN' from *Bacillus amyloliquefaciens* and cutinase from *F. solani pisi* [143, 144]. Furthermore, changes in protein targeting pathways enhanced the secretion of maltose binding protein (MalE11) and alkaline phosphatase (PhoA) [145] and modifications in the secretory machine (SecA) improved secretion of alkaline cellulase (Egl-237) and human interferon α (hIFN-α2b) [146]. Additionally, fusing hIFN-α2b with the AmyE propeptide also increased its production, secretion and activity [147]. Furthermore, mutations in the ECF sigma factor SigW or CssRS two-component system significantly improved production of membrane proteins [65]. Altogether, already many studies contributed to the improvement of *B. subtilis* as a "cell factory".

Central carbon metabolism

In order to allow for the most efficient protein production using *B. subtilis* as a production host, not only modifications in the secretion machinery and the stress responsive systems are of importance, but also the optimal growth rate should be assured. This is achieved by growing the cells in rich growth medium with the most preferred carbon and energy source like, in case of *B. subtilis*, glucose, fructose or mannose. *B. subtilis* and other bacteria are able to maximize the metabolic efficiency through activation of expression of the genes encoding enzymes necessary for preferred carbon source utilization (carbon catabolite activation, CCA) and simultaneous repression of the genes involved in utilization of secondary carbon sources (carbon

catabolite repression, CCR). Both mechanisms, together called carbon catabolite control (CCC), occur simultaneously and in *B. subtilis* they are exerted by a phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS) and a global regulator of carbon metabolism genes, CcpA (catabolite control protein A) (Fig. 2) [148, 149].

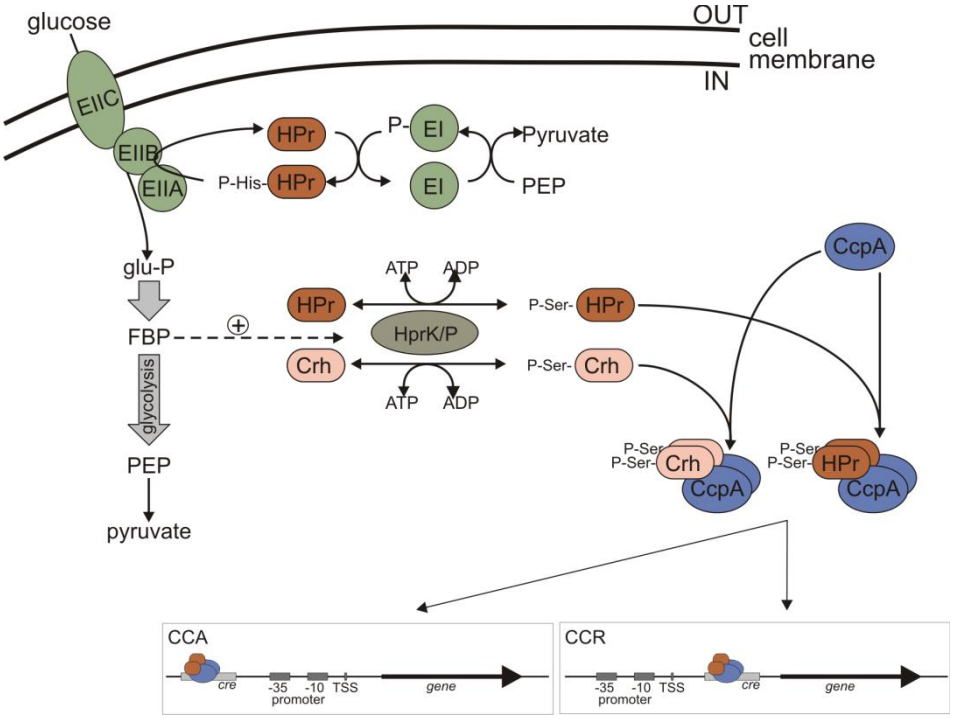

Figure 2. The mechanism of carbon catabolite control in *B. subtilis*. EI and HPr, non-sugar-specific enzymes of PEP-dependent phosphotransferase system (PTS); EIIA, EIIB and EIIC, domains of the sugar-specific PEP-dependent PTS; glu-P, glucose-6-phosphate; FBP, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; Crh, HPr-like protein; HPrK/P, HPr kinase/phosphatase; CcpA, carbon catabolite protein A; cre, catabolite responsive elements; TSS, transcriptional start site; CCA, carbon catabolite activation; CCR, carbon catabolite repression. The uptake of glucose, or other favored carbohydrate, results in an increase of FBP concentration in the cell, which triggers ATP-dependent HPrK/P-catalyzed phosphorylation of HPr and Crh proteins at the conserved serine (Ser) residue. Seryl-phosphorylated forms of HPr and Crh bind to CcpA. Active CcpA-[HPr-Ser-P] and CcpA-[Crh-Ser-P] complexes can bind to the DNA at cre sites and trigger CCA or CCR depending on the cre position in relation to the promoter (for simplicity, CcpA-[Crh-Ser-P] complexes are removed from cre sites). See text for details. This figure was adapted from **[149, 150]**.

The PTS system is the main system for carbohydrate uptake and it involves the general non-sugar-specific proteins enzyme I (EI) and HPr (histidine-containing protein), and sugar-specific enzyme II (EII) consisting of three domains: EIIA, EIIB and EIIC. The EIIA and EIIB domains are involved in transfer of the phosphoryl group and the EIIC domain – in translocation of the sugar substrate [151]. The signaling intermediate

protein HPr is phosphorylated at the conserved histidine residue (His_{15}) by EI at the expense of the high-energy metabolite phosphoenolpyruvate (PEP). Next, the phosphoryl group is transferred to EIIA and then further to EIIB. During transport through the membrane by the transporter domain EIIC, glucose is phosphorylated by EIIB, yielding glucose-6-phosphate (glu-P), which is further metabolized in glycolysis [151–153]. The intermediate product of this pathway, fructose-1,6-bisphosphate (FBP) activates HPr kinase/phosphatase (HprK/P) that phosphorylates the HPr protein at conserved serine residue (Ser_{46}) at the expense of ATP. A seryl-phosphorylated form of HPr (HPr-Ser-P) interacts with several PTS and non-PTS sugar permeases, resulting in a reduced uptake of sugars [152, 154]. In addition, HPr-Ser-P interacts with CcpA, which induces binding of the CcpA-[HPr-Ser-P] complex to operator sequences in the promoter regions of the regulated genes [155, 156]. Next to HPr, an HPr-like protein, Crh (catabolite repression Hpr) can also be phosphorylated at the serine residue by HPrK/P at the expense of ATP, bind to CcpA and cause carbon catabolite control. Crh, however, lacks the conserved His_{15} residue, therefore it has no function in PTS transport [157, 158]. The CcpA activity is also modulated by low molecular weight molecules like fructose-1,6-bisphosphate, glucose-6-phosphate and NADP/NADH, which enhance CcpA affinity for HPr-Ser-P, trigger cooperative CcpA binding to DNA and enhance the CcpA interaction with the transcription machinery, respectively [155, 159–162].

CcpA is a member of the LacI/GalR transcriptional regulators [163]. In complex with HPr-Ser-P (CcpA-[HPr-Ser-P]) or Crh-Ser-P (CcpA-[Crh-Ser-P]), CcpA binds to DNA at operator sequences called *cre* (catabolite responsive elements). *Cre* boxes are pseudo-palindromic, highly degenerate sequences with the consensus WTGNNARCGNWWWCAW [164–166]. In general, if the *cre* box is localized upstream from the -35 box of the promoter, the downstream gene/operon is subject to CCA. Otherwise, CcpA binding to *cre* boxes either overlapping with the promoter or located downstream, results in CCR [149].

CcpA is a global regulator assumed to control expression of roughly 300 genes [149]. The CcpA regulon was defined in time, i.e., at different stages of growth in glucose-containing medium [167]. New potential CcpA target genes were identified recently and used to improve regulatory network topology [168]. In the presence of the favored carbon source, CcpA in *B. subtilis* exerts repression of many operons involved in the secondary carbon sources catabolism, e.g., *araABDLMNPQ-abfA*, *bglPH*, *galKT*, *glpFK*, *trePAR*, involved in utilization of arabinose, β-glucoside, galactose, glycerol and trehalose, respectively [165, 169–173]. Also *amyE* coding for the extracellular α-amylase hydrolyzing starch is subject to CcpA-mediated carbon catabolite repression

[163, 174]. Besides, various amino acids and nucleotides serve as carbon and nitrogen sources. The *drm-pupG* operon involved in metabolism of deoxyribonucleoside and the *hutPHUIGM* operon for histidine utilization are direct targets of CcpA [175–177]. CcpA inhibits the tricarboxylic acid (TCA) cycle by repressing expression of *citZ* encoding the first gene of the cycle, citrate synthase [178, 179]. This prevents the cells from producing an excess ATP, while they can derive enough ATP from glycolysis. Also transport of the TCA cycle intermediates is suppressed by CcpA-dependent repression of the *citM-yflN* operon involved in citrate transport as well as the *citST* operon coding for two-component regulatory system positively regulating *citM-yflN* [180, 181]. Transport systems for other carbohydrates like malate, fumarate or succinate are shut down by CcpA-mediated repression of the encoding gene, *dctP* [182]. CcpA directly represses expression of the *resABCDE* operon required for respiration [183]. CcpA mostly acts as a repressor and there are only few cases of CCA: *ackA* and *pta* encoding enzymes catalyzing the conversion of acetyl-CoA to acetate, *alsSD* involved in acetoin biosynthesis, and *ilv-leu* playing a role in the biosynthesis of branched-chain amino acids (BCAA) (isoleucine, leucine and valine) [184–187]. Enhancement of these processes prevents accumulation of pyruvate, which is produced to high levels when cells are grown in a rich medium supplemented with a rapidly metabolizable carbon source such as glucose.

Altogether, CCA and CCR in a CcpA-dependent manner result in a very efficient metabolism. Therefore, knowledge on the metabolome is also of importance in the process of optimization of conditions for *B. subtilis* cultivation in large fermentors for industry purposes.

Scope of this thesis

B. subtilis is an attractive organism used for commercial production of enzymes as well as for protein production for fundamental research. As described in this chapter (**Chapter 1**), several attempts have been made over the last years to improve this organism in protein production to gain higher production yields and proteins of a better quality. Yet, protein production may face limitations. The research described in this thesis was initiated to gain a better and more comprehensive view on the response of *B. subtilis* cells to secretion stress caused by overproduction of proteins (**Chapters 2, 3** and **4**) as well as on carbon catabolite control by CcpA (**Chapter 5**), which is an important aspect of the optimization of cells' cultivation in big fermentations.

In **chapter 2**, responses of *B. subtilis* to protein production stress caused by overproduction of proteins with different (subcellular) destination are studied extensively on the transcriptome level. This work revealed general stress responses activated by overproduction of many proteins as well as responses specific to overproduction of certain proteins. One such specific effect was strong upregulation of *ykrL* encoding a protein with high similarity to a membrane protein quality control protease, i.e., HtpX of *E. coli*. YkrL function and *ykrL* regulation was a further subject of **chapter 3**. It shows that YkrL is involved in responses to membrane stress and that its expression is regulated by the Rok repressor and, even stronger, by the so far unidentified regulator, YkrK.

The subject of **chapter 4** is the post-translocational molecular chaperone essential for the stability of secreted proteins, i.e., the PrsA lipoprotein anchored with its N-terminus to the membrane and with its C-terminus exposed to the extracytoplasmic site of the cell membrane. In this chapter, the PrsA localization pattern as well as cell wall synthesis defect in a *prsA* mutant are revealed, which contributed to a broader study (published elsewhere), where it was shown that several penicillin binding proteins (PBPs), which are membrane-bound proteins involved in cell wall synthesis, are folded in a PrsA-dependent manner. It also gives insight to the immunofluorescence technique as an alternative method for determination of the localization pattern of membrane proteins.

Chapter 5 focuses on carbon catabolite control by the global regulator CcpA. In particular, attempts were made to determine the hierarchy in which the CcpA target genes are regulated in the presence of a preferable carbon source for *B. subtilis*, glucose. This was achieved by detailed analysis of the sequence and location of the CcpA binding boxes, *cre* (catabolite responsive elements). Slight, but interesting differences between *cre* sites to which CcpA shows higher affinity and those to which CcpA seems to bind with lower affinity are revealed.

Chapter 6 provides a general discussion with focus on the most important results that will be useful for further improvement of *B. subtilis* as a protein production and/or secretion host.

CHAPTER 2

Comparative transcriptional responses of *Bacillus subtilis* cells overproducing either secreted proteins, lipoproteins or membrane proteins

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Bacillus subtilis **is a favorable host for the production of industrially relevant proteins because of its capacity of secreting proteins into the medium to high levels, its GRAS (Generally Recognized As Safe) status, its genetic accessibility and its capacity to grow in large fermentations. However, production of heterologous proteins still faces limitations.**

This study aimed at the identification of bottlenecks in secretory protein production by analyzing the response of *B. subtilis* at the transcriptome level to overproduction of eight secretory proteins of endogenous and heterologous origin and with different subcellular or extracellular destination: secreted proteins (NprE and XynA of *B. subtilis*, Usp45 of *Lactococcus lactis*, TEM-1 β-lactamase of *Escherichia coli*), membrane proteins (LmrA of *L. lactis* and XylP of *Lactobacillus pentosus*) and lipoproteins (MntA and YcdH of *B. subtilis*). Responses specific for proteins with a common localization as well as more general stress responses were observed. The latter include upregulation of genes encoding intracellular stress proteins (*groES/EL*, CtsR regulated genes). Specific responses include upregulation of the *liaIHGFSR* operon under Usp45 and TEM-1 β-lactamase overproduction; *cssRS*, *htrA* and *htrB* under all secreted proteins overproduction; *sigW* and SigW-regulated genes mainly under membrane proteins overproduction; and *ykrL* (encoding an HtpX homologue) specifically under membrane proteins overproduction.

The results give better insight to *B. subtilis* response to protein overproduction stress and provide potential targets for genetic engineering in order to further improve *B. subtilis* as a protein production host.

Introduction

The Gram-positive bacterium *Bacillus subtilis* is widely used in large scale production of endogenous and heterologous proteins used in food- and other industries. It is particularly favored as a production host since it has the capacity of secreting proteins to high levels into the medium enabling easy isolation and purification, it can be grown in large fermentations and is considered as a GRAS (Generally Recognized As Safe) organism by the US Food and Drug Administration. In addition, *B. subtilis* is still the most studied Gram-positive organism in fundamental research and is therefore a good model organism in the search for bottlenecks in protein overproduction. There are several cellular mechanisms that can hamper secretion of heterologous proteins on particular stages of the *B. subtilis* secretion pathway. At early stages of protein secretion, like synthesis of secretory pre-proteins, pre-protein interactions with cellular chaperones and binding to the translocase, the limitations may potentially result from, e.g., low transcription levels, inefficient translation, presence of intracellular proteases, deficiency in chaperones, poor targeting to the translocase, *etc*. [125]. The second stage of the protein secretion, i.e., translocation across the membrane via the Sec- or Tat- [27] translocase, may be confined by secretion machinery jamming [125]. At the late stages, which include removal of the signal peptide, release from the translocase, folding and passing the cell wall, deficiency in signal peptidases, foldases, chaperones and presence of extracellular proteases resulting in incorrect folding of proteins and protein's instability may also set limits to the secretion efficiency [29, 125]. The focus on identification and later manipulation of factors involved in protein secretion have led to the improvement of *B. subtilis* as a production host, for example by deletion of extracellular and/or intracellular proteases [126, 128, 133], use of strong or inducible promoters [135–137], overproduction of chaperones [127, 132] or signal peptidases [138, 188], modification of the cell wall [139, 140], protein modification [141, 142] and deletion of stress responsive systems [65].

Next to overproduction of proteins secreted into the medium, the overproduction of membrane proteins in *B. subtilis* is of a particular interest [189]. Membrane proteins are potential drug targets as they are exposed to and accessible from the extracytoplasmic environment, and therefore interesting for the pharmaceutical industry. Rational drug design, however, requires a three-dimensional structure, usually obtained from protein crystals, which can only be obtained when sufficient amounts of membrane protein of high quality are available [189].

In this work, a comparative transcriptomics approach was followed to study cellular responses to secretory proteins overproduction at the transcriptional level, in order to reveal so far unidentified possible production bottlenecks and thus potential targets for productive host engineering. Endogenous and heterologous proteins with different subcellular localization, i.e., secreted proteins, membrane proteins and lipoproteins were overproduced in *B. subtilis.* At least two proteins of each localization class were chosen in order to be able to discriminate between effects specific for one protein and effects common to one localization type. Transcriptomes were analyzed using DNA microarrays and subsequent use of appropriate bioinformatics tools. General responses as well as responses specific to proteins with a particular localization were identified.

Results and discussion

Transcriptome analysis of lipoproteins, membrane proteins or secreted proteins overproduction stress

B. subtilis remains a powerful host for the (industrial) production of secreted or membrane proteins but expression of heterologous proteins in particular has met limitations. These may occur at different levels of the production and secretion pathway. Here, the response of *B. subtilis* on the transcriptional level to overproduction of secretory proteins of endogenous or heterologous origin and with different subcellular localization, i.e., membrane proteins, lipoproteins and secreted proteins, was determined by transcriptome analysis.

Table 1. Proteins overproduced in *B. subtilis* NZ8900 host using SURE system.

Protein	Function	Organism	Subcellular localization
XylP	Xyloside transporter	*Lb. pentosus*	Membrane
LmrA (inactive mutant)	ABC-transporter	*L. lactis*	Membrane
MntA	Manganese binding	*B. subtilis*	Lipoprotein
YcdH	Zinc binding	*B. subtilis*	Lipoprotein
XynA	Xylanase	*B. subtilis*	Secreted
NprE	Neutral protease	*B. subtilis*	Secreted
Usp45	Unknown	*L. lactis*	Secreted
TEM1 β-lactamase	β-lactamase	*E. coli*	Secreted

Eight genes encoding heterologous and endogenous proteins (Table 1) with different subcellular localization were cloned using the SURE system overexpression vector pNZ8902 or pNZ8901 [135]: *lmrA* of *Lactococcus lactis*, encoding the membrane embedded putative multidrug transporter LmrA [190]; *xylP* of *Lactobacillus pentosus*

encoding a membrane embedded xyloside transporter XylP [191], *mntA* and *ycdH* of *B. subtilis* encoding the manganese binding lipoprotein MntA [192] and the putative zinc binding lipoprotein YcdH [193], respectively; *bla* of *Escherichia coli* encoding the periplasm located TEM-1 β-lactamase (Bla) [194]; *usp45* of *L. lactis*, encoding the cell wall-associated Usp45 [195]; and *nprE* and *xynA* of *B. subtilis*, encoding the secreted neutral protease NprE [196] and the secreted xylanase XynA [197], respectively. The genes were fused to C-terminal 6His-tag encoding sequences. *B. subtilis* NZ8900 harboring these constructs or the empty vector pNZ8902 or pNZ8901 were grown to mid-exponential phase and expression was induced with subtilin. Samples were taken 30 minutes after induction for microarray analyses and after two hours for testing protein production. SDS-PAGE analysis of whole-cell, membrane, cytoplasm and medium fractions together with His-tag immunodetection demonstrated that XylP, LmrA, MntA, YcdH, TEM-1 β-lactamase and Usp45 were overproduced to levels varying from high for LmrA, YcdH and Usp45 to hardly visible on a Coomassie stained gel but well detectable using immunodetection (XylP) (Fig. 1). Distinct localization patterns were observed for each class of protein (Fig. 1). XynA and NprE were efficiently produced and secreted into the medium (Fig. 1 B), whereas Usp45 and TEM-1 β-lactamase were detected mainly in whole cell fractions (Fig. 1A, left panel). Since the latter two were not or hardly detectable in the cytoplasmic and membrane fractions (Fig. 1C and D), it is likely that they accumulated in the cell wall or membrane/cell wall interface. In accordance, TEM-1 β-lactamase expressed in *B. subtilis* was previously shown to accumulate in the membrane/cell wall interface due to inefficient passage through the cell wall [198]. Usp45 shows homology with proteins involved in cell wall metabolism, e.g., peptidoglycan hydrolases of *Streptococcus mutans*, *Streptococcus oralis*, *Lactococcus lactis subsp. lactis* [199–201], which may explain localization in or at the cell wall. Overexpression of *usp45* did not inhibit growth, whereas overexpression of *bla* resulted in growth inhibition as well as cell lysis, possibly due to interference with cell wall metabolism.

LmrA and XylP were exclusively found in the membrane fraction (Fig. 1C). Similarly, the lipoproteins MntA and YcdH were present mainly in the membrane fraction (Fig. 1C), but immunodetection also indicated their presence at a low level in the medium (Fig. 1B, right panel) and cytoplasmic fraction (Fig. 1D, right panel). Immunodetection using His-tag antibodies proved to be of limited use in comparing levels of the different proteins, since they were detected with very different efficiencies (compare Fig. 1B, left and right panel). Especially TEM1 β-lactamase and Usp45 were hardly detectable in general.

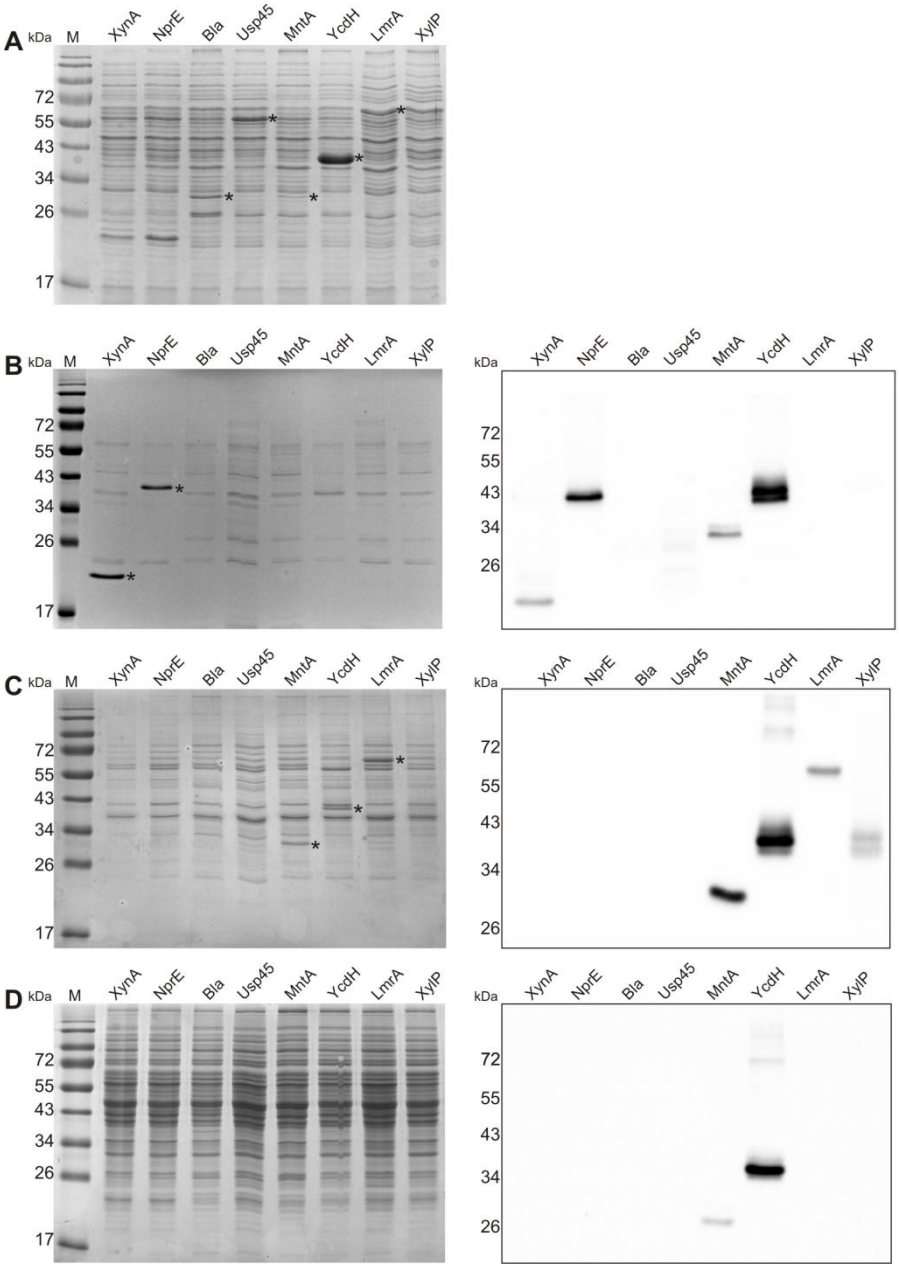

Figure 1. Overproduction of different secretory proteins in *B. subtilis*. (**A**) Whole cell extracts, (**B**) medium fractions, (**C**) membrane fractions, (**D**) cytoplasmic fractions. Left panels show SDS-PAGE gels; right panels (B, C and D) show immunodetection of the 6his-tagged proteins using Penta-His HRP conjugate antibodies (Qiagen). Asterisks indicate protein bands corresponding to the overproduced proteins, assigned on basis of calculated molecular mass and/or immunodetection. Calculated molecular masses of proteins, with and without signal peptide, in kDa: XynA, 32.3 (preprotein), 20.4 (matured); NprE, 56.5 (preprotein), 53.9 (matured); Bla (TEM-1 β-lactamase), 32.3 (preprotein), 29.7 (matured); Usp45, 48.2 (preprotein), 45.5 (matured); MntA, 33.4 (preprotein), 32.4 (matured); YcdH, 36.5 (preprotein), 34.3 (matured); LmrA, 66.2; XylP, 55.3.

The mRNA levels of each overproducing strain were compared with those of the control strain using DNA microarrays. Fold-changes in the expression level of genes that were at least 2.5 times up- or downregulated in response to overproduction of both proteins of the same subcellular localization, or to overproduction of at least 4 proteins with other destinations, are summarized in Table 2. The complete microarray data is available at GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34505) under accession number GSE34505.

General effects

Overproduction of all secreted proteins, except NprE, caused upregulation of class I heat-shock genes coding for molecular chaperons, *groES* and *groEL* (Table 2). Overproduction of the same proteins, except for XynA and MntA, resulted in activation of class III heat-shock genes, which code for components of protease complexes (ClpXP, ClpEP, *etc*.), and other genes regulated by CtsR, a stress and heat-shock response regulator [107, 114] (Table 2). This intracellular stress response may be caused by a high protein production rate in combination with a limited capacity in protein secretion or membrane insertion, and/or, in case of the heterologous proteins, a lower compatibility of the secretion signal with the host secretion machinery. However, accumulation of the proteins was not observed (Figure 1D). This suggests that, although the proteins were apparently secreted with good efficiency, their presence at lower levels were enough to induce the general cytoplasmic stress response. Increased expression of chaperones like GroES/EL and Clp proteases can protect the cell from toxic accumulation of mis- or unfolded protein [117, 202]. However, high expression and activity of proteases may also set a limit for production of heterologous proteins in *B. subtilis* on large scale.

The *nfrA-ywcH* operon, encoding a nitro/flavin reductase and a monooxygenase, respectively [123], was upregulated in 5 of the 8 cases (Table 2). NfrA is believed to be involved in a response to stress-induced protein damage and its corresponding gene is induced upon a wide range of stresses [203]. Therefore the coproduction of NfrA can be considered in the improvement of protein overproduction.

Another observed effect in case of most overproduced proteins was strong induction of the *yhaSTU* operon. It codes for a K^+ efflux system and has been shown to be induced by alkaline pH, which has been suggested to be a secondary effect of compromised membrane function and bioenergetic integrity of the cell [204, 205], and salt stress [206].

Table 2. Genes with significantly altered expression as a result of endogenous and heterologous proteins overproduction in *B. subtilis* cells [a,b]

Gene	Description	Overproduced protein							
		Membrane proteins		Lipoproteins		Secreted proteins			
		XylP	LmrA	MntA	YcdH	XynA	NprE	Usp45	Bla
UPREGULATED									
Cell envelope stress response									
htrB	similar to HtrA-like serine protease	0.67	1.03	1.70	**7.37**	**10.86**	1.12	0.44	**7.52**
cssR	two-component response regulator	0.96	1.06	**6.82**	1.38	**5.97**	1.08	**2.90**	1.85
cssS	two-component sensor histidine kinase	0.95	1.11	**3.10**	1.54	**2.67**	0.99	2.37	1.66
htrA	Quality control serine protease (heat-shock protein)	0.76	1.17	**3.17**	**5.18**	**14.88**	0.78	**46.70**	**7.23**
yxlC	sigma-Y antisigma factor	**6.61**	1.73	1.64	0.96	1.49	0.90	**4.03**	1.07
yxlD	putative sigma-Y antisigma factor component	**5.49**	1.98	1.56	1.03	1.09	0.97	**5.34**	1.13
yxlE	negative regulator of sigma-Y activity	**8.75**	1.18	1.57	1.03	1.35	1.20	**4.07**	1.09
yxlF	putative ABC transporter component	**5.77**	1.50	1.88	0.89	1.02	0.94	**6.67**	0.95
yxlG	putative ABC-transporter (permease)	**3.76**	1.79	1.67	0.99	1.52	0.99	1.91	1.01
sigM	RNA polymerase ECF-type sigma factor	1.17	0.94	**3.01**	**2.51**	0.62	1.49	1.67	1.21
liaI	permease	2.38	1.07	1.31	**2.55**	1.59	2.03	**8.96**	**23.44**
liaH	modulator of liaIHGFSR operon expression	2.32	1.08	1.19	**3.40**	1.47	**2.51**	**9.43**	**29.75**
liaG	hypothetical protein	1.48	1.06	2.27	**2.67**	0.82	**2.65**	**4.55**	**13.38**
liaF	integral inner membrane protein	0.89	1.07	2.14	1.94	0.92	**2.56**	**4.16**	**10.93**
liaS	two-component sensor histidine kinase	1.14	NA	2.42	1.93	0.89	NA	**5.88**	**6.61**
liaR	two-component response regulator	0.94	1.04	1.91	**2.57**	0.79	1.96	2.19	**4.76**
ykrL	Homolog of HtpX, membrane protease	**10.35**	**3.62**	1.66	0.88	2.15	0.90	**2.98**	1.85
Cell envelope stress response/SigW regulon									
sigW	RNA polymerase ECF-type sigma factor	**4.52**	**5.50**	1.95	1.77	1.62	1.05	1.73	2.44
rsiW	sigmaW anti-sigma factor	**5.54**	**4.11**	2.43	1.72	2.31	0.84	1.89	**2.61**
yuaF	putative integral inner membrane protein	**6.93**	**2.65**	1.09	0.23	1.41	1.31	2.15	2.36
yuaG	similar to flotillin 1	**12.21**	**3.43**	0.66	0.75	1.87	1.00	**4.52**	2.40
yuaI	putative acetyl-transferase	**15.16**	**6.62**	0.40	0.81	**2.80**	0.78	**5.01**	**2.70**
pspA	phage shock protein A homolog	**5.53**	2.38	0.55	0.26	1.14	1.79	1.42	1.50
ydjG	putative phage replication protein	**7.70**	**2.85**	0.59	1.85	1.93	1.01	**3.09**	1.46
ydjH	hypothetical protein	**6.88**	**3.92**	0.60	0.48	1.68	1.09	2.19	1.86
ydjI	hypothetical protein	**6.78**	**4.55**	0.59	0.75	1.86	0.57	**2.84**	2.40
yeaA	conserved hypothetical protein	**5.58**	**3.28**	0.93	0.42	1.41	2.36	1.35	2.10
ydjP	similar to chloroperoxydase	**7.41**	**4.67**	1.16	0.91	**2.55**	1.53	**4.56**	**3.00**
ydjO	unknown	**3.81**	**7.59**	1.14	1.08	1.99	1.46	**2.97**	**2.57**
sppA	signal peptide peptidase	**4.25**	**3.12**	0.33	0.26	1.18	0.39	1.70	1.80
yteJ	integral inner membrane protein	**4.48**	**4.16**	0.31	0.29	1.27	0.34	2.27	1.77
pbpE	penicillin-binding protein 4	**11.25**	**8.56**	0.95	0.97	2.08	0.49	**6.37**	**2.72**
racX	amino acid racemase	**8.99**	**8.09**	0.78	0.95	2.06	0.45	**6.00**	2.39
yaaN	hypothetical protein	**4.03**	**6.82**	1.07	0.43	1.15	**2.64**	1.51	1.82
yceC	putative stress adaptation protein	2.44	**3.05**	2.06	NA	NA	0.54	NA	1.64
yceD	putative stress adaptation protein	**5.29**	**7.02**	0.81	0.76	2.04	0.92	**3.14**	1.55
yceE	putative stress adaptation protein	**4.55**	**5.36**	0.82	0.67	2.08	0.89	**3.71**	2.34
yceF	putative stress adaptation protein	**5.30**	**4.91**	0.70	0.78	2.07	0.73	**4.27**	1.79
yceG	hypothetical protein	**3.99**	**3.02**	0.79	1.07	1.94	1.04	**3.29**	1.22
yceH	hypothetical protein	**3.72**	**4.09**	0.64	0.99	1.99	0.60	**3.68**	2.15
yjoB	ATPase possibly involved in protein degradation	**7.16**	**2.98**	1.28	0.65	1.45	0.94	1.74	2.44
yknW	permease	**2.51**	**3.06**	0.67	0.36	1.12	0.84	1.14	1.46
yknY	ABC transporter ATP-binding protein	**2.63**	**2.54**	0.49	0.33	1.31	0.57	1.51	1.64
ythQ	putative ABC transporter (permease)	**10.01**	**5.75**	0.80	NA	2.21	NA	**3.21**	**3.19**
yqfB	hypothetical protein	**6.37**	**6.24**	1.12	0.56	1.66	0.39	**3.10**	**2.93**
yvlA	hypothetical protein	**4.11**	**3.06**	1.49	0.59	1.23	1.34	1.39	2.04
yvlB	hypothetical protein	**6.13**	**9.56**	0.99	0.66	1.76	0.91	**3.84**	2.10
yvlC	putative regulator (stress mediated)	**3.83**	**7.71**	0.99	0.61	1.61	0.76	2.24	2.05
yvlD	putative integral inner membrane protein	**4.26**	**6.66**	1.60	0.65	1.22	0.74	**2.98**	1.87
yxjI	unknown	**10.12**	**5.20**	0.96	0.55	1.60	1.46	1.38	1.83
Intracellular stress response									
hrcA	transcriptional repressor of class I stress genes	**11.08**	2.23	1.89	**12.62**	0.96	1.30	2.33	2.12
groES	class I heat-shock protein (chaperonin)	**8.37**	**7.17**	0.10	**6.85**	**3.15**	1.11	**3.68**	**3.07**
groEL	class I heat-shock protein (chaperonin)	**8.38**	**4.17**	0.35	**5.07**	**2.73**	1.14	**3.00**	**2.66**
nfrA	NADPH-linked nitro/flavin reductase	**7.36**	**3.32**	0.47	1.67	**10.37**	0.98	**13.77**	1.85

Table 2. Continued.

Gene	Description	Overproduced protein							
		Membrane proteins		Lipoproteins		Secreted proteins			
		XylP	LmrA	MntA	YcdH	XynA	NprE	Usp45	Bla
ywcH	putative monooxygenase	**10.29**	**2.91**	1.04	**6.03**	**5.29**	0.86	NA	2.46
trxB	thioredoxin reductase	**4.71**	1.59	0.88	**2.98**	**3.97**	0.72	**3.56**	1.36
trxA	thioredoxin	2.29	1.94	0.86	1.68	**3.08**	1.13	**3.11**	1.31
ctsR	transcriptional regulator of class III stress genes	0.71	0.55	1.39	NA	NA	NA	NA	NA
mcsA	modulator of CtsR repression	**10.38**	**3.47**	0.87	**5.12**	1.72	1.57	**4.63**	**3.11**
mcsB	modulator of CtsR repression	**6.92**	**4.11**	0.83	**5.09**	2.46	1.09	**2.68**	**2.63**
clpC	class III stress response-related ATPase	**5.65**	**3.24**	0.65	**4.63**	2.03	0.80	**3.28**	**2.80**
radA	DNA repair protein	**2.79**	1.97	0.75	**3.86**	1.82	1.26	**3.14**	2.19
clpE	Class III, ATP-dependent Clp protease-like	**74.08**	1.89	0.98	**4.54**	0.77	1.24	**5.95**	**4.79**
Membrane bioenergetics									
fdhD	required for formate dehydrogenase activity	**2.89**	1.26	**2.99**	**4.65**	1.93	1.65	1.67	0.94
cydB	cytochrome bd ubiquinol oxidase (subunit II)	0.75	2.28	1.39	0.51	**2.93**	**4.50**	0.74	0.64
Miscellaneous									
kinD	TCS sensor histidine kinase; initiation of sporulation	1.35	1.20	**3.18**	**2.76**	1.49	1.81	1.05	1.05
yabT	putative serine/threonine-protein kinase	2.19	1.14	NA	1.56	**2.55**	**2.65**	1.26	1.04
yceK	putative transcriptional regulator (ArsR family)	**2.58**	**2.54**	0.81	1.35	1.07	1.06	2.10	1.92
yjbI	putative thiol management oxidoreductase component	**3.93**	0.99	1.45	**2.95**	**3.23**	1.33	**3.61**	1.46
yloC	unknown	1.65	1.09	**3.56**	**3.51**	1.47	1.36	1.57	1.05
yndN	fosfomycin resistance protein FosB	**6.66**	**13.17**	0.94	0.32	1.60	0.56	**3.49**	**3.04**
yrkA	putative membrane associated protein	**3.20**	**3.75**	1.51	0.96	1.48	0.97	1.13	1.30
yvdT	uncharacterized transcriptional regulator	**2.94**	**3.28**	1.82	2.19	1.37	1.80	2.26	1.92
yvdS	similar to molecular chaperone	**7.12**	2.12	1.20	1.14	1.08	1.26	**3.43**	1.96
yvdR	similar to molecular chaperone	**6.32**	**2.66**	2.46	1.39	1.01	1.36	**3.99**	1.81
Transport/binding proteins									
cydC	ABC membrane transporter ATP-binding protein	**3.22**	**3.76**	0.87	0.32	**2.92**	2.36	2.25	1.12
yceI	putative transporter	**2.92**	**3.55**	1.49	0.80	1.75	0.69	**2.89**	2.26
yhaS	K+/H+ antiporter for K+ efflux	**9.48**	2.13	2.08	1.76	2.42	0.96	**3.41**	1.94
yhaT	K+/H+ antiporter for K+ efflux	**15.45**	**3.48**	2.16	**2.91**	2.47	1.22	**4.80**	**2.54**
yhaU	K+/H+ antiporter for K+ efflux	**15.49**	2.19	2.38	1.84	**2.56**	1.00	**5.14**	1.97
Unknown									
ydiN	unknown	**4.44**	**2.76**	0.22	**5.11**	1.75	1.75	2.41	**3.51**
yomP	hypothetical protein	**3.57**	**3.21**	0.92	**3.33**	0.89	**2.60**	1.40	0.82
yomT	hypothetical protein	**2.92**	**2.60**	0.83	**2.90**	0.72	2.47	1.24	0.85
yomV	hypothetical protein	**3.59**	**3.37**	1.05	**3.92**	0.73	**2.55**	1.43	0.88
yomW	hypothetical protein	**3.19**	**3.38**	0.97	**4.57**	0.65	**2.79**	1.31	0.78
yomY	hypothetical protein	**3.66**	**2.53**	1.09	**3.64**	0.77	**2.60**	1.66	0.82
yonB	hypothetical protein	**3.22**	**2.92**	0.96	**2.61**	1.01	2.26	1.24	0.79
yonC	hypothetical protein	**2.92**	**2.64**	0.83	**2.84**	0.96	2.41	1.43	0.84
yvkN	hypothetical protein	**3.21**	**2.69**	0.89	0.61	1.83	1.07	1.88	1.69
ywmB	hypothetical protein	1.32	1.18	**3.00**	**2.67**	1.30	2.43	0.55	1.16
DOWNREGULATED									
Starvation response									
sdpA	export of killing factor SdpC	**15.28**	**2.78**	**4.29**	**3.96**	**3.54**	1.54	**14.19**	**4.35**
sdpB	exporter of killing factor SdpC	**19.87**	**4.06**	**3.26**	**5.34**	**3.96**	1.49	**13.85**	**4.00**
sdpC	killing factor SdpC	**8.12**	**2.75**	2.45	**12.90**	**11.51**	1.21	**26.62**	**5.56**
Protein folding and modification									
bdbA	bacteriophage SPbeta thiol-disulfide oxidoreductase	**3.14**	1.56	**7.83**	1.37	2.42	1.36	**15.73**	2.38
bdbB	bacteriophage SPbeta thiol-disulfide oxidoreductase	**3.59**	**2.79**	**6.00**	**2.57**	**3.05**	1.16	**14.00**	2.00
Cell envelope stress/SigW regulon									
sppA	signal peptide peptidase	0.24	0.32	**3.03**	**3.89**	0.85	**2.56**	0.59	0.56
yteJ	putative integral inner membrane protein	0.22	0.24	**3.18**	**3.51**	0.79	**2.93**	0.44	0.56
Transport/binding proteins and lipoproteins									
gltT	proton/sodium-glutamate symport protein	**3.36**	**3.12**	**2.52**	1.63	0.82	1.96	**8.35**	1.20
pbuX	xanthine permease	**3.32**	**2.74**	0.45	1.76	1.47	1.50	**4.45**	1.61
yhaQ	Na+-efflux ABC transporter, ATP-binding protein	2.07	1.55	**3.73**	**4.51**	2.19	2.10	1.65	1.32
yoaG	putative permease	0.13	0.67	**3.13**	**3.68**	1.20	1.15	0.66	0.53

Table 2. Continued.

Gene	Description	Overproduced protein							
		Membrane proteins		Lipoproteins		Secreted proteins			
		XylP	LmrA	MntA	YcdH	XynA	NprE	Usp45	Bla
Membrane bioenergetics									
ctaB	cytochrome caa3 oxydase assembly factor	1.18	1.63	0.66	1.16	1.34	1.15	**3.48**	1.17
ctaC	cytochrome caa3 oxidase subunit II	**4.37**	1.13	**4.74**	**2.68**	1.08	1.91	**5.52**	1.20
ctaD	cytochrome caa3 oxidase subunit I	**3.71**	1.37	**3.58**	**3.14**	NA	1.81	**3.33**	1.27
ctaE	cytochrome caa3 oxidase subunit III	**3.57**	1.44	2.40	**3.02**	1.90	2.08	**4.21**	1.10
ctaF	cytochrome caa3 oxidase subunit IV	**4.33**	1.91	2.05	**3.86**	**2.68**	2.32	**4.24**	1.41
ctaG	cytochrome aa3 assembly factor	**4.61**	NA	2.40	1.83	NA	1.19	**2.67**	NA
Miscellaneous									
wapA	cell wall-associated protein precursor	**2.88**	1.07	1.15	**8.19**	**2.50**	0.91	**6.38**	1.15
yxxG	hypothetical protein	**4.04**	0.99	1.22	**8.68**	**4.20**	0.87	**5.25**	1.08
wprA	cell wall-associated protease	2.45	1.05	**4.87**	**4.19**	2.27	1.85	**9.13**	1.27
Unknown									
yisL	hypothetical protein	2.00	1.13	**2.58**	**4.59**	1.41	1.96	0.73	1.11
yokE	hypothetical protein	1.56	1.76	**7.19**	**2.84**	**2.74**	1.50	**4.35**	1.28
ytxG	hypothetical protein	1.80	0.76	**2.98**	**2.56**	0.83	1.00	0.48	0.93
yukE	hypothetical protein	1.37	1.56	**3.25**	**2.67**	0.92	2.04	1.60	1.02
yxbC	hypothetical protein	**3.55**	0.69	**3.22**	**5.71**	0.72	1.30	2.06	1.11

[a] Significant changes (p-value < 0.01, fold > 2.5) are shown in bold.
[b] Endogenous proteins: MntA, YcdH, XynA, NprE; heterologous proteins: XylP (*Lb. pentosus*), LmrA and Usp45 (*L. lactis*), Bla (TEM-1 β-lactamase, *E. coli*).

The genes *trxA* and *trxB* were upregulated in the majority of the cases, without a bias towards a particular localization of the overproduced protein. *trxA* and *trxB* are members of Spx regulon involved in thiol-specific oxidative stress and they code for thioredoxin and thioredoxin reductase, respectively [207]. These genes are thought to be required for keeping proteins in a reduced state which, once secreted, form disulfide bonds during folding [207]. However, there was no correlation between presence of (putative) disulfide bonds in an overproduced protein and induction of *trxA* or *trxB* (only TEM-1 β-lactamase, YcdH and XylP possess putative disulfide bonds, out of which overproduction of only YcdH resulted in *trxB* induction). Therefore, upregulation of *trxA* and *trxB* is most likely induced by thiol stress as a result of secondary effects of overproduction of secretory proteins, such as a compromised membrane function.

An effect that was observed in case of all overexpressed proteins was strong downregulation of the *sdpABC* operon (sporulating delay protein operon) involved in production and secretion of the killing factor SdpC (Table 2). It plays a role in programmed cell death (PCD), a mechanism of sporulation delay by killing nonsporulating siblings and feeding on the dead cells under conditions of nutrient limitation [208, 209]. This effect may be related to nutrient limitation which was shown to induce the sporulation process in a subpopulation of a *B. subtilis* culture with concomitant activation of the *sdpABC* and *sdpRI* immunity operons [209].

Another general effect, but less pronounced than for *sdpABC*, was downregulation of the *ctaCDEF* genes coding for cytochrome *c* oxidase *caa3* [210].

Overproduction of none of the proteins caused upregulation of genes coding for components of the secretion (Sec) machinery, like *secA*, *secDF*, *ffh, etc*., which are responsible for translocation of unfolded pre-proteins across or insertion into the membrane (for review see [27]). Apparently, increasing its protein secretion capacity is not a strategy of the cell to deal with an accumulation of secretory proteins. This may indicate either that the SecYEG channel does not form a bottleneck in secretion in the experiments performed here, or that expression of the genes encoding the SecYEG components is simply not upregulated by (the consequences of) an artificially imposed overproduction of secretory proteins. The latter suggests that SecYEG should not necessarily be excluded as a potential target for production strain improvement. In agreement, overexpression of *prsA*, encoding the extracellular foldase PrsA, was shown to increase the secretion of an α-amylase fourfold [132], while *prsA* was not upregulated in any of the tested cases here. This however does not detract from the value of the data as a source of new potential targets for strain improvement. For some of these genes, induced by overexpression of many of the tested secretory proteins, it was indeed shown previously that either their deletion or overexpression improved specific protein production yields, e.g., *sigW* and *cssRS* [65] and genes encoding intracellular chaperones [128].

Proteins with extracytosolic destination induce the CssRS mediated secretion stress response

Overproduction of the secreted protein XynA of *B. subtilis*, the cell wall-associated proteins Usp45 of *L. lactis* and TEM-1 β-lactamase of *E. coli*, as well as lipoproteins MntA and YcdH of *B. subtilis* resulted in significant upregulation of the secretion stress genes: *htrA*, *htrB* and *cssRS* (Table 2). CssR and CssS encode a response regulator and its cognate, membrane embedded sensor, respectively, and control the expression of *htrA* and *htrB* [43, 47]. These encode membrane-anchored HtrA and HtrB proteins, which have their active site on the *trans* side of the membrane and are thought to have proteolytic as well as chaperone activity for removal of misfolded protein or for assisting in folding of newly secreted proteins, respectively [48]. The CssRS two component system is activated by accumulation of mis- or unfolded secreted protein at the membrane/cell wall interface as a result of, e.g., overexpression of these proteins or heat stress [41, 46]. In this study, overproduction of the membrane proteins LmrA and XylP did not significantly induce *htrA* or *htrB*. This is in agreement with previous results from an analysis of the activation of the *htrA* promoter in response to

overproduction of secretory proteins, including MntA, XynA, TEM-1 β-lactamase, Usp45 and LmrA, showing that the stress signal is sensed on the outside of the cell and not from within the membrane [46]. In agreement, it was shown recently that the CssS extracellular loop domain functions in signal perception and/or transduction [45]. Surprisingly, NprE overproduction did not induce the CssRS response. Possibly, NprE can be produced and secreted to high levels without accumulation of misfolded protein.

Usp45 and TEM-1 β-lactamase specifically induce the LiaRS-dependent response

The two proteins which were detected mainly in the whole cell fractions, but not in the membrane and cytoplasmic fractions, Usp45 and TEM1-β-lactamase (Fig. 1), specifically induced the *liaIHGFSR* (*yvqIHGFEC*) operon (Table 2), a cell envelope stress operon which is under control of the LiaRS (YvqCE) two-component system [10, 67, 78, 211]. The fact that LiaRS is strongly induced by cell wall-active antibiotics [11], suggests that Usp45 and TEM1-β-lactamase had accumulated in or at the cell wall, as noted earlier, and thereby interfered with cell wall metabolism. Since the other secretory proteins did not, or to a much lesser extent, induce LiaRS (Table 2), it appears that the signal which is sensed by the sensor LiaS originates from cell wall metabolism related processes, rather than for example cell membrane integrity.

Membrane protein overproduction induces a SigW response and *ykrL* expression

The overproduction of the membrane proteins LmrA and XylP and, to a lesser extent, the cell wall-associated proteins Usp45 and TEM-1 β-lactamase caused significant upregulation of *sigW* and many genes belonging to the SigW regulon (Table 2). The SigW regulon has been shown to be induced by a variety of cell envelope stresses like treatment with detergents (Triton X-100), antibiotics (vancomycin, penicillin) [78], alkaline stress [72] or membrane protein overproduction [65]. Activation of SigW depends on proteolytic degradation of the anti-SigmaW factor RsiW by a multipass membrane protease, PrsW and, subsequently, other proteases [81, 82], but the exact signal triggering this cascade is not known. The induction by membrane protein overexpression suggests that the stress signal is sensed from within the membrane.

Next to SigW response, an unknown gene, *ykrL,* was significantly upregulated under LmrA and XylP overproduction (Table 2). YkrL shows high homology to the *E. coli* HtpX, a membrane embedded metalloprotease which has been implied in membrane protein quality control [212]. The upregulation of *ykrL* suggests a similar role in *B. subtilis*. It would be of interest to test the effect of different levels of YkrL on the level and quality of overproduced membrane protein. Expression of *htpX* in *E. coli* is regulated by the CpxRA two component system that regulates a number of genes

involved in cell envelope stress, including *degP* (or *htrA*), encoding a close homologue of *B. subtilis* HtrA and HtrB [213]. Here, no correlation between expression of the CssRS targets and *ykrL* was observed, suggesting that *ykrL* expression does not depend on CssRS and is regulated differently from *htpX* in *E. coli*.

In *E. coli*, the membrane located ATP-dependent metalloprotease FtsH is involved in the membrane protein stress response [60]. A similar role of *B. subtilis* FtsH, sharing 47% identity with *E. coli* FtsH, was suggested before [189]. However, *ftsH* was not significantly upregulated in response to overproduction of membrane proteins or to any of the other secretory proteins. Previous results revealing the sporulation control proteins SpoVM and Spo0E as substrates of FtsH [61, 62] may therefore be examples of a more specific role of FtsH in *B. subtilis*, rather than a general protein quality control system.

An operon of unknown function, *yvdTSR*, encoding a putative transcriptional regulator and two membrane proteins with homology to small multidrug resistance (SMR) proteins, was also specifically upregulated, but its role in membrane stress is unclear.

Like in case of the other secretory proteins, overproduction of LmrA and XylP led to induction of the class I heat shock protein genes *groES*, *groEL* and class III heat shock protein genes, e.g., *clpE*, *clpC*, which suggests that some fraction of overproduced membrane proteins is targeted by chaperones or proteases for degradation in the cytoplasm before translocation through the Sec machinery and insertion into the membrane. Alternatively, a protein that is incorrectly inserted into the membrane may be subject to Clp-mediated proteolysis, although it is not known whether membrane embedded proteins are accessible to Clp complexes.

Other extracytoplasmic function (ECF) sigma factors

Next to the SigW response, induced by overproduction of the LmrA, XylP, Usp45 and TEM1 β-lactamase, upregulation of SigM and SigY RNA polymerase ECF (extracytoplasmic function)-type sigma factors, was observed in some cases (Table 2). SigM has been shown to be involved in a response to salt, low pH, ethanol, heat and oxidative stress and cell wall synthesis inhibiting antibiotics [85, 214]. In this study, *sigM* was upregulated under conditions of overproduction of the lipoproteins MntA and YcdH. However, known SigM targets [215] were not upregulated. Expression of SigY and some of the SigY target genes [89] was induced upon XylP and Usp45 overproduction.

Conclusions

This comparative study revealed differential responses of *B. subtilis* to stress caused by overproduction of secretory proteins with different subcellular localization. New insights in (specificity of) stress responses, in particular at the membrane and cell wall level were obtained. The data reveal possible bottlenecks in the protein production process, which can be targeted in the future development of the improved production strains.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3. *L. lactis* NZ9000 [216] was used as intermediate cloning hosts for pNZ8901 and pNZ8902 based vectors. *B. subtilis* strains were grown in TY medium [217] at 37° C with vigorous shaking. TY medium was supplemented with kanamycin (5 μg/ml), erythromycin (0.5 μg/ml) or chloramphenicol (5 μg/ml) when needed. *L. lactis* strains were transformed by electroporation as described before [218] using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, California). *B. subtilis* strains were transformed as described before [219].

Table 3. List of strains and plasmids used in this study.

Strain/plasmid	Description	Reference
L. lactis		
NZ9000	MG1363 derivative, *pepN*::*nisRK*	[216]
B. subtilis		
NZ8900	168, *amyE*::*spaRK*, KanR, SURE expression system host	[135]
Plasmids		
pNZ8901	SURE expression vector, P*spaSpn*, CmR	[135]
pNZ8902	SURE expression vector, P*spaSpn*, EmR	[135]
pNZ-xynA	pNZ8902 carrying *xynA* of *B. subtilis*	[46]
pNZ-usp45	pNZ8902 carrying *usp45* of *L. lactis* MG1363	[46]
pNZ-mntA	pNZ8902 carrying *mntA* of *B. subtilis*	[46]
pNZ-lmrA	pNZ8902 carrying *lmrA* of *L. lactis* MG1363	[46]
pNZ-nprE	pNZ8901 carrying *nprE* of *B. subtilis*	This work
pNZ-bla	pNZ8902 carrying *bla* of *E. coli,*	This work
pNZ-ycdH	pNZ8902 carrying *ycdH* of *B. subtilis*	This work
pNZ-xylP	pNZ8902 carrying *xylP* of *Lb. pentosus*	This work

Plasmid and strain construction

Molecular techniques were carried out as described before [220]. All primers used in this study are listed in Table 4. To construct overexpression vectors, the genes *nprE*, *bla*, *ycdH* and *xylP* were amplified using primers nprE-fw and nprE-rv, bla_F and bla_R, ycdH-Fw and ycdH-rv, xylP-fw and xylP-rv, respectively. Template DNA for amplification of *nprE* and *ycdH* was *B. subtilis* chromosomal DNA. The *bla* gene was amplified from pUC18 plasmid DNA [221] and *xylP* from chromosomal DNA of *Lb. pentosus*. The PCR products of *bla* and *xylP* were digested with *Pag*I and *Xba*I and ligated to pNZ8902, which was digested with *Nco*I and *Xba*I, resulting in pNZ-bla and pNZ-xylP. The *nprE* PCR product was digested with *Nco*I and *Xba*I and ligated to pNZ8901 digested with the same enzymes, resulting in pNZ-nprE. The *ycdH* PCR product was digested with *Bst*EII and *Xba*I and ligated to pNZ8902 digested with the same enzymes, yielding pNZ-ycdH. Restriction enzymes were obtained from Fermentas. The sequences of all constructs were confirmed by DNA sequence analysis (ServiceXS, Leiden, The Netherlands).

Table 4. Primers used in this study.

Name	Target	Sequence (5'- 3')[a]	Restriction enzyme site
nprE-fw	*nprE*	CGCAAACCATGGGTTTAGGTAAGAAATTGTCTGTTGC	*Nco*I
nprE-rv	*nprE*	GCGAAATCTAGATTAATGGTGATGGTGATGGTGCAATCCAACAGCATTCCAGGC	*Xba*I
bla_F	*bla*	AAACCCTCATGAGTATTCAACATTTCCGTGTCG	*Pag*I
bla_R	*bla*	ATACGCTCTAGATTAATGGTGATGGTGATGGTGCCAATGCTTAATCAGTG	*Xba*I
ycdH-fw	*ycdH*	GCGAAAGGTGACCGATATGTTTAAAAAATGGAGCGG	*BstE*II
ycdH-rv	*ycdH*	GCGAAATCTAGATTAATGGTGATGGTGATGGTGTGATTTAACCAATAGTGAATCT TTCAGGGC	*Xba*I
xylP-fw	*xylP*	CGCATATCATGAGCGTTAGTATGCAGC	*Pag*I
xylP-rv	*xylP*	GCGAAATCTAGATTAATGGTGATGGTGATGGTGCTTTTGATCGTCAGCAA	*Xba*I

[a] Restriction enzyme sites are underlined.

DNA microarray analysis

The overexpressed endogenous proteins were XynA, NprE, MntA and YcdH (Table 1). The overexpressed heterologous proteins were TEM1 β-lactamase from *E. coli*, Usp45 and LmrA (inactive mutant) from *L. lactis* and XylP from *Lb. pentosus* (Table 1). For the overproduction of the proteins, the SURE overexpression system was used [135]. The transcription profile of the control *B. subtilis* strain NZ8900 with empty pNZ8902 vector was compared to an isogenic target strain carrying one of the overexpression constructs: pNZ-xynA, pNZ-bla, pNZ-usp45, pNZ-mntA, pNZ-ycdH, pNZ-lmrA or pNZ-xylP. The target strain containing pNZ-nprE was compared to NZ8900 carrying empty pNZ8901. In total, eight independent microarray experiments were conducted.

Strains harboring overexpression constructs or the empty vector pNZ8901 or pNZ8902 were grown overnight in 10 ml TY broth supplemented with appropriate antibiotics and diluted the next day in 50 ml of fresh medium to an OD_{600} of 0.05. At an OD_{600} of 0.6, 0.1% (vol/vol) subtilin-containing supernatant of *B. subtilis* strain ATCC 6633 [222] was added to the growth medium to induce gene expression. After 30 minutes, 10 OD units (1 OD_{600} unit corresponds to 1 ml of a culture of an OD_{600} of 1.0) of each culture were collected for RNA isolation. All the microarray experiments were performed in three biological replicates essentially as described before [167]. Total RNA was isolated using a High Pure RNA isolation Kit (Roche Applied Science). RNA quantity and quality were tested with a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer 2100 (Agilent Technologies Netherlands BV), respectively. Amino allyl-modified cDNA was synthesized using the Superscript III Reverse Transcriptase Kit (Invitrogen), purified with the CyScribe GFX purification kit (Amersham Biosciences) and labeled with Cy3- or Cy5-monoreactive dye (Amersham Biosciences). Labeled cDNA was purified with the CyScribe GFX purification kit (Amersham Biosciences). Labeled cDNA concentration and dye incorporation were assessed with a Nano Drop ND-1000 spectrophotometer. The labeled cDNA was hybridized to oligonucleotide microarrays in Ambion Slidehyb #1 buffer (Ambion Europe Ltd) at 48ºC for 18-20 hours. Next, microarray slides were washed for 5 min in 2 × SSC (300 mM NaCl, 30 mM sodium citrate) with 0.5% SDS, twice for 5 min in 1 × SSC with 0.25% SDS and for 5 min in 1 × SSC with 0.1% SDS, and dried by centrifugation. The slides were scanned with a GeneTac LS V confocal laser scanner (Genomic Solutions Ltd). ArrayPro 4.5 software (Media Cybernetics Inc., Silver Spring, Md., USA) was used to determine intensities of each spot on the microarrays using a local corners background correction method. Resulting expression levels were processed and normalized using the Lowess method with Micro-Prep [223]. The ln-transformed ratios of the expression levels were subject to a *t*-test using Cyber-T tool [224] resulting in expression ratios and Cyber-T (Bayesian) *p*-values.

SDS-PAGE and Western blotting

In order to determine the subcellular localization of overproduced proteins XylP, LmrA, MntA, YcdH, XynA, NprE, Usp45 and TEM1-β-lactamase (Bla) in *B. subtilis*, fractionation experiments were performed essentially as described before [65]. Cells were grown in TY medium. At the OD_{600} of 0.6, protein production was induced by adding 0.1% subtilin containing supernatant of *B. subtilis* strain, ATCC 6633 [135, 222] and cultures were further incubated. After two hours, cells were collected by centrifugation (4,000 × g, 4ºC, 10 min), resuspended in protoplast buffer (PBS pH 7.2,

20 mM MgCl2, 20% sucrose, 2 mg/ml lysozyme, and Complete protease inhibitors Roche) and incubated 30 minutes at 37ºC. Protoplasts were collected by centrifugation (4,000 × g, 4ºC, 10 min), resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 2.5 mM EDTA) and disrupted by sonication (Sonics Vibra Cell, Beun De Ronde). Unbroken protoplasts and cellular debris were removed by centrifugation (4,000 × g, 4ºC, 10 min). Supernatant was ultracentrifuged (200,000 × g, 4ºC, 30 min). The supernatant fraction containing cytosolic proteins was collected and an aliquot was used to prepare SDS-PAGE samples. The pellet was resuspended in solubilization buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 50 mM NaCl, 1% Triton-X-100) over night on a rotor at 4ºC. Nonsolubilized membranes were removed by ultracentrifugation (100,000 × g, 4ºC, 15 min). Supernatant with solubilized membrane proteins was collected and used for SDS-PAGE sample preparation.

The whole cell extracts were prepared as fallows. 1 OD unit of a culture was collected by centrifugation, resuspended in 150 µl of buffer containing 10 mM Tris-HCl pH 8.1, 20% sucrose, 10 mM EDTA, 50 mM NaCl and 2 mg/ml lysozyme, and incubated at 37ºC for 30 min. An equal volume of 2 × SDS-PAGE sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 1% DTT, 20% glycerol, 0.05% bromophenol blue) was added and the samples were boiled for 5 min.

The extracellular proteins present in the medium were precipitated by adding 200 µl of ice-cold 100% TCA to 1.8 ml of medium and incubation on ice for 1 hour. The mixture was centrifuged and the pellet was then washed with acetone, dried by air and resuspended in 100 µl 1 × SDS-PAGE sample buffer. Proteins from the whole cell extracts and the cell and medium fractions were separated on SDS-PAGE gels and transferred to a PVDF membrane. The immunodetection of His-tagged proteins was performed using the Penta-His HRP Conjugate Kit (Qiagen) and ECL detection reagents (Amersham).

Acknowledgements

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CHAPTER 3

Regulation of *ykrL* (*htpX*) by Rok and YkrK, a novel type of regulator in *Bacillus subtilis*

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Expression of *ykrL* of *Bacillus subtilis*, encoding a close homologue of the *Escherichia coli* membrane protein quality control protease HtpX, was shown to be upregulated under membrane protein overproduction stress. Using DNA affinity chromatography, two proteins were found to bind to the promoter region of *ykrL*: Rok, known as a repressor of competence and genes for extracytoplasmic functions, and YkrK, a novel type of regulator encoded by the gene adjacent to *ykrL*, but divergently transcribed. Electrophoretic mobility shift assays showed Rok and YkrK binding to the *ykrL* promoter region as well as YkrK binding to the *ykrK* promoter region. Comparative bioinformatic analysis of the *ykrL* promoter regions in related *Bacillus* species revealed a consensus motif, which was demonstrated to be the binding site of YkrK. Deletion of *rok* and *ykrK* in a P*ykrL-gfp* reporter strain showed that both proteins are repressors of *ykrL* expression. In addition, conditions which activated P*ykrL* (membrane protein overproduction, dissipation of the membrane potential, salt- and phenol stress) point to the involvement of YkrL in membrane protein quality control.

Introduction

The quality of bacterial membrane proteins, essential for the viability of the cell, may be challenged during environmental stresses that can eventually lead to accumulation of misfolded proteins in the membrane. Therefore, the membrane protein folding state must be constantly monitored and damaged proteins must be repaired or eliminated. This is facilitated by membrane protein quality control systems. In *Escherichia coli*, two structurally unrelated membrane-integrated metalloproteases, HtpX and the ATP-dependent protein FtsH, are involved in membrane protein quality control by facilitating proteolytic degradation of proteins (Reviewed in [60]). Involvement of HtpX in membrane quality control is supported by the observation that disruption of both *htpX* and *ftsH* in a strain with the *ftsH*⁻ suppressor mutation *sfhC21* results in thermo-sensitivity, while single disruptions are silent [213]. The *htpX* gene of *E. coli* is under control of the CpxR/CpxA extracytoplasmic stress response system [213], which also regulates expression of a protein involved in secretion stress, DegP (HtrA) [56] and has a homologue in the Gram-positive model organism *Bacillus subtilis*, i.e., CssRS [47]. Little is known about how the quality of membrane proteins is monitored in *B. subtilis* and how cells respond to membrane stress. In another study [225] we found that membrane stress caused by membrane protein (*Lactobacillus pentosus* xyloside transporter XylP and *Lactococcus lactis* multidrug transporter LmrA) overproduction in *B. subtilis* led to an increase of *sigW* and *ykrL* expression. The *B. subtilis* SigW regulon consists of ~60 genes [205] and is induced in response to cell envelope stress [72, 78]. *ykrL* codes for a homolog of *E. coli* HtpX and is also predicted to be a membrane protein itself. In this study, the regulation of *ykrL* expression in *B. subtilis* was investigated and turned out to be very different from that of *htpX* in *E. coli*. We show that *ykrL* expression is repressed by Rok and by a novel type of DNA binding protein, YkrK, encoded by a gene adjacent to *ykrL*, but divergently transcribed, and that expression of *ykrL* is stimulated under conditions potentially influencing membrane protein structure.

Results

Deletion of *ykrL* leads to a higher sensitivity to membrane protein overproduction and to dissipation of the membrane potential

Heterologous overproduction of the membrane proteins XylP, a xyloside transporter from *Lb. pentosus*, and LmrA, a multidrug transporter from *L. lactis*, in *B. subtilis*

caused significant upregulation of *ykrL,* encoding a close homologue of HtpX of *E. coli* (44% identity), *sigW* and most of the SigW regulon members [225]. Knock-out mutants of *ykrL* and *sigW* were constructed, yielding strains JW8940 and KB100, respectively, which were tested for sensitivity to membrane stress (Fig. 1A and B) caused by overexpression of *xylP* from pNZ-xylP. Overproduction of XylP resulted in a significantly higher inhibition of growth for both the Δ*ykrL* and Δ*sigW* mutants than for the control cells harboring the empty vector pNZ8902. The Δ*sigW* mutant was most sensitive, showing cell lysis after 1 hour of induction of expression of *xylP*. Overproduction of LmrA was also tested and showed highly similar effects (data not shown). The stronger effect of the *sigW* deletion is not surprising, as it regulates the expression of approximately 60 genes [205].

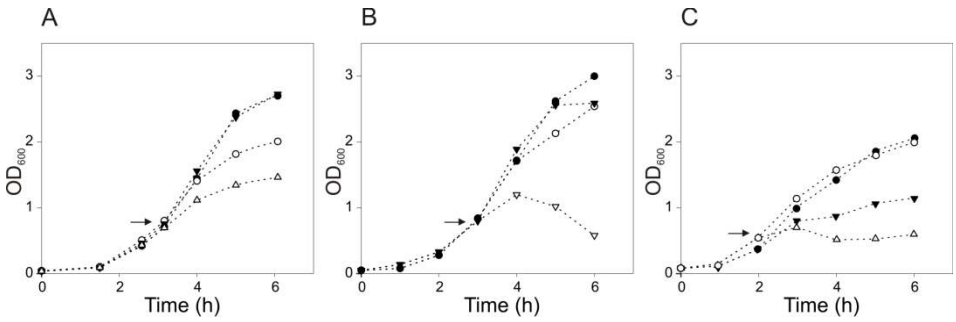

Figure 1. Sensitivity of *ykrL* (A and C) and *sigW* (B) deletion mutants to membrane protein (XylP) overproduction (A and B) or to membrane potential (ΔΨ) dissipation (C). (**A** and **B**) Growth of wild type (circles) and Δ*ykrL* (JW8940, A) or Δ*sigW* (KB100, B) strains (triangles) overexpressing *xylP* (open symbols) or harboring the empty vector pNZ8902 (closed symbols). Expression of *xylP* was induced at an OD_{600} of 0.8 (arrow) with 0.1% subtilin. (**C**) Growth of wild type (JW8900, closed symbols) and Δ*ykrL* (JW8940, open symbols) in LB with 200 mM KCl with (triangles) or without (circles) addition of 0.5 μM valinomycin. Lower growth rates are due to addition of KCl. Arrow indicates timepoint of valinomycin addition. All growth curves are representative of at least three independent experiments.

HtpX of *E. coli* has proteolytic activity on membrane proteins and has been suggested to be involved in degradation of misfolded membrane proteins [60]. Since the transmembrane electrical potential (ΔΨ) has been shown to be important for correct insertion of membrane proteins into the membrane via the protein secretion machinery [226, 227], the effect of membrane potential dissipation using valinomycin was tested on the *ykrL* mutant (JW8940) (Fig. 1C). Valinomycin is an antibiotic that acts as a K^+-carrier specifically dissipating ΔΨ in the presence of a sufficiently high extracellular concentration of K^+ ions [228]. When added at a final concentration of 0.5 μM (sublethal concentration for wild type cells) to exponentially growing cells in LB medium supplied with 200 mM KCl, the Δ*ykrL* strain showed a much higher sensitivity than wild type cells. In a control experiment without addition of KCl, in which

valinomycin leads to hyperpolarization due to K^+ efflux rather than depolarization, the Δ*ykrL* strain was not more sensitive than the wild type. After the addition of different concentrations of nigericin, which dissipates the proton gradient across the membrane (ΔpH) by H^+/K^+ exchange [229], no difference in sensitivity between the mutant and wild type strains was observed. This indicates that the stress caused by membrane protein overproduction against which YkrL is acting, is not proton leakage through the membrane via misfolded membrane proteins, but possibly the accumulation of misfolded protein itself. In agreement, experiments in which the membrane potential was measured in Δ*ykrL* and wild type cells, with or without overexpression of *xylP*, did not show differences in the ability to maintain or build up membrane potential (data not shown).

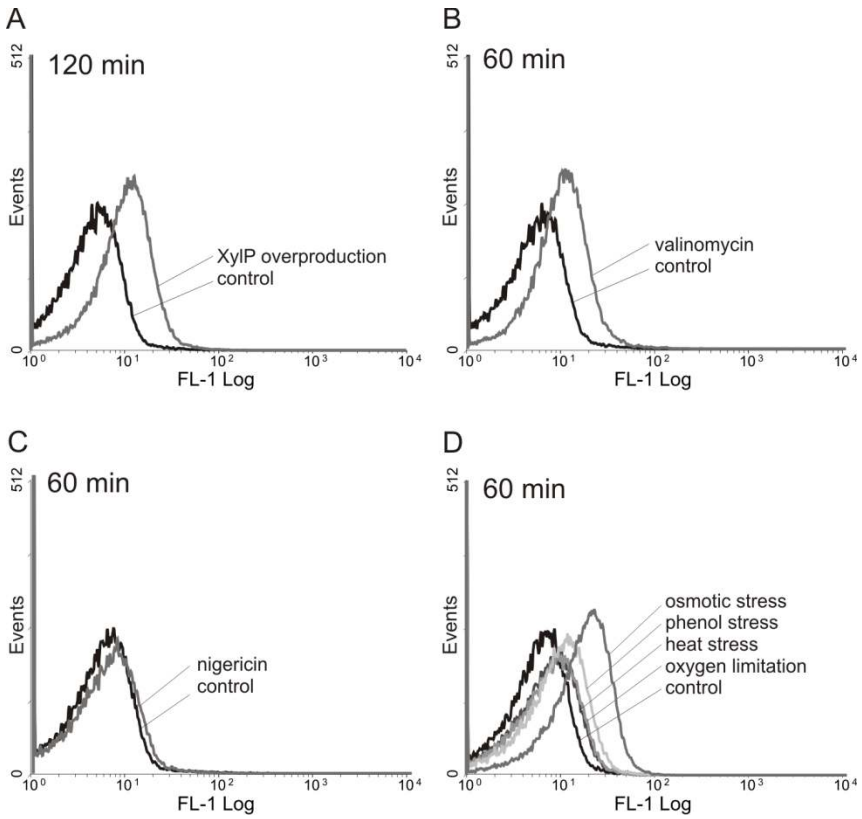

Figure 2. Flow cytometry histograms of the P*ykrL-gfp* reporter strains BC300 (A) or BC400 (B, C, D) showing P*ykrL* activity under different stress conditions. (**A**) *xylP* overexpression from pNZ-xylP induced by subtilin; control: strain carrying empty pNZ8902. (**B**) Membrane potential (ΔΨ) dissipation using 0.5 μM valinomycin. (**C**) ΔpH dissipation with 7.5 nM nigericin. (**D**) Oxygen limitation (vessel containing 20% air), heat stress (42°C), phenol stress (16 mM) and salt stress (650 mM NaCl). Stress conditions were applied to cultures at OD_{600} = 0.5 and GFP fluorescence was monitored at time intervals. Shown are measurements 120 (A) and 60 (B, C, D) minutes after applying stress. The shift in fluorescent signal (FL-1) to the right indicates higher P*ykrL-gfp* activity.

Stress conditions activating the *ykrL* promoter

To investigate the regulation of *ykrL* expression, a reporter strain was constructed containing a fusion of the *ykrL* promoter region to *gfp* (P*ykrL-gfp*) integrated at the native locus. As *ykrL* was induced by membrane protein overproduction in the transcriptome analyses, activation of P*ykrL* by overexpression of *xylP* in the P*ykrL-gfp* reporter strain (BC300) was tested using flow cytometry (Fig. 2A). P*ykrL* clearly showed a higher activity upon XylP overproduction compared to cells not producing XylP.

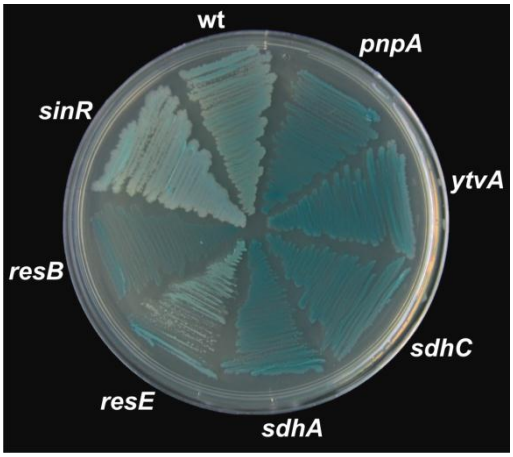

Figure 3. Random mutagenesis of the P*ykrL-lacZ* reporter strain HT400. Strains carrying TnYLB-1 transposon mutations with positive (*pnpA*, *ytvA*, *sdhC*, *sdhA*, *resE*, *resB*) and negative (*sinR*) effects on P*ykrL* activity. WT, HT400; *pnpA*, polynucleotide phosphorylase; *ytvA*, positive regulator of sigma-B activity; *sdhC* and *sdhA*, succinate dehydrogenase subunits; *resE*, two-component sensor histidine kinase, global regulation of aerobic and anaerobic respiration; *resB*, cytochrome *c* biogenesis protein; *sinR*, master regulator of biofilm formation. Strains were plated on LB solid medium supplemented with 0.008% X-gal.

Dissipation of membrane potential (ΔΨ) by addition of a sublethal concentration of valinomycin to the P*ykrL-gfp* reporter strain (BC400) growing in LB supplied with 200 mM KCl resulted in a strong induction of P*ykrL* (Fig. 2B). In the absence of KCl, no induction was observed (not shown). In contrast, dissipation of the proton concentration gradient (ΔpH) by addition of a sublethal concentration of nigericin did not affect P*ykrL* activity, not even after prolonged incubation (Fig. 2C). This, as well as the sensitivity of the Δ*ykrL* strain to valinomycin but not nigericin (previous section), shows a relation of the membrane potential, but not the proton concentration gradient, with stress caused by membrane protein overproduction. A relation between the P*ykrL* activity and membrane energetics was suggested by results from a random transposon mutagenesis screening in a P*ykrL-lacZ* reporter strain (HT400), which showed that P*ykrL* is induced by mutations in *resE*, *resB*, *sdhC*, and *sdhA* (Fig. 3). *resE* codes for a sensor histidine kinase of the ResDE two-component system which regulates expression of genes involved in aerobic and anaerobic respiration [230], including its upstream genes *resABC* [231]; *resB* is essential for cytochrome *c* synthesis [232]; *sdhC* and *sdhA* encode components of succinate dehydrogenase which plays a role in the electron transport chain [233].

Although the effects of these mutations are pleiotropic, they constituted almost two-third of the identified mutations that induced P*ykrL* (not shown) and the genes all play a role in generation of membrane potential. This, together with the above mentioned results, suggests that the membrane potential component of the proton motive force could affect the activity of P*ykrL* either directly or indirectly.

In addition, P*ykrL* activity was tested under different stress conditions that can influence the membrane protein structure or were reported to induce *ykrL* expression previously (phenol stress [234], and salt stress [206]). The conditions tested were addition of 16 mM phenol, addition of 650 mM NaCl, submission to heat stress at 42ºC and 50ºC and anaerobic growth (a closed vessel filled up to 80% with culture) (Fig. 2D). Salt stress had a strong effect on P*ykrL*, while phenol, heat and anaerobic stress resulted in milder activation of P*ykrL*.

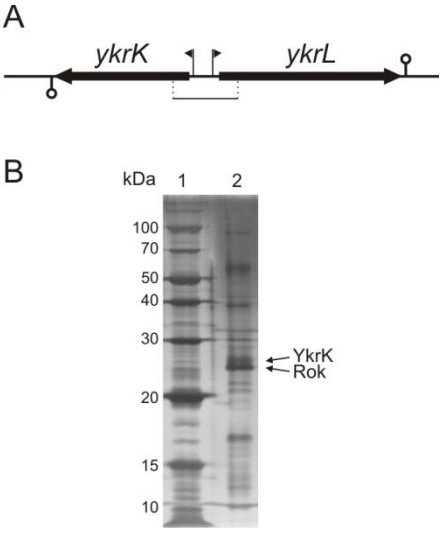

Figure 4. Identification of proteins binding to *ykrK-ykrL* intergenic region using DNA affinity chromatography. (**A**) Schematic organization of the *ykrK-ykrL* locus in *B. subtilis*. Lollipops indicate terminator structures. Flags indicate predicted promoters. The line below indicates the fragment used for the affinity chromatography. (**B**) SDS-PAGE and silver stain analysis of the proteins enriched from the cytoplasmic fraction of a *B. subtilis* NZ8900 culture using the *ykrK-ykrL* intergenic region immobilized on Streptavidin beads. Lane 1, protein ladder; lane 2, proteins enriched with the *ykrK-ykrL* intergenic region of *B. subtilis*.

Rok and YkrK bind to the intergenic region of *ykrK* and *ykrL*

In order to find proteins binding to the promoter of *ykrL*, DNA affinity chromatography was performed. A 542 bp DNA fragment containing the *ykrK-ykrL* intergenic region with the predicted *ykrK* and *ykrL* promoters (Fig. 4A), was amplified, biotinylated, immobilized on magnetic Streptavidin beads and incubated with a cytoplasmic fraction of *B. subtilis* cells. Proteins bound to the DNA were eluted and analyzed on an SDS-PAGE gel (Fig. 4B) followed by in gel-digestion and identification by mass spectrometry. Among the most abundant proteins that bound to the *ykrK-ykrL* intergenic DNA fragment were Rok and YkrK. Rok is known to act as a negative regulator of

competence [235] and genes coding for membrane-localized and secreted proteins [236], and was shown to bind to A+T-rich DNA [237]. The other protein, YkrK, is an unknown 233 amino acid protein encoded by *ykrK*, the gene adjacent to, but divergently transcribed from *ykrL* (Fig. 4A). Apart from close homologs in closely related *Bacillus* species, no significant homology was found with any other known protein. It shows minor local similarity to MerR family regulators, but can be considered a novel type of DNA binding protein. The *Bacillus* species that harbor YkrK homologs share the same genetic organization of the divergent *ykrL* and *ykrK* corresponding genes.

Other, less abundant, proteins identified in the elution fraction were 30S and 50S ribosomal proteins, dihydrolipoamide acetyltransferase (*acoC*), lipoamide acyltransferase (*bkdB*) and dihydrolipoyl dehydrogenase (*lpdV*). Since these proteins were not (potential) DNA binding proteins, their role in *ykrL* regulation was not further analyzed.

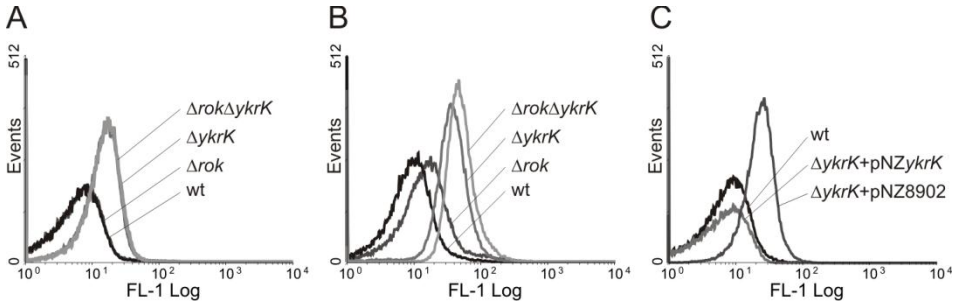

Figure 5. Flow cytometry histograms showing P*ykrL* activity in P*ykrL-gfp* reporter strains. Strains: wild type (BC400), Δ*rok* (BC401), Δ*ykrK* (BC402), Δ*rok*Δ*ykrK* (BC403), Δ*ykrK*+pNZ-ykrK (BC404 carrying pNZ-ykrK), Δ*ykrK*+pNZ8902 (BC404 carrying pNZ8902). (**A**) GFP signal in cells growing exponentially, (**B**) GFP signal in cells in stationary phase, (**C**) GFP signal in cells growing exponentially 30 min after inducing *ykrK* expression with subtilin. The shift in fluorescent signal (FL-1) to the right indicates higher P*ykrL-gfp* activity.

Rok and YkrK are repressors of *ykrL* expression

To test how YkrK and Rok regulate expression of *ykrL*, i.e., by activation or repression, strains containing the P*ykrL-gfp* fusion combined with a deletion of *rok* (BC401), *ykrK* (BC402) or both *rok* and *ykrK* (BC403) were constructed. GFP fluorescence was measured every hour throughout growth (only chosen time points are shown, Fig. 5). During early exponential phase, P*ykrL* activity was clearly higher in the Δ*ykrK* and Δ*rok*Δ*ykrK* mutants (Fig. 5A). Later in growth (transition from exponential to stationary phase and early stationary phase) the difference in GFP signal between wild type and these two mutants became larger and P*ykrL* activity also increased in the Δ*rok* mutant at late stationary growth phase (Fig. 5B). Overexpression of *ykrK* from pNZ-ykrK using the SURE system [135] in the Δ*ykrK* mutant (BC404) resulted in full repression of

P*ykrL-gfp* (Fig. 5C). Altogether, we demonstrate that both Rok and YkrK are repressors of the *ykrL* promoter.

Rok binds to the *ykrL* promoter region; YkrK binds to both the *ykrK* and *ykrL* promoter regions

To determine more precisely the regions to which Rok and YkrK bind, four fragments, A, B, C and D (Fig. 6A), covering the *ykrK-ykrL* intergenic region, were amplified. The DNA fragments A and B contained the predicted *ykrK* promoter (P*ykrK*) and the predicted *ykrL* promoter (P*ykrL*), respectively. Fragment C covered the region between these two promoters including predicted -35 boxes, and fragment D covered the whole region. The Rok and YkrK proteins were expressed in *E. coli* and *L. lactis*, and purified using a C-terminal 6His- and Strep-tag, respectively. Binding of Rok-His_6 and YkrK-Strep to these fragments was tested by electrophoretic mobility shift assays (EMSAs) (Fig. 6B). Binding affinities were estimated from the decrease in signal of the unbound DNA using binding kinetics curve fitting. Both proteins bound to fragment B (P*ykrL*), although YkrK with much higher affinity than Rok (apparent K_d values of 0.1 μM and 1.2 μM, respectively). YkrK also showed significant binding to fragment A (K_d app = 3.4 μM) and C (K_d app = 2.7 μM), albeit with much lower affinity than to fragment B, indicating that, next to *ykrL*, YkrK might regulate its own expression. The mobility shift patterns of fragment D were consistent with one binding site for Rok in the intergenic region and three for YkrK, the latter appearing as a sum of the YkrK binding patterns of fragment A and B.

A YkrK binding motif is present within P*ykrK*, P*ykrL* and in between the two promoters

In order to identify a consensus sequence within the *ykrK-ykrL* intergenic region that may represent a YkrK binding motif, we compared the sequences of this region from five *Bacillus* species which contain a corresponding, divergent *ykrK-ykrL* pair: *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. halodurans* and *B. pumilus* , using the Motif Sampler tool [238]. A conserved sequence TGAWCTTA (W = A/T) was found (Fig. 7A). This 8-nt motif is present in the *ykrK-ykrL* intergenic region of *B. subtilis* in three places (Fig. 7B): overlapping with the predicted -35 box of P*ykrK*, downstream of the -10 box of P*ykrL* and, with more deviation from the consensus, in between these two promoters. The locations of the motif correlates with the observed YkrK binding to the four DNA fragments in the EMSA experiment (compare Fig. 6).

To confirm experimentally the result obtained with the YkrK binding motif search, a YkrK binding assay was performed with DNA fragment B, covering the predicted

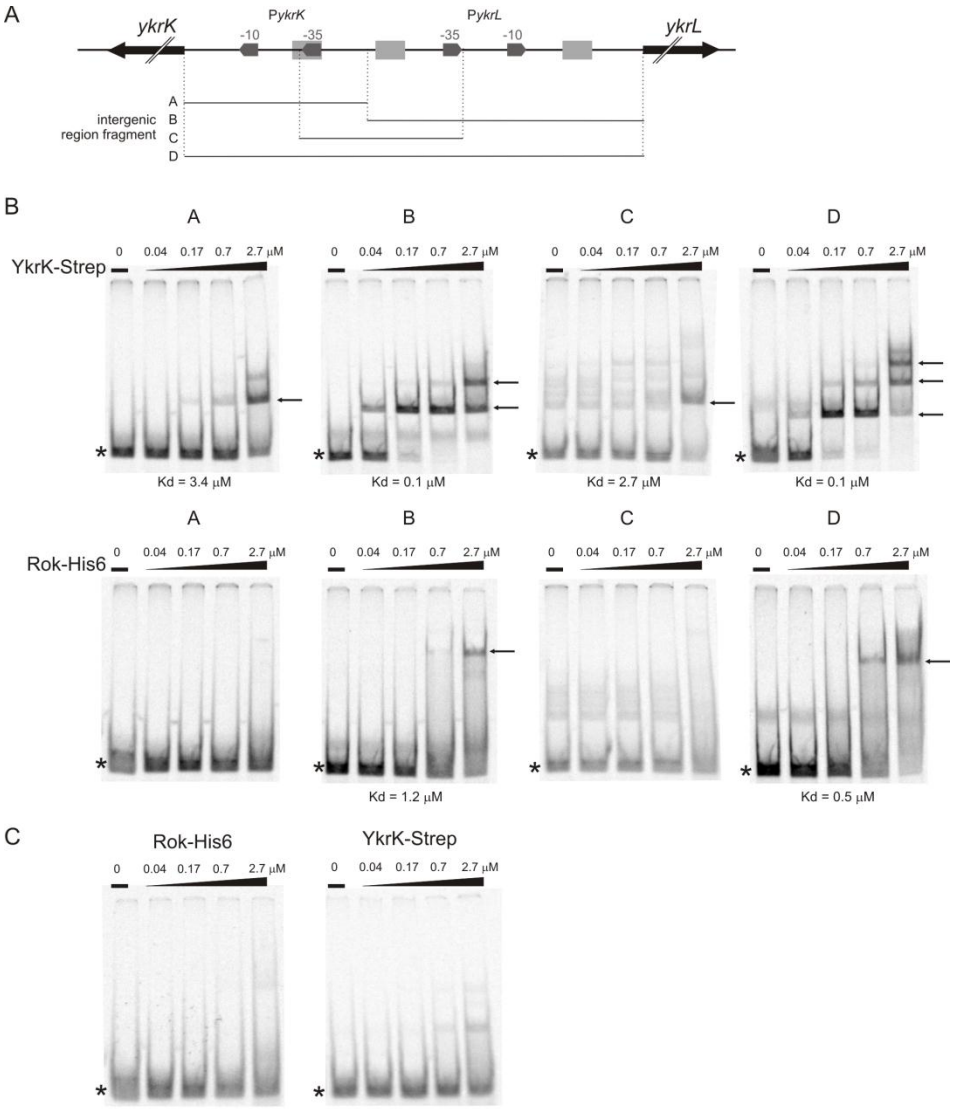

Figure 6. ***In vitro*** **binding of YkrK-Strep and Rok-His$_6$ to fragments of the** ***ykrK-ykrL*** **intergenic region.** (**A**) Schematic representation of the *ykrK-ykrL* intergenic region and fragments A (P*ykrK*), B (P*ykrL*), C and D used for EMSA. Predicted -35 and -10 boxes of P*ykrK* and P*ykrL* are shown as boxed arrows and putative YkrK binding sites as light grey rectangles. (**B**) EMSAs of YkrK-Strep (top panel) and Rok-His$_6$ (bottom panel) binding to fragments A, B, C and D. (**C**) Negative control: Rok-His$_6$ and YkrK-Strep binding to P*secA* DNA. Asterisks indicate free ^{33}P-DNA probes and arrows indicate the position of the shifted probe. The calculated K_d values are indicated below each binding assay graph.

P*ykrL*, in its original sequence and with three variants carrying point mutations in the predicted binding motif: B-mut1 (G2A), B-mut2 (C5T) and B-mut3 (G2A + C5T) (Fig. 7C). YkrK bound to the wild type DNA fragment B with an estimated K_d of 60 nM. The single point mutations on the second (B-mut1) and fifth (B-mut2) position of the

predicted motif caused at least a ten-fold decrease in affinity of YkrK binding (apparent K_d values of 600 nM and 960 nM, respectively). When both mutations were present simultaneously (B-mut3), affinity decreased even more substantially (K_d app = 1.65 μM). This result confirms that the conserved sequence TGAWCTTA (W = A/T) is the YkrK binding motif, or at least constitutes the major part of the binding site. Up- and downstream base pairs may be involved as well, which could explain the difference of the YkrK binding affinity between the *ykrL* promoter and the other two binding sites present in the *ykrK* promoter and in the region in between.

Since *ykrL* was possibly not the only target of YkrK regulation, *ykrK* was overexpressed in *B. subtilis* and the transcriptome was compared to control cells using DNA microarrays. The upstream regions of significantly regulated genes (listed in Table 1) were searched for the YkrK binding motif using the genome-scale dna-pattern tool [239] with default parameters. Next to *ykrK* and *ykrL*, only two genes were found to possess the putative YkrK binding motif (TGAWCTTA) in their promoter regions: *yrkA*, coding for a putative membrane protein of unknown function, and *penP*, encoding a secreted penicillinase. The motif of *penP*, however, is in the reverse orientation, while the expression of the adjacent but divergently transcribed gene, *yoaZ*, was not changed upon YkrK overexpression. It appears that YkrK regulates only a small number of genes including *ykrL* and possibly *ykrK*, but this needs further research.

Discussion

The regulation of *ykrL* of *B. subtilis*, encoding a close homologue of the *E. coli* HtpX, is described. In addition, insight in the physiological role of YkrL was obtained from a *ykrL* knock-out mutant as well as from stress conditions inducing *ykrL* expression. YkrL is a membrane protein with four predicted transmembrane segments and contains all the conserved residues present in the active site of the 44% identical HtpX of *E. coli*, a zinc dependent metalloprotease involved in membrane protein quality control [60, 212]. It was shown that a *ykrL* knock-out mutant was more sensitive to membrane protein overproduction stress and dissipation of transmembrane electrical potential (ΔΨ) than the wild type mother strain. These conditions also induced expression from the *ykrL* promoter, P*ykrL*. In contrast, the *ykrL* mutation did not increase sensitivity to dissipation of the chemical proton gradient, ΔpH, which together with the ΔΨ constitutes the proton motive force (pmf). Neither did this condition induce P*ykrL*. This suggests that the stress that is sensed and leads to induction of *ykrL* expression is

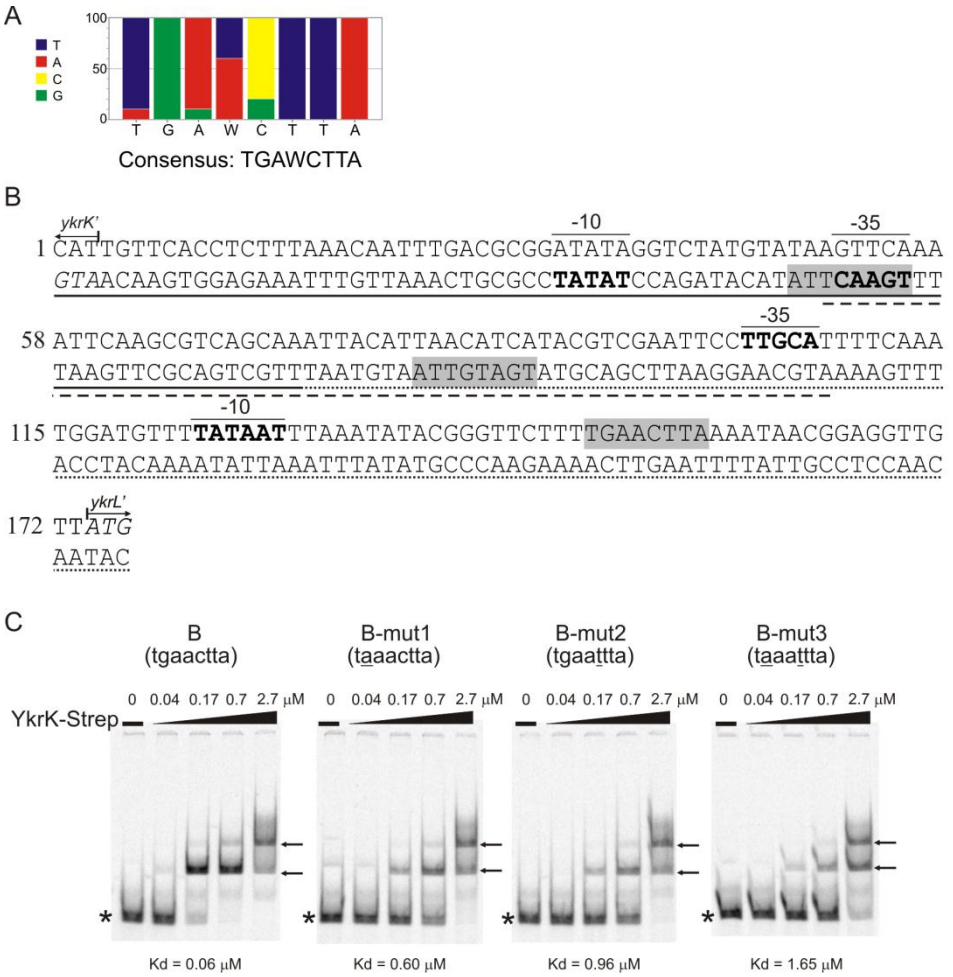

Figure 7. YkrK binding motif search and validation. (**A**) Weight matrix of consensus sequence present within *ykrK-ykrL* intergenic region in different *Bacilli* species. (**B**) Sequence of the *ykrK-ykrL* intergenic region of *B. subtilis* with putative YkrK binding sites in grey shading, start codons of *ykrL* and *ykrK* in italics and predicted -10 and -35 boxes in bold. The fragments used for EMSA experiments shown in Fig. 6 are underlined: solid line, fragment A (P*ykrK*); dotted line, fragment B (P*ykrL*); dashed line, fragment C. (**C**) EMSA of YkrK-Strep binding to fragment B containing the consensus motif and to its derivatives containing point mutations in the motif (B-mut1, B-mut2, B-mut3). Point mutations are underlined. Asterisks indicate free ^{33}P-DNA probes and arrows indicate the position of the shifted probe. The calculated K_d values are indicated below each binding assay graph.

the presence or accumulation of misfolded proteins in the membrane, rather than proton leakage as a result of a disturbed membrane integrity. In this case, the observed activation of P*ykrL* by membrane potential dissipation can be explained by the requirement of membrane potential for correct insertion of membrane proteins [226, 227].

Table 1. Genes with significantly changed expression ratio (0.4 > ratio > 2.5, Bayesian p < 0.01) upon YkrK overproduction.

Gene	Ratio	Product	YkrK binding motif	
			Sequence [a]	Start; end [b]
ykrK	136.94	hypothetical protein	atttTGAACTTAtaca	-52; -45
			atttTGAACTTAtaca*	-153; -146
ykzE	6.75	hypothetical protein		
guaC	5.43	guanosine 5'-monophosphate oxidoreductase		
bmrU	5.05	diacylglycerol kinase		
yqjF	4.07	hypothetical protein		
purC	3.89	phosphoribosylaminoimidazole-succinocarboxamide synthase		
purS	3.59	phosphoribosylformylglycinamidine synthase subunit PurS		
purB	3.46	adenylosuccinate lyase		
amyC	2.96	maltose and multiple sugars ABC transporter permease		
amyD	2.91	carbohydrate ABC transporter permease		
ytlD	2.82	permease		
purQ	2.79	phosphoribosylformylglycinamidine synthase I		
ywjC	2.76	hypothetical protein		
purE	2.76	phosphoribosylaminoimidazole carboxylase I		
msmE	2.69	multiple sugar-binding lipoprotein		
purK	2.61	phosphoribosylaminoimidazole carboxylase ATPase subunit		
yfnF	0.40	glycosyltransferase		
yktB	0.38	hypothetical protein		
penP	0.36	beta-lactamase precursor	caatTGATCTTAtatt*	-106; -99
sspG	0.36	small acid-soluble spore protein		
ykrL	0.22	heat shock protein HtpX	ctttTGAACTTAaaat	-25; -18
			atttTGAACTTAtaca*	-126; -119
yrkA	0.21	membrane associated protein	ctttTGAACTTAtaat	-88; -81
ykoX	0.19	integral inner membrane protein		

[a] Predicted YkrK binding motif (in capitals) with flanking sequences.
[b] Distance calculated from the first nucleotide of the start codon.
* Motifs found in the direction reverse to the gene.

We suggest that YkrL, similar to HtpX of *E. coli*, is involved in a membrane protein stress response, likely by proteolytic degradation of misfolded membrane proteins, and serves as a membrane protein quality control system. Supporting this hypothesis is the strongly increased amount of YkrL observed in the membrane proteome in a conditional double knock-out of *spoIIIJ* and *yqjG*, encoding YidC homologues involved in membrane protein insertion [240]. However, differences between the HtpX and YkrL function may exist. The *htpX* gene is induced in *E. coli* by heat stress [213], whereas the effect of heat stress on *ykrL* expression in *B. subtilis*, albeit significant, was less strong than that of other tested conditions such as salt stress. The strong induction of P*ykrL* by salt stress and phenol, observed before [206, 234], may be explained by misfolding of proteins induced by the hyperosmotic conditions and denaturation, respectively. Indeed, Hahne *et al.*, [241] observed that salt stress induced cytosolic as well as membrane

protein quality control proteins in *B. subtilis*, although *ykrL* was not indicated as significantly regulated in that study.

In *E. coli*, next to HtpX, the membrane located ATP-dependent metalloprotease FtsH is involved in membrane protein quality control [60]. The gene encoding FtsH of *B. subtilis*, sharing 47% amino acid sequence identity with *E. coli* FtsH, was, unlike *ykrL*, not induced by membrane stress caused by membrane protein overproduction [225]. Although we cannot exclude a general role of FtsH in membrane protein quality control, its previously described functions [61, 62] may represent a more specific role in the physiology of *B. subtilis*.

YkrL has recently been shown to associate with the MreB cytoskeleton [242] forming a spiral structure immediately underneath the cell membrane and coordinating cell wall synthesis together with MreC and MreD [243]. This spiral localization of MreB is disrupted under dissipation of the membrane potential [244]. The site where YkrL is recruited may be close to the cell wall synthesis machinery (MreB complex), where, during rapid growth, cells could benefit from a membrane protein quality control system.

In *E. coli*, expression of *htpX* is regulated by the CpxR/CpxA two component system [213], which regulates many genes with an important role in envelope protein folding [59], including the secretion stress protein DegP (HtrA) [56]. The corresponding system in *B. subtilis*, CssRS, regulating *htrA* and *htrB* expression [47], appeared not to be involved in *ykrL* expression. Instead, two proteins binding to the *ykrL* promoter region were identified by DNA affinity chromatography: the unknown protein YkrK, encoded by *ykrK*, located upstream of *ykrL*, but divergently transcribed, and Rok, a known repressor of competence and of genes with extracytoplasmic function [235, 236]. Both proteins were demonstrated to act as repressors of *ykrL* expression. Electrophoresis mobility shift assays showed that Rok binds to the *ykrL* promoter region, whereas YkrK binds at three positions in the *ykrK-ykrL* intergenic region: with high affinity to the *ykrL* promoter region, with low affinity to the *ykrK* promoter region and with low affinity to a region in between these. A consensus motif for YkrK binding was identified by comparative bioinformatic analysis of homologous *ykrK-ykrL* intergenic regions of related *Bacillus* species. The motif, TGAWCTTA (w = a/t), was confirmed by making point mutations, which led to a drastically lowered affinity for YkrK. The presence of the binding motif in the *ykrK* promoter region, overlapping with its predicted -35 sequence, and the weak but significant binding of YkrK to this region, suggests that YkrK may be involved in its own regulation, but this needs further research.

The exact binding site for Rok in the *ykrK-ykrL* intergenic region was not determined, but recent work demonstrated that Rok binding occurs at A+T-rich DNA [237]. The fragment of the *ykrK-ykrL* intergenic region to which Rok bound contains an uninterrupted 18 bp stretch of A+T, overlapping with the predicted -10 box of P*ykrL* (Fig. 7B). The fragments to which Rok did not bind do not contain such uninterrupted stretches, although the average A+T content of the fragments did not significantly differ.

The functioning of YkrK is an interesting target for further research, as it may be either directly or indirectly involved in the sensing of the stress signal that leads to its dissociation from the *ykrL* promoter. As this stress is occurring and sensed at or in the membrane, interaction of YkrK with other membrane associated or embedded proteins can be expected. At the C-terminus of YkrK, a 34 amino acid sequence containing a large proportion of hydrophobic amino acids is predicted as a relatively hydrophobic α-helix by secondary structure prediction tool POLYVIEW [245], which may play a role in such interaction.

In conclusion, this study addressed the so far relatively unexplored area of membrane protein stress in *B. subtilis*, in particular the regulation of the membrane stress responsive gene *ykrL*. Two regulators were identified, Rok and the novel YkrK protein, for which a binding motif was revealed. Results on an *ykrL* knock-out strain and P*ykrL* controlled expression data together with other studies discussed above, indicate YkrL as a quality control system for membrane proteins.

Materials and Methods

Bacterial strains and growth conditions

B. subtilis strains used in this study are isogenic derivatives of strain 168 [1] and are listed in Table 2. *L. lactis* NZ9000 [216] was used as an intermediate cloning host for pNZ-xylP and pNZ-ykrK-strep and *E. coli* DH5α for pPykrL-gfp. *B. subtilis* and *E. coli* strains were grown in LB medium [246] at 37°C with shaking. *L. lactis* was grown at 30°C in M17 broth (Oxoid, Basingstone, England) supplemented with 0.5% (wt/vol) glucose. LB medium was supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin for *E. coli*, 5 μg/ml kanamycin, 100 μg/ml spectinomycin, 0.5 μg/ml erythromycin, 5 μg/ml chloramphenicol or 9 μg/ml tetracycline for *B. subtilis* and 5 μg/ml chloramphenicol or 5 μg/ml erythromycin for *L. lactis*, when needed. Solid media were prepared by adding 1.5% agar.

The membrane located xyloside transporter XylP from *Lb. pentosus* was overexpressed in *B. subtilis* using the subtilin-regulated gene expression (SURE) system [135].

Molecular techniques were carried out as described before [220]. Primers used in this study are listed in Table 3.

Table 2. Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Reference
Bacterial strains		
E. coli		
DH5α	F⁻ *endA1 glnV44 thi*-1 *recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ–	[247]
ED428	*rok-his*$_6$	[235]
L. lactis		
NZ9000	*pepN*::*nisRK*	[216]
B. subtilis		
168	*trpC2*	[1]
NZ8900	*trpC2 amyE*::*spaRK* (Kan^R)	[135]
JW8900	*trpC2 thrC*::*spaRK* (Erm^R)	J. W. Veening, unpublished
JW8940	*trpC2 thrC*::*spaRK ykrL* (Erm^R, Spc^R)	This study
RH100	*trpC2 thrC*::*spaRK* (Spc^R)	R. Nijland, unpublished
KB100	*trpC2 thrC*::*spaRK sigW* (Spc^R, Kan^R)	This study
BC300	*trpC2 thrC*::*spaRK* (Spc^R) P*ykrL-gfp* (Cm^R)	This study
BC400	*trpC2* P*ykrL-gfp* (Cm^R)	This study
BC401	*trpC2* P*ykrL-gfp rok* (Cm^R, Kan^R)	This study
BC402	*trpC2* P*ykrL-gfp ykrK* (Cm^R, Tet^R)	This study
BC403	*trpC2* P*ykrL-gfp rok ykrK* (Cm^R, Kan^R, Tet^R)	This study
BC404	*trpC2* P*ykrL-gfp amyE*::*spaRK ykrK* (Cm^R, Kan^R, Tet^R)	This study
HT400	*trpC2 amyE*::P*ykrL-lacZ* (Cm^R)	This study
Plasmids		
pNZ8902	P*spaSpn* (Erm^R)	[135]
pNZ-xylP	P*spaSpn-xylP* (Erm^R)	This study
pNZ-ykrK-strep	P*spaSpn-ykrK-strep* (Erm^R)	This study
pNZ-ykrK	P*spaSpn-ykrK* (Erm^R)	This study

Plasmid and strain construction

All primers used in this study are listed in Table 3. The *ykrL* deletion was constructed as follows. First, the *ykrL* locus including 735 bp upstream the start codon and 584 bp downstream the stop codon, was amplified using primers ykrLdel-fw and ykrLdel-rv. The PCR product was ligated into pCR2.1-TOPO (Invitrogen). The resulting vector, pTOPO-DykrL-1, was digested with *Swa*I and *Eco*47III enzymes. The spectinomycin resistance gene was amplified from pDG1726 [248] using primers specBsu-F and specBsu-R and ligated into pTOPO-DykrL-1. The resulting plasmid pTOPO-DykrL-2 carries *ykrL* flanking regions and the spectinomycin resistance gene replacing 874 bp of the *ykrL* gene. Strain JW8940 was constructed by transforming JW8900 with pTOPO-

DykrL-2 resulting in replacement of the *ykrL* gene with the spectinomycin cassette by double recombination.

The *sigW* deletion mutant was constructed as follows. The *sigW* locus including 540 bp upstream the start codon and 376 bp downstream the stop codon was amplified using primers sigW-del-fw and sigW-del-rv. The PCR product was ligated into pCR2.1-TOPO (Invitrogen) resulting in pTOPO-DsigW-1. Next, the kanamycin resistance marker was amplified from pDG780 [248] using the primers Km-dsigW-fw and Km-dsigW-rv. After digestion with *Sac*I and *Acc*I, the product was ligated into pTOPO-DsigW-1, from which a 374 bp internal fragment of *sigW* was removed by digestion with the same enzymes. The resulting vector, pTOPO-DsigW-2, was transformed to the *B. subtilis* RH100, giving rise to strain KB100 which contains the kanamycin resistance cassette replacing the 374 bp internal *sigW* fragment.

To disrupt *ykrK*, the deletion construct pDG1514-ykrK was made in two steps. An upstream flanking region of *ykrK* was amplified with primers ykrK::tc-o1 and ykrK::tc-o2, digested with *Bam*HI and *Pst*I and ligated to the corresponding sites of pDG1514 [248], upstream the tetracycline resistance cassette. Next, a downstream flanking region of *ykrK* was amplified with primers ykrK::tc-o3 and ykrK::tc-o4, digested with *Hind*III and *Xho*I and ligated at the same sites downstream the resistance cassette, resulting in pDG1514-ykrK. The construct was then transformed to BC400 and BC401, which gave strains BC402 and BC403, respectively, with a disrupted *ykrK* gene.

The P*ykrL-gfp* reporter strains were obtained as follows. First, a DNA fragment of 510 bp upstream of *ykrL* containing the promoter region of *ykrL* was amplified using primers PykrL-fw and PykrL-rv. The PCR product was digested with *Kpn*I and *Pst*I and ligated into the same sites of pSG1151 [249], which resulted in pPykrL-gfp. pPykrL-gfp was transformed to the strains 168 and RH100 resulting, by means of Campbell-type integration, in strains BC400 and BC300, respectively.

The BC401 strain carrying the P*ykrL-gfp* fusion and *rok* deletion was made by transforming BC400 with chromosomal DNA from *B. subtilis* BD3196, carrying a *rok* mutation [236].

Strain HT400 was constructed as follows. A 463 bp *ykrL* promoter region of *B. subtilis* was amplified using primers PykrLpDL-fw and PykrLpDL-rv, digested with *BamH*I and *Kpn*I and ligated upstream of the β-galactosidase gene (*bgaB*) in pDL, an *amyE* integration vector [103], which was cut with corresponding enzymes. The resulting plasmid pDL-PykrL was transformed to *B. subtilis* 168, resulting in integration of the P*ykrL-bgaB* (P*ykrL-lacZ*) fusion in the *amyE* locus.

Table 3. Primers used in this study.

Name	Sequence (5'- 3')
ykrLdel-fw	GCTCTGTCTCCTGTTCGGCG
ykrLdel-rv	GATCAGAAACAGTACCTGCTTC
specBsu-F	GGGCTTGGATCCCAACGCTGTCGACGTTGTAAAACGACGG
specBsu-R	CGCATAGCTTTCCGGTCGCCGCAGCTATGACCATGATTACGC
sigW-del-fw	GCGAAAGGTACCTCTGCCTTACAAGCAGAGGG
sigW-del-rv	GCGAAATCTAGAATTCGGCTGCTTGGACACGC
Km-dsigW-fw	TAATACGACTCACTATAGGGC
Km-dsigW-rv	ACCGGGCCCCCCCTCGAGGTATACGGTATCGATACAAATTCC
ykrK::tc-o1	GCGAAAGGATCCCGATACTGCGACAAGTGAACG
ykrK::tc-o2	GCGAAACTGCAGTGACATGTTCGCTGATTGGACG
ykrK::tc-o3	GCGAAAAAGCTTAGATTATCGCCATTACAGTCC
ykrK::tc-o4	GCGAAACTCGAGAAGAAGAGAAAGCGGATGACC
PykrL-fw	GCGAAAGGTACCGTTCAATTGAGTATTTTTTGCCC
PykrL-rv	GCGAAACTGCAGCGCCATAACAACCTCCGTTATTTT
PykrLpDL-fw	GCGAAAGGATCCTTGCCCAGCTCAGGGTGTCCG
PykrLpDL-rv	GCGAAAGGTACCAAAAGAACCCGTATATTTAAATTA
ykrK-strep-F	CGTAGTCATGATGAACATTTTTAAACTCTCTCGAACCGATATGG
ykrK-strep-R	CGTCCGGTACCTCATTTTTCAAATTGTGGATGGCTCCATAGAATGTCTGCTGTCATTTTATG
ykrK-fw	GCGAAATCATGAACATTTTTAAACTCTCTCGAACCG
ykrK-rv	GCGAAAAAGCTTTTATTTATTGCTGGCAATTGTTGTGG
xylP-fw	CGCATATCATGAGCGTTAGTATGCAGC
xylP-rv	GCGAAATCTAGATTAATGGTGATGGTGATGGTGCTTTTGATCGTCAGCAA
PykrL-oligo4	ACAAAACGATCCCGATTGTTG
PykrL-oligo5	TGTCCGATCAGATCTTTGATATCAC
SQ_PsecA_o1	CAAATTCTTTGGAAATAACAAAAGGTATGATATGATAATGAGAGGTATACATGGACTAG
SQ_PsecA-o2	CTAGTCCATGTATACCTCTCATTATCATATCATACCTTTTGTTATTTCCAAAGAATTTG
KL1-o1	ATGTTCATTGTTCACCTC
KL1-o2	TTGCTGACGCTTGAATTTTG
KL2-o1	ATTACATTAACATCATACGTCG
KL2-o2	TTCGCCATAACAACCTCC
KL3-f	AGTTCAAAATTCAAGCGTC
KL3-r	ATGCAAGGAATTCGACGTATG
KL2-o2-a	TTCGCCATAACAACCTCCGTTATTTTAAGTTTAAAAGAACCCGTATATTTAAATTATA
KL2-o2-t	TTCGCCATAACAACCTCCGTTATTTTAAATTCAAAAGAACCCGTATATTTAAATTATA
KL2-o2-at	TTCGCCATAACAACCTCCGTTATTTTAAATTTAAAAGAACCCGTATATTTAAATTATA

Plasmid pNZ-ykrK-strep was made by amplifying *ykrK* using primers ykrK-strep-F and ykrK-strep-R carrying the Strep-tag sequence for a C-terminal fusion. The product was digested with *Pag*I and *Kpn*I and ligated into pNZ8948 [216], cut beforehand with *Nco*I and *Kpn*I.

To construct pNZ-ykrK, *ykrK* gene was amplified using primers ykrK-fw and ykrK-rv. The product was digested with *Pag*I and *Hind*III and ligated into pNZ8902 digested with *Nco*I and *Hind*III.

To make pNZ-xylP, *xylP* of *Lb. pentosus* was amplified using primers xylP-fw and xylP-rv. The PCR product was digested with *Pag*I and *Xba*I and ligated into pNZ8902 digested with *Nco*I and *Xba*I.

Expression and purification of YkrK and Rok

YkrK-strep was expressed in *L. lactis* NZ9000 from pNZ-ykrK-strep, using the NICE system [250]. Cells were grown in GM17 medium to mid-exponential phase (OD_{600} = 0.5) and induced with 5 ng/ml nisin (Sigma). After 2 hours cells were harvested, washed and resuspended in buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl). 5 mg/ml lysozyme was added and, after 10 minutes incubation at 45ºC, cells were disrupted by bead beating for 2 minutes at 4ºC in a Mini-Beadbeater-16 (Biospec Products). Cell debris was removed by centrifugation and the supernatant was incubated with Strep-Tactin Sepharose (IBA) with gentle rotation for 2 hours at 4ºC. The mixture was loaded onto a Bio-Spin disposable chromatography column (Bio-Rad) and washed three times with buffer W. The protein was eluted from the column with 100 mM Tris-HCl pH 8.0, 150 mM EDTA, and 2.5 mM desthiobiotin. The protein concentration was determined using a ND-1000 Spectrophotometer (NanoDrop Technologies). Fractions containing pure YkrK were used in gel retardation analysis.

Rok-His_6 was expressed in *E. coli* ED428 and purified using a Superflow Ni-nitrilotriacetic acid resin column (QIAGEN) as described before [236].

DNA affinity chromatography

DNA affinity chromatography was performed as described before [251, 252] with modifications. The 542 bp *ykrK-ykrL* intergenic region was amplified using primers PykrL-oligo4 and PykrL-oligo5, of which PykrL-oligo4 was provided in a biotinylated form on the 5'-end (Biolegio). The amplified fragment covered a region from -412 to +70 with respect to the translational start site of *ykrL*. The PCR product labeled with biotin was immobilized on SiMag-Streptavidin magnetic beads (Bio-Nobile). For cytoplasmic fraction isolation, *B. subtilis* NZ8900 was grown in 600 ml LB. Cells were collected at late exponential phase, washed with TGED buffer [20 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 0.01% (v/v) Triton X-100, 100 mM NaCl, 1 mM DTT], resuspended in the same buffer supplemented with 60 mg of lysozyme and protease inhibitor cocktail Complete (Roche) and incubated for 1 hour at 37°C. Cells were passed three times through a French press operated at 13 kpsi. Preparation of a cytoplasmic fraction, incubation with immobilized and biotinylated DNA and subsequent washing and elution steps were performed as described before [251, 252]. Elution fractions were analyzed by SDS polyacrylamide gel electrophoresis and detected by silver staining. Gel slices were excised from the gel and destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate. Proteins were identified by in-gel trypsin digestion followed by liquid chromatography-mass spectrometry (LC-MS/MS) as described before [253]. For protein identification the MS

data were submitted to Mascot (version 2.1, Matrix Science, London, UK) and searched against the *B. subtilis* proteome sequence.

Flow cytometry

Overnight cultures of the *B. subtilis* reporter strain carrying the P*ykrL-gfp* fusion were diluted to an OD_{600} of 0.05, grown to exponential phase, diluted again and grown under the desired conditions. For flow cytometry, cells were washed and diluted in filter sterilized PBS buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.3) and analyzed using a BD FACSCanto (BD Biosciences) operating on an argon laser at 488 nm. The GFP signal (FL-1) was collected through a FITC filter. The photomultiplier tube voltage was set at 700 V. In each measurement, 50 000 events (cells) were counted. The data was then analyzed using WinMDI software (version 2.9; http://facs.scripps.edu/software.html).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed essentially as described before [254]. DNA probes were amplified using Phusion High-Fidelity DNA polymerase (NEB) and primers listed in Table 3: KL1-o1 and KL1-o2 primers were used to amplify DNA fragment A, KL2-o1 and KL2-o2 were used for fragment B, and KL3-f and KL3-r for fragment C. Mutated fragments, B-mut1, B-mut2 and B-mut3, were amplified using primer KL2-o1 in pair with KL2-o2-a, KL2-o2-t or KL2-o2-at, respectively. The PCR fragments were end-labeled with T4-polynucleotide kinase using [γ-^{33}P]ATP. Varying amounts of protein were mixed on ice with 5000 cpm (corresponding to approximately 4 ng) of probe DNA in binding buffer (20 mM Tris-HCl pH 8, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiotreitol, 0.05 mg/ml poly[d(I-C)], and 0.05 mg/ml BSA) and subsequently incubated for 30 min at room temperature. Glycerol was added to a final concentration of 10% and the samples were loaded on a nondenaturing 6% polyacrylamide gel. Gels were run in 1 × TAE buffer (40 mM Tris-acetate pH 8.0, 2 mM EDTA) at 100 V for 45 or 55 min depending on the probe size, and dried in a vacuum gel dryer (Bio-Rad). Radioactivity was visualized using phosphor-screens and a Cyclone PhosphorImager (Packard). In all the EMSA experiments, *secA* promoter (P*secA*) amplified with primers SQ_PsecA_o1 and SQ_PsecA_o2 was used as a negative control.

Protein-DNA binding affinities (dissociation constant K_d) were determined by fitting a binding kinetics to the data derived from radiographs. Bound and unbound fractions were determined by measuring band intensities using ImageJ software (version 1.44i; National Institutes of Health, USA, http://rsb.info.nih.gov/nih-image/).

YkrK binding motif search

In order to find a YkrK binding motif, the intergenic sequences between *ykrK* and *ykrL* homolog genes in *B. subtilis, B. amyloliquefaciens*, *B. licheniformis*, *B. halodurans* and *B. pumilus* were compared using Motif Sampler tool [238]. The motif weight matrix was generated using Genome2D [255].

DNA microarray analysis

The transcriptional profile of *B. subtilis* NZ8900 carrying pNZ-ykrK was compared to the profile of strain NZ8900 strain carrying the empty vector pNZ8902. For *ykrK* induction, SURE expression system was used [135]. Strains were grown in TY broth and at OD_{600} of 0.8, 0.1% subtilin was added to both cultures. After 30 minutes further incubation, 10 OD units of each culture was collected for RNA isolation. The microarray experiment was performed in three biological replicates and one technical replicate.

RNA isolation, amino allyl-modified cDNA synthesis, cDNA labeling with Cy3 and Cy5 dyes, hybridization to oligonucleotide microarrays, washing, scanning, image analysis and normalization of data as well as statistical analysis were performed as described

previously [167]. The complete microarray data is available at GEO data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) under accession number GSE33456.

Transposon mutagenesis

Detection of transposition events. Random mutagenesis of the *B. subtilis* strain HT400 was performed using the TnYLB-1 transposon following the protocol described before [256] with modifications. The HT400 strain was transformed with pMarA carrying the TnYLB-1 transposon. Transformants were selected on plates for erythromycin resistance at 30°C, permissive for plasmid replication. A positive clone was grown for three hours at 30°C, whereupon the temperature was shifted to 45°C (nonpermissive temperature) and grown for additional 4 hours. Proper dilutions were plated on TY agar containing 5 μg/ml kanamycin and 0.01% X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma) and incubated at 45°C. To confirm that the transposition event was efficient, the same dilutions were plated on TY agar containing 1 μg/ml erythromycin.

Identification of transposon insertion sites. 1 μg of chromosomal DNA from transposon mutants was digested with *Taq*I or *Hind*III, purified and circularized in a ligation reaction using T4 DNA ligase. 100 ng of ligated DNA was used as a template for

inverse-PCR (iPCR) using primers oIPCR1 and oIPCR2 [256]. iPCR products were purified and sequenced with oIPCR3 primer [256] by ServiceXS (Leiden, The Netherlands).

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CHAPTER 4

Determination of the *Bacillus subtilis* PrsA chaperone/foldase localization pattern and assessment of the cell wall synthesis defect in a *prsA* mutant

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The PrsA protein is a membrane-anchored peptidylprolyl *cis-trans* isomerase (PPIase) in *Bacillus subtilis* and most other Gram-positive bacteria. It catalyzes the post-translocational folding of exported proteins and is essential for normal growth of *B. subtilis*. Here, the mechanism behind this indispensability is studied. A viable *prsA* null mutant could be constructed in the presence of a high concentration of magnesium. Van-FL labeling of peptidoglycan precursors in PrsA-depleted and *prsA* mutant cells, and fluorescence microscopy revealed changes in cell morphology as well as a defect in cell wall biosynthesis. Immunostaining of cells carrying PrsA-Myc showed a helical pattern of PrsA localization in the lateral cell membrane. The results presented in this chapter were a contribution to a broader study where it was shown that PrsA is essential for normal growth as folding of penicillin-binding proteins (PBPs) that are involved in the lateral cell wall synthesis is dependent on this PPIase.

Introduction

Intracellular folding of a protein into a native functional structure is assisted by molecular chaperones and foldase enzymes. A class of foldases ubiquitous in all types of cells and cell compartments is formed by peptidyl-prolyl *cis-trans* isomerases (PPIases), which catalyze the isomerization of peptide bonds immediately preceding proline residues [257–259].

PrsA is a lipoprotein bound to the outer face of the cytoplasmic membrane in *Bacillus subtilis* and other Gram-positive Firmicutes [130, 260]. It consists of a diacylglycerol membrane anchor, a large functionally unknown N-terminal domain, followed by a PPIase domain and a small functionally unknown C-terminal domain, all of which were shown to be essential for PrsA function [260]. *B. subtilis* PrsA exhibits PPIase activity but may also have a chaperone-like activity *in vivo* [260]. Several extracellular proteins in various Gram-positive bacteria are secreted or maturated in a PrsA-dependent manner [130–132, 139, 261–265]. Overexpression of PrsA enhances α-amylase secretion from *Bacillus* and *Lactococcus lactis* cells including the biotechnically important thermoresistant AmyL α-amylase of *Bacillus licheniformis* [130–132, 262]. Some other extracellular proteins are also secreted at increased levels from PrsA overexpressing cells [127, 266]. In *B. subtilis*, PrsA is an essential cell component in normal growth conditions indicating that it has an indispensable role in protein folding at the membrane/cell wall interface [131]. In contrast to the rod-shaped *B. subtilis*, PrsA is a dispensable protein in several cocci, *L. lactis* [261], *Streptococcus pyogenes* [263] and *Staphylococcus aureus* (Vitikainen *et al.*, unpublished).

In this study our purpose was to identify the indispensable cell components which are folded in a PrsA-dependent manner and to elucidate why PrsA is an essential protein in the rod-shaped bacterium *B. subtilis,* but non-essential in cocci. A hypothesis explaining this difference could be that PrsA catalyzes the folding of a protein(s) involved in the biosynthesis of the cylindrical (lateral) cell wall and determination of the rod cell shape. The bacterial cell shape is maintained by a peptidoglycan cell wall (murein sacculus) and the actin-like proteins Mbl, MreB and MreBH, which form helical cables (cytoskeleton) that encircle the cell immediately beneath the cell membrane [243, 267–271]. The rod shape of *B. subtilis* is also dependent on other proteins including MreC and MreD, which are membrane proteins and interact with each other and Mbl [272, 273]. In the absence of any of these Mre proteins, cells are spherical or aberrant in shape or non-viable in normal growth conditions [243, 267, 274]. Studies on the cell shape determination of *Caulobacter crescentus* and *B. subtilis* have also shown that MreB,

MreC and MreD interact with penicillin-binding proteins (PBPs) [243, 273, 275, 276]. Peptidoglycan precursors are incorporated into the wall at distinct sites organized in a helical pattern along the lateral wall [277–279]. The Mre proteins and two PBP1-associated cell division proteins, EzrA and GpsB, are involved in the determination of the spatial organization and dynamics of the peptidoglycan synthesis [243, 280].

PBPs are membrane-bound transglycosylase and transpeptidase enzymes which use peptidoglycan precursors to synthesize peptidoglycan chains and cross-link adjacent glycan chains to form a murein sacculus [281, 282]. The *B. subtilis* genome sequence has revealed 16 PBP-encoding genes, many of which are functionally redundant [283]. The PBPs have several distinct localization patterns in the cell suggesting dedicated functional roles for them in cell wall growth or cell division [283]. The PBP3 and PBP4a monofunctional transpeptidases and the PBP5 D-alanyl-D-alanine carboxypeptidase are localized in distinct spots or bands in the region of the lateral cell wall, suggesting their involvement in the elongation of the lateral wall. The bifunctional PBP1 is involved in the growth of both the lateral wall and the septum [243, 280, 283, 284]. The PBP2a and PbpH transpeptidases have activity in the lateral wall synthesis and rod-shape determination [285], whereas the septal localization of PBP2b suggests a specific role for this transpeptidase in cell division [283].

MreC and PBPs possess a relatively high number of proline residues (about 3% of its amino acid residues) and the functional domains of these proteins are localized in the same cell compartment as PrsA suggesting that their folding could be dependent on PrsA. In the study presented in this chapter, labeling of peptidoglycan precursors with the fluorescent antibiotic vancomycin (Van-FL) was used to characterize the cell wall biosynthesis defect of PrsA-depleted and *prsA* mutant cells. Also the PrsA localization pattern along the cell membrane was assessed with immunostaining with Cy3-labelled antibodies. The results shown in this chapter, in combination with other results (membrane proteome analyses, MreC and PBPs stability in PrsA-depleted cells study as well as better characterization of the cell wall biosynthesis defect in PrsA-depleted cells and the functional role of PrsA in cell shape determination using various methods) published by Hyyryläinen *et al*. [286], showed that several PBPs are folded in a PrsA-dependent manner, suggesting that this is the likely cause for the growth arrest in the absence of PrsA.

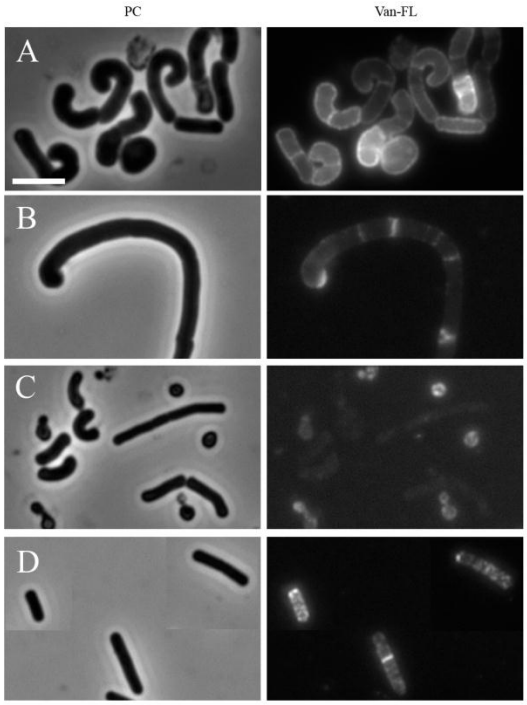

Figure 1. Van-FL staining of *B. subtilis* strain IH9024 (Δ*prsA*) (A-C) and 168 (D). (**A** and **D**) Logarithmic growth phase, (**B**) stationary phase, (**C**) late stationary phase. Left panels – phase contrast images, right panels – Van-FL staining. Scale bar represents 6 μm (the same for all the images).

Results

Depletion or deletion of PrsA affects lateral cell wall biosynthesis

The *prsA* null mutant (IH9024) could be constructed on plates supplemented with magnesium [286]. The Δ*prsA* strain grew on Antibiotic Medium 3 agar plates supplemented with 20 mM $MgCl_2$ forming very small homogenous colonies. Microscopic inspection of cells in corresponding liquid cultures showed the presence of large deformed cells in exponentially growing cultures, which changed in overnight grown cultures to small motile coccoid cells, short bent rods, which were thinner than those in the exponential growth phase, and fairly normal-looking rod-shaped cells (Fig. 1). The appearance of viable coccoid *prsA* null mutant cells corroborates the evidence on the involvement of PrsA in lateral cell wall biosynthesis and cell elongation, which was suggested by the fact that the stability of several PBPs involved in the lateral cell wall synthesis is dependent on PrsA [286]. However, the presence of some rod-shaped bacteria in overnight cultures suggests that they were capable of synthesizing the cylindrical lateral wall in the absence of PrsA. Any fast-growing suppressors that would

have overgrown the more slowly growing Δ*prsA* mutant on plates were not observed [286].

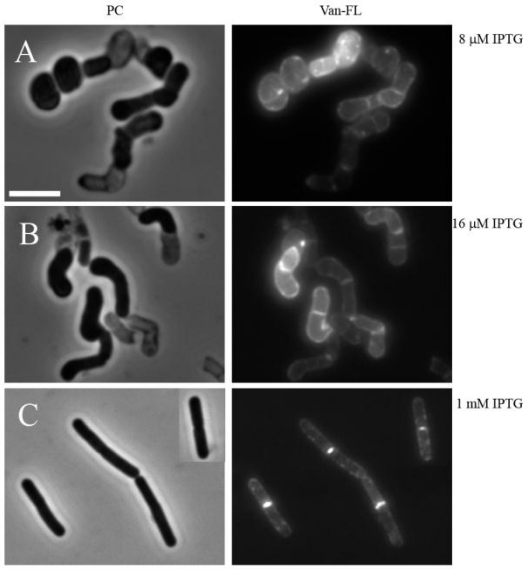

Figure 2. Van-FL staining of *B. subtilis* strain IH7211 (P*spac-prsA*). *prsA* expression was induced with 8 μM IPTG (**A**), 16 μM IPTG (**B**) and 1 mM IPTG (**C**). Left panels – phase contrast images, right panels – Van-FL staining. Scale bar represents 6 μm (the same for all the images).

Van-FL imaging of the cell wall defect of *prsA* mutants

The cell wall defect was further characterized by imaging peptidoglycan biosynthesis in the *prsA* null mutant (IH9024) and PrsA-depleted cells (IH7211) of *B. subtilis* with fluorescent vancomycin (Van-FL) (Figs 1 and 2). Van-FL binds to the terminal D-Ala-D-Ala moieties in non-cross-linked peptidoglycan precursors and growing glycan chains [277, 278]. Using Van-FL staining and fluorescence microscopy, it has been shown that lateral wall peptidoglycan polymers are synthesized in distinct spots organized in a spiral pattern [277, 278]. PBPs that are located in the lateral wall in a similar spiral organization pattern are responsible for the synthesis of the lateral wall peptidoglycan. On the other hand, PBPs that normally synthesize the division septum are also capable of synthesizing lateral wall peptidoglycan [277]. In cells of *B. subtilis* 168 and IH7211 (P*spac-prsA*) induced with 1 mM IPTG, fluorescence was mainly seen in the division septum, but the spiral synthesis pattern of lateral wall peptidoglycan was also observed (Figs 1D and 2C, right panels, respectively). The *prsA* null mutant and PrsA-depleted cells of IH7211 (P*spac-prsA* induced with 8 mM and 16 mM IPTG) were more intensively fluorescent than wild-type and non-depleted cells. In thick rods and spherical severely PrsA-depleted cells, fluorescence was strongly increased in the entire wall. This result suggests that peptidoglycan (lipid II) precursors are more abundant in

the membrane of PrsA-depleted than nondepleted cells and distributed evenly around whole deformed cells. Stationary-phase cells of the *prsA* null mutant, including the small cocci-like ones (Fig. 1B and C), were less fluorescent than the deformed exponential-phase cells.

PrsA is localized in spots with a spiral-like pattern of organization along the cell membrane

In order to find out whether the PrsA lipoprotein is distributed evenly around the cell membrane or in an uneven manner like MreC and several PBPs [272, 283], we constructed the *B. subtilis* IH8478 strain which expresses PrsA modified with a C-terminal Myc-tag (PrsA-Myc). This strain was subjected to the immunofluorescence procedure (see Materials and Methods) in which PrsA-Myc was stained with anti-c-Myc antibodies, secondary antibodies conjugated with biotin and ExtrAvidin conjugated with Cy3. The stained PrsA-Myc was visualized by fluorescence microscopy. The localization pattern of PrsA-Myc was determined both in cells from the exponential and stationary phase of growth (Fig. 3). Specificity of anti-c-Myc antibodies used as primary antibodies in the immunofluorescence technique was shown with Western blotting (Fig. 4). The fluorescence images showed that PrsA is not distributed evenly in the membrane but it is localized in distinct spots that are lined up in spirals (Fig. 5A). This pattern is stable throughout vegetative growth until stationary phase. However, the spiral structures are better resolved in exponentially growing cells than in stationary phase cells. To show specific binding of the antibodies used for immunostaining and to exclude false PrsA-Myc staining result, *B. subtilis* strain 168 (PrsA) was used as a negative control. Indeed, no Cy3 signal was detected in the control strain (Fig. 5B).

Discussion

PrsA peptidyl-prolyl *cis-trans* isomerase has an essential role in extracytoplasmic protein folding in rod-shaped bacteria. The localization of the enzyme domain at the membrane/cell wall interface suggests that PrsA may assist the folding of membrane proteins which have large functional domains on the outer surface of the membrane. The work presented in this chapter was a contribution to a broader study, where the mechanism of PrsA indispensability was revealed [286].

The morphological changes of *B. subtilis* cells in the absence of PrsA suggest involvement of PrsA in lateral cell wall biosynthesis. PrsA-depleted cells are severely

deformed, spherical in shape. Also small cocci-like cells were observed in stationary phase cultures of the *prsA* null mutant. In the wild type-like rods of the *prsA* null mutant, the lateral wall synthesis may have been restored by some compensation mechanism for instance a secondary suppressor mutation [286]. A high concentration of magnesium restores the growth of mutants with defects in different aspects of cell wall synthesis (Δ*ponA*, Δ*mreB*, Δ*mreC*, Δ*mreD*) [274, 287]. Similarly, magnesium restored the growth of the PrsA-depleted strain IH7211. Magnesium probably stabilizes peptidoglycan enabling the bacterium to maintain its rod shape (thick) at very low PrsA levels [286].

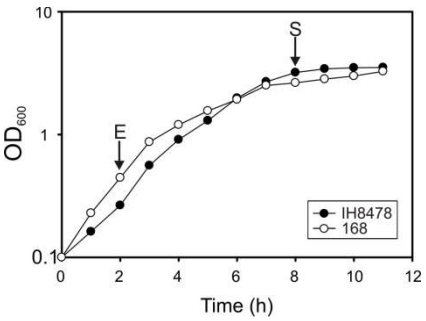

Figure 3. Growth curves of *B. subtilis* IH8478 (*prsA-myc*) and 168. Arrows labeled "E" and "S" indicate sample collection for Western blotting and immunofluorescence procedure (see following figures). E, exponential growth phase; S, stationary growth phase.

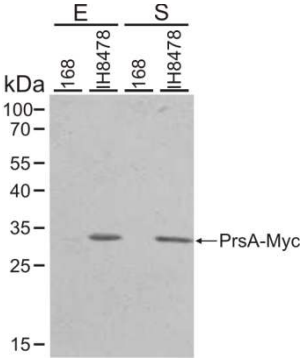

Figure 4. Western blot detection of PrsA-Myc using anti-c-Myc and anti-mouse-HRP antibodies. Samples were collected from *B. subtilis* IH8478 (*prsA-myc*) and 168 (negative control) cultures at exponential (E) and stationary (S) growth phase.

As shown by Van-FL staining, peptidoglycan biosynthesis was impaired in PrsA-depleted cells and in the *prsA* null mutant. In contrast to wild type cells, Van-FL stained strongly Δ*prsA* and PrsA-depleted exponential-phase cells and the fluorescence was fairly uniformly distributed around the whole cell membrane. The increased number of remaining pentapeptide side-chains and their even distribution in the wall might explain the increased Van-FL staining. An alternative hypothesis might be that the level of membrane-bound peptidoglycan precursors was increased in these deformed cells and that the precursors either moved freely in the membrane or were translocated uniformly across the membrane.

The localization of PrsA in the membrane was also determined using the immunofluorescence technique by taking advantage of a *B. subtilis* strain that expresses Myc-tagged PrsA. Because the *prsA-myc* fusion gene is present as a single copy in the chromosome and under the control of the native *prsA* promoter, artifacts due to

overproduction were avoided. The advantage of the immunofluorescence technique over translational fusions to a fluorescent protein is the very small size of Myc tag. Moreover, a fluorescent protein, like for instance GFP, folds properly only in the cytoplasm and is not fluorescent on the *trans* side of the membrane [288–290]. The results showed that PrsA is not randomly distributed in the membrane, but is localized to the lateral cell membrane in which it forms distinct spots organized in a helical pattern. To our knowledge this is the first lipoprotein which has been shown to have a helical organization pattern. The helical organization of PrsA raises the question whether it is associated with any of the other proteins with a similar organization pattern including the cell-shape determining proteins and PBPs [243, 267, 268, 270–272, 283]. The helical pattern might be dependent on them. It has been shown that a cytoskeleton protein, MreBH, can determine the helical organization of a protein, LytE, on the extracytoplasmic side of the membrane [291].

Another finding published by Hyyryläinen *et al.* [286] which supports the hypothesis of PrsA being required for lateral cell wall synthesis is that the folding and stability of those PBPs which are involved in the lateral cell wall synthesis (PBP2a, PBP2b, PBP3, PBP4) are dependent on PrsA. The primary reason for the growth inhibition and cell wall synthesis defect in PrsA-depleted or *prsA* mutant cells is probably insufficient amounts of active PBPs.

Despite of the rod cell shape, *Corynebacteria* such as *Corynebacterium glutamicum* and *Corynebacterium diphtheriae*, which belong to Gram-positive bacteria, do not possess PrsA [292]. Obviously PrsA-like foldases/chaperones are not needed to synthesize the lateral cell wall and maintain the rod cell shape of these bacteria. The PrsA-independency may be due to the different mode of lateral cell wall synthesis as compared to *B. subtilis* and most probably other rod-shaped Firmicutes. In *C. glutamicum*, peptidoglycan is incorporated into the wall via cell poles in a manner dependent on the DivIVA protein [277, 293]. PrsA is also dispensable in cocci [261, 263] probably because in cocci peptidoglycan is assembled at the division septum and the hemispherical poles derived from it [294].

The PrsA foldase/chaperone catalyzes post-translocational folding of exported proteins [54, 260, 295]. Overexpression of PrsA enhances secretion of some extracellular proteins, particularly α-amylases of *Bacillus sp*. from industrial Gram-positive bacteria [130–132, 262, 266]. Therefore, PrsA is an important tool for increasing yields in industrial protein production. Now, we have shown that PrsA also has a housekeeping role in the cell – it is required directly or indirectly for PBP folding and lateral cell wall biosynthesis. Since many important current antibiotics, e.g., β-lactams, exert their

antimicrobial effect by inhibiting PBPs, our results suggest that inhibiting PrsA might be an alternative way to inhibit cell wall biosynthesis of pathogenic rod-shaped bacteria and treat infectious diseases.

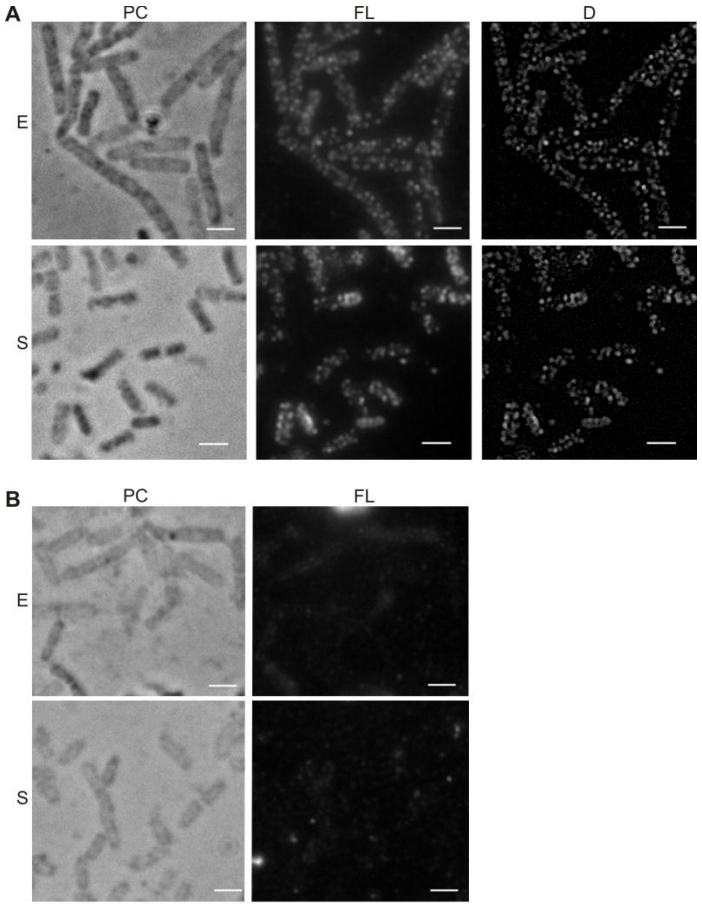

Figure 5. Immunolocalization of PrsA-Myc in *B. subtilis* cells. (**A**) Immunolocalization of PrsA-Myc in *B. subtilis* IH8478; (**B**) negative control of immunostaining, *B. subtilis* 168. Samples for immunofluorescence microscopy were collected at exponential (E) and stationary (S) growth phase. PC, phase contrast pictures; FL, fluorescence pictures of Cy3-stained cells; D, fluorescence pictures after deconvolution. Scale bar – 2 µm. Immunofluorescence procedure has been described in Materials and Methods.

Materials and Methods

Strains and growth conditions

The *B. subtilis* strains used in this study are listed in Table 1. Strains were grown in TY liquid medium with shaking or on TY agar plates at 37°C. Bacteria were cultivated in

Antibiotic medium 3 (Difco) when the effect of magnesium on PrsA-depleted cells and Van-FL staining of peptidoglycan synthesis in *prsA* null mutant were studied. When needed, the growth media were supplemented with appropriate antibiotics: 5 μg/ml chloramphenicol, 1 μg/ml erythromycin. The expression of P*spac-prsA* was induced with 1 mM (full induction) or with 8 or 16 μM (PrsA depletion) IPTG.

Table 1. Strains used in this study.

Strain	Description	Reference
B. subtilis		
168	*trpC2*	[1]
IH9024	168 Δ*prsA*	[286]
IH7211	168 *prsA*::pKTH3384 *Pspac-prsA*	[131]
IH8478	168 *prsA*::pMUTIN-cMyc *prsA-myc*	[286]

Van-FL staining

B. subtilis strains 168 and IH9024 (Δ*prsA*) were grown overnight in 10 ml Antibiotic medium 3 (Difco) supplemented with 20 mM MgCl2 and chloramphenicol 5 μg/ml when needed. Overnight cultures were diluted in 20 ml fresh medium to OD_{600} = 0.1 and grown 24 hours. Cells were collected for Van-FL staining at exponential, stationary and late stationary phase. *B. subtilis* strain IH7211 (P*spac-prsA*) was grown overnight on a TY agar plate supplemented with 1 μg/ml erythromycin. Material from a plate was suspended in phosphate-buffered saline (PBS) to OD_{600} = 1.0 and washed three times with 1 ml PBS. 20 μl of the suspension was used to inoculate 10 ml TY containing 1 μg/ml erythromycin and IPTG at three different concentrations: 8 μM, 16 μM and 1 mM. At OD_{600} = 0.6, samples were collected for Van-FL staining.

0.5 ml of a culture was incubated 20 minutes with 1 μg/ml fluorescently labeled vancomycin (BODIPY® FL vancomycin, Invitrogen) mixed in 1:1 ratio with unlabeled vancomycin (Sigma). The cells were spotted on microscope slides (Knittel Gläser, Germany). The Van-FL stained cells were viewed immediately under the fluorescence microscope (Olympus IX71) equipped with a Cool Snap HQ2 camera (Photometrics). Van-FL fluorescence was visualized with a bandpass 470/40 nm excitation filter and a bandpass 525/50 nm emission filter. Images were analyzed using ImageJ (http://rsb.info.nih.gov/nih-image/) and Adobe Photoshop CS2 Version 9.0.

Immunofluorescence

Single colonies of *B. subtilis* 168 and IH8478 (*prsA-myc*) grown on TY agar plates were used to inoculate 5 ml TY liquid medium. Overnight cultures were diluted in 20 ml TY medium to OD_{600} = 0.1 and grown till stationary phase. TY medium was supplemented with erythromycin to a final concentration of 1 μg/ml when needed. Samples for

immunofluorescence assay were collected at exponential and stationary growth phase. Immunofluorescence staining was performed according to the method described by Harry and collaborators [296] with modifications described below.

Cells were fixed and permeabilized as follows. 0.5 ml of bacterial culture in TY was mixed with an equal volume of 2 × fixative solution containing 2.68% paraformaldehyde and 0.005% glutaraldehyde and incubated for 15 min at room temperature (21-23°C) and 30 min on ice. After fixation the cells were washed three times in PBS and resuspended in GTE (50 mM glucose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA). A fresh lysozyme solution in GTE was added to an aliquot of cells to a final concentration of 2 mg/ml and cells were immediately spotted on multiwell slides (MP Biomedicals, LLC) coated with 0.01% poly-L-lysine (Sigma). After 5 min incubation wells were washed with PBS and left to dry in the air.

For immunostaining, cells were blocked with blocking solution (PBS containing 2% BSA and 0.01% Tween) for 15 min at room temperature. Next, cells were incubated with mouse anti-c-Myc antibodies (Gentaur) diluted 1:1000 in the blocking solution for 1 hour at room temperature. After washing the cells 10 times with PBS, secondary anti-mouse biotin-conjugated antibody (Sigma-Aldrich) diluted 1:500 in the blocking solution was added and cells were incubated at room temperature for 1 hour. Cells were washed again 10 times with PBS and incubated 1 hour with 1:25 diluted ExtrAvidin Cy3 conjugate (Sigma-Aldrich) at room temperature in the dark. Samples were washed 10 times with PBS and mounted with Vectashield mounting medium (Vector Laboratories, Inc.). Slides were stored at -20ºC.

Sample imaging was performed using a wide-field Zeiss Axioscop50 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Princeton Instruments 1300Y digital camera. Cy3 fluorescence was visualized with a bandpass (546 ⁄ 12 nm) excitation filter, a 560 nm dichromatic mirror, and a bandpass (575–640 nm) emission filter. Images were analyzed using ImageJ (http://rsb.info.nih.gov/nih-image/) and Adobe Photoshop CS2 Version 9.0. Wide-field images were corrected for bleaching and unstable illumination using the Huygens Professional deconvolution software by Scientific Volume Imaging (http://www.svi.nl/).

Western blot

1 OD unit of a *B. subtilis* culture was harvested by centrifugation for 10 min at 5000 × g, resuspended in 150 µl of protoplast buffer (20% sucrose, 10 mM Tris-HCl pH 8.1, 10 mM EDTA, 50 mM NaCl, 2 mg/ml lysozyme) and incubated at 37ºC for 30 min. An equal volume of 2 × SDS-PAGE sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS,

1% DTT, 20% glycerol, 0.05% bromophenol blue) was added and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The immunodetection of PrsA-Myc was performed using mouse anti-c-Myc (Clontech) and anti-mouse Horseradish Peroxidase linked antibody (GE Healthcare UK, Ltd.). Subsequently, membranes were incubated with ECL detection reagent (Amersham) and proteins were visualized using Curix 60 AGFA Film Processor (Siemens AG).

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CHAPTER 5

High- and low-affinity *cre* boxes for CcpA binding in *Bacillus subtilis* revealed by genome-wide analysis

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In *Bacillus subtilis* and its relatives carbon catabolite control, a mechanism enabling to reach maximal efficiency of carbon and energy sources metabolism, is achieved by the global regulator CcpA (carbon catabolite protein A). CcpA in a complex with HPr-Ser-P (seryl-phosphorylated form of histidine-containing protein, HPr) binds to operator sites called catabolite responsive elements, *cre*. Depending on the *cre* box position relative to the promoter, the CcpA/HPr-Ser-P complex can either act as a positive or a negative regulator. The *cre* boxes are highly degenerate semi-palindromes with a lowly conserved consensus sequence. So far, studies aimed at revealing how CcpA can bind such diverse sites were focused on the analysis of single *cre* boxes. In this study, a genome-wide analysis of *cre* sites was performed in order to identify differences in *cre* sequence and position, which determine their binding affinity.

The transcriptomes of *B. subtilis* cultures with three different CcpA expression levels were compared. The higher the amount of CcpA in the cells, the more operons possessing *cre* sites were differentially regulated. The *cre* boxes that mediated regulation at low CcpA levels were designated as strong (high affinity) and those which responded only to high amounts of CcpA, as weak (low affinity). Differences in the sequence and position in relation to the transcription start site between strong and weak *cre* boxes were revealed.

Certain residues at specific positions in the *cre* box as well as, to a certain extent, a more palindromic nature of *cre* sequences and the location of *cre* in close vicinity to the transcription start site contribute to the strength of CcpA-dependent regulation. The main factors contributing to *cre* regulatory efficiencies, enabling subtle differential control of various subregulons of the CcpA regulon, are identified.

Introduction

A well-known phenomenon among bacteria is the sole utilization of the most favored carbon source (e.g., glucose, fructose or malate) over other sugars present in the environment. The regulatory mechanism coordinating the metabolism of carbon and energy sources in order to maximize the metabolic efficiency is called carbon catabolite control, i.e., carbon catabolite repression (CCR) and carbon catabolite activation (CCA). Carbon catabolite control in *Bacillus subtilis* and other low-GC Gram-positive bacteria is exerted by the CcpA protein (catabolite control protein A) [149]. CcpA is a member of the LacI/GalR family of transcriptional regulators [163] and it can act either as a positive or negative regulator of genes that are in most cases involved in carbon acquisition or metabolism [297]. CcpA is synthesized constitutively, regardless to the availability of preferred carbon sources [298], it forms a dimer [299] and its activity is modulated by a complex interaction with either one of the corepressors, HPr or Crh [158, 298–301]. In the presence of glucose or other rapidly metabolized carbon sources, the histidine-containing protein (HPr) and an HPr-like protein (Crh) are phosphorylated on a conserved serine (Ser46) residue by HPr kinase [302, 303]. Binding of the seryl-phosphorylated HPr (HPr-Ser-P) or Crh (Crh-Ser-P) to CcpA stimulates the activity of CcpA [158, 300, 301, 303]. During growth on carbohydrates there is much more HPr than Crh in the cell [304]. Notably, the Crh-specific function in the regulation of expression during growth on substrates other than carbohydrates was recently revealed [149]. Hence, Crh seems to play a secondary role in CCR. Next to HPr and Crh, low-molecular-weight molecules like NADP, glucose-6-phosphate (G6P), and fructose-1,6-bisphosphate (FBP) modulate CcpA activity by either stimulation of HPr kinase activity (FBP) [159, 160], enhancement of CcpA affinity for HPr-Ser-P (FBP) [155], triggering cooperative CcpA binding to DNA (G6P) [162], or enhancing the CcpA interaction with the transcription machinery (NADP/NADPH) [161].

CcpA binds to DNA at *cis*-acting sequences called catabolite responsive elements (*cre*) located in the promoter region or within open reading frames of the regulated genes and operons. So far more than 50 *cre* sites were identified in the *B. subtilis* genome [149]. A general rule was deduced, stating that genes with *cre* boxes located upstream of -35 sequences of the promoter are subject to activation by the CcpA complex, as shown for *ackA* [184], *pta* [185] and *ilvB* [186, 187]. However, *ackA* is cooperatively activated by CcpA and CodY [305, 306] and full activation of *ackA* requires also an additional conserved sequence present upstream of the *cre* box [307]. Moreover, the *lev* operon is subject to CcpA repression, although the *lev cre* site is located upstream of the

promoter. However, regulation of the *lev* operon involves also the LevR transcriptional activator: binding of CcpA to the *lev cre* site prevents a productive interaction between LevR and RNA polymerase [308]. Binding of CcpA to *cre* boxes overlapping the promoter leads to transcriptional repression by interfering with the transcription machinery binding, as for *amyE*, *bglP*, *cccA*, *dctP*, *glpF*, *phoP*, *acuA* [170, 171, 174, 182, 309–311]. The binding of the protein complex to *cre* boxes that are located downstream of the transcription start site blocks transcription elongation, as is the case for most of the genes and operons regulated by CcpA [149, 301].

Cre boxes are highly degenerate pseudo-palindromes with the consensus sequence WTGNNARCGNWWWCAW, where the strongly conserved residues are underlined [164–166]. Little is known about how CcpA can bind to such diverse *cre* sequences. Our hypothesis was that CcpA can bind with different affinities to *cre* boxes with particular sequence and/or position in relation to the transcription start site (TSS). In order to identify *cre* boxes with different affinities, CcpA expression was induced to three different levels using a tetracycline-dependent gene regulation system [312] and genome wide analysis of *cre* boxes was performed using transcriptome analyses combined with bioinformatics tools. High- and low-affinity *cre* boxes with subtle differences in their sequence and/or position in relation to the TSS are revealed.

Results

Tight regulation of CcpA production level

In order to enable very tight control of the CcpA expression level in *B. subtilis*, strain MP902 (P*tet-ccpA*, P*xyl-tetR*) was constructed. Strain MP902 carries the *ccpA* gene under control of the tetracycline-inducible promoter, P*tet*, integrated in the native promoter locus and the P*tet* repressor, *tetR*, under control of the xylose-inducible promoter, P*xyl*, located on the plasmid pWH119 [312]. To show tight regulation of the CcpA expression level, the MP902 strain was grown in rich TY medium [217] supplemented with 0.2% xylose and a wide range of concentrations (0.1 – 20 nM) of P*tet* inducer, anhydrotetracycline (ATc) which is a non-bacteriostatic tetracycline analog. As demonstrated in Fig. 1, the system allows obtaining several distinct expression levels of CcpA.

In order to test the influence of the different CcpA amounts in the cells on the CcpA regulon, three representative CcpA expression levels (hereafter referred to as low, medium and high) were chosen and the cultures were used for microarray experiments.

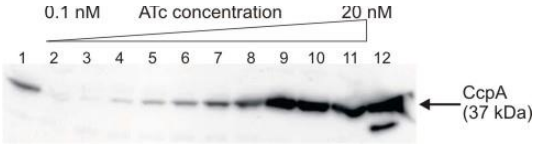

Figure 1. Tight regulation of the CcpA expression level in *B. subtilis* strain MP902 (P*tet-ccpA*, P*xyl-tetR*). Lane 1, wild type strain 168; lanes 2 – 11, MP902 grown in the presence of 0.2% xylose and increasing concentration of anhydrotetracycline (ATc): 0.1, 0.2, 04, 0.7, 1, 2, 4, 8, 10 and 20 nM, respectively; lane 12, 200 ng of purified CcpA. The representative graph of three reproducible experiments is shown.

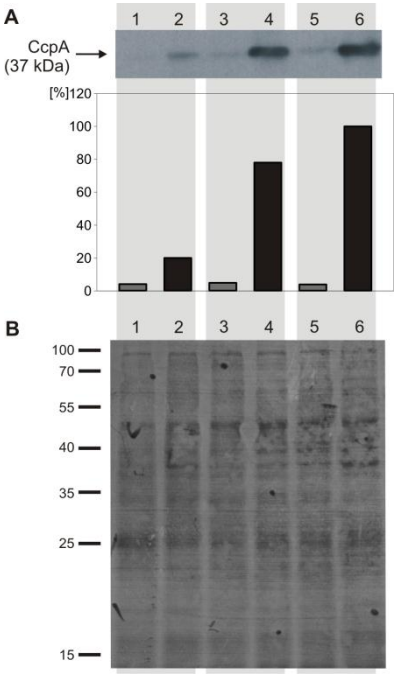

Figure 2. CcpA expression levels in *B. subtilis* cultures used for DNA microarray experiments. (**A**) *upper panel*, CcpA detection using anti-CcpA antibody; *lower panel*, signal quantification with ImageJ. Four CcpA expression levels were achieved by growing *B. subtilis* strain MP902 (P*tet-ccpA*, P*xyl-tetR*) in absence (lanes 1, 3 and 5) and in the presence of 0.1, 2 and 20 nM ATc (lanes 2, 4 and 6), respectively. All cultures were grown in the presence of 0.2% xylose and 1% glucose. Shadows in the background of the picture indicate culture pairs used in microarray experiments (**B**) Ponceau S control membrane staining for protein load verification. Lane numbers correspond to lane numbers in panel A. The representative graphs of three reproducible experiments are shown.

For transcriptome analyses, the MP902 strain was grown in rich TY medium [217], since most likely it contains inducers for secondary regulators which could hide CCR in minimal medium, and the samples were taken during exponential growth because CCR is expected to be strongest during maximal cell growth. The strain was grown in the presence of 0.2% xylose to induce TetR expression and a high concentration of glucose (1%) in order to ensure sufficient production of CcpA cofactors like HPr-Ser-P, NADP, glucose-6-phosphate (G6P) or fructose-1,6-bisphosphate (FBP) and optimal activity of CcpA. The medium was supplemented with different concentrations of ATc, exerting different CcpA production levels in the different cultures: 0.1 nM ATc (low CcpA induction level), 2 nM ATc (medium CcpA induction level) and 20 nM ATc (high CcpA induction level). The control culture was grown without ATc leading to no or

only residual CcpA production. The CcpA production levels of the different MP902 cultures used for microarray experiments were assessed by Western blotting (Fig. 2).

Effect of different CcpA amounts on gene regulation

The transcriptional profiles of exponentially growing cells of *B. subtilis* MP902 (P*tet-ccpA*, P*xyl-tetR*) grown in rich medium supplemented with glucose and xylose and expressing CcpA at low, medium and high levels (Fig. 2) due to the presence of different concentrations of the P*tet* inducer, ATc, were compared to the transcriptional profile of MP902 cells grown in the corresponding medium but without ATc (no CcpA expression induction). Our first observation was that the more CcpA present in the cells the more genes were found to be significantly regulated (Table 1). Genes were considered to be regulated if they were at least 1.8 fold up- or downregulated. When CcpA was expressed at low, medium and high levels, 128, 343 and 408 genes were found to be differentially expressed, respectively. CcpA is known to act, depending on the *cre* box position in relation to the transcriptional start site (TSS), as a repressor or activator [313–315], but many more cases of repression than of activation are known [316]. Consistently, most of the regulated genes found in the microarray analyses with different CcpA induction levels were downregulated.

Table 1. Number of analyzed predicted *cre* boxes and regulated genes in response to different CcpA expression levels.

	Level of the CcpA expression		
	Low (0.1 nM ATc)	Medium (2 nM ATc)	High (20 nM ATc)
N° of all genes	4106	4106	4106
N° of regulated genes	128	343	408
N° all predicted *cre* boxes	418	418	418
N° *cre* boxes of operons [a]	161	161	161
N° regulated operons with *cre* box [a]	30	58	67

[a] *cre* boxes within -500 and +100 nucleotides from start codon of first genes of operons.

The first genes of operons known from the literature to possess *cre* boxes (DataBase of Transcriptional Regulation in *B. subtilis*, DBTBS [317] and reviewed by Fujita [149]) and which were differentially expressed at least under the high CcpA production level were extracted from the microarray data. Since it is estimated that the CcpA regulon includes more members than known so far [149], as also shown recently [168], a prediction of putative *cre* boxes was performed. Using Genome2D [255] and a list of described *cre* boxes in the literature (reviewed by Fujita [149]) a Weight Matrix of *cre* boxes was generated: $T_1G_2A_3A_4A_5R_6C_7G_8Y_9T_{10}W_{11}W_{12}C_{13}A_{14}$. This *cre* motif was used to search the whole *B. subtilis* genome for putative *cre* boxes. As a result, 418 putative

Table 2. High- and low-affinity *cre* boxes of the first genes of operons.

	Gene	Strand	*cre* sequence	Gene expression fold change [a]			*cre* to TSS distance [b]
				Low CcpA induction	Medium CcpA induction	High CcpA induction	
High affinity cre *boxes*							
1	*acoR*	upper	TGAAAGCGCTTTAT	**-4.8**	**-18.7**	**-21.7**	-27
2	*acsA*	lower	TGAAAGCGTTACCA	**-2.3**	**-2.5**	**-2.7**	+44
3	*acuA*	upper	TGAAAACGCTTTAT	**-2.2**	**-4.6**	**-7.7**	-26
4	*amyE*	upper	TGTAAGCGTTAACA	**-2.6**	**-10.7**	**-12.9**	+4
5	*bglP*	lower	TGAAAGCGTTGACA	**-2.5**	**-4.7**	**-4.6**	-36
6	*cccA*	lower	TGTAAGCGTATACA	**-2.2**	**-1.8**	**-2.8**	-29
7	*citM*	upper	TGTAAGCGGATTCA	**-2.6**	**-2.7**	**-2.9**	+46
8	*cstA*	lower	TGAATGCGGTTACA	**-2.2**	**-1.9**	**-2.4**	+32
9	*dctP*	upper	TGAAAACGCTATCA	**-7.4**	**-12.3**	**-16.6**	-14
10	*glpF*	upper	TGACACCGCTTTCA	**-4.3**	**-21.9**	**-35.6**	-27
11	*gmuB*	upper	TGTAAGCGTTTTAA	**-3.0**	**-15.6**	**-35.8**	+6
12	*iolA-1*	lower	TGAAAGCGTTTAAT	**-1.8**	**-1.9**	**-2.1**	+93
13	*iolA-2 (iolB)*	lower	TGAAAACGTTGTCA	**-2.2**	**-2.5**	**-2.4**	+2404
14	*manR*	upper	TGTAAACGGTTTCT	**-2.0**	**-3.7**	**-8.0**	0
15	*msmX*	lower	AGAAAGCGTTTACA	**-2.0**	**-2.6**	**-3.1**	-15
16	*rbsR*	upper	TGTAAACGGTTACA	**-6.7**	**-15.2**	**-23.1**	+6
17	*rocG*	lower	TTAAAGCGCTTACA	**-2.6**	**-3.5**	**-3.1**	+43
18	*sacP*	lower	CGAAAACGCTATCA	**-2.1**	**-7.9**	**-8.1**	-19
19	*sucC*	upper	TGAAAGCGCAGTCT	**-2.0**	**-5.8**	**-3.4**	0
20	*treP*	upper	TGAAAACGCTTGCA	**-3.2**	**-13.0**	**-17.5**	+372
21	*uxaC*	upper	TGAAAGCGTTATCA	**-2.5**	**-3.7**	**-8.9**	+1237
22	*xsa*	lower	TAAAAGCGCTTACA	**-1.9**	**-1.8**	**-2.6**	+7
23	*xylA*	upper	TGGAAGCGCAAACA	**-2.4**	**-11.9**	**-11.1**	+144
24	*xynP*	upper	TGAAAGCGCTTTTA	**-4.0**	**-11.0**	**-17.9**	+230
25	*yisS*	upper	AGAAAACGCTTTCT	**-1.9**	**-3.5**	**-3.7**	+74
26	*yjmD*	upper	TGAAAGCGGTTCAA	**-2.2**	**-2.4**	**-8.8**	ND
27	*ykoM*	upper	TGCAAGGGCTTTCA	**-2.0**	**-3.4**	**-3.5**	+150
28	*yrpD*	upper	TGATAGCGTTTTCT	**-1.9**	**-8.0**	**-6.8**	+127
29	*ytkA*	lower	TGTAAGCGTTTGCT	**-1.9**	**-6.4**	**-6.8**	ND
30	*yulD*	lower	TGAAAGCGCTATCT	**-2.3**	**-4.9**	**-5.3**	ND
31	*yvfK*	lower	TTAAAGCGCTTTCA	**-4.0**	**-6.1**	**-10.6**	+5
Low affinity cre *boxes*							
1	*abnA*	lower	TGTAAGCGCTTTCT	-1.8	-1.7	**-2.5**	+85
2	*acoA*	lower	TGTAAGCGTTTGCT	-1.1	-1.0	**-1.8**	+462
3	*citZ*	lower	TGTAAGCATTTTCT	-1.5	**-1.8**	**-2.1**	+88
4	*csbX*	lower	TGAAAACGGTGCCA	-1.4	**-2.8**	**-2.1**	-401
5	*cydA*	lower	TGAAATGAATCGTT	1.6	1.0	**-2.7**	-21
6	*drm*	lower	TGAAAACGGTTTAT	-1.3	**-3.6**	**-3.2**	-16
7	*gntR-1*	upper	TGAAAGTGTTTGCA	-1.3	**-2.8**	**-3.2**	-41
8	*gntR-2*	upper	TGAAAGCGGTACCA	-1.3	**-2.8**	**-3.2**	+148
9	*hutP*	upper	TGAAACCGCTTCCA	-1.3	**-1.9**	**-2.6**	+209
10	*lcfA*	lower	TGAAAACGTTATCA	-1.4	**-2.6**	**-2.6**	+450
11	*levD*	lower	TGAAAACGCTTAAC	-1.5	-1.2	**-2.2**	-45
12	*malA*	upper	TGTAAACGTTATCA	-1.7	**-2.0**	**-2.6**	+6
13	*mleN*	lower	TGAAAGCGTTTTAG	-1.5	**-3.5**	**-2.4**	+21
14	*msmR*	upper	TGTAACCGCTTACT	-1.7	**-4.2**	**-12.2**	-28
15	*mtlR*	upper	TGAAAGCGTTTTAT	-1.5	**-2.7**	**-2.5**	-16
16	*odhA*	lower	TGGAAGCGTTTTTA	-1.6	**-6.6**	**-3.4**	+21
17	*pbuG*	upper	TGAAAACGTTTTTT	-1.1	-1.5	**-1.9**	+245
18	*pta*	lower	TGAAAGCGCTATAA	1.3	**-3.2**	**-2.7**	-55
19	*resA*	lower	TAAAAACGCTTTCT	-1.1	**-1.9**	**-1.9**	-72

Table 2. Continued.

	Gene	Strand	*cre* sequence	Gene expression fold change [a]			*cre* to TSS distance [b]
				Low CcpA induction	Medium CcpA induction	High CcpA induction	
20	*sigL*	lower	GGAAAACGCTTTCA	-1.1	**-3.1**	**-3.3**	ND
21	*wprA*	upper	TGTAAGCGGTATCT	-1.6	**-5.5**	**-4.2**	+43
22	*yckB*	lower	TGAAAACGCGATCA	-1.4	**-3.5**	**-2.1**	-48
23	*ycsA*	upper	AGAAAGCGCTTACG	-1.7	**-6.0**	**-10.3**	+67
24	*ydzA*	lower	TGAAAACGTGTCCA	-1.3	**-6.4**	**-6.4**	+9
25	*yesL*	upper	TGAAAGCGTTTTCC	-1.3	-1.6	**-2.0**	+125
26	*yfiG*	upper	AGAAAGCGGTTACA	-1.6	**-2.7**	**-4.6**	+38
27	*yncC*	upper	TGTAAACGGTTACA	-1.3	**-2.4**	**-3.8**	+84
28	*yojA*	lower	TGAAAGCGCTTTCT	1.1	-1.5	**-1.8**	+57
29	*yqgW*	upper	TGAAAACGCTATCG	-1.1	**-4.5**	**-4.2**	-39
30	*yqgY*	upper	TGAAAATGTTTACA	-1.4	**-5.4**	**-4.1**	-38
31	*ysbA*	lower	TGTAAGCGCTTTAT	1.0	**-3.8**	**-7.6**	ND
32	*ysfC*	upper	TGAAAGCGTTTTTT	-1.5	-1.5	**-2.0**	+196
33	*yugN*	lower	TGAATGCGCTTTCT	-1.7	**-2.4**	**-2.3**	ND
34	*yuxG*	lower	TGAAAACGGATACA	-1.2	**-4.2**	**-6.1**	0
35	*yvdG*	lower	TGTAACCGCTTTCT	-1.4	-1.5	**-2.1**	-28
36	*yxlH*	upper	TTGAAACGCTTTCA	-1.4	**-2.0**	**-2.3**	+260
37	*yydK*	upper	TGTAAGCGGTTTAT	-1.5	**-3.2**	**-2.4**	-21
38	*yyzE*	lower	TGAAAGCGTAACCA	-1.2	**-3.0**	**-2.1**	0
Activating cre *boxes*							
1	*ilvB*	lower	TGAAAGCGTATACA	**3.0**	**6.2**	**2.7**	+88
2	*opuE*	lower	TGAAAGCGTTTTAT	**2.3**	**2.5**	**2.3**	-103
3	*ycbP*	lower	TGAAAGCGCTCGCT	**2.5**	**3.3**	**2.6**	+30

[a] In bold – genes significantly regulated (1.8 < fold < -1.8).
[b] *cre* box distance to transcriptional start site calculated from the conserved G residue in the middle CpG of the *cre* box.

cre boxes were found: 200 in the upper and 218 in the lower strand (Table 1). Most of the predicted *cre* boxes may not be functional taking into account their large distance from the promoter. Therefore, *cre* boxes located within -500 and +100 nucleotides relative to the start codon of the first gene of an operon were extracted. There were 161 genes possessing *cre* boxes that met these criteria (Table 1). Since the search did not entirely cover the list of the known *cre* sites (for review see [149]), *cre* sites known from literature were also added to the analyzed *cre* sites. In total, there were 30, 58 and 67 operons possessing (known and predicted) *cre* sites and which were significantly downregulated under low, medium or high CcpA induction level, respectively. Three operons with known and predicted *cre* sites were activated under all these conditions (Table 2 and, in more detail, Appendix). The increase in amount of CcpA-regulated operons upon increasing amounts of CcpA indicates the presence of high-affinity *cre* boxes titrating away CcpA from the weaker *cre* boxes, which can trigger regulation of additional genes only when more functional CcpA is present in the cell. Therefore, the 31 *cre* boxes of the 30 operons (*iol operon* possesses two *cre* boxes: within *iolA* and *iolB*) repressed when CcpA was present in low amounts were designated as strong (high affinity to CcpA) and the other 38 *cre* sites of 37 operons (*gntR* possesses two *cre* sites),

which were repressed only in the presence of higher amounts of CcpA in the cells (medium and high CcpA induction levels), were designated as weak (low affinity to CcpA) (Table 2). The high- and low-affinity, and the three activating *cre* boxes (Table 2) were analyzed with respect to their sequence and their position relative to the TSS. The term 'affinity' in this study is contractual, as direct binding assays were not performed in this study, and it is used to denote hierarchy in CcpA target genes regulation. From other (mutational) studies it is however apparent that strong regulation commonly coincides with high affinity and vice versa, so the term affinity appears to be adequate to describe differences in strong or weak regulation.

Analysis of *cre* box affinities in relation to their sequence

In order to detect differences within the sequence between different *cre* boxes, which putatively determine the *cre* box affinity, separate Weight Matrices for high- and low-affinity *cre* boxes that are responsible for gene repression were generated using Genome2D [255] (Fig. 3). The resulting consensus sequences are $T_1G_2A_3A_4A_5G_6C_7G_8C_9T_{10}T_{11}T_{12}C_{13}A_{14}$ and $T_1G_2A_3A_4A_5R_6C_7G_8Y_9T_{10}T_{11}T_{12}C_{13}W_{14}$, for strong and weak *cre* boxes, respectively. *Cre* boxes from both groups have very conserved G_2, C_7 and G_8 residues, as in *cre* motifs proposed before [164–166]. Although the differences between high- and low-affinity *cre* are not very pronounced, the *cre* boxes with high affinity to CcpA seem to have a more conserved sequence around the middle CpG (conserved GCpGC instead of RCpGY) and at the C_{13} and A_{14} positions (Fig. 3). To analyze the differences in the *cre* sequences in more detail, the high- and low-affinity *cre* boxes were aligned. The alignments show that the strong *cre* boxes (Table 3) have, on average, more palindromic residues than the weak *cre* boxes (Table 4) particularly at the external residues and in the middle CpG.

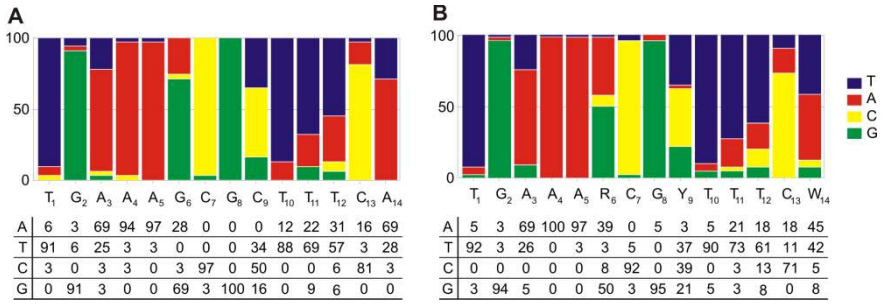

Figure 3. Analysis of high- (A) and low-affinity (B) *cre* boxes responsible for gene repression. Weight Matrix (*upper panels*) and *cre* box consensus with Position Frequency Matrix (PFM) (*lower panels*). In the consensus sequence: R is A or G, Y is T or C.

Table 3. Analysis of *cre* boxes with apparent high affinity to CcpA. *Cre* boxes of repressed genes are aligned.

Gene	Cre sequence														Score [a]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
acoR	T	G	A	A	A	G	C	G	C	T	T	T	A	T	5
acsA	T	G	A	A	A	G	C	G	T	T	A	C	C	A	4
acuA	T	G	A	A	A	A	C	G	C	T	T	T	A	T	4
amyE	T	G	T	A	A	G	C	G	T	T	A	A	C	A	5
bglP	T	G	A	A	A	G	C	G	T	T	G	A	C	A	4
cccA	T	G	T	A	A	G	C	G	T	A	T	A	C	A	5
citM	T	G	T	A	A	G	C	G	G	A	T	T	C	A	4
cstA	T	G	A	A	T	G	C	G	G	T	T	A	C	A	4
dctP	T	G	A	A	A	A	C	G	C	T	A	T	C	A	5
glpF	T	G	A	C	A	C	C	G	C	T	T	T	C	A	5
manR	T	G	T	A	A	A	C	G	G	T	T	T	C	T	4
iolA-1	T	G	A	A	A	G	C	G	T	T	T	A	A	T	3
iolA-2	T	G	A	A	A	A	C	G	T	T	G	T	C	A	6
msmX	A	G	A	A	A	G	C	G	T	T	T	A	C	A	4
rbsR	T	G	T	A	A	A	C	G	G	T	T	A	C	A	6
rocG	T	T	A	A	A	G	C	G	C	T	T	A	C	A	5
sacP	C	G	A	A	A	A	C	G	C	T	A	T	C	A	4
sucC	T	G	A	A	A	G	C	G	C	A	G	T	C	T	4
treP	T	G	A	A	A	A	C	G	C	T	T	G	C	A	5
uxaC	T	G	A	A	A	G	C	G	T	T	A	T	C	A	5
xsa	T	A	A	A	A	G	C	G	C	T	T	A	C	A	5
xylA	T	G	G	A	A	G	C	G	C	A	A	A	C	A	4
xynP	T	G	A	A	A	G	C	G	C	T	T	T	T	A	6
ydhM	T	G	T	A	A	G	C	G	T	T	T	T	A	A	4
yisS	A	G	A	A	A	A	C	G	C	T	T	T	C	T	6
yjmD	T	G	A	A	A	G	C	G	G	T	T	C	A	A	4
ykoM	T	G	C	A	A	G	G	G	C	T	T	T	C	A	5
yrpD	T	G	A	T	A	G	C	G	T	T	T	T	C	T	4
ytkA	T	G	T	A	A	G	C	G	T	T	T	G	C	T	4
yulD	T	G	A	A	A	G	C	G	C	T	A	T	C	T	5
yvfK	T	T	A	A	A	G	C	G	C	T	T	T	C	A	6
palindrome % [b]	68	71	52	61	84	32	97	97	32	84	61	52	71	68	Average score = 4.6

[a] In bold – palindromic residues.
[b] Score - a number of palindromic pairs.
[c] Occurrence of palindromic residue at each position.

The *cre* sites of the genes that were activated in this study (*ilvB*, *opuE* and *ycbP*) were not included in the Weight Matrix generation nor *cre* alignment as *cre* boxes that are responsible for gene expression activation may need additional (upstream) sequences, as shown for instance for *ackA* [307]. Moreover, their sequence might putatively differ from the repressing *cre* sites, but the population of activating *cre* sites is too small to perform statistically significant analysis. However, taking into account the fact that all three genes that were activated in the microarray experiments in this study are regulated

already in the presence of low amounts of CcpA in the cell, the activating *cre* sites seem to take a higher place in the hierarchy of the genes regulated by CcpA. Additionally, the *cre* sites of *ilvB* and *ycbP* appear to match the consensus of the high-affinity *cre* boxes better compared to the consensus of low-affinity *cre* boxes (Table 2).

Table 4. Analysis of *cre* boxes with apparent low affinity to CcpA. *Cre* boxes of repressed genes are aligned.

Gene	Cre sequence														Score [a]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
abnA	T	G	T	A	A	G	C	G	C	T	T	T	C	T	5
acoA	T	G	T	A	A	G	C	G	T	T	T	G	C	T	4
citZ	T	G	T	A	A	G	C	A	T	T	T	T	C	T	3
csbX	T	G	A	A	A	A	C	G	G	T	G	C	C	A	4
cydA	T	G	A	A	A	T	G	A	A	T	C	G	T	T	2
drm	T	G	A	A	A	A	C	G	G	T	T	T	A	T	4
gntR-1	T	G	A	A	A	G	T	G	T	T	T	G	C	A	4
gntR-2	T	G	A	A	A	G	C	G	G	T	A	C	C	A	4
hutP	T	G	A	A	A	C	C	G	C	T	T	C	C	A	5
lcfA	T	G	A	A	A	A	C	G	T	T	A	T	C	A	6
levD	T	G	A	A	A	A	C	G	C	T	T	A	A	C	3
malA	T	G	T	A	A	A	C	G	T	T	A	T	C	A	5
mleN	T	G	A	A	A	G	C	G	T	T	T	T	A	G	4
msmR	T	G	T	A	A	C	C	G	C	T	T	A	C	T	5
mtlR	T	G	A	A	A	G	C	G	T	T	T	T	A	T	4
odhA	T	G	G	A	A	G	C	G	T	T	T	T	T	A	4
pbuG	T	G	A	A	A	A	C	G	T	T	T	T	T	T	5
pta	T	G	A	A	A	G	C	G	C	T	A	T	A	A	5
resA	T	A	A	A	A	A	C	G	C	T	T	T	C	T	4
sigL	G	G	A	A	A	A	C	G	C	T	T	T	C	A	5
wprA	T	G	T	A	A	G	C	G	G	T	A	T	C	T	3
yckB	T	G	A	A	A	A	C	G	C	G	A	T	C	A	4
ycsA	A	G	A	A	A	G	C	G	C	T	T	A	C	G	5
ydzA	T	G	A	A	A	A	C	G	T	G	T	C	C	A	5
yesL	T	G	A	A	A	G	C	G	T	T	T	T	C	C	4
yfiG	A	G	A	A	A	G	C	G	G	T	T	A	C	A	4
yncC	T	G	T	A	A	A	C	G	G	T	T	A	C	A	6
yojA	T	G	A	A	A	G	C	G	C	T	T	T	C	T	6
yqgW	T	G	A	A	A	A	C	G	C	T	A	T	C	G	4
yqgY	T	G	A	A	A	A	T	G	T	T	T	A	C	A	5
ysbA	T	G	T	A	A	G	C	G	C	T	T	T	A	T	4
ysfC	T	G	A	A	A	G	C	G	T	T	T	T	T	T	4
yugN	T	G	A	A	T	G	C	G	C	T	T	T	C	T	5
yuxG	T	G	A	A	A	A	C	G	G	A	T	A	C	A	4
yvdG	T	G	T	A	A	C	C	G	C	T	T	T	C	T	5
yxlH	T	T	G	A	A	A	C	G	C	T	T	T	C	A	4
yydK	T	G	T	A	A	G	C	G	G	T	T	T	A	T	3
yyzE	T	G	A	A	A	G	C	G	T	A	A	C	C	A	3
palindrome % [b]	43	68	40	70	88	33	90	90	33	88	70	40	68	43	Average score = 4.3

[a] In bold – palindromic residues.
[b] Score - a number of palindromic pairs.
[c] Occurrence of palindromic residue at each position.

Analysis of the influence of relative *cre* box position on regulation

To find out whether the *cre* box position in relation to the promoter plays a role in determining the affinity to CcpA, the distance between *cre* boxes and the corresponding transcription start sites (TSS) was analyzed. The TSS of the regulated genes possessing a *cre* box were extracted from the literature or, when this information was lacking, predicted in this study (Table 2 and Appendix). The calculated *cre* to TSS distance (counting form the conserved G residue in the middle of the *cre* boxes to the TSS) was plotted against expression level fold change of the regulated genes under high levels of CcpA, separately for the genes with either high (Fig. 4A) and low affinity *cre* boxes (Fig. 4B). The majority of high affinity *cre* boxes are localized in close vicinity to the TSS (*cre*-TSS distance from 0 to +7, that is a TSS within the *cre* box) and around positions -27, -14 and +44. Repression of the genes with *cre* sites located with increment of approximately 10 - 11 nt (full helix turn) was significantly stronger, such as found for *cre* boxes of *acoR*, *glpF*, *dctP*, *gmuB*, *xynP*, *treP*, which are localized at positions -27, -27, -14, +6, +230, +372, respectively. Further downstream from the TSS, there are more low affinity *cre* boxes than high affinity ones.

Discussion

CcpA is a global regulator of carbon catabolism [297] controlling expression of genes by binding to cognate operator sequences, *cre*, which is characterized by a low-conserved consensus sequence [164–166]. Hence, it seems possible that CcpA binds some *cre* sites with higher affinity than others. So far, the global studies of CcpA-dependent carbon catabolite repression were focused on identification of the members of the CcpA regulon [167, 168, 316], while the analysis of *cre* boxes in respect to their sequences, position and affinities in CcpA binding have been focused only on single examples [164, 165, 184, 301, 318]. A broader comparison of 32 *cre* boxes sequences and function was published by Miwa Y. *et al.* and it was deduced that a lower mismatching of *cre* sequences to the query sequence in the same direction as that of transcription of the target genes and a more palindromic sequence of *cre* boxes are desirable for their better function [165]. The goal of our study was to perform a genome-wide analysis of *cre* boxes in order to reveal *cre* boxes with high and low binding affinities by comparing the CcpA regulon under three distinct conditions, where different amounts of CcpA were present in the cells, and to identify *cre* features that determine this affinity.

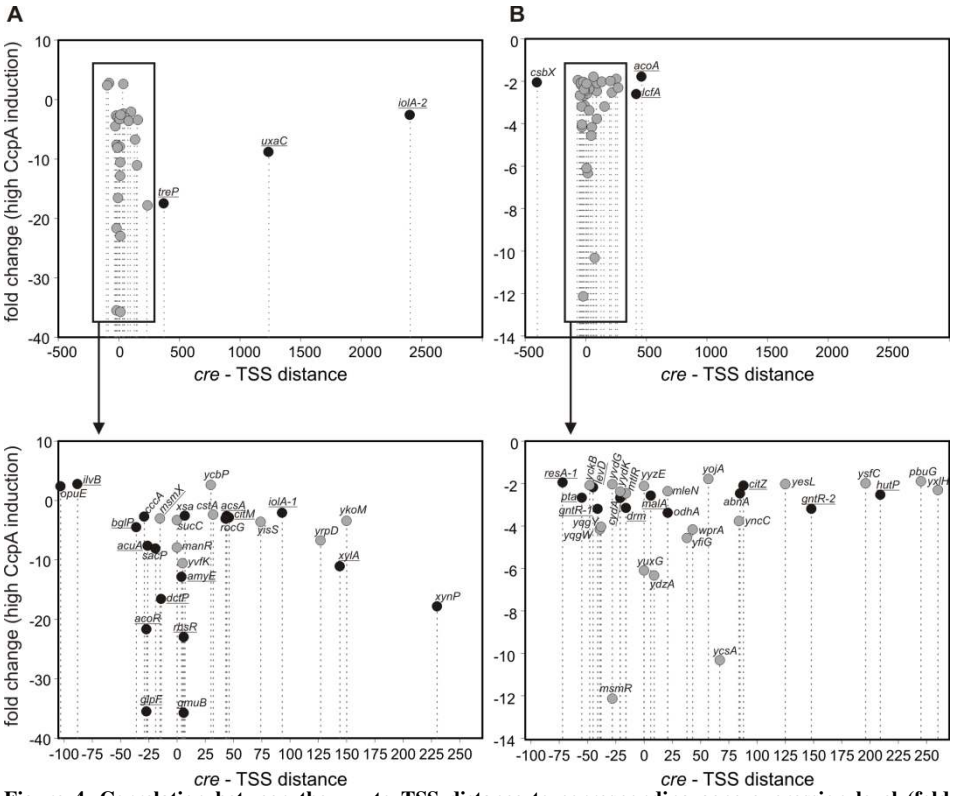

Figure 4. Correlation between the *cre* to TSS distance to corresponding gene expression level (fold change). (**A**) High-affinity *cre* boxes, (**B**) low affinity *cre* boxes. Black circles - *cre* boxes of the genes for which TSSs were detected experimentally; grey circles - *cre* boxes of the genes for which TSSs were predicted in this study, underlined gene names – genes with *cre* sites known from literature. "0" on the X ax represents the TSS position, negative numbers – *cre* boxes upstream TSS, positive numbers – *cre* boxes downstream TSS. For clarity, the outliers were removed (for the full list of *cre*-TSS distance, see Appendix).

Using a tetracycline-dependent gene regulation system [312] we achieved a tightly-controlled *ccpA* expression, leading to a wide range of CcpA amounts in the cells. *B. subtilis* cultures with relative low, medium or high amounts of CcpA in the cells were subjected to transcriptome analyses. The cells were grown in the presence of glucose to ensure sufficient production of low-molecular-weight modulators of CcpA activity (NADP, glucose-6-phosphate, fructose-1,6-bisphosphate). As expected, higher levels of CcpA protein lead to more genes significantly up- or downregulated. Most of the regulated genes, however, were affected indirectly, as they were lacking a *cre* site. Genes regulated indirectly in a CcpA-dependent manner (no *cre* or unfunctional *cre*) were already observed before and were proposed to be grouped in class II, next to class I that includes genes regulated by CcpA directly [178, 316, 319]. In our analysis, only genes belonging to class I were taken into account as the subject of this study was the nature of discriminating *cre* boxes. Many repressed genes are σA-dependent and do not

need another inducing protein for their expression. However, expression of some genes is regulated by more than one regulator. In those rare cases of multiple regulation, the full extent of regulation would not be observed in our transcriptome analysis, but this does not affect our analysis since we are looking at the relative strength of repression at different CcpA concentrations.

The search for putative *cre* boxes in the *B. subtilis* genome, using a *cre* motif generated from the *cre* boxes known from DBTBS [317], $T_1G_2A_3A_4A_5R_6C_7G_8Y_9T_{10}W_{11}W_{12}C_{13}A_{14}$, resulted in 418 putative *cre* boxes. The majority of the predicted *cre* boxes were within ORFs far away from promoters and, although functional *cre* boxes located within coding sequences are present in the *B. subtilis* genome, a lot of the predicted *cre* sites seemed to be at a too large distance from the promoter to possibly be able to play a role in regulation of gene expression. Therefore, *cre* boxes located within -500 and +100 nucleotides from the first nucleotide of a start codon of the first genes of an operon were extracted. Also *cre* boxes triggering gene regulation that are known from the literature, but not predicted by our method, were included in our analysis. The genes differentially expressed at least at a high CcpA production level and possessing *cre* box(es) known from literature [149, 317] and/or predicted in this study were selected. Among the selected genes, 30 were downregulated and 3 were upregulated at a low CcpA induction level, while the other 37 genes were downregulated only when CcpA was produced at higher levels (medium and high CcpA induction levels). For all these genes, expression fold changes were calculated as ratios of the amounts of transcripts downstream of *cre* boxes as the microarray chip probes were synthesized upstream from them. Of the regulated first genes of operons possessing known and/or predicted *cre* box, chip probes of only *kdgR* and *resA* were upstream from *kdgR-cre* and second *cre* of *resA* (located 1709 bp downstream from TSS). Therefore, these *cre* boxes were not included in the sequence and position analysis of *cre* boxes. Since regulation depends on CcpA-*cre* binding, *cre* boxes causing significant regulation of downstream operons already when a small amount of CcpA is available are supposed to have a high affinity to CcpA and titrate CcpA away from low-affinity *cre* sites, which are able to exert regulation of other operons only when more CcpA is present. Notably, over a dozen of known *cre's* fell out of our data set, because the corresponding genes were not significantly regulated in any of the three microarray experiments. Despite of the fact that they could be considered as very low-affinity sites, they were not included in the analysis as lack of the differential expression might have been a false negative result due to, e.g., high background signal, bad spot quality on the microarray slides, mRNA degradation, growth conditions, more complex regulation or yet unidentified factors. Moreover, it should be noted that division of *cre* boxes to two

affinity groups is a simplification necessary for this analysis. Very likely a gradient distribution of *cre* site affinities occurs in nature, which would be difficult to assess.

The detailed analysis of the sequences of high- and low-affinity *cre* boxes, led to a few interesting observations. The G_2 and middle C_7 and G_8 residues (Fig. 3), known as highly conserved residues [164–166] are conserved in both high- and low-affinity *cre* boxes. Interestingly, the high-affinity *cre* boxes have more conserved G_6 and C_9 surrounding the middle CpG and C_{13} (palindromic to the conserved G_2) and A_{14} (palindromic to T_1) and their sequences are significantly more palindromic overall. It was observed before that a more palindromic sequence of *cre* sites contributes to a better function [165]. The more palindromic nature of the high-affinity *cre* sites (in comparison to low-affinity *cre* sites) might create a more symmetric DNA conformation, preferred for CcpA binding. Although the bases at positions 4 and 11 are more often palindromic to each other in the weak *cre* boxes, this is obviously less important for the *cre* strength. In a previous study [164] it was shown that CcpA binds with similar affinities to different *cre* boxes, which explains well the role of CcpA as a global regulator. However, the three *cre* boxes tested in that work differ very little around the middle CpG and in their symmetry (palindromic sequence) and they did not differ at the residues corresponding to our C_{13} nor A_{14}.

Comparison of the high- and low-affinity *cre* boxes location in relation to the TSS also shows some trends. While the low-affinity *cre* sites can be located at any position from the TSS, the high-affinity *cre* sites cluster around the TSS, 14 and 27 base pairs upstream from TSS and 44 base pairs downstream from TSS. Simultaneously, the strongest repression by CcpA was observed for the genes with *cre* sites located around the TSS (*amyE, rbsR*, *gmuB*) and at positions -27 (*acoR*, *glpF*), -14 (*dctP*), +230 (*xynP*) and +372 (*treP*) base pairs from the TSS, which are separated by approximately 10 - 11-nt increments (corresponding with a full helical turn). This observation is in agreement with previous findings that activation or repression by CcpA binding to *cre* boxes is helix-face-dependent [184, 318]. Also in *Lactococcus lactis* the strongest repression by CcpA was shown to occur when the center of *cre* box was located -39, -26, -16, +5 and +15 from the TSS [320].

It was shown before that genes with *cre* boxes located further upstream from -35 sequences of the promoter are subject to activation by the CcpA complex as in case of *ackA* [184], *pta* [185] and *ilvB* [186, 187]. In our work, however, under the tested conditions, only three genes were activated: *ilvB*, *opuE* and *ycbP* (the two latter genes with *cre* sites predicted in this study). We did not observe activation of *ackA* in this study. This is probably due to the very low basal expression of CcpA from the TetR

repressed promoter that might be high enough for binding of CcpA to the *ackA cre* box and for full activation of the *ackA* promoter. In this case, a further increase of CcpA does not result in an additional increase of *ackA* expression. Surprisingly, *pta* was downregulated. However, in this study both test and control cultures were grown in medium supplemented with glucose. The mechanism of *pta* regulation in this case is thus different from low glucose-dependent CCA. Based on our criteria, the *cre* boxes of all three activated genes are of the high affinity type. Although the *ycbP cre* box appears to be downstream to the TSS (+30), both the *cre* box and the TSS in this case are not experimentally confirmed.

Some genes and operons possess multiple *cre* boxes. Since DNA microarray technology was used in this study to assess expression fold changes of genes and operons in the presence of different amounts of CcpA, we were not always able to judge whether the effect is due to one *cre* box (and which one) or more. In our set (Table 2) there were only two operons with two *cre* boxes (the first genes of these operons are: *iolA* and *gntR*). *gntR* was weakly regulated (low-affinity *cre* boxes), suggesting that the regulatory effects of the two *cre* boxes do not add up to exert strong regulation. In case of the *iolA* operon, each of the two *cre* boxes is located within another gene of the operon (*cre*-1 within *iolA* and *cre*-2 within the second gene of the operon, *iolB*). In this case, the regulatory effects of these *cre* boxes could be assessed independently. Based on the fold changes of *iolA* (*cre*-1) and *iolB* (*cre*-2), both *cre*-1 and *cre*-2 seem to be of high affinity. Multiple *cre* boxes could serve for fine tuning of CcpA-regulated genes and operons.

For the genes with *cre* boxes located close to the TSS and downstream, distinct repression mechanisms were proposed. Elongation blockage (roadblock) was shown for *xyl*, *ara* and *gnt* operons, as well as *sigL* and *acsA* [321–325]. Prevention of binding RNAP to the promoter sequence was demonstrated for the *acuABC* and *bglPH* operons possessing *cre* partially overlapping with the promoter region [169, 326]. Transcription inhibition by direct interaction of CcpA with the σ-subunit of RNAP already bound to the promoter was shown in case of the *amyE* gene and *xyl* operon [318]. The presence of a high-affinity *cre* box in close vicinity to the TSS shown in this study, suggests that repression by inhibition of RNAP binding is one of the most effective mechanism of negative regulation by CcpA.

In conclusion, we propose that besides the strongly conserved G_2 residue and the middle CpG, the residues G_6 and C_9 (surrounding the middle CpG), C_{13} and A_{14} and, to a certain extent a more palindromic sequence and a location of *cre* in close vicinity to the TSS, contribute to the high affinity of CcpA for certain *cre* boxes. This finding

contributes to further understanding how CcpA binding to *cre* boxes is modulated and how subregulons can be formed. However, not all the *cre* boxes behave strictly according to this rule, suggesting that *cre* affinity is possibly determined in an even more complicated way. The *cre* sequence and position may play a role simultaneously and/or more factors may be involved, for instance additional conserved sequences as shown for *ackA* [307] or sequences flanking *cre* sites as in case of *acsA* [325].

It will be interesting to use these predictions for other Gram-positive organisms employing CcpA, like other *Bacilli*, lactic acid bacteria, or pathogenic *Streptococci* and *Staphylococci*.

Materials and Methods

Bacterial strains and growth conditions

B. subtilis strain MP902 (*trpC2*, P*tet-ccpA*, pWH119, Km^R, Em^R) was grown in rich TY medium [217] in the dark at 37ºC with shaking. The medium was supplemented with 15 µg/ml kanamycin, 2.5 µg/ml erythromycin, 1% glucose, 0.2% xylose and anhydrotetracycline (ATc) at different concentrations. For inoculation, synchronized stocks were used. Synchronized stocks were prepared by growing the strain in TY medium with a corresponding composition as described before [167]. At OD_{600} = 0.8, the cells were collected for determination of the CcpA production level with Western blot and for RNA isolation to be used for microarray analysis.

Construction of the MP902 strain

All primers used in this work are listed in Table 5. To replace the *ccpA* promoter by a tetracycline-inducible promoter at the natural locus on the chromosome, the integration vector pWH849 was constructed as follows. A *ccpA* fragment truncated at the 3' end was amplified from plasmid pWH1533 [327] using primers ccpAmut1 and Accout, restricted with *BsrG*I and *Kpn*I and cloned into vector pWH618 [327]. The resulting vector was named pWH700 and contains the terminal 246 bases of *aroA*, the intergenic region between *aroA* and *ccpA* and 689 bases of *ccpA*. Next, a kanamycin resistance cassette was amplified from plasmid pDG792 [248], using primers KmkfwR and KmkbwR, and inserted in the intergenic region between *aroA* and *ccpA* via the restriction sites *BsrG*I and *Acc*I. The resulting vector was named pWH800. The tetracycline-inducible promoter, P*tet* was amplified from the plasmid pWH1935-2 [328] with primers tetPccpAfw and tetPccpAbw. The resulting PCR fragment was used as a

primer together with the primer Accout in order to fuse the tetracycline-inducible promoter P*tet* with *ccpA* at the intergenic region between *aroA* and *ccpA* in an overlap PCR with pWH800 as a template. The resulting PCR fragment was restricted with *BsrG*I and *Kpn*I and cloned into vector pWH800, resulting in pWH849. *B. subtilis* 168 [1] was transformed with pWH849, linearized with *Sca*I, to replace the *ccpA* promoter on the chromosome by the tet inducible promoter via double homologous recombination. Positive candidates were selected on TY plates with kanamycin and verified by PCR screening. The resulting strain was named MP901. Strain MP901 was transformed with pWH119 plasmid [312] carrying tetracycline repressor gene, *tetR*, under control of xylose-inducible promoter, P*xyl* (P*xyl-tetR*), resulting in MP902 strain.

Table 5. Primers used in this study.

Name	Sequence (5'- 3')
ccpAmut1	ATAATATCTAGAACCAAGTATACGTTTTCATC
Accout	ATAATAATAGGTACCGCTTCGAGTCCGGAATC
KmkfwR	ATAATAATATGTACAGATAAACCCAGCGAACCA
KmkbwR	AATAATAATAATAGTATACTATAAAACATCAGAGTATGGA
tetPccpAfw	ATAATAATATGTACAGCATGGTCCTAATTTTTGTT
tetPccpAbw	TACTGGATACACTTATCCTTCTGCAGGCATGCAAGCTA

Quantification of the CcpA production level with sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

B. subtilis MP902 cells were grown in LB medium with 0.2% xylose and 0.1 to 20 nM ATc after one overnight culture with the respective xylose and ATc concentrations. In the mid log phase, 0.5 OD_{600} equivalents of the cells were sedimented and resuspended in SBT buffer (50mM TrisHCl, 200mM NaCl, 10mM β-mercaptoethanol pH 7.5). After sonification, 0.05 OD_{600} equivalents of the crude protein extracts and 200ng wild-type CcpA purified as described previously [327] were subjected to SDS-PAGE on a 10% polyacrylamide gel. Proteins were then transferred to a PVDF membrane by electroblotting. After blocking, the membrane was incubated with a 1:10,000 dilution of rabbit polyclonal anti-CcpA antibodies [329]. For detection of CcpA on an X-ray film the membrane was incubated with anti-rabbit horseradish peroxidase conjugate and a luminol containing reagent mixture from an ECL+ kit (GE Healthcare, Munich, Germany) according to manufacturer's instructions.

To analyze the CcpA production level in the cultures used for microarray experiments, the cells were collected at an optical density of OD_{600} = 0.8 (simultaneously with collection of the cells for total RNA isolation for microarray experiments). The signal

on Western blot was quantified using ImageJ gel analyzer (http://rsb.info.nih.gov/ij/). For gel loading verification, the control blots were stained with 0.1% Ponceau S dissolved in 5% acetic acid. Images of Ponceau S – stained membranes were obtained using GS-800 calibrated densitometer (Bio-Rad).

DNA microarray analysis

16 ml of a culture was harvested at optical density of OD_{600} = 0.8 by centrifugation at 8,000 × g for 2 min. The pellet was rapidly frozen in liquid nitrogen and stored at -80ºC until RNA isolation. DNA microarray experiments were performed in general as described before [167]. Total RNA was isolated using High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. RNA quantity and quality were tested with a ND-1000 spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer 2100 with RNA 6000 LabChips (Agilent Technologies Netherlands BV), respectively. The amino allyl modified cDNA was synthesized with the Superscript III Reverse Transcriptase kit (Invitrogen), purified with Cyscribe GFX purification kit (Amersham Biosciences), labeled with Cy3 and Cy5 dyes and purified again. The labeled cDNA was hybridized to oligonucleotide microarrays in Ambion Slidehyb #1 buffer (Ambion Europe Ltd). Slides were washed, dried by centrifugation and scanned with a GeneTac LS V confocal laser scanner (Genomic Solutions Ltd). Scans were analyzed with ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, Md., USA). The resulting expression levels were normalized with Micro-Prep [223] and subjected to a *t*-test using the Cyber-T tool [224]. All microarray experiments were performed in three biological
replicates. The complete microarray data is available at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE35154.

The sequences of the *cre* boxes known from DBTBS [317] were used to generate a weight matrix in Genome2D [255]. The resulting Weight Matrix was fed into the Genome2D [255] to find the potential *cre* boxes in the whole genome of *B. subtilis*. In this search, a cut-off of 8.96 was used. The promoters (-35 and -10 boxes) and transcriptional start sites (TSS) were predicted using PePPER (Prediction of Prokaryote Promoter Elements and Regulons) tool [330] and sequence analysis. For the annotation, GenBank file NC000964.gbk last modified on the 19th of October 2011 available at http://www.ncbi.nlm.nih.gov/guide/ was used. The operons from DBTBS database [317] were confirmed with experimental evidence from microarray results obtained in this study (clustered up- or downregulation of genes belonging to one operon).

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CHAPTER 6

Summary and general discussion

Bacillus subtilis is a rod-shaped model Gram-positive bacterium that is commonly used as a "cell factory" for protein production, thanks to the ability to efficiently secrete proteins to the growth medium and other features like the absence of the endotoxin lipopolysaccharide and the possibility of growing in large fermentors. *B. subtilis* and its close relatives are used to produce more than half of the commercially available enzymes used for the detergent-, food- and beverage industries and in the development of pharmaceuticals [29–35]. Also for fundamental research, homologous and heterologous proteins are often produced using *B. subtilis* as a host. However, under conditions of protein production and secretion at high levels, intracellular- and cell envelope stress responses turn on, which may set limits to the production on a large scale. Over the years, the rational manipulation of the limiting factors that hamper different stages of protein production [27, 29, 65, 125] resulted in improvement of *B. subtilis* as a protein production host. This thesis contributes to a further understanding of the regulatory responses to overproduction of different classes of extracytosolic proteins, in particular membrane proteins, which can be used in further protein production improvement. Moreover, it adds to the fundamental knowledge on membrane stress responses and also on the *cis*-acting factors involved in carbon catabolite control by the global regulator CcpA. The major findings of this thesis are depicted in Fig. 1 (summarizing **Chapter 2, 3** and **4**) and Fig. 2 (summarizing **Chapter 5**).

For many years responses of *B. subtilis* cells to various stress factors like osmotic shock, heat shock, starvation, antibiotics, detergents, *etc*. have been studied. This resulted in a great collection of knowledge on stress response mechanisms involving extracytoplasmic function (ECF) sigma factors, two component systems (TCS), and many, commonly called, heat- and cold-shock genes. As *B. subtilis* is frequently used in industry and in fundamental research for production of proteins at large scale, cell physiology under extracellular protein overproduction conditions also gained great interest. Extracellular proteins are very interesting for the industry because of their relatively easy purification. It has been shown that (next to heat shock) high level production of secreted homologous and heterologous proteins induce a specific stress response in *B. subtilis* through a two-component signal transduction system called CssS-CssR [41–43]. **Chapter 2** of this thesis provides an additional, extensive study of the responses on the transcriptional level of *B. subtilis* cells overproducing different classes of secretory proteins with endogenous and heterologous origin, involving proteins secreted to the medium, cell wall-attached proteins, as well as membrane proteins and lipoproteins inserted to the membrane via the Sec-translocation machinery

[8] (Fig. 1). By testing the effects of overproduction of several of these proteins, we could gain more knowledge on cellular responses to protein overproduction that potentially take place during fermentations on an industrial scale. The overproduced proteins were the membrane embedded putative multidrug transporter LmrA of *Lactococcus lactis* [190], the *Lactobacillus pentosus* membrane embedded xyloside transporter XylP [191], the *B. subtilis* manganese binding lipoprotein MntA [192], the putative zinc binding lipoprotein YcdH [193], the *Escherichia coli* periplasm located TEM-1 β-lactamase (Bla) [194], the *L. lactis* cell wall-associated protein Usp45 [195], and two *B. subtilis* secreted proteins: neutral protease NprE [196] and xylanase XynA [197]. Due to the fact that more than one protein of each localization class was chosen, effects specific for one protein and effects common to one localization class could be discriminated. Responses specific for proteins with a common localization as well as more general stress responses were observed. A future study of *B. subtilis* cells overproducing different classes (localization) of proteins, where within each class there would be both hetero- and homologous proteins, could give an even better insight on the *B. subtilis* cells responses to protein overproduction stress. It would enable to distinguish responses that are activated only when nonnative proteins are overproduced, which is particularly interesting for the industry. It has been shown that enhanced production of intracellular molecular chaperones like GroES/EL and DnaK/DnaJ/GrpE resulted in a better production, and subsequently better characterization, of antidigoxin and fibrin-specific single-chain antibody fragments [127, 128]. Consistently in our study, the most pronounced effect that was common to overproduction of most of the tested proteins was upregulation of genes encoding intracellular stress proteins. Those were class I heat-shock genes coding for molecular chaperons (GroES/EL), class III heat-shock genes coding for components of protease complexes (ClpXP, ClpEP, *etc*.) and other genes regulated by CtsR, a stress and heat-shock response regulator [107, 114]. Although in our study none of the genes encoding components involved in protein secretion, like for instance SecA, SecYEG, Ffh, signal peptidases, *etc*., were upregulated, it has been shown that overproduction of the extracellular molecular chaperone PrsA and one of the signal peptidases, SipT, improved heterologous protein production [127–129, 132, 138]. Also changes in protein targeting pathways and modifications in the secretory machine (SecA) improved secretion of heterologous proteins [145, 146]. This suggests that, although (in our study) the genes encoding secretion machinery components were not upregulated under artificially imposed overproduction of secretory proteins, manipulations of these components may also improve protein production and that there might be more yet unidentified potential bottlenecks in protein production.

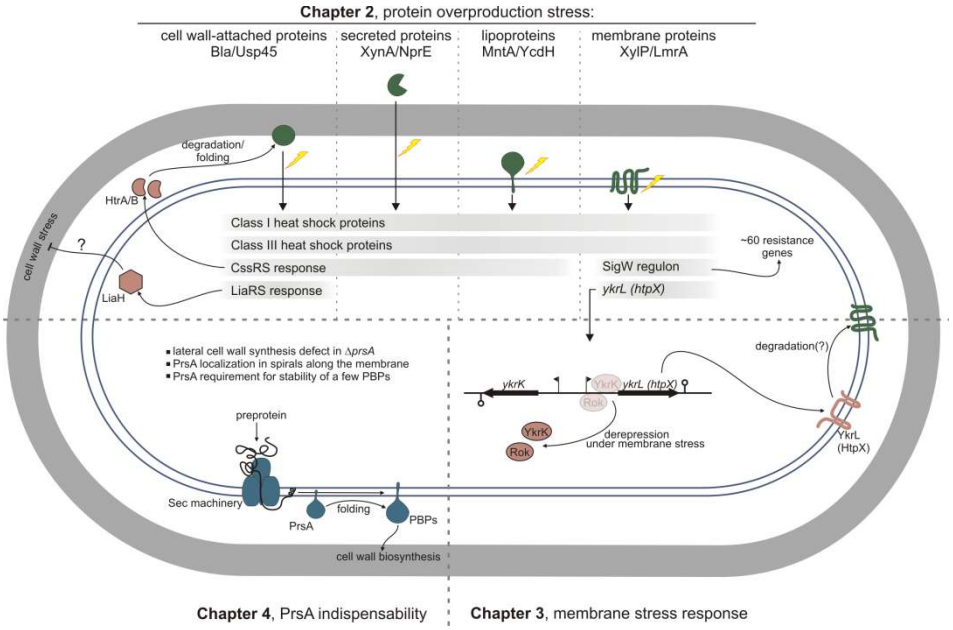

Figure 1. Major findings described in Chapters 2, 3 and 4. Chapter 2, cellular stress responses activated under overproduction of proteins of different localization class: cell wall-attached, secreted, lipoproteins and membrane proteins. Common effects (Class I and III heat shock proteins activation) and effects specific for a few or only one class of overproduced proteins (CssRS, LiaRS, SigW regulon, *ykrL*) are indicated. **Chapter 3**, response to membrane stress caused by membrane overproduction, ΔΨ dissipation, salt stress and phenol. Expression of *ykrL*, which encodes a putative membrane protease, is regulated by two repressors, Rok and a novel regulator YkrK. Under stress conditions, *ykrL* expression is derepressed by an unknown mechanism, YkrL is produced and probably recruited for degradation of malfunctioning membrane proteins. **Chapter 4**, elucidation of PrsA chaperone/foldase indispensability. The listed main findings led to conclusion that PrsA is vital for the cell survival as it is crucial for the stability of the penicillin binding proteins (PBPs) that are required for the lateral cell wall biosynthesis (PBP2a, PBP2b, PBP3 and PBP4). See text for details.

The other responses were activated only during overproduction of particular classes of proteins. Specifically, the *liaIHGFSR* operon was upregulated upon overproduction of cell wall-attached proteins. Overproduction of secreted and cell wall-attached proteins and lipoproteins, consistent with previous results [46, 47], led to induction of the CssRS response; and membrane protein overproduction resulted in specific upregulation of the *sigW*-regulon and *ykrL*. Hypothetically, deletion of these stress responses could improve production yield of proteins with particular destination in the cell. Zweers *et al*. showed before [65] that deletion of *sigW* has a positive effect on membrane protein production, which is in agreement with our observation that the SigW response was elevated under membrane protein overproduction stress. In the same study it was shown that also deletion of the CssRS response improved production of membrane proteins, which, taking into account that in our study the CssRS response was not activated in case of membrane protein overproduction, is rather surprising. Moreover, as shown in **chapter 3** of this thesis, CssRS seems not to be involved in membrane protein quality control.

The explanation for improved membrane protein production in a Δ*cssS* strain could be the interdependence between CssRS and other stress response system(s), like for instance the SigW regulon (as noticed by Zweers *et al*. [65]).

The *ykrL* gene encodes a protein with high homology to the *E. coli* HtpX, a membrane embedded metalloprotease, which has been implied in membrane protein quality control [212]. The upregulation of *ykrL* in response to overproduction of membrane proteins suggests a similar role of YkrL in *B. subtilis*. While there is quite a lot of knowledge collected on the membrane quality control in *E. coli*, there is not much known about this mechanism in *B. subtilis*. The membrane stress response in *B. subtilis* was subject of this study (**Chapter 3**) and it was shown to be different from the membrane protein quality control mechanism in *E. coli* (Fig. 1). Firstly, *htpX* of *E. coli* is regulated by the CpxRA two-component system that regulates a number of genes involved in cell envelope stress, including *degP* (*htrA*) encoding a homologue of *B. subtilis* HtrA and HtrB [213]. *B. subtilis htrA* and *htrB* are regulated by the CssR regulator [47]. However, using DNA affinity chromatography, EMSA and flow cytometry analysis of cells carrying promoter-*gfp* fusions, we showed that *ykrL* of *B. subtilis* is regulated by two negative regulators, YkrK and Rok, and not by CssR. Consistently, no correlation between expression of the CssR target genes and *ykrL* under membrane protein overproduction was observed (**Chapter 2**). Moreover, in *E. coli,* next to HtpX, the membrane located ATP-dependent metalloprotease FtsH is also involved in the membrane protein stress response [60]. Also in the Gram-positive bacterium *L. lactis*, the homologous protein FtsH [331] is crucial for membrane protein biogenesis [332] and it was shown to be upregulated upon membrane protein overproduction stress [333]. Yet, *ftsH* was not upregulated in response to overproduction of membrane proteins in *B. subtilis* (**Chapter 2**). It was shown before that *B. subtilis* FtsH is involved in sporulation by degrading the sporulation control proteins SpoVM and Spo0E [61, 62], which suggests that FtsH plays a more specific role, rather than serving as a general protein quality control system.

As mentioned above, in **chapter 3** two transcriptional negative regulators that control *ykrL* expression were identified. These are YkrK, encoded by an uncharacterized gene adjacent to *ykrL* but divergently transcribed, and Rok, a known repressor of competence and of genes with extracytoplasmic function [235, 236]. As shown by EMSA experiments, YkrK binds to the *ykrL* promoter region with much higher affinity than Rok, which identifies YkrK as the major regulator of *ykrL*. This is also the first time that the function of the YkrK protein is described. Moreover, apart from minor local similarity to MerR family regulators, YkrK appears to represent a novel type of regulator. A unique feature of YkrK as a cytoplasmic transcriptional regulator is the

presence of a C-terminal highly hydrophobic α-helix domain. This could be a putative cell membrane-docking domain via which YkrK could be recruited to the cell membrane under stress conditions resulting in a very unusual derepression mechanism. It would be desired to test the subcellular localization of YkrK. Localization of YkrK at the cell membrane under membrane stress conditions and localization in the cytosol when no stress is applied would prove this hypothesis and describe YkrK as a novel regulator. Preliminary attempts to test the localization pattern of YkrK using GFP (green fluorescence protein) protein fusion (YkrK-GFP) have been taken. Indeed, under certain conditions, membrane localization of YkrK-GFP could be observed (data not shown). However, the results were not entirely consistent and aggregation of the YkrK-GFP fusion protein (or only GFP after YkrK-GFP degradation) could not be excluded, making indisputable conclusions impossible.

Not only the regulation of the membrane stress-responsive *ykrL,* but also the mechanism of this response was a subject of this thesis. The role of YkrL in membrane protein quality control was first proposed by Zweers *et al.* [189] purely based on the homology of YkrL to HtpX of *E. coli*. In **chapter 2**, upregulation of *ykrL* in response to overproduction of membrane proteins (but not proteins with other subcellular localization) was shown by means of DNA microarray analyses. Further experiments using other methods (**Chapter 3**) confirmed this effect and revealed other factors causing *ykrL* activation, namely dissipation of the transmembrane electrical potential (ΔΨ), salt stress and phenol. Interestingly, dissipation of the chemical proton gradient (ΔpH), which together with the ΔΨ constitutes the proton motive force (pmf), did not have any effect on *ykrL* expression. This suggests that the stress stimulus that is sensed and leads to induction of *ykrL* expression is the presence of misfolded proteins accumulating in the membrane as a consequence of artificial protein overproduction or of other factors disturbing membrane protein structure, rather than proton leakage as a result of a disturbed membrane integrity. The observed activation of P*ykrL* by membrane potential dissipation can be explained by the requirement of the membrane potential for correct insertion of membrane proteins [226, 227].

Proteins secreted to the medium are particularly convenient for industrial production due to the easy isolation of the product from the growth medium. Many industrially important secreted proteins are produced using *B. subtilis* as a production host, e.g., subtilisin BPN' from *Bacillus amyloliquefaciens*, cutinase from *Fusarium solani pisi* [143, 144], human interleukin-3 (hIL-3) and interferon α (hIFN-α2b) [133, 146], recombinant lipoxygenase from *Anabaena sp*. [129], α-amylase from *B.*

amyloliquefaciens [138]. Years of research resulted in identifying genetic modifications that improved production of proteins by *B. subtilis* cells (for review see Chapter 1). Great impact on developing a better protein producer was achieved by deletion of six extracellular proteases encoded by *aprA*, *nprE*, *nprB*, *epr*, *bpf* and *mpr* [126]. On the other hand, enhanced production of the extracytoplasmic molecular chaperone, PrsA, was shown to facilitate production of an antidigoxin and fibrin-specific single-chain antibody fragment [127, 128], the above mentioned lipoxygenase [129] and α-amylase [130–132].

PrsA is a lipoprotein bound to the outer face of the cytoplasmic membrane in *B. subtilis* and other Gram-positive Firmicutes [130, 260]. *B. subtilis* PrsA exhibits activity of peptidyl-prolyl *cis-trans* isomerases (PPIase) catalyzing the isomerization of peptide bonds immediately preceding proline residues [257–259], but may also have a chaperone-like activity *in vivo* [260]. The first (unsuccessful) attempts to delete the *prsA* gene indicated that it is an essential cell component suggesting that it has an indispensable role in protein folding at the membrane/cell wall interface [130, 131].

The indispensability of PrsA is the subject of **chapter 4** of this thesis (Fig. 1). The purpose of this study was to identify the essential cell components, the correct folding of which depends on PrsA, in order to establish why PrsA is a vital protein in *B. subtilis*. Our hypothesis was that PrsA catalyzes the folding of proteins involved in the biosynthesis of the cylindrical (lateral) cell wall and the determination of the rod shape. This hypothesis was supported by the fact that, in contrast to the rod-shaped *B. subtilis*, PrsA is dispensable in several cocci [261, 263] and by the fact that the enzyme domain of PrsA is located at the membrane/cell wall interface, suggesting that PrsA may assist the folding of membrane proteins possessing large functional domains on the outer surface of the membrane. Indeed, the results presented in **chapter 4** (and [286]) confirmed this hypothesis. It appeared that the *prsA* gene could be deleted in a medium with high concentration of magnesium. This already was a direct indication that PrsA could be involved in cell wall biosynthesis as it was shown before that high concentrations of magnesium restores growth of mutants with defects in different aspects of cell wall synthesis (Δ*ponA*, Δ*mreB*, Δ*mreC*, Δ*mreD*) [274, 287]. It is assumed that magnesium could indirectly affect peptidoglycan structure or turnover [287], but the exact mechanism of this suppression is unknown. Other circumstantial evidence for PrsA being possibly involved in cell wall synthesis was the subcellular localization pattern of the PrsA-Myc protein (**Chapter 4**). The immunolocalization showed that PrsA is not randomly or homogenously distributed in the membrane but that it is rather localized in distinct spots organized in a helical pattern at the lateral cell membrane, as it was also observed for the cell-shape determining proteins, Mbl, MreB, MreBH, MreC

and MreD, and some PBPs (PBP3, PBP4, PBP4a) [243, 267, 268, 270–272, 283]. Moreover, depletion and deletion of PrsA affected the morphology of *B. subtilis* cells, which again suggests involvement of PrsA in lateral cell wall biosynthesis. *In vitro* assays also showed that PrsA is crucial for stability of penicillin binding proteins that are involved in the lateral cell wall synthesis, i.e., PBP2a, PBP2b, PBP3 and PBP4 [286]. Interestingly, it has been shown before that *B. subtilis* MreB, MreC and MreD interact with penicillin binding proteins [243, 273]. Altogether, the results explain why PrsA is indispensable for the cell viability: the reason for the growth inhibition of PrsA-depleted or *prsA* mutant cells appears to be the insufficient amount of active PBPs formed and the subsequent cell wall synthesis defect.

The subject of **chapter 5** is the carbon catabolite control mechanism allowing *B. subtilis* cells to achieve maximal metabolic efficiency when more carbon sources are available in the environment. This mechanism is exerted through activation of expression of the genes encoding enzymes necessary for preferred carbon source utilization (carbon catabolite activation, CCA) and simultaneous repression of the genes involved in utilization of secondary carbon sources (carbon catabolite repression, CCR) and it ensures the optimal growth, which is also an important aspect of large fermentation for industrial purposes. The global regulator of carbon metabolism genes, CcpA, binds to operator sequences called *cre* (catabolite responsive elements) resulting in activation or repression of the genes depending on the position of the *cre* box in relation to the promoter [149, 164–166, 297]. The specific goal of the study described in **chapter 5** was to determine the hierarchy in which the CcpA target genes are regulated in the presence of a preferable carbon source for *B. subtilis*, glucose, to gain better knowledge on the nature of *cre* sites and the gene regulation mechanism by CcpA on the molecular level (Fig. 2).

From a list of *cre* boxes described in the literature (reviewed in [149]), a Weight Matrix of *cre* boxes was generated: $T_1G_2A_3A_4A_5R_6C_7G_8Y_9T_{10}W_{11}W_{12}C_{13}A_{14}$ (where R is G or A, Y is C or T and W is A or T). By extensive DNA microarray analysis of *B. subtilis* cells producing CcpA at different levels, *cre* sites to which CcpA exhibits higher or lower affinity were identified (**Chapter 5**). The thorough analysis of the sequences of high- and low-affinity *cre* boxes led to the observation that the high-affinity *cre* boxes, comparing to the low-affinity ones, are more conserved at the G_6 and C_9 residues around the middle CG and at the distal A_{14} residue (Fig. 2). Moreover, the overall sequence of the high-affinity *cre* boxes has more palindromic nature when compared to the low-affinity *cre* boxes, consistent with the previous observation that a more palindromic sequence of *cre* boxes is desired for their better function [165]. Although in a previous study [164] it was shown that CcpA binds with similar affinities to different

cre boxes, the *cre* boxes tested in that work differ very little within the residues that were shown by our analysis to play a particular role in the CcpA affinity. Moreover, analysis of the *cre* boxes position in relation to the transcriptional start site (TSS) revealed that high-affinity *cre* boxes cluster around the TSS and at positions located approximately 10 – 11-nucleotide increments (corresponding with a full helical DNA turn) from the TSS (Fig. 2), which is in agreement with a previous finding showing that activation or repression by CcpA binding to *cre* boxes is helix-face-dependent [184, 318]. The presence of high-affinity *cre* boxes in close vicinity to the TSS, suggests that repression by inhibition of RNA polymerase binding is one of the most effective mechanisms of negative regulation by CcpA. In addition, the more palindromic sequence of the high-affinity *cre* boxes might create a more symmetric DNA conformation, preferred for CcpA-dimer binding.

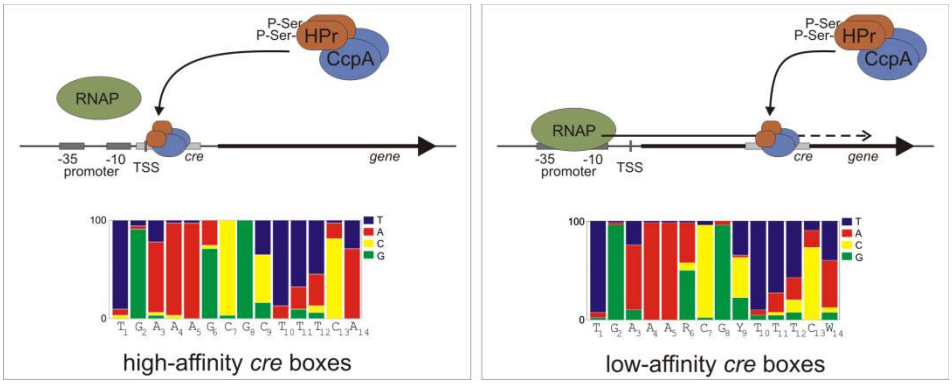

Figure 2. Simplified scheme representing main features of high- and low-affinity *cre* boxes (Chapter 5). Relative position and sequence motif of *cre* boxes revealing high (left panel) and low (right panel) affinity for CcpA binding and triggering strong and weak CcpA-dependent repression, respectively, are shown. High-affinity *cre* boxes localize at close vicinity to transcriptional start sites (TSS) and at positions ca. 10 – 11-nucleotide increments (full helical DNA turn) downstream (not depicted) and their overall sequence has more conserved and more palindromic nature. Low-affinity *cre* boxes occupy helix face-independent distal positions. CcpA binding to high-affinity *cre* boxes localized close to TSS, resulting in inhibition of RNA polymerase (RNAP) binding to the promoter, is probably more effective mechanism of repression than roadblock by binding to distal low-affinity *cre* boxes. See text for details.

In conclusion, this thesis revealed differential responses of *B. subtilis* to stress caused by overproduction of secretory proteins with different subcellular localization with a focus on membrane stress response, which can be potentially used in (further) improvement of *B. subtilis* as a protein production host for industry. A novel regulator, YkrK, involved in membrane stress response regulation was found and the mechanism of the executive gene (*ykrL*) regulation was described. Moreover, the reason for the indispensability of the PrsA lipoprotein catalyzing the post-translocational folding of exported proteins was studied. PrsA appeared to be crucial for the cell viability due to involvement in folding

of some PBPs and, consequently, in lateral cell wall biosynthesis. In addition, a better insight on the carbon catabolite control mechanism by the global regulator CcpA was obtained, in particular on the role of cognate *cre*-sites in determining the strength of regulation.

APPENDIX

Supplementary material to Chapter 5

Appendix. List of significantly regulated first genes of operons with predicted *cre* boxes within -500 and +100 nucleotides from start codon and *cre* boxes known from literature (DBTBS). Significant fold changes (1.8 < fold change < -1.8) are shown in bold. *cre* box to transcriptional start site (TSS) distance is calculated from conserved G residue in the middle of *cre* box to TSS. For *cre* box affinity determination criteria see Chapter 5.

Gene name	Gene ID	Strand	*cre* box sequence	*cre* box score	Fold change			*cre* box affinity	*cre* to TSS distance[a]	TSS source reference[b]
					Low CcpA induction	Medium CcpA induction	High CcpA induction			
abnA	BSU28810	lower	TGTAAGCGCTTTCT	9,8	-1,78	-1,67	**-2,49**	low	85	[334]
acoA	BSU08060	lower	TGTAAGCGTTTGCT	DBTBS	-1,08	-1,03	**-1,80**	low	462	[335]
acoR	BSU08100	upper	TGAAAGCGCTTTAT	9.59/DBTBS	**-4,78**	**-18,74**	**-21,75**	high	-27	P
acsA	BSU29680	lower	TGAAAGCGTTACCA	9.83/DBTBS	**-2,28**	**-2,47**	**-2,68**	high	44	[326]
acuA	BSU29690	upper	TGAAAACGCTTTAT	9.35/DBTBS	**-2,17**	**-4,63**	**-7,70**	high	-26	[326]
amyE	BSU03040	upper	TGTAAGCGTTAACA	9.72/DBTBS	**-2,57**	**-10,73**	**-12,91**	high	4	[174]
bglP	BSU39270	lower	TGAAAGCGTTGACA	9.85/DBTBS	**-2,48**	**-4,70**	**-4,56**	high	-36	[336]
cccA	BSU25190	lower	TGTAAGCGTATACA	9,28	**-2,21**	**-1,82**	**-2,78**	high	-29	[309]
citM	BSU07610	upper	TGTAAGCGGATTCA	9.37/DBTBS	**-2,57**	**-2,71**	**-2,93**	high	46	[180]
citZ	BSU29140	lower	TGTAAGCATTTTCT	DBTBS	-1,53	**-1,84**	**-2,12**	low	88	[337]
csbX	BSU27760	lower	TGAAAACGGTGCCA	9,2	-1,42	**-2,78**	**-2,07**	low	-401	[338]
cstA	BSU28710	lower	TGAATGCGGTTACA	9,15	**-2,20**	**-1,88**	**-2,44**	high	32	P
cydA	BSU38760	lower	TGAAATGAATCGTT	DBTBS	1,64	1,02	**-2,70**	low	-21	[339]
dctP	BSU04470	upper	TGAAAACGCTATCA	10.2/DBTBS	**-7,41**	**-12,26**	**-16,64**	high	-14	[182]
drm	BSU23500	lower	TGAAAACGGTTTAT	9.11/DBTBS	-1,35	**-3,65**	**-3,17**	low	-16	[175]
glpF	BSU09280	upper	TGACACCGCTTTCA	DBTBS	**-4,32**	**-21,95**	**-35,58**	high	-27	[340]
gmuB	BSU05810	upper	TGTAAGCGTTTTAA	9,67	**-3,02**	**-15,57**	**-35,82**	high	6	[341]
gntR-1	BSU40050	upper	TGAAAGTGTTTGCA	DBTBS	-1,31	**-2,76**	**-3,21**	low	-41	[342]
gntR-2	BSU40050	upper	TGAAAGCGGTACCA	DBTBS	-1,31	**-2,76**	**-3,21**	low	148	[342]
hutP	BSU39340	upper	TGAAACCGCTTCCA	DBTBS	-1,26	**-1,87**	**-2,55**	low	209	[343]
ilvB	BSU28310	lower	TGAAAGCGTATACA	DBTBS	**3,02**	**6,20**	**2,71**	high	88	[344]
iolA-1	BSU39760	lower	TGAAAGCGTTTAAT	9.26/DBTBS	**-1,79**	**-1,87**	**-2,14**	high	93	[345]
iolA-2 (iolB)[c]	BSU28310	lower	TGAAAACGTTGTCA	DBTBS	**-2,21**	**-2,53**	**-2,40**	high	2404	[345]
lcfA	BSU28560	lower	TGAAAACGTTATCA	DBTBS	-1,38	**-2,62**	**-2,60**	low	450	[346]
levD	BSU27070	lower	TGAAAACGCTTAAC	8.98/DBTBS	-1,53	-1,22	**-2,20**	low	-45	[347]
malA	BSU08180	upper	TGTAAACGTTATCA	9.74/DBTBS	-1,68	**-2,01**	**-2,59**	low	6	[348]
manR	BSU12000	upper	TGTAAACGGTTTCT	9,33	**-2,04**	**-3,73**	**-7,97**	high	0	P
mleN	BSU23560	lower	TGAAAGCGTTTTAG	9,35	-1,50	**-3,46**	**-2,38**	low	21	P
msmR	BSU30260	upper	TGTAACCGCTTACT	9,04	-1,72	**-4,21**	**-12,16**	low	-28	P
msmX	BSU38810	lower	AGAAAGCGTTTACA	9.59/DBTBS	**-1,97**	**-2,60**	**-3,09**	high	-15	P
mtlR	BSU04160	upper	TGAAAGCGTTTTAT	9,52	-1,46	**-2,74**	**-2,48**	low	-16	P
odhA	BSU19370	lower	TGGAAGCGTTTTTA	9,43	-1,62	**-6,57**	**-3,40**	low	21	[349]
opuE	BSU06660	lower	TGAAAGCGTTTTAT	9,52	**2,33**	**2,49**	**2,32**	high	-103	[350]
pbuG	BSU06370	upper	TGAAAACGTTTTTT	9,2	-1,12	-1,54	**-1,91**	low	245	P
pta	BSU37660	lower	TGAAAGCGCTATAA	DBTBS	1,27	**-3,22**	**-2,69**	low	-55	[185]
rbsR	BSU35910	upper	TGTAAACGGTTACA	9.57/DBTBS	**-6,67**	**-15,21**	**-23,05**	high	6	[351]
resA	BSU23150	lower	TAAAAACGCTTTCT	DBTBS	-1,06	**-1,90**	**-1,97**	low	-72	[231]
rocG	BSU37790	lower	TTAAAGCGCTTACA	9,48	**-2,60**	**-3,49**	**-3,11**	high	43	[352]
sacP	BSU38050	lower	CGAAAACGCTATCA	9,3	**-2,05**	**-7,91**	**-8,14**	high	-19	[353]
sigL	BSU34200	lower	GGAAAACGCTTTCA	DBTBS	-1,13	**-3,15**	**-3,28**	low	ND	
sucC	BSU16090	upper	TGAAAGCGCAGTCT	8,98	**-1,99**	**-5,84**	**-3,36**	high	0	P
treP	BSU07800	upper	TGAAAACGCTTGCA	DBTBS	**-3,23**	**-13,02**	**-17,54**	high	372	[354]
uxaC	BSU12300	upper	TGAAAGCGTTATCA	DBTBS	**-2,55**	**-3,69**	**-8,88**	high	1237	[355]
wprA	BSU10770	upper	TGTAAGCGGTATCT	9,3	-1,63	**-5,47**	**-4,19**	low	43	P
xsa	BSU28510	lower	TAAAAGCGCTTACA	9,43	**-1,90**	**-1,80**	**-2,64**	high	7	[334]
xylA	BSU17600	upper	TGGAAGCGCAAACA	DBTBS	**-2,44**	**-11,93**	**-11,14**	high	144	[356]
xynP	BSU17570	upper	TGAAAGCGCTTTTA	10/DBTBS	**-3,98**	**-10,99**	**-17,87**	high	230	[315]
ycbP	BSU02590	lower	TGAAAGCGCTCGCT	9,13	**2,54**	**3,32**	**2,55**	high	30	P
yckB	BSU03380	lower	TGAAAACGCGATCA	9,3	-1,41	**-3,53**	**-2,10**	low	-48	P
ycsA	BSU04000	upper	AGAAAGCGCTTACG	8,98	-1,75	**-6,02**	**-10,35**	low	67	P
ydzA	BSU04240	lower	TGAAAACGTGTCCA	9	-1,29	**-6,39**	**-6,35**	low	9	P
yesL	BSU06940	upper	TGAAAGCGTTTTCC	9,98	-1,33	-1,59	**-2,05**	low	125	P
yfiG	BSU08260	upper	AGAAAGCGGTTACA	9,41	-1,62	**-2,69**	**-4,59**	low	38	P
yisS	BSU10850	upper	AGAAAACGCTTTCT	9,17	**-1,90**	**-3,55**	**-3,67**	high	74	P
yjmD	BSU12330	upper	TGAAAGCGGTTCAA	9,35	**-2,18**	**-2,36**	**-8,76**	high	ND	

Appendix. Continued.

Gene name	Gene ID	Strand	*cre* box sequence	*cre* box score	Fold change			*cre* box affinity	*cre* to TSS distance[a]	TSS source reference[b]
					Low CcpA induction	Medium CcpA induction	High CcpA induction			
ykoM	BSU13340	upper	TGCAAGGGCTTTCA	9,17	**-2,04**	**-3,39**	**-3,49**	high	150	P
yncC	BSU17630	upper	TGTAAACGGTTACA	9,57	-1,30	**-2,44**	**-3,79**	low	84	P
yojA	BSU19520	lower	TGAAAGCGCTTTCT	10,2	1,06	-1,50	**-1,81**	low	57	P
yqgW	BSU24800	upper	TGAAAACGCTATCG	9,48	-1,11	**-4,47**	**-4,18**	low	-39	P
yqgY	BSU24780	upper	TGAAAATGTTTACA	9,15	-1,39	**-5,44**	**-4,06**	low	-38	P
yrpD	BSU26820	upper	TGATAGCGTTTTCT	9,11	**-1,90**	**-8,00**	**-6,80**	high	127	P
ysbA	BSU28910	lower	TGTAAGCGCTTTAT	9,24	1,02	**-3,83**	**-7,57**	low	ND	
ysfC	BSU28680	upper	TGAAAGCGTTTTTT	9,43	-1,47	-1,49	**-2,01**	low	196	P
ytkA	BSU30660	lower	TGTAAGCGTTTGCT	9,24	**-1,86**	**-6,42**	**-6,83**	high	ND	
yugN	BSU31330	lower	TGAATGCGCTTTCT	9,15	-1,66	**-2,43**	**-2,30**	low	ND	
yulD	BSU31190	lower	TGAAAGCGCTATCT	9,89	**-2,27**	**-4,85**	**-5,31**	high	ND	
yuxG	BSU31220	lower	TGAAAACGGATACA	9,22	-1,21	**-4,17**	**-6,12**	low	0	P
yvdG	BSU34610	lower	TGTAACCGCTTTCT	9,3	-1,37	-1,53	**-2,05**	low	-28	P
yvfK	BSU34160	lower	TTAAAGCGCTTTCA	9,74	**-3,99**	**-6,14**	**-10,63**	high	5	P
yxlH	BSU38640	upper	TTGAAACGCTTTCA	9	-1,41	**-1,96**	**-2,33**	low	260	P
yydK	BSU40130	upper	TGTAAGCGGTTTAT	9	-1,47	**-3,25**	**-2,40**	low	-21	P
yyzE	BSU40120	lower	TGAAAGCGTAACCA	9,13	-1,17	**-2,97**	**-2,13**	low	0	P

[a] ND, not determined.

[b] P, prediction from this study.

[c] *iolA cre*-2 is located within *iolB*, the second gene of the *iol* operon. Fold changes of *iolB* are given.

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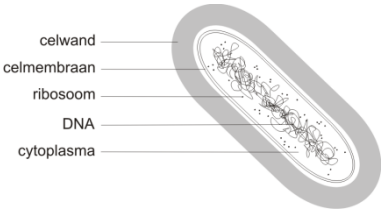
SAMENVATTING VOOR DE LEEK

Dit proefschrift beschrijft het onderzoek naar mechanismen die bacteriële cellen vertonen als reactie op overproductie van eiwitten die (potentieel) nuttig zijn in de was- en levensmiddelenindustrie en het onderzoek aan de regulatie voor efficiënter gebruik van koolstofbronnen door bacteriële cellen.

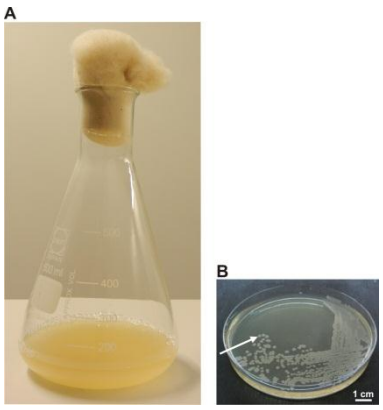
De modelorganisme in deze studie is een niet-pathogene bacterie die in de grond leeft, *Bacillus subtilis*. *B. subtilis* (de gebruikelijke afkorting) cellen zijn staafvormig en 2 - 5 μm lang (1 μm = 0,001 mm). De belangrijkste elementen waaruit *B. subtilis* cellen zijn opgebouwt zijn de celmembraan, cytoplasma en celwand (Figuur 1). De celmembraan bestaat uit een dubbele laag van lipiden met daarin eiwitten. Het scheidt de binnenkant van een cel met de externe omgeving. Het cytoplasma is een gel-achtige substantie die grotendeels bestaat uit water en vult het binnenste van de cel met daarin het DNA (de drager van genetische informatie), eiwitten, ribosomen (die een rol spelen bij de productie van eiwitten) en andere elementen. De celwand bevindt zich buiten het celmembraan en het bestaat voornamelijk uit peptidoglycaan, een complex netwerk van lange gecrosslinkte polysaccharideketens gebonden door korte ketens van aminozuren.



Figuur 1. Schematische weergave van een *Bacillus subtilis* cel (longitudinale doorsnede).

Dankzij sommige eigenschappen wordt *B. subtilis* veel gebruikt in het onderzoek als modelorganisme. Deze handige eigenschappen omvatten: niet pathogeen, gemakkelijk te kweken onder laboratoriumomstandigheden (Figuur 2), kennis van het genoom (het volledige genetische materiaal van cellen) en de beschikbaarheid van ontwikkelde methoden voor genetische manipulatie. *B. subtilis* is in staat om ongunstige omstandigheden (bijvoorbeeld geen beschikbaarheid van voedingsstoffen of een te hoge temperatuur) te overleven door: de productie van zeer resistente sporen (sporulatie), te reizen op zoek naar voedingsstoffen (chemotaxis), het verzamelen van vreemd genetisch materiaal uit de omgeving (competentie) daarmee het verwerven van nieuwe functies en mogelijkheden, enzymeiwitten te exporteren naar de omgeving (secretie) om

organische stoffen te degraderen in kleinere moleculen die gemakkelijker gemetaboliseerd kunnen worden. Deze laatste overlevingsstrategie van *B. subtilis* wordt algemeen toegepast in de industrie voor de productie van verschillende eiwitten/enzymen van *B. subtilis* (endogeen eiwit) of andere organismen (heteroloog eiwit). Deze enzymen kunnen vervolgens gebruikt worden bij de productie van voedingsmiddelen, waspoeder of medicijnen. Een bekend voorbeeld waarbij een bacterie gebruikt wordt voor de productie van enzymen is *Escherichia coli* die insuline produceert. Er zitten nadelen aan het gebruik van *E. coli* als productie stam, zo heeft *E. coli* geen mogelijkheden om de eiwitten te exporteren en bovendien is het een pathogene bacterie. De zuivering van eiwitten gemaakt door *E. coli* is veel moeilijker dan wanneer de eiwitten geproduceert worden door bijvoorbeeld *B. subtilis* aangezien de cellen kapot gemaakt moeten worden om het gewenste proteïne te krijgen. Verder moeten de aanwezige pathogene toxinen grondig vernietigd worden zodat het eindprodukt veilig voor de mens is. Helaas is de productie van eiwitten met behulp van *B. subtilis* als een "fabriek" ook niet optimaal. Als reactie op verhoogde productie en secretie van eiwitten, activeren de cellen van *B. subtilis* verschillende afweermechanismen zoals een verhoogde productie van proteolytische eiwitten die verantvoordelijk zijn voor afbraak van andere eiwitten. Deze proteases breken vervolgens ook het voor industriële productie gewenste eiwit af.



Figuur 2. Culture van *Bacillus subtilis*. (**A**) Een culture in 100 ml vloeibaar medium. Honderden miljarden bacteriële cellen zijn aanwezig in de vloeistof en veroorzaken een vertroebeling van het medium die zichtbaar is voor het blote oog. Deze bacterie culturen kunnen gemaakt worden in honderden liters wat voornamelijk voor de industrie bruikbaar is. (**B**) *B. subtilis* cultures op een vast medium. Afzonderlijke kolonies, clusters van vele miljoenen cellen, zijn zichtbaar (pijl).

In **hoofdstuk twee** staat de identificatie beschreven van afweermechanismen die geactiveerd worden als reactie op verhoogde productie van acht verschillende eiwitten in *B. subtilis* cellen. Dit werd bereikt door de analyse van de expressie van alle genen van de cellen die een eiwit produceren met een complexe techniek (zogenaamd microarrays). Kunstmatige veranderingen van de geïdentificeerde afweermechanismen (zoals de verhoogde productie van speciale eiwitten die verantwoordelijk zijn voor de

afbraak van andere niet-functionele eiwitten) door middel van genetische manipulatie kan leiden tot een nieuwe stam van *B. subtilis* die betere prestaties kan leveren wat betreft de prodcutie en de kwailiteit van geproduceerde eiwitten.

Het doel van de studie beschreven in het **derde hoofdstuk** is het ontraffelen van de cellulaire reactie op stress geinduceerde door een verhoogde productie van eiwitten die verankerd zijn in het celmembraan. Tevens wordt dezelfde reactie veroorzaakt door andere schadelijke factoren die de structuur van membraaneiwitten afbreken zoals zout of een hoge temperatuur. Deze factoren veroorzaken denaturatie (schade aan de structuur) van het eiwit. Tijdens deze studie werd een nieuw regulerend eiwit (YkrK) gevonden die de expressie regeelt (productie op basis van de informatie in het DNA) van een ander eiwit, namelijk YkrL (of HtpX). Dit YkrL eiwit is verankerd in het celmembraan en is waarschijnlijk verantwoordelijk voor de afbraak van beschadigde membraaneiwitten. Deze afbraak heeft een positieve invloed op de algemene structuur en de werking van het celmembraan, waar veel eiwitten aanwezig zijn die essentiële functies hebben voor het overleven van de cel. Bovendien, bacteriële membraaneiwitten zijn gedeeltelijk blootgesteld aan de buitenkant van de cel, waardoor ze gemakkelijk toegankelijk zijn voor geneesmiddelen zoals gebruikt in chemotherapie. Voor het produceren van nieuwe geneesmiddelen met behulp van *B. subtilis* cellen is zuivering vereist. De afbraak van misvormde eiwitcomplexen in het membraan maakt deze zuivering eenvoudiger. Echter, een te efficiënte afbraak van membraaneiwitten door de werking van YkrL kan ook de hoeveelheid van het geproduceerde membraaneiwit verlagen. Door genetische manipulatie van het defensieve mechanisme (YkrL, YkrK) kan *B. subtilis* efficiënter membraaneiwitten produceren.

Het onderwerp van **hoofdstuk vier** is een lipoproteïne, een eiwit met een lipide (vet) staart, PrsA. Het lipoproteïne PrsA is verankerd in het celmembraan en een deel van het enzym is gericht naar buiten (naar de ruimte tussen de celmamembraan en celwand). PrsA speelt rol bij het correct en volledig vouwen van gesecreteerde eiwitten en, net als veel andere eiwitten, is een essentieel onderdeel van de cel. De resultaten toonden aan dat PrsA essentieel is voor de cel vanwege de betrokkenheid bij de vouwing van eiwitten die een belangrijke rol spelen in de celwand biosynthese. Deze door PrsA gevouwen eiwitten heten penicilline-bindende eiwitten (PBPs) en zijn betrokken bij het maken van de celwand. De celwand is een belangrijk element in de constructie van de cel. Het fungeert als een soort exoskelet die fysische krachten opvangt en zo de cel in zijn vorm houdt.

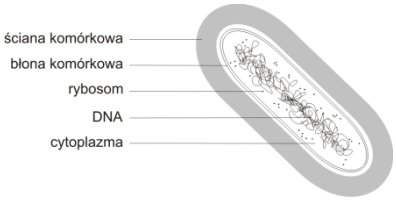
B. subtilis, zoals andere bacteriën, heeft de mogelijkheid om een groot aantal stoffen te gebruiken als koolstofbron voor het bouwen van eiwitten en andere elementen van de

cel. Voor een optimale groei en energieverbruik is het nuttig om alleen genen tot expressie te brengen die op dat moment nodig zijn. CcpA regelt de productie (expressie) van enzymatische eiwitten die betrokken zijn bij het metabolisme (afbraak) van deze koolstofbronnen. Genen die coderen voor enzymen die nodig zijn voor benutting van energetisch gunstigste koolstofbronnen (glucose, fructose, mannose) worden geactiveerd door CcpA, terwijl de genen die coderen voor enzymen die functies uitvoeren in het gebruik van andere koolstofbronnen, die minder de voorkeur hebben worden gedempt (repressie) door CcpA. Activering en repressie van genen door de CcpA wordt bewerkstelligt doordat CcpA bindt aan specifieke korte fragmenten DNA, zogenaamde *cre* boxen. Ze zijn gekenmerkt door een specifieke DNA-sequentie (de volgorde van de samenstellende nucleotiden waaruit DNA is opgebouwd) en positie (afstand) in het DNA van een bepaald gen die gereguleerd wordt door CcpA. In **hoofdstuk vijf** zijn de sequentie en positie van de *cre* boxen van veel genen gereguleerd door de CcpA grondig geanalyseerd. De resultaten toonden aan dat kleine verschillen in de specifieke sequentie en de positie van *cre* boxen en de impact van deze verschillen op het vermogen van CcpA om aan het DNA van deze boxen te binden, het regulerende vermogen van CcpA bepalen. Deze resultaten dragen bij tot een beter begrip van het metabolisme van koolstofbronnen en het regelen daarvan door CcpA.

STRESZCZENIE DLA LAIKÓW

Tematem tej pracy naukowej są mechanizmy obronne uruchamiane przez komórki bakteryjne *Bacillus subtilis* w odpowiedzi na intensywną produckję białek (potencjalnie) użytecznych w przemyśle oraz mechanizm kontroli metabolizmu źródeł węgla.

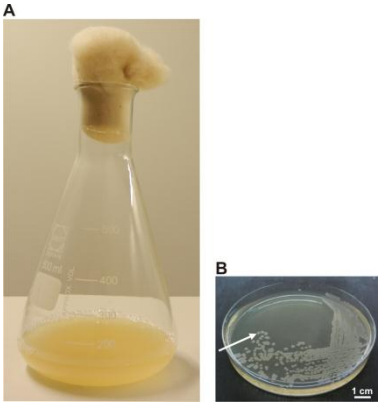
Organizmem badawczym w tej pracy jest glebowa, niechorobotwórcza bakteria, *Bacillus subtilis* (*Laseczka sienna*). Komórki *B. subtilis* charakteryzują się pałeczkowatym kształtem i długością 2 - 5 μm (1 μm = 0.001 mm). Główne elementy budowy komórki *B. subtilis* to błona komorkowa, cytoplazma i ściana komórkowa (Ryc. 1). Błona komórkowa zbudowana jest z podwójnej warstwy lipidów (tłuszczy) i białek. Oddziela ona wnętrze komórki od środowiska zewnętrznego. Cytoplazma to substancja żelowa składająca się w większości z wody. Wypełnia ona wnętrze komórki, gdzie znajdują się nośnik informacji genetycznej w postaci DNA, białka, rybosomy pełniące funkcje w ekspresji (produkcji) białek i inne elementy. Ściana komórkowa zbudowana jest z peptydoglikanu czyli skomplikowanej sieci łańcuchów polisacharydowych (wielocukrowych) połączonych krótkimi łańcuchami aminokwasowymi.



Rycina 1. Schemat budowy komórki *Bacillus subtilis* (przekrój podłużny).

Dzięki pewnym cechom *B. subtilis* zdobyła sławę jako organizm modelowy w badaniach naukowych. Są to między innymi niechorobotwórczość, łatwa hodowla w warunkach laboratoryjnych (Ryc. 2), znajomość genomu (całego materiału genetycznego komórki), dostępność opracowanych metod manipulacji genetycznych. *B. subtilis* jest zdolna do przetrwania niesprzyjających warunków (np. niedostępność składników odżywczych, wysoka temperatura) poprzez produkcję wysoko odpornych przetrwalników (sporulacja), przemieszczanie się w poszukiwaniu substancji odżywczych (chemotaksja), pobieranie obcego materiału genetycznego ze środowiska (kompetencja) a tym samym nabywanie nowych cech i zdolnośći, eksport do

środowiska białek enzymatycznych (sekrecja) degradujących związki organiczne na mniejsze łatwiej metabolizowane cząsteczki. Ta ostatnia właściwość *B. subtilis* jest szeroko wykorzystywana w przemyśle do produkcji różnego typu białek/enzymów pochodzących z *B. subtilis* (białka endogeniczne) lub innych organizmów (białka heterologiczne) i mających zastosowanie w produkcji żywności, proszków do prania czy też leków. Również dobrze znana *Escherichia coli* (*Pałeczka okrężnicy*) jest wykorzystywana np. do produkcji insuliny. *E. coli* jednak nie posiada zdolności sekrecji białek, a ponadto jest to bakteria chorobotwórcza. Oczyszczanie białek z hodowli *E. coli* jest w związku z tym znacznie trudniejsze, gdyż wymaga zniszczenia komórek, aby dotrzec do pożądanego białka, oraz dokładnego zniszczenia chorobotwórczych toksyn, aby końcowy produkt był bezpieczny dla człowieka. Niestety produkcja białek z wykorzystaniem *B. subtilis* jako swoistej „fabryki" również nie przebiega bez przeszkód. W odpowiedzi na intensywną produkcję i sekrecję białek, komórki *B. subtilis* uruchamiają różnego rodzaju mechanizmy obronne w postaci np. wzmożonej produkcji białek proteolitycznych degradujących inne białka, również to białko pożądane w produkcji przemysłowej.



Rycina 2. Hodowle bakterii *Bacillus subtilis*. (**A**) 100 ml hodowli w podłożu płynnym. Zawieszone w płynie setki bilionów komórek bakteryjnych powodują widoczne gołym okiem zmętnienie pożywki. Możliwe są rónież hodowle o znacznie większej objętości, tj. setki litrów. Wykonuje się je głównie na potrzeby przemysłu. (**B**) hodowla na podłożu (pożywce) stałym. Widoczne są pojedyncze kolonie bakteryjne (strzałka) stanowiące skupiska wielu milionów komórek.

W **rozdziale drugim,** poprzez analizę całego genomu komórek *B. subtilis* produkujących różne biłka, zostały zidentyfikowane mechanizmy obronne uruchamiane w odpowiedzi na intensywną produkcję białek. Do tego celu wykorzystano skomplikowaną technikę, tzw. mikromacierze. Dalsze modyfikacje zidentyfikowanych mechanizmów obronnych (takich jak na przykład wzmożona produkcja specjalnych białek odpowiedzialnych za degradację innych niefunkcjonalnych białek) poprzez manipulacje genetyczne mogą zaowocować wygenerowaniem nowego szczepu *B. subtilis*, który umożliwi wyższą wydajność i lepszą jakość produkowanych białek.

Przedmiotem badań w **rozdziale trzecim** jest swoista odpowiedź komórkowa na stres błonowy wywołany intensywną produkcją białek zakotwiczonych w błonie komórkowej i innych szkodliwych czynników wpływających negatywnie na strukturę białek błonowych, takich jak sól czy temperatura. Czynniki te powodują denaturację (uszkodzenie struktury) białka. W wyniku tych badań zostało odkryte nowe białko regulatorowe, YkrK, kontrolujące ekspresję (produkcję na podstawie informacji zawartej w DNA) innego białka, a mianowicie YkrL (lub HtpX). Białko to jest zakotwiczone w błonie komórkowej i najprawdopodobniej jest odpowiedzialne za degradację uszkodzonych białek błonowych. Ma to korzystny wpływ na ogólną strukturę i funkcjonowanie błony komórkowej, gdzie znajduje się wiele białek pełniących istotne funkcje dla przeżycia komórki. Ponad to białka błonowe bakterii są częściowo wyeksponowane na zewnątrz komórki, co sprawia, że są one łatwo dostępne dla leków np. stosowancyh w chemoterapii. Do wygenerowania nowych leków wymagana jest jednak uprzednia produkcja białek docelowych (np. z wykorzystaniem *B. subtilis*), ich oczyszczenie i charakteryzacja. Jakkolwiek, za wysoka aktywność YkrL może doprowadzić do degradacji produkowanych przez *B. subtilis* białek błonowych i, w efekcie, do obniżenia wydajności produkcji. Manipulacje genetyczne również w zakresie tego mechanizmu obronnego (YkrL, YkrK) mogą przyczynić się do wygenerowania szczepu *B. subtilis* lepszego w produkcji białek błonowych.

Tematem **rozdziału czwartego** jest lipoproteina, czyli białko posiadające rdzeń lipidowy (tłuszczowy), PrsA. Lipoproteina PrsA jest zakotwiczona w błonie komórkowej i posiada część enzymatyczną skierowaną na zewnątrz (w przestrzeń pomiędzy błoną komórkową a ścianą komórkową). PrsA pełni rolę w przybieraniu dojrzałej struktury białek sekrecyjnych i, jak wiele innych białek, jest niezbędnym komponentem komórki. Wyniki wykazały, że PrsA jest esencjonalnym elementem komórki w zwiazku z zaangażowaniem w dojrzewanie biełek, które pełnią kluczową rolę w biosyntezie ściany komórkowej. Są to tzw. białka wiążące penicylinę, PBP (*ang*. penicillin binding proteins). Ściana komórkowa stanowi istotny element w budowie komórki; pełni rolę swoistego szkieletu zewnętrznego nadającego kształt komórce oraz chroniącego przed czynnikami środowiska zewnętrznego a także przed pęcznieniem w wyniku pobierania wody ze środowiska. Dlatego też PrsA jest niezbędna do przeżycia komórki.

B. subtilis, jak inne bakterie, ma zdolność do wykorzystywania szerokiego zakresu związków jako źródło węgla do budowy białek i innych elemntów komórki. Aby jednak zapewnić optymalne zużycie energii i tempo wzrostu, białko regulatorowe CcpA kontroluje produkcję (ekspresję) białek enzymatycznych zaangażowanych w metabolizm (rozkład) tychże źródeł węgla. Geny kodujące białka enzymatyczne

potrzebne do utylizowania energetycznie najkorzystniejszych źródeł węgla (glukoza, fruktoza, jabłczan) są uaktywniane (aktywacja), podczas gdy geny kodujące enzymy pełniące funkcje w utylizacji innych, mniej preferowanych źródeł węgla, są wyciszane (represja). Aktywacja i represja tych genów odbywa się poprzez wiązanie CcpA do określonych krótkich fragmentów DNA. Są to tzw. boksy *cre*. Charakteryzują się one specyficzną sekwencją DNA (porządek składowych cząsteczek budujących nić DNA) oraz pozycją (odległość) na nici DNA względem danego genu regulowanego przez CcpA. W **rozdziale piątym** boksy *cre* wielu genów regulowanych przez CcpA zostały dokładnie przeanalizowane pod względem sekwencji i pozycji. Wyniki wykazały specyficzne drobne różnice w sekwencji i pozycji boksów *cre* oraz wpływ tych różnic na zdolność CcpA do wiązania się z DNA w obrębie tych boksów, tym samym zdolność regulacyjną CcpA. Wyniki te przyczyniają się do lepszego zrozumienia mechanizmu regulacji metabolizmu źródeł węgla przez CcpA.

LIST OF PUBLICATIONS

Marciniak BC, Pabijaniak M, de Jong A, Dühring R, Seidel G, Hillen W, Kuipers OP: **High- and low-affinity *cre* boxes for CcpA binding in *Bacillus subtilis* revealed by genome-wide analysis**. *BMC Genomics* 2012, **13**:401.

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