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Title and subtitle: On the role of penicillin-binding protein SpoVD in endospore cortex assembly.


Abstract

Bacteria of the genera *Bacillus* and *Clostridium* can form endospores as a strategy to survive unfavourable environmental conditions. Endospore formation involves synthesis of cortex, a thick layer of modified peptidoglycan that surrounds the spore. This layer is required for heat resistance of the spore and mutant spores lacking the cortex layer can be identified by a simple heat shock assay.

*B. subtilis* SpoVD is a class B, high molecular weight, penicillin-binding protein (PBP) essential for spore cortex peptidoglycan synthesis. The exact role of the protein in cortex assembly is unknown but it most likely catalyses the formation of cross-links between glycan strands in nascent peptidoglycan. SpoVD deficient strains produce heat sensitive spores without cortex layer. Two conserved cysteine residues (Cys332 and Cys351) in the transpeptidase domain of SpoVD seem important for activity of the enzyme. They can form an intramolecular disulfide bond and this is catalysed by the membrane-bound thiol-disulfide oxidoreductase BdbD. The disulfide bond in SpoVD is located close to the transpeptidase active site and blocks the function of the protein. The bond is broken by the action of StoA, a sporulation-specific membrane-bound thiol-disulfide oxidoreductase. Based on these findings a thiol-based redox switch regulation of SpoVD activity was proposed in 2010.

The aim of this PhD project was to elucidate the function of SpoVD in cortex synthesis and to find out the physiological role of the proposed switch and the two cysteine residues in SpoVD. In depth investigation of the process of cortex assembly contribute to our understanding of peptidoglycan synthesis in general. This is of considerable medicinal interest since, e.g., bacterial cell wall synthesis is an effective target for many antibiotics in clinical use, such as penicillins and cephalosporins and eventual new drugs.

I demonstrate, by the use of a constructed SpoVD active site mutant strain, that synthesis of cortex explicitly depends on the transpeptidase activity of the protein. I show that the C-terminal PASTA domain of SpoVD is not important for the function of the protein in cortex synthesis. My results from *in vitro* experiments with several isolated protein variants strengthen the view that SpoVD is a specific target for StoA. My findings, supported by data available in the literature, indicate that the two cysteine residues in SpoVD affect the dynamics of the transpeptidase domain. Finally, I propose a revised model for the function of BdbD and StoA in modulation of the redox state of SpoVD, where BdbD and StoA are suggested to act (mainly) during the folding of newly synthesised SpoVD.



Key words: sporulation, cortex, *Bacillus subtilis*, penicillin-binding proteins, SpoVD, peptidoglycan

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# On the role of penicillin-binding protein SpoVD in endospore cortex assembly

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*To my grandparents, Wanda and Stefan*

# Contents

# List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-V):

I **Bukowska-Faniband, E. and Hederstedt, L.** (2013) Cortex synthesis during *Bacillus subtilis* sporulation depends on the transpeptidase activity of SpoVD. FEMS Microbiol. Lett. **346:** 65-72.

II **Bukowska-Faniband, E. and Hederstedt, L.** (2014) The PASTA domain of penicillin-binding protein SpoVD is dispensable for endospore cortex peptidoglycan assembly in *Bacillus subtilis*. Microbiology. In press, **doi:10.1099/mic.0.000011**.

III **Bukowska-Faniband, E. and Hederstedt, L.** Interaction between *Bacillus subtilis* thiol-disulfide oxidoreductase StoA and penicillin-binding protein SpoVD. Manuscript.

IV **Li, D., Hederstedt, L., Bukowska-Faniband, E.** Properties of *Bacillus subtilis* penicillin-binding protein Pbp4b encoded by the *pbpI* gene. Manuscript

V **Bukowska-Faniband, E. and Hederstedt, L.** Role of cysteine residues in *Bacillus subtilis* SpoVE for endospore cortex synthesis. Manuscript.

# List of contributions

**Paper I**

E. Bukowska-Faniband is the corresponding author. She performed all the experiments and wrote the paper under supervision of L. Hederstedt. The authors together planned the experiments and interpreted the results.

**Paper II**

E. Bukowska-Faniband is the corresponding author. She performed all the experiments and wrote the paper under supervision of L. Hederstedt. The authors together planned the experiments and interpreted results.

**Paper III**

E. Bukowska-Faniband performed all the experiments and wrote the paper under supervision of L. Hederstedt. The authors together planned experiments and interpreted results.

**Paper IV**

D. Li performed all the experiments under supervision of L. Hederstedt and E. Bukowska-Faniband. All authors together planned experiments and interpreted the results. D. Li and L. Hederstedt wrote the paper.

**Paper V**

E. Bukowska-Faniband performed all initial experiments and wrote the paper. The authors together planned experiments, supervised the project and interpreted results.

# Introduction

With relatively few exceptions, bacterial cells possess a cell wall that determines their shape and provides protection against mechanical damage and osmotic lysis. The major component of the cell wall in *Eubacteria* is peptidoglycan, a polymer consisting of amino sugars and short peptides. Assembly of peptidoglycan is achieved by the activity of penicillin-binding proteins (PBPs) that are active on the outer side of the cytoplasmic membrane (1). Since PBPs are essential components of the peptidoglycan synthesis machinery and unique to bacteria, they constitute an effective target for many drugs. β-lactam antibiotics, such as penicillin, that inhibit activity of PBPs have been successfully used for the past 70 years to fight infections caused by bacteria (2). However, the number of antibiotic resistant clinical isolates increases every year. Bacteria develop mechanisms that combat the action of common antibiotics, leading to ineffective treatment and prolonged illnesses (3-5). A need for new drug targets drives research on bacterial cell wall morphogenesis.

Despite many years of extensive studies, the process of peptidoglycan synthesis is far from being completely understood at the molecular level. The complex macromolecular structure of peptidoglycan and the essential nature of many proteins involved in its synthesis make experimental studies difficult. Interestingly, synthesis of spore cortex peptidoglycan during sporulation in *Bacillus subtilis* is not essential (6).This enables analysis of mutants defective in enzymes that otherwise are essential for growth, and offers a unique system to elucidate principles of bacterial cell wall assembly. Heat resistance of endospores depends on the presence of the cortex layer (7). Loss or gain of heat resistance of spores therefore provides a convenient quantitative assay to screen for mutants that lack or possess cortex layer.

SpoVD is a class B high molecular weight (HMW) penicillin-binding protein (PBP) that is essential for cortex synthesis in *B. subtilis* sporulating cells. SpoVD deficient mutants form heat sensitive endospores, completely lacking the cortex layer (8).The detailed functions of the protein are unknown, but it presumably catalyses the formation of peptide cross-links between glycan strands in nascent cortex. The activity of the transpeptidase domain of SpoVD has been suggested to be regulated by a thiol-based redox switch not previously reported for any other PBP (9).

My PhD thesis work aims to find out the physiological role of this proposed switch and the two cysteine residues in SpoVD, and to increase our understanding of peptidoglycan biogenesis by investigation of SpoVD and its interacting proteins.

The first section of this PhD thesis gives the reader the background information needed to understand the second part of the thesis. Section I provides an overview of topics such as endospore formation in the model organism *B. subtilis*, differences between vegetative and cortical peptidoglycan, synthesis of peptidoglycan by penicillin-binding proteins and subcellular localization of proteins during sporulation. Section II is focused on the current knowledge about SpoVD and includes my own findings, conclusions and proposals about the molecular details of the protein. It also connects the reader to my attached Papers I, II, III, IV and V.

# Section I. General background

# 1. Sporulation in *Bacillus subtilis*

Bacteria of the genera *Bacillus* and *Clostridium* can form endospores to survive unfavourable environmental conditions. *Bacillus subtilis* is the most extensively studied endospore-forming microorganism. This rod-shaped Gram-positive bacterium is most commonly found in soil. Due to relative ease of genetic manipulation, complete genome sequence availability and a toolbox of experimental techniques, *B. subtilis* has become a model organism for basic research (10). Although *B. subtilis* is not pathogenic or toxigenic to humans, in depth research with this model organism can provide insight into cellular processes of related pathogenic species, such as *B. anthracis* or *B. cereus*.

## 1.1. Morphological stages of sporulation

Sporulation, i.e. formation of an endospore, takes many hours to complete and involves a series of cell morphological changes (Figure 1). In response to starvation for a carbon, nitrogen or phosphorus source, and if the cell density is high enough, vegetative cells cease to grow and at outset of sporulation an asymmetrically located septum near one pole of the cell is formed. Two compartments result from the asymmetric division, the smaller forespore and the larger mother cell. Each of them will contain one copy of the chromosome. In subsequent development, the forespore is engulfed by the mother cell in a phagocytosis-like process. This results in formation of a double-membrane enclosed forespore inside the mother cell cytoplasm, i.e. a cell within a cell. Following engulfment two protective layers, the cortex and the coat, are assembled around the forespore. In the final stage of sporulation, the mother cell lyses and the mature spore is released into the environment. Endopores are metabolically dormant and can survive for many hundreds of years without nutrients. They are resistant to high temperatures, desiccation, ionizing radiation, organic solvents, detergents and hydrolytic enzymes that normally kill cells. When nutrients become available, spores can quickly germinate and begin vegetative growth (11, 12).

As a common practice, the sporulation process is described in eight stages (designated 0-VII). These stages are defined based on the morphological features of the sporulating cell. A vegetatively growing cell is defined as stage 0. In stage I, two copies of the chromosome condense and elongate to form a single filament that stretches

across the long axis of the cell. Stage II is defined by the formation of an asymmetrically positioned septum. Stage III indicates completion of engulfment. During stage IV the cortex layer is deposited in the intermembrane space of the forespore, and in stage V the spore coat is laid down on the surface of the developing spore. Stage VI is when the spore has acquired resistance to high temperatures and UV radiation. Stage VII is defined by the release of the spore via lysis of the mother cell. The nomenclature for sporulation genes is associated with the stage at which the sporulation is blocked due to mutation of the particular gene (e.g. *spoII* mutants complete asymmetric septation but fail to complete engulfment) (13, 14).

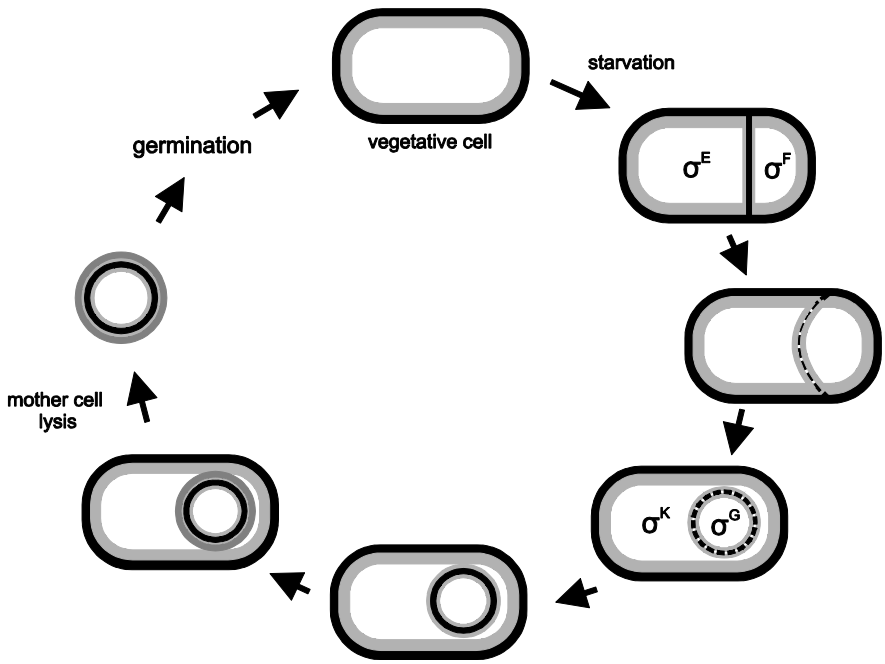



**Figure 1.** Illustration of the morphological changes occurring during *B. subtilis* sporulation (for a detailed description see text). The drawing presents also the cellular location and the time point in sporulation at which different sporulation-specific sigma factors become active. Structures are presented by different colours: black – peptidoglycan, light grey – membranes, dark grey – protein coat. A dashed line represents peptidoglycan remodelling during engulfment of the forespore as suggested by Tocheva *et. al*, 2011 (15).

## 1.2. Regulation of gene expression during sporulation

Initiation and coordination of sporulation are under complex regulation, including several checkpoints which ensure that previous developmental stages are completed. The switch from vegetative growth to sporulation is initiated by the activation in the cell of the master transcription regulator Spo0A through a phosphorelay. Activated Spo0A triggers the formation of the asymmetric division septum and transcription of several key sporulation-specific genes, particularly *spoIIA*, *spoIIE* and *spoIIG*. The morphological changes that occur during sporulation are mediated by global changes in gene expression. Programs of gene expression are initiated in each cell compartment under control of sporulation-specific RNA polymerase σ factors (Figure 1). The stationary phase σ factor, $\sigma^H$, controls expression of several genes during the earliest stages of sporulation, prior to polar septum formation. Immediately after asymmetric division, $\sigma^F$ becomes active in the forespore, rapidly followed by activation of $\sigma^E$ in the mother cell. Next, forespore engulfment occurs and the late compartment-specific σ factors become active. $\sigma^G$ in the forespore and $\sigma^K$ in the mother cell drive the final stages of sporulation (12, 13, 16, 17).

## 1.3. *B. subtilis* endospore morphology

A mature *B. subtilis* endospore consists of a central core, surrounded by inner membrane (corresponding to the cytoplasmic membrane), germ cell wall, cortex, outer membrane and coat (Figure 2). The core corresponds to the cytoplasmic compartment of a vegetative cell, but there is essentially no metabolic activity and the water content is low compared to vegetative cells. A thin layer of germ cell wall, present on the surface of the inner spore membrane, serves as primordial cell wall during germination. The cortex is a thick layer composed of modified peptidoglycan. It is required for spore core dehydration and essential for spore heat resistance. The proteinaceous coat, composed of many different proteins, is deposited on the outer surface of the outer membrane and important for resistance to chemicals and hydrolytic enzymes (18-20). *B. subtilis* spores are ovoid with an average size of about 1μm in length and 0.5 μm in diameter (21).

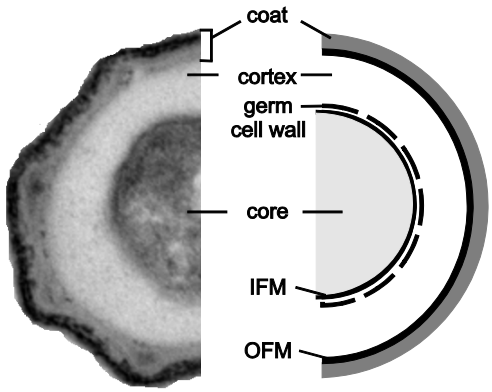



**Figure 2.** Thin section electron micrograph (left side) and schematic representation (right side) of a mature *B. subtilis* endospore. IFM – inner forespore membrane; OFM – outer forespore membrane.

## 1.4. Problematic and beneficial aspects of endospores

Endospores of some pathogenic *Bacillus* and *Clostridium* species can be problematic for medicine and the food industry. The remarkable resistance properties, described above, make spores very difficult to decontaminate. Endospores are the main reason for the need of autoclaves to sterilize liquids. Spores in food products can lead to food poisoning. The major endospore-forming pathogens that are causative agents of foodborne diseases are *C. perfingens*, *C. botulinum* and *B.cereus* (22). One major problem faced in the clinic is difficulty to effectively decontaminate large areas. Therefore, long-term hospitalized patients are at risk of infection with *C. difficile*, which is a leading cause of antibiotic-associated diarrhoea (23).On the other hand, spores have a number of potential applications. Since they are able to survive the acidity of the stomach, spores of several *Bacillus* species are commercially used as probiotics (24). In Japan, spores of the *B. subtilis* variant *natto* are used as the starter culture for preparation of the traditional dish "natto" (24). Use of spores as a display surface for heterologous antigens and enzymes is currently investigated by several research groups. It is a potentially powerful tool to, for example, deliver antigens to mucosal surfaces and an alternative to several currently used vaccines (25).

# 2. Peptidoglycan of *B. subtilis*

Peptidoglycan synthesis occurs in *B. subtilis* during both vegetative growth and sporulation, resulting in the cell wall and the cortex, respectively. Peptidoglycan of these layers, although synthesized from a common precursor molecule (lipid II), differs somewhat in structure and chemical composition.

## 2.1. Peptidoglycan in vegetative cells

Vegetative cell wall peptidoglycan consists of linear glycan strands that are cross-linked via short peptide chains (Figure 3). The glycan strands contain repeating disaccharide residues of one N-acetyl-glucosamine (NAG) and one N-acetyl-muramic acid (NAM) linked by β-1,4 bonds. The average length of glycan strands in *B.subtilis* is 1300 disaccharides, but individual strands of up to 5000 disaccharides can be found in purified glycan (26). A peptide side chain is attached to the carboxyl group of NAM. The most common stem peptide found in *B. subtilis* is L-Ala$_{(1)}$-D-Glu$_{(2)}$-Dpm$_{(3)}$-D-Ala$_{(4)}$-D-Ala$_{(5)}$(Dpm is diaminopimelic acid), with the L-Ala$_{(1)}$ attached to NAM. The peptide cross-bridge is between Dpm$_{(3)}$ in one peptide and the D-Ala$_{(4)}$ in another peptide. The terminal D-Ala residue of the peptide that had its D-Ala$_{(4)}$ cross-linked is removed during the transpeptidation reaction, whereas the two terminal D-Ala residues on the other stem peptide are removed by a carboxypeptidase. Stem peptides which have not been cross-linked are usually trimmed and present as tripeptides (27, 28). The released amino acids are typically imported by the cell and reused (29).

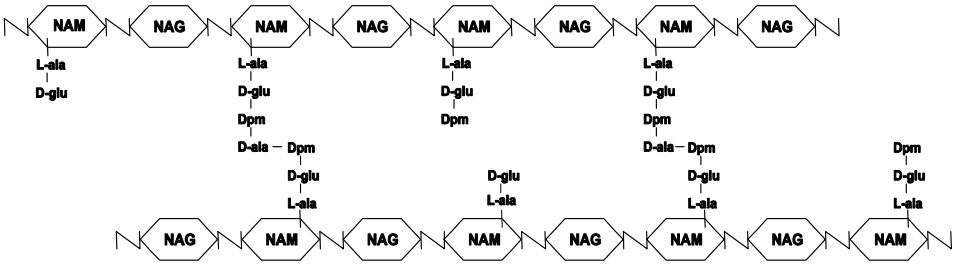



**Figure 3.** Basic structure of *B. subtilis* cell wall peptidoglycan shown in two multiple cross-linked glycan strands. NAG – N-acetyl-glucosamine; NAM – N-acetyl-muramic acid; Dpm – diaminopimelic acid.

## 2.2. Endospore peptidoglycan

Two contiguous layers can be distinguished in endospore peptidoglycan. A thin peptidoglycan layer adjacent to the inner forespore membrane, called the germ cell wall (Figure 2), has the same composition as the vegetative cell wall. It presumably constitutes a template for the synthesis of cortex during sporulation and is regarded as primer for cell wall synthesis during germination and outgrowth (30). The germ cell wall is surrounded by a much thicker layer of peptidoglycan, known as the cortex (Figure 2). Cortex peptidoglycan has a modified structure compared to vegetative cell wall, which determines its ability to carry out roles specific to the spore. The major modification of the cortex peptidoglycan is the absence of a peptide side chain on ~50% of the NAM residues and conversion of these residues to muramic-δ-lactam (MAL) (Figure 4). The presence of MAL results in a lower number of possible peptide cross-links (6, 27, 31). Additionally, it is suggested that the action of sporulation-specific D,D-carboxypeptidases generates a gradient of peptide cross-links across the span of the cortical peptidoglycan. In this model, the innermost layers of the cortex are loosely cross-linked and the level of cross-linking increases towards the outermost layer. The apparent gradient and general low degree of cross-linking give the spore peptidoglycan properties that largely contribute to spore core dehydration (32). Cortex peptidoglycan is rapidly degraded during germination, while the germ cell wall is left intact. The presence of MAL in the cortex is a structural determinant for the specificity of lytic enzymes during germination (33).

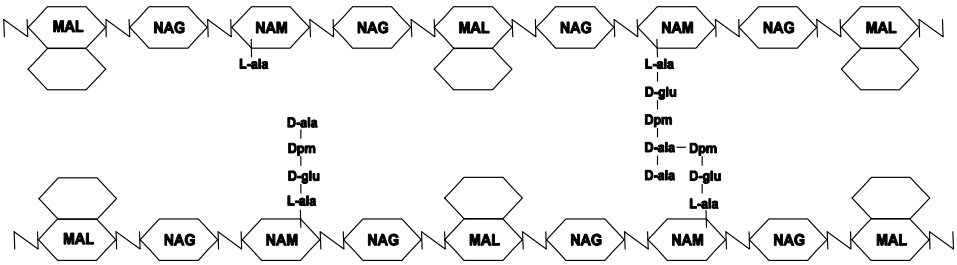



**Figure 4**. Basic structure of *B. subtilis* cortex peptidoglycan. About fifty percent of the NAM residues are modified into muramic-δ-lactam (MAL).

## 2.3. Peptidoglycan synthesis

Synthesis of peptidoglycan can be divided into three stages. The first stage comprises synthesis of the precursor molecules (UDP-N-acetyl-glucosamine and UDP-N-acetyl-muramyl pentapeptide) and takes place in the cytoplasm. In the second stage, the UDP-N-acetyl-muramyl pentapeptide is coupled to a special lipid (bactoprenol) at the cytoplasmic membrane and the N-acetyl-glucosamine is then added. This yields lipid II which is the immediate precursor for the polymerization of peptidoglycan. The coupling of the precursor to a lipid molecule enables the cell to transport the hydrophilic substrate through the hydrophobic membrane. It is still unresolved how the lipid II translocation (flipping) is mediated. Several *B. subtilis* proteins are proposed to function as flippase: FtsW, RodA, and SpoVE. However, firm biochemical evidence for such a role of these proteins has not been presented so far. The final stage of peptidoglycan assembly, which takes place at the outer side of the cytoplasmic membrane, involves the incorporation of the disaccharide-pentapeptide unit into growing glycan strands followed by cross-linking of strands in three dimensions. This is achieved through the action of PBPs (1, 28, 34).

# 3. Penicillin-binding proteins

*Eubacteria* generally contain multiple PBPs which function in the synthesis and remodelling of the cell wall. PBPs were first identified as proteins that covalently bind penicillin and related β-lactam antibiotics. Two major enzymatic activities are carried out by PBPs; polymerization of glycan strands (transglycosylation) and peptide cross-linking between glycan strands (transpeptidation). Some PBPs can hydrolyze the last D-Ala of the peptidoglycan stem pentapeptide (D,D-carboxypeptidation) or hydrolyze the peptide bond connecting two glycan strands (endopeptidation). The major functional parts of PBPs are localized on the outer side of the cytoplasmic membrane, where peptidoglycan assembly takes place (2, 35, 36).

Based on their size, two main categories of PBPs can be distinguished: low molecular weight (LMW) PBPs (≤60 kDa) and high molecular weight (HMW) PBPs (>60kDa). LMW PBPs are monofunctional and appear to be dispensable for cell viability. This group of PBPs catalyse D,D-carboxypeptidase or endopeptidase reactions. HMW PBPs are multimodular and responsible for polymerization and cross-linking of peptidoglycan strands (2). They consist of an N-terminal cytoplasmic peptide, a transmembrane segment, and two much larger C-terminal domains joined by a β-rich linker. Depending on the structure and catalytic activity of the C-terminal domains, they belong either to the class A or the class B HMW PBPs. Those of class A have a glycosyltransferase domain followed by a transpeptidase domain, whereas the transpeptidase domain is preceded by a domain of unknown function in those of class B (5, 36). Some class B HMW PBPs display one or two so-called PASTA (PBP and serine-threonine kinase associated) domains at their C-terminal end (37).

## 3.1. Transpeptidation,β-lactam action and bacterial resistance to β-lactams

The transpeptidase domain of PBPs is defined by three conserved sequence motifs; the SXXK tetrad (where X is any amino acid) that contains the active site serine (underlined) and the SXN and the KTG triads. During transpeptidation (Figure 5A), the active site serine attacks the carbonyl carbon of the second last alanine in the stem pentapeptide (D-Ala$_{(4)}$), which leads to formation of a covalent acyl-enzyme complex and release of the terminal alanine residue (D-Ala$_{(5)}$). Subsequently in the enzyme

mechanism, the ester bond of the acyl-enzyme complex is attacked by the amine of the third amino acid residue of another stem pentapeptide ($Dpm_{(3)}$ in *B. subtilis*), resulting in a peptide bond between the two adjacent peptidoglycan strands and release of the enzyme (3, 38).

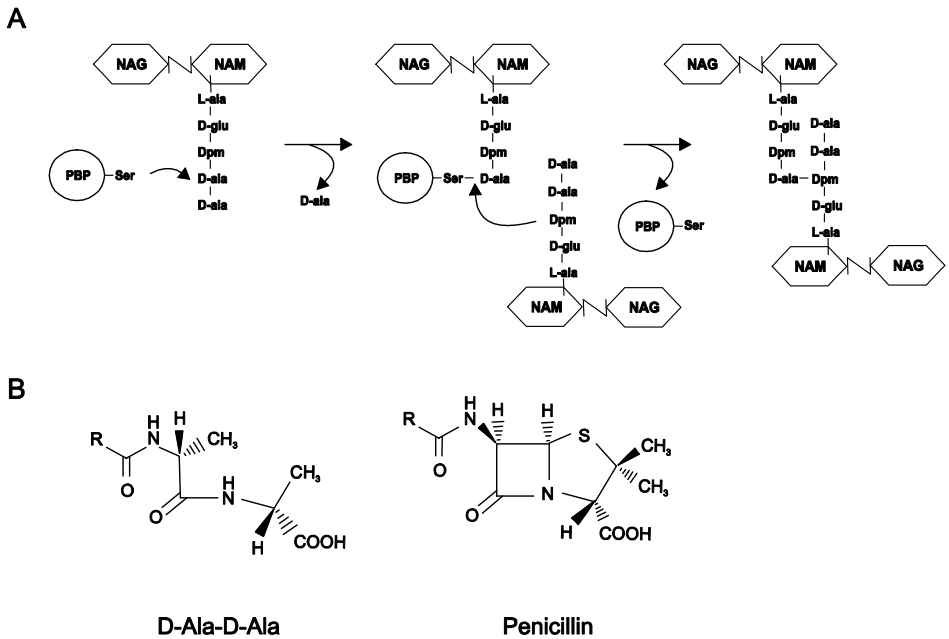



**Figure 5.** (**A**) Transpeptidase reaction catalysed by PBPs (for a detailed description see text). (**B**) Structural comparison of D-Ala-D-Ala dipeptide and penicillin.

β-lactam antibiotics structurally mimic the natural D-Ala-D-Ala substrate of PBPs (Figure 5B). The attack of the active site serine of the PBP on the carbonyl carbon of the β-lactam ring results in opening of the ring and formation of covalent acyl-enzyme complex. This complex is hydrolyzed very slowly, thus blocking the normal transpeptidase activity of the PBP (3).

Many bacteria develop resistance towards commonly used β-lactam antibiotics. Such resistance can be achieved by several mechanisms such as decreased permeability of the cell envelope, production of enzymes that inactivate the antibiotic (β-lactamases) or production of altered, drug-insensitive, PBPs. PBPs with decreased affinity towards β-lactams usually possess mutations in the transpeptidase domain (4). For example, a number of mutations that reduce affinity to β-lactams have been found in the Pbp2x of different *Streptococcus pneumoniae* clinical isolates (3). Analysis of such mutant

variants contributes to a detailed picture of how remodelling of the structure of the transpeptidase domain lowers the affinity to β-lactams without significantly affecting the native activity of the PBP in peptidoglycan synthesis.

## 3.2. Penicillin-binding proteins of *Bacillus subtilis*

Analysis of the *B. subtilis* strain 168 genome sequence reveals 16 genes encoding 17 PBPs. These PBPs function in vegetative growth, sporulation or germination and comprise five class A HMW PBPs, six class B HMW PBPs and six LMW PBPs (Table 1).

### 3.2.1. HMW PBPs

Class A PBP 1a and 1b are both encoded by the *ponA* gene. The difference between these two proteins is probably a result of carboxyl-terminal processing (39). PBP1a/b is part of the cell division machinery and plays a role during formation of both the division and the sporulation septum (40, 41). Inactivation of *ponA* does not block cell division during vegetative growth. However, *ponA* defective strains grow more slowly than the wild type, along with abnormal cell morphology (longer and thinner cells with atypical septal structures). It seems that the function of PBP1a/b is more critical at asymmetric cell division during sporulation than during vegetative growth. Sporulation efficiency of *ponA* defective mutants is reduced to 14% as compared to a wild type strain (39, 42). As determined by fluorescence microscopy such cells are strongly inhibited in formation of the asymmetric division septum (41).

Vegetatively growing cells contain two other class A HMW PBPs, PBP2c and PBP4, encoded by the *pbpF* and *pbpD* genes, respectively. Absence of either one or both of these proteins causes no obvious phenotype. Combined with PBP1a/b deficiency it results in further reduction in growth rate and changes in cell morphology. The ability of such mutants to grow suggests the presence of other, to date unknown, enzymes with transglycosylase activity that assemble peptidoglycan in the absence of PBPs 1a/b, 2c and 4 (42, 43). PBP2c is produced also in the forespore compartment under the control of $\sigma^G$, suggesting a potential role in cortex synthesis or spore germination. However, *pbpF* deletion mutants sporulate normally and endospore outgrowth has normal kinetics (44).

The last class A PBP, PBP2d encoded by the *pbpG* gene (previously named *ywhE*), is produced exclusively during sporulation, under the control of $\sigma^F$, and to a lesser degree $\sigma^G$. The precise function of PBP2d remains unknown, since *pbpG* negative mutants sporulate normally, produce heat resistant spores and show no defect in germination or outgrowth (45). Interestingly, a *pbpF pbpG* double deficient mutant

strain has a severe sporulation defect resulting in a >$10^4$-fold decrease in spore production. Electron-microscopic analysis revealed that the majority of these cells possess a defect in development, visible as a highly disorganized forespore. It has been proposed that either PBP2c or PBP2d can carry out synthesis of germ cell wall peptidoglycan, which in turn is used as a template for proper synthesis of the spore cortex (30).

*B. subtilis* six class B PBPs play roles in septation, sporulation and regulation of cell shape. PBP2b, encoded by *pbpB*, is the only known essential PBP in *B. subtilis*. It localizes to the septum and is involved in the formation of septal peptidoglycan. It seems that PBP2b is also recruited to the asymmetric septum during sporulation, but its presence there is transient (46). PBP2a, encoded by the *pbpA* gene, plays an important role in cell elongation during germination and outgrowth. Absence of PBP2a does not result in any phenotypic changes during vegetative growth, but mutant spores are unable to convert from an ovoid to a cylindrical cell shape during outgrowth (47). After several hours, these cells finally recover their rod shape thanks to increased production of another class B PBP, PbpH. It was observed that in a *pbpA pbpH* double deficient mutant, outgrowing cells never achieve rod shape and eventually lyse (48). The class B PBP SpoVD is produced exclusively during sporulation under the control of $\sigma^E$ and is required for synthesis of the spore cortex. Endospores of mutants deficient in SpoVD, or with an inactive SpoVD transpeptidase domain, lack the cortex layer and are heat sensitive (8)(Paper I). PBP4b, encoded by the *pbpI* gene (previously named *yrrR*), is another mother-cell specific class B PBP which does not seem to play any significant role in spore peptidoglycan synthesis (49)(Paper IV). The last PBP of class B is PBP3, encoded by the *pbpC* gene. It is expressed during exponential growth phase and in low amounts during sporulation. Lack of PBP3 does not cause any phenotypic changes (50).

As presented above, several single PBP deficient *B. subtilis* mutants lack a distinct phenotype. This observation suggests that the various PBPs have overlapping functions in the cell and can compensate for each other to maintain peptidoglycan assembly.

### 3.2.2. LMW PBPs

Six genes encoding LMW PBPs have been found in the genome of *B. subtilis*; *dacA*, *dacB*, *dacC*, *dacF*, *pbpE* and *pbpX* (Table 1). The sequences of the four Dac polypeptides are highly similar to known D,D-carboxypeptidases. For PBP5 (DacA) and PBP5* (DacB) this activity has been shown *in vitro*. D,D-carboxypeptidase activity of Pbp4a (DacC) and DacF is still to be demonstrated (32). PBP5 is produced in *B. subtilis* during vegetative growth, although a large amount of this protein remains in the cell during spore formation. The D-alanine carboxypeptidase activity of membranes isolated from a PBP5 deficient mutant was decreased to about

5% of the wild type level (7). Exponentially growing cells of strains lacking PBP5 show normal morphology, but at entry into stationary growth phase the cells become significantly shorter. Inactivation of the *dacA* gene has no effect on sporulation, spore phenotype or spore germination (7, 32). PBP5* is unique to sporulating cells, since its production is under the control of $\sigma^E$ (51). This enzyme localizes to the outer forespore membrane and has a function in cortex synthesis. Lack of PBP5* leads to a large increase in peptidoglycan cross-linking of spores. This, in turn, is associated with an inability to maintain spore core dehydration upon heating and therefore slightly decreased heat resistance of spores (32, 52). Expression of *dacC*, encoding PBP4a, is initiated after the end of exponential growth and is dependent on $\sigma^H$. PBP4a seems to have a redundant role under normal growth conditions, since a *dacC* null mutation result in no obvious phenotype and spores show the same heat resistance and cortex structure as that of wild type (53). The *dacF* gene is expressed only within the forespore under the control of $\sigma^F$. There is no phenotypic change associated with *dacF* mutations alone. The function of DacF can however be observed in the absence of PBP5*. The spore peptidoglycan of a *dacB dacF* double deficient mutant is much more cross-linked than that in PBP5* deficient spores, indicating that DacF participate in cortical peptidoglycan synthesis (32). The D,D-endopeptidase PBP4* (product of *pbpE*) and PbpX (product of *pbpX*) are implicated in sporulation, but their functions remain unknown (54).

## 3.3. Localization of *B. subtilis* PBPs

Subcellular localization of *B. subtilis* PBPs in vegetative and sporulating cells has been studied mainly by making use of fusions of PBPs to green fluorescent protein (GFP) (54, 55)(Paper II) or mCherry (56)(Paper I, Paper II) and to a lesser extent by immunofluorescence (40, 46). Localization data for *B. subtilis* PBPs (as well as of other bacterial species) is reviewed in Scheffers and Pinho (57), and an overview is presented in Table1.

**Table 1.** *B. subtilis* PBPs and their subcellular localization. Data derived from (54, 55) unless otherwise specified.

| Gene | Protein | Expression[1]/ σ-factor dependency[2] | Localization[3] |
|---|---|---|---|
| Class A PBPs (transglycosylase/transpeptidase) | | | |
| *ponA* | PBP1a/b | veg<br>early spor† | division septum, low fluorescence at cell periphery<br>sporulation septum |
| *pbpF* | PBP2c | veg<br>spor ($\sigma^G$) | division septum, low fluorescence at cell periphery<br>forespore |
| *pbpD* | PBP4 | veg | division septum, distinct spots at cell periphery |
| *pbpG* | PBP2d | spor ($\sigma^F$, $\sigma^G$) | forespore |
| Class B PBPs (transpeptidase) | | | |
| *pbpA* | PBP2a | veg | evenly distributed along the cell membrane |
| *pbpB* | PBP2b | veg<br>early spor† | division septum<br>sporulation septum (transient) |
| *pbpC* | PBP3 | veg<br>spor (low expression)¶ | distinct foci and bands at cell periphery<br>*no data* |
| *spoVD* | SpoVD | spor ($\sigma^E$) | Forespore (56) (Paper I, Paper II) |
| *pbpH* | PbpH | veg | evenly distributed along the cell membrane |
| *pbpI* | PBP4b | spor ($\sigma^E$, $\sigma^F$ (58) ) | *no data* |
| LMW PBPs (carboxypeptidase) | | | |
| *dacA* | PBP5 | veg<br>spor‡ | division septum, distinct spots at cell periphery<br>*no data* |
| *dacB* | PBP5* | spor ($\sigma^E$) | *no data* |
| *dacC* | PBP4a | late stationary phase ($\sigma^H$) | *no data* |
| *dacF* | DacF | spor ($\sigma^F$) | *no data* |
| LMW PBPs (endopeptidase) | | | |
| *pbpE* | PBP4* | early spor ($\sigma^A$) | *no data* |
| *pbpX* | PBPX | not known | veg: division septum, low fluorescence at cell periphery;<br>spor: sporulation septum and the forespore§ |

1. Veg, expression during vegetative growth; Spor, expression during sporulation.
2. σ-factor dependency is presented for PBPs which are involved in sporulation. σA- vegetative growth and early sporulation genes; σH- postexponential gene expression; σE- early mother cell gene expression; σF- early prespore gene expression; σG- late forespore gene expression.
3. Localization pattern of PBPs at the time of expression specified in the column "Expression/σ-factor dependency". PBPs were detected in cells by light microscopy as fluorescent fusion protein or immunostained protein.
† The protein produced during vegetative growth is also present in the cells during asymmetric division.
¶ σ-factor dependency unknown.
‡ Based on the presence of the protein in sporulating cells.
§ Since the time of expression of the native PBPX is not known, the data shown in the table represent the localization of GFP-PbpX fusion protein expressed from a xylose-inducible promoter during both vegetative growth and sporulation.

# 4. The SEDS family of proteins

The SEDS (shape, elongation, division and sporulation) family comprises homologous integral membrane proteins, essential and conserved in most *Eubacteria* that synthesize peptidoglycan as a part of their cellular envelope (59). The best known members of this family of proteins are *Escherichia coli* FtsW (60) and RodA (61, 62), and *B. subtilis* SpoVE (56, 63) which function in cell division, cell elongation, and spore formation, respectively.

SEDS proteins are found associated with a cognate class B PBP, most likely in order to control the synthesis of peptidoglycan during specific periods of the bacterial cell cycle (59). In *E. coli*, FtsW is a partner to the septation-specific PBP3 (60), whereas RodA is functionally associated with the elongation-specific PBP2 (64).*B. subtilis* possesses three SEDS proteins; FtsW, RodA and SpoVE. FtsW is a partner to PBP2b, RodA most likely accompanies one or more elongation PBPs, whereas SpoVE interacts with SpoVD. Each protein pair apparently is a component of different peptidoglycan synthesis complexes (56, 65).

Several lines of evidence indicate *E. coli* RodA and FtsW as transporters (flippases) of lipid II during peptidoglycan synthesis (64, 66). *In vitro* studies by Mohammadi *et al.* (67) provided the first biochemical evidence for function of *E. coli* FtsW in the transport of lipid II across the membrane. However, recent *in vivo* studies by Sham *et. al* (68) did not confirm flippase activity of FtsW. Instead, MurJ was proposed to be the lipid II flippase in *E. coli*.

# 5. Different mechanisms for proper localization of proteins during sporulation in *B. subtilis*

During sporulation in *B. subtilis*, many membrane proteins that are produced in the mother-cell cytoplasm localize selectively to the forespore outer membrane and function in spore morphogenesis. The mechanism behind targeting of these proteins to the forespore is poorly understood. There are at least three alternate models for how these proteins become properly localized (Figure 6) (69). In the first model (A), called targeted insertion, the protein is directly and selectively inserted into the outer membrane that surrounds the forespore. The second model (B), selective degradation, assumes random insertion of the protein into membranes in the cell and proteolytic elimination of the protein from the mother cell cytoplasmic membrane. The third model (C) involves random insertion of the protein into membranes, followed by lateral diffusion and capture in the forespore outer membrane (69).

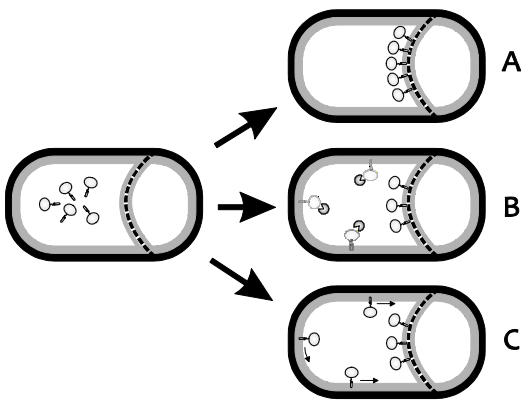



**Figure 6.** Drawing presenting three models for the selective localization of mother cell-synthesized proteins to the forespore outer membrane during sporulation. (**A**) targeted insertion; (**B**) selective degradation; (**C**) random insertion, lateral diffusion and capture. The scheme is adopted from Figure 1 in Rudner *et al.* (69).

SpoVM, a small protein (26 amino acid residues) which plays a role in cortex and coat morphogenesis, recognizes the positive membrane curvature present at the surface of the forespore (70). Such a geometric cue for the recruitment of curvature-sensing proteins supports the targeted insertion model (Figure 6A).

SpoIVFB is one of the best-studied proteins that localize specifically to the outer forespore membrane. It processes pro-$\sigma^K$ to active $\sigma^K$ at the late stage of sporulation. Proper localization of SpoIVFB depends on another membrane integral protein called SpoIVA. The collective data presented by Rudner *et al*. (69) are compatible with a model in which SpoIVFB is inserted into the cytoplasmic membrane followed by diffusion to, and capture in, the outer forespore membrane (Figure 6C). Another example of a protein which localizes to the forespore via a similar mechanism is SpoIIIAH. However, SpoIIIAH is a protein which reaches its proper localization through an interaction with the inner forespore membrane protein SpoIIQ. Extracellular domains of these two proteins interact in the space between the inner and outer forespore membranes and thus SpoIIIAH become localized by a zipper-like mechanism (71).

# 6. Determinants for the subcellular localization of PBPs

PBPs are considered to localize within the cell mainly through protein-protein interactions. These interactions can be either between the PBP and other proteins involved in cell wall synthesis, or between different PBPs that make up a multienzyme complex.

Localization of *B. subtilis* PBP1 to the vegetative septum is dependent on several cell division proteins, including FtsZ, PBP2b, DivIB and DivIC (41). Also localization of the division-specific PBP3 in *E. coli* has been reported to be dependent on the prior localization of FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsBL, and FtsW (72). The first 56 amino acid residues of *E. coli* PBP3, which comprise the transmembrane segment, are sufficient for interaction with FtsW and localization of the protein to the septum (60, 73). Another interaction between a SEDS protein and its cognate PBP was shown in *B. subtilis* sporulating cells. SpoVD requires the putative lipid II flippase SpoVE for its recruitment to the outer forespore membrane. The two proteins appear to interact directly, suggesting that the localization of SpoVD is dependent on this protein-protein interaction specifically (56).

Several lines of evidence demonstrate that recruitment of some PBPs to subcellular sites requires recognition of enzyme substrate. It was observed that *E. coli* PBP3 did not localize to the septum in the presence of furazlocilin, a β-lactam antibiotic that binds with high specificity to the transpeptidase active site of PBP3. This suggested that PBP3 has to be enzymatically active in order to be localized (74).Similar results were obtained for S*taphylococcus aureus* PBP2, involved in cell division, which became dispersed over the entire surface of the cell upon addition of oxacillin. Furthermore, modifying the substrate in such a way that it can no longer be recognized by the protein (D-cycloserine treatment) or by blocking the access of PBP2 to its substrate by addition of vancomycin resulted in PBP2 delocalization (75). Localization of PBP3 in *Caulobacter crescentus* also seems to rely on a functional transpeptidase domain. Inactivation of the catalytic site by substitution of serine-296 for alanine impaired the ability of PBP3 to localize at the pole and to the FtsZ ring (76).

It was recently demonstrated that PASTA domains at the C-terminal end of *S. pneumoniae* Pbp2x are involved in the localization of the protein to division sites (77). The exact role of PASTA domains in PBPs is unknown, but they are thought to

interact with uncross-linked peptidoglycan (37). So, PASTA domains could drive localization of PBSs to the sites of active peptidoglycan synthesis through recognition of the substrate.

In summary, the collective data presented above support two models for PBP localization to division sites; protein-protein interactions and enzyme substrate recognition. The idea of substrate recognition as a targeting factor shines new light on how the data obtained for *E. coli* PBP3 could be interpreted. For example, the requirement of FtsW for recruitment of PBP3 is based on the fact that depletion of FtsW results in miss-localization of PBP3. This could be due to the loss of protein-protein interactions, but it could also be explained by lack of transpeptidation substrates (assuming that FtsW functions as a flippase) (57). Although the transmembrane segment of *E. coli* PBP3 is sufficient to direct fused GFP to the septum, removal of the transpeptidase domain from full length protein also reduces its proper localization. This clearly demonstrates the importance of the transpeptidase domain for localization (57, 78).

One possible model could encompass a combination of protein-protein interactions and substrate recognition as localization factor. PBPs could be first recruited to the septum through protein-protein interaction with division proteins. Then, this multienzyme cell wall synthesis machinery would be separated from the other division proteins to maintain localization through substrate recognition. The fact that the cell wall synthesis machinery does not co-localize with the FtsZ ring during the entire synthesis of division septum supports this "dual" model (57).

# Section II. On the role of penicillin-binding protein SpoVD in endospore cortex assembly

# 7. *B. subtilis* SpoVD

## 7.1. Overall function

*B. subtilis* SpoVD is, as mentioned in the first section, a class B HMW PBP essential for cortex peptidoglycan synthesis during sporulation. The exact role of the protein in the process of cortex assembly is unknown but it most likely catalyses cross-linking between peptide side chains of different glycan strands. As determined by electron microscopy of negatively stained fixed cells, SpoVD deficient strains produce spores without a trace of cortex layer (8)(Paper I). Also, SpoVD deficiency results in accumulation of peptidoglycan synthesis precursors in the cytoplasm of the mother cell (79). Altogether, this suggests that SpoVD acts early in cortical peptidoglycan synthesis. The germ cell wall is apparently assembled in SpoVD deficient mutants (as concluded from the normal germination of spores) indicating that SpoVD is not important for the synthesis of this layer. We recently demonstrated, by the use of a SpoVD active-site mutant strain, that synthesis of cortex explicitly depends on the transpeptidase activity of SpoVD (Paper I). Since heat resistance of spores depends on the presence of cortex (7), cells that lack functional SpoVD produce heat sensitive spores (9)(Paper I). Heat resistance is therefore one easy way to test strains for the presence of spore cortex.

## 7.2. Genetics

The *spoVD* gene is located at 133° on the *B. subtilis* chromosome (80). It is positioned in a cluster of genes involved in peptidoglycan synthesis and is flanked by the *pbpB* and *murE* genes (81, 82) (Figure 7A). *pbpB* encodes Pbp2b, a SpoVD paralogue involved in the synthesis of septal peptidoglycan during vegetative growth (Table 1)(82). Presumably *pbpB* and *spoVD* have sometime in the past originated via a gene duplication event and the products of the two genes became specialized in different stages of the *B. subtilis* life cycle (82). The enzymes encoded by *murD* and *murE* are involved in biosynthesis of the peptidoglycan precursor UDP-NAM-pentapeptide in the cytoplasm. MurD catalyses addition of the D-Glu residue at the second position, and MurE catalyses addition of the Dpm residue at the third position in what is to become the pentapeptide side chain. MraY, encoded by *mraY*, is a transferase that

catalyses formation of undecaprenyl pyrophosphoryl NAM-pentapeptide (lipid I) from UDP-NAM-pentapeptide and undecaprenyl phosphate (81).

Transcription of *spoVD* is driven by a mother cell-specific $\sigma^E$-dependent promoter (8). *spoVD* mRNA appears in the cells between hours 1 and 2 of sporulation, and peaks at about the third hour of sporulation (Figure 7B) (83). This is consistent with the time of $\sigma^E$ activity (84)(Figure 1). As shown in Figure 7B, *spoVD* mRNA remains in sporulating cells for many hours. Expression of *spoVD* is downregulated by the SpoIIID protein that binds to the *spoVD* promoter (85).

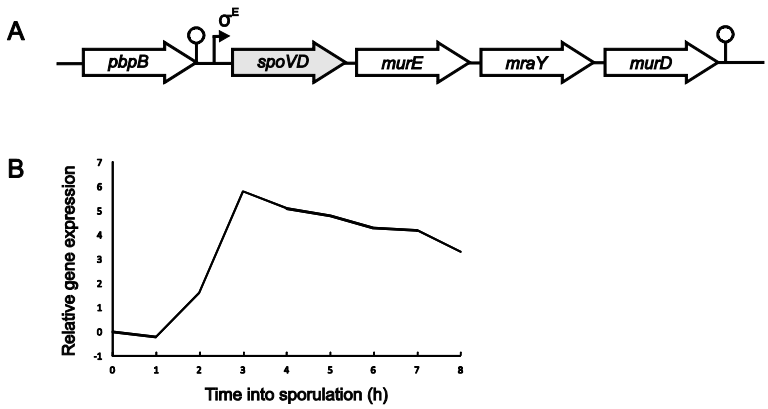



**Figure** 7. (**A**) Map of the *spoVD* region in the chromosome of *B. subtilis* strain 168. (**B**) Relative cellular level of *spoVD* mRNA during sporulation at 37°C. Time point "0"indicates initiation of sporulation by the resuspension method. The graph was prepared using data from (83).

## 7.3. Protein structure

The SpoVD protein (71 kDa) has 646 amino acid residues and can be regarded as having four domains (86); an N-terminal membrane-spanning domain (with a predicted single transmembrane segment comprising residues 12 to 31; (87)), a domain of unknown function (residues 54-206), the transpeptidase domain (residues 246-557) and the C-terminal PASTA domain (residues 584-638) (Figure 8A).

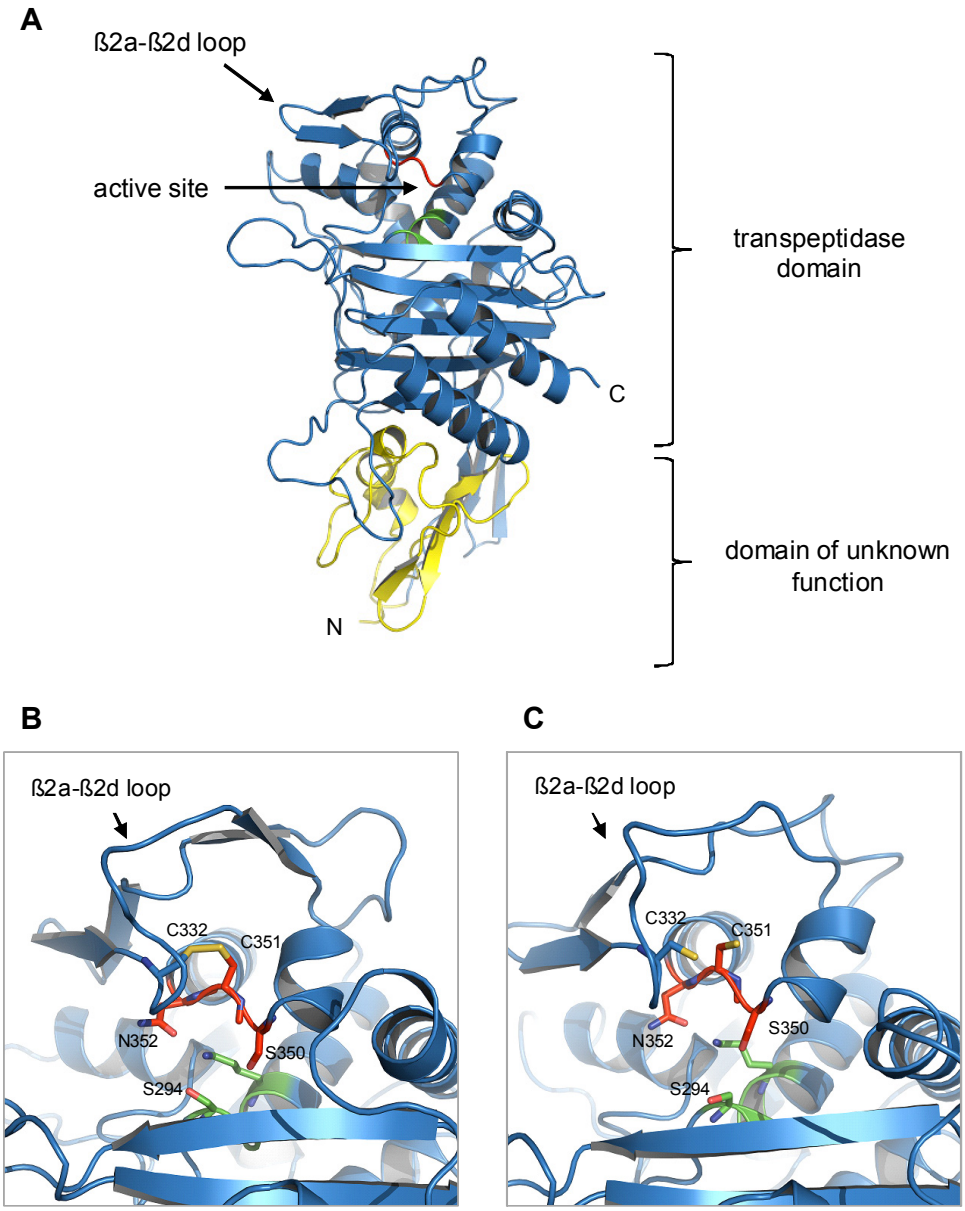



**Figure 8.** (**A**) Model of the structure of SpoVD (residues 54-411). The SXXK and SXN motifs in the active site region are indicated in green and red, respectively; N- and C-terminal ends are highlighted. (**B**) and (**C**) A close-up of the active site region of oxidized (B) and reduced (C) SpoVD. Residues S294 (the active-site serine), S350-C351-N352 (the SXN motif) and C332 (in the β2a-β2d loop) are highlighted. Details on the structural model are presented in reference (9).

The membrane-spanning domain, which is presumably an α-helix, anchors the protein to the membrane (Figure 9A, strain LMD107). The domain of unknown function is believed to interact with other proteins involved in peptidoglycan synthesis. We recently showed that the predicted active-site serine residue (Ser294) of the transpeptidase domain is, as expected, required for the formation of the acyl-enzyme complex (Paper I). The role of the small PASTA domain is unclear. It is not required for cortex synthesis but appears important for protein stability (Paper II).

There is no X-ray crystal structural data available for SpoVD. A three dimensional structure model of the membrane-extrinsic part (presented in Figure 8) has been built (9) based on the crystal structure of the homologous *S. pneumoniae* Pbp2x (the soluble parts of the two proteins are ~33% identical).

## 7.4. Protein trafficking

SpoVD appears in the cell membranes at about the second hour of sporulation at 37°C and is detectable by immuno-blotting until at least the fifth hour of sporulation (Figure 9A, strain LMD105). The protein is produced in the mother cell cytoplasm and can, theoretically, be inserted into both the mother cell membrane and the outer forespore membrane. Fluorescence microscopy of strains producing SpoVD fused to GFP or mCherry unambiguously show that the protein localizes to the forespore (Figure 9B) (56)(Paper I, Paper II). The structural determinants and the mechanism for SpoVD localization remain unknown but, as mentioned in section I of this thesis, the presence of SpoVE (a putative lipid II flippase) seems crucial for recruitment of SpoVD to the outer forespore membrane (56). As determined by fractionation of the mother cell and forespore membranes, SpoVD is also found in the mother cell membrane fraction (Figure 9C). Therefore, SpoVD is most likely enriched at the forespore through random insertion of the protein into cell membranes, followed by diffusion and capture in the forespore outer membrane (Figure 6C).

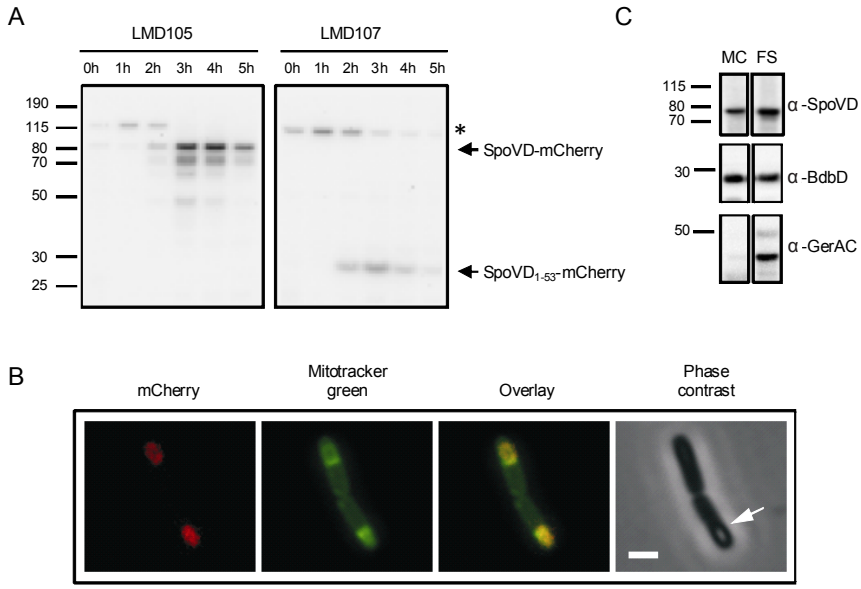



**Figure 9.** Subcellular localization of SpoVD in *B. subtilis* sporulating cells.

(**A**) Immuno-blot showing the presence of full length SpoVD fused to mCherry (LMD 105), and the membrane spanning domain of SpoVD (amino acid residues 1-53) fused to mCherry (LMD107) in membranes isolated from *B. subtilis* sporulating cells. Fusion to mCherry was to allow immuno-detection of protein using dsRed antibodies (Clontech). Cells were sporulated by resuspension at 37°C and samples were taken at hourly intervals after the resuspension as indicated. The membrane fraction was isolated from the cells. Approximately 15 μg of proteins were loaded in each lane. An asterisk indicates a non-specific band independent of mCherry. Molecular mass markers, in kDa, are indicated.

(**B**) Localization of SpoVD-mCherry in *B. subtilis* LMD101/pLEB7 (Paper II) sporulating cells. Cells were sporulated by resuspension at 30°C and imaged at 7.5 h after the resuspension. Mother cell and forespore membranes were stained with the membrane-permeable fluorescent dye Mitotracker green. An arrow in the phase contrast image indicates a maturing spore that has turned phase grey. Scale bar is 2 μm.

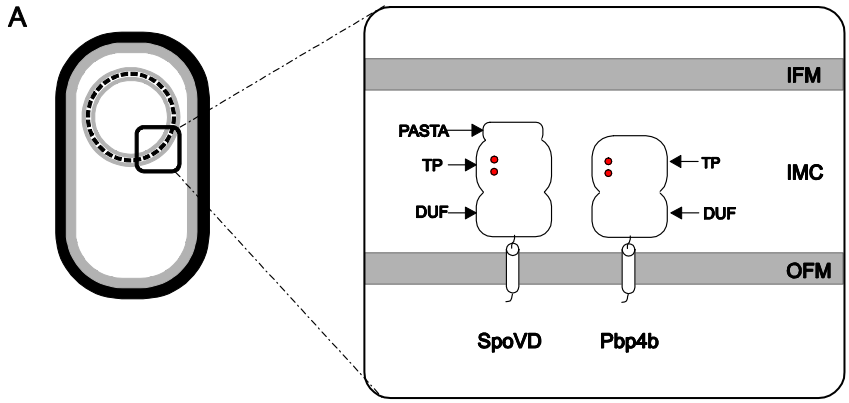
(**C**) Immuno-blot for SpoVD, BdbD and GerAC in cell fractions enriched for mother cell (MC) and forespore (FS) membranes. *B. subtilis* 1A1 (wild type) cells were sporulated by resuspension at 37°C and collected 5h after the resuspension. Cell fractions enriched for the mother cell and forespore membranes were prepared according to a protocol kindly provided by Prof. J. Dworkin (Columbia University). Approximately 7.5 μg of proteins were loaded in each lane. Samples were probed with anti-SpoVD (9), anti-BdbD (88) and anti-GerAC ((89), a gift from Prof. P. Setlow, University of Connecticut) sera. Immuno-blots for BdbD and GerAC served as controls (BdbD is expected to be distributed to both membrane fractions and GerAC to be present exclusively in the forespore membrane fraction). Molecular mass markers, in kDa, are indicated.

# 8. Dithiol in the transpeptidase domain of SpoVD and similar PBPs

## 8.1. Bioinformatic data

Comparative sequence analysis of SpoVD orthologues revealed two conserved cysteine residues (Cys332 and Cys351) in the transpeptidase domain (9). We found that *B. subtilis* PBP4b, which is a SpoVD paralogue, also contains two conserved cysteine residues (Cys328 and Cys353) in the transpeptidase domain (Figure 10A). In both SpoVD and Pbp4b, the second cysteine residue (Cys351 in SpoVD and Cys353 in Pbp4b) is residue X within the conserved SXN motif of the active site region (Figure 10B). The position of the first cysteine residue is variable, but is always found upstream from the SCN motif.

The two conserved cysteine residues were initially thought to be present exclusively in PBPs that are involved in synthesis of spore cortex (9). However, after an extensive database screen of transpeptidase-domain sequences containing cysteine residues, we have revised this hypothesis. The search aimed to find transpeptidase domains of HMW PBPs that contain a SC motif and one additional cysteine residue in the upstream region up to 100 amino acid residues away. The obtained data showed that 20% of the class B PBPs matched the specified criteria. Among them, there are PBPs of non-sporulating bacteria (e.g. PBPA of *Mycobacterium tuberculosis*), indicating that the presence of conserved cysteine residues in the transpeptidase domain extends beyond the sporulation-specific PBPs. Also, results of our search show that the position of the first cysteine residue is 12 to 56 residues (most frequently 19 or 32 residues) upstream of the SCN motif (L. Hederstedt and E. Bukowska-Faniband, unpublished).




B

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Bs_SpoVD |1-646|  302 AAALEEQKVNLKRDQFYDKGHAEVDGARLRCWKR-GGHGLQTYLEVVQNSCNPGFVEL-GE
Bs_Pbp4b |1-584|  312 AAAIENNMVKPSQT--FNCN-LNLYG------EPGDDKGTLSFDESFAQSCNYTFTSLAEQ
Bs_Pbp2b |1-716|  320 AAAMQENVFNA--NEKYKSGTFEVGGAPVKDHNNGVGWGPTTYHDGVLRSSNVAFAKLAKE
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**Figure 10.** Conserved cysteine residues in the transpeptidase domain of *B. subtilis* SpoVD and PBP4b.
(**A**) Schematic representation of SpoVD and Pbp4b in the intermembrane compartment of the forespore. Conserved cysteine residues in the transpeptidase domain are shown as red-filled circles. PASTA – PASTA domain, TP – transpeptidase domain, DUF – domain of unknown function, IFM – inner forespore membrane, IMC – intermembrane compartment, OFM – outer forespore membrane. The dashed line surrounding the forespore indicates the intermembrane compartment where cortex is synthesized.
(**B**) Sequence alignment of part of *B. subtilis* SpoVD [Q03524], Pbp4b [O32032] and Pbp2b [Q07868]. Pbp2b is a SpoVD paralogue involved in cell division (Table 1). Conserved cysteine residues in SpoVD and Pbp4b are highlighted in red. The alignment was carried out using CLUSTAL-W (www.ebi.ac.uk).

## 8.2. Structural and biochemical data

The two cysteine residues in the transpeptidase domain of *B. subtilis* SpoVD can form an intramolecular disulfide bond. This was first shown by structural modelling and supported by biochemical analysis of purified soluble SpoVD using the thiolate-specific reagent Mal-PEG (methoxypolyethylene glycol maleimide) (9). *In vivo* studies, of which details are given below, provided evidence that the oxidized (i.e. disulfide bond-containing) SpoVD is inactive in cortex synthesis and requires reduction of its thiols to become active (9). As shown in Figure 8B, the disulfide bond connects the SCN motif with the β2a-β2d loop that is adjacent to the active site region. The disulfide bond in SpoVD restricts the dynamics of the loop and may interfere with access of substrate to the active site and consequently block cortex synthesis (9). Only a small difference in covalent binding of penicillin was observed

between oxidized and reduced SpoVD (9). This little effect can be explained by the size difference between penicillin and the much larger native substrate of SpoVD, and does not rule out the possibility that the disulfide bond blocks binding of uncross-linked peptidoglycan.

Several attempts to form a covalent conjugate of purified reduced soluble Pbp4b (Paper IV) with thiolate-specific reagents were unsuccessful, suggesting that the two conserved cysteine residues are not accessible for the reagent (unpublished data). Hence, the presence of a disulfide bond in Pbp4b is still to be proved.

Remarkably, the crystal structure of *M. tuberculosis* PBPA shows the presence of two disulfide bonds in the transpeptidase domain (90, 91). One of these (between Cys266 and Cys282 in the $S_{281}XN_{283}$ motif) is analogous to that in SpoVD. This finding provided the first crystallographic evidence that a disulfide bond can be formed between the two conserved cysteine residues of some PBPs. The second disulfide bond was found between Cys154 and Cys158. All four cysteines are conserved in PBPA orthologues in different mycobacterial species (90, 91).

## 8.3. *In vivo* oxidation and reduction of SpoVD

### 8.3.1. Oxidation of SpoVD by BdbD

In *B. subtilis* sporulating cells, disulfide bond formation in SpoVD is catalysed by BdbD (9), a general oxidizing membrane-bound thiol-disulfide oxidoreductase (TDOR) of 222 amino acid residues (88, 92). A scheme of the general reaction catalysed by TDORs is shown in Figure 11. BdbD has broad substrate specificity and acts on the outer side of the cytoplasmic membrane. Correct folding of several proteins, e.g. ComGC, ComGG and ComEC, depends on the BdbD activity. On the other hand, disulfide bonds that are non-specifically introduced into some proteins by BdbD may block or interfere with their folding or function (92, 93).

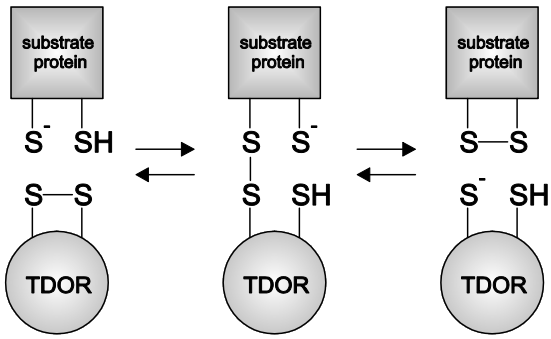


**Figure 11.** Schematic representation of the reaction mechanism of thiol-disulfide oxidoreductases (TDORs). TDORs catalyse formation and disruption of disulfide-bonds in substrate proteins. This reaction involves formation of a transient mixed disulfide intermediate between the TDOR and the substrate.

The two forespore membranes originate as an invagination of the vegetative cell cytoplasmic membrane. Thus, BdbD is active also in the forespore intermembrane space. BdbD-catalysed disulfide bond formation is not required for sporulation because BdbD deficient cells sporulate normally (94) (Table 2, strain LUL10). Activity of BdbD in the forespore intermembrane space leads to undesired disulfide bond formation, as is observed for example in the case of SpoVD.

### 8.3.2. Oxidized SpoVD is a substrate for StoA

As mentioned above, oxidized SpoVD is inactive in cortex synthesis and this is most likely due to decreased transpeptidase activity in peptidoglycan synthesis and/or blocked interaction with partner proteins. Oxidized SpoVD is activated by the action of StoA, a membrane-bound TDOR of 165 amino acid residues which breaks (reduces) the disulfide bond in SpoVD (9). StoA deficient strains produce heat sensitive spores without cortex layer (94, 95) (Table 2). This phenotype is consistent with the suggested model (9) in which StoA functions to keep SpoVD reduced, i.e. in its active state. StoA is present in the cells exclusively during sporulation. Expression of *stoA* (*ykvV*) is driven by $\sigma^E$- and $\sigma^G$-dependent promoters (95), indicating that StoA is most likely present in both the outer and the inner forespore membranes. Intriguingly, it was demonstrated that expression of *stoA* exclusively in the forespore compartment (from the $\sigma^G$-dependent promoter only) is sufficient to produce a wild type level of heat resistant spores, while a strain in which StoA is produced solely in the mother cell (from the $\sigma^E$-dependent promoter) is somewhat defective in production of heat resistant spores (yield of heat resistant spores in this strain was reduced to 13%) (95). These findings suggest that, for so far unknown reason, StoA

must be produced in the forespore compartment to effectively accomplish its function in sporulation.

After reduction of the disulfide bond in SpoVD, StoA becomes oxidized and has to be re-reduced in order to perform another round of catalysis. CcdA, a membrane integral reducing TDOR of 235 amino acid residues, is suggested to break the disulfide bond in oxidized StoA (93). The majority of spores produced by a CcdA deficient strain is heat sensitive (Table 2) and lacks cortex layer (94), indicating involvement of CcdA in cortex synthesis. In the absence of CcdA, StoA is active but not efficiently re-reduced. A CcdA/StoA double mutant shows an accumulative phenotype (94), i.e. produces only about 0.006% heat resistant spores (Table 2).

Figure 12 shows a model for the function of BdbD, StoA and CcdA in regulation of the redox state of SpoVD. No other substrate than SpoVD has been identified for StoA. Interaction between StoA and SpoVD has recently been demonstrated *in vitro* by using purified proteins and a substrate-trapping approach (Paper III).

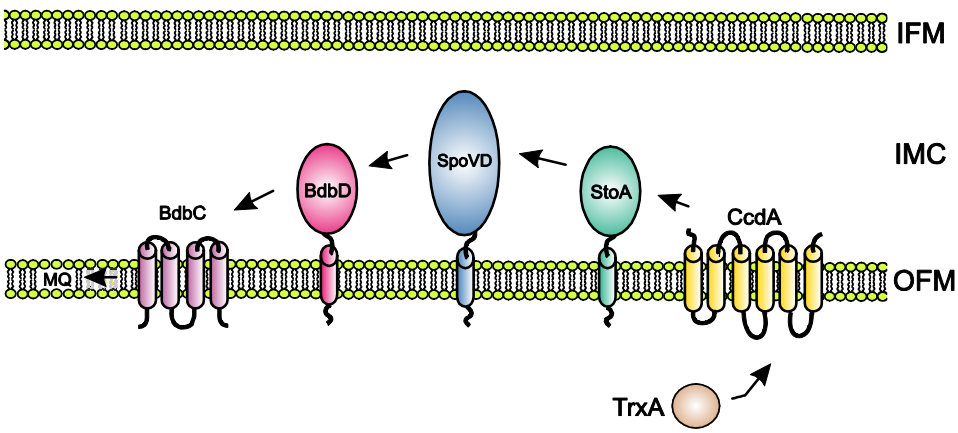


**Figure 12.** Model for the function of BdbD, StoA and CcdA in tuning of the redox state of SpoVD in the intermembrane space of the forespore. A disulfide bond in SpoVD is formed by the action of BdbD, leading to inactivation of SpoVD. StoA breaks the bond in SpoVD, restoring activity. StoA is kept reduced by CcdA which in turn is reduced by thioredoxin (TrxA). BdbC oxidizes BdbD and reduces menaquinone (MQ) in the respiratory chain. Arrows indicate the direction of electron flow. IFM – inner forespore membrane, IMC – intermembrane compartment, OFM – outer forespore membrane. The illustration is based on Figure 6 in Liu *et al.* (9).

**Table 2.** Yield of heat resistant spores of various *B. subtilis* strains. Data obtained from (94) and (9).

| Strain | Relevant phenotype | Yield (%) of heat resistant spores [a] |
|---|---|---|
| 1A1 | Wild type | ≥ 75 |
| LMD19 | SpoVD$^-$ | $< 1 \times 10^{-5}$ |
| LUL20 | StoA$^-$ | 0.05 |
| LUL10 | BdbD$^-$ | 94 |
| LUL23 | BdbD$^-$ StoA$^-$ | 98 |
| LU60A1 | CcdA$^-$ | 4 |
| LUL21 | StoA$^-$ CcdA$^-$ | 0.006 |
| LMD21 | SpoVD(Cys332Asp) | 65 |
| LMD22 | SpoVD(Cys332Ser) | 0.4 |
| LMD24 | SpoVD(Cys351Ser) | $< 1 \times 10^{-3}$ |
| LMD32 | SpoVD(Cys332Asp) StoA$^-$ | 54 |
| LMD48 | SpoVD(Cys332Asp) CcdA$^-$ | 56 |
| LMD52 | SpoVD(Cys332Asp) StoA$^-$ CcdA$^-$ | 61 |

[a]Cells were sporulated by growth in NSMP for 2 days at 37°C. Yield of heat resistant spores was assayed by incubation of the culture at 80°C for 15 min. Percentage of heat resistant spores was calculated as colony-forming units (CFU) after heating divided by CFU of not heated culture. Sporulation and heat resistant assays were performed at least four times. Representative results are shown.

## 8.4. Does the disulfide bond have a regulatory function in SpoVD?

It was originally assumed that oxidation and reduction of SpoVD could occur repeatedly, i.e. that the activity of SpoVD can be switched off and on by disulfide bond formation and breakage (9). To date, SpoVD is the only known example of a PBP which activity is possibly regulated by a thiol-disulfide redox switch.

Intriguingly, the putative redox switch does not seem important to the cell. BdbD deficient or BdbD/StoA double deficient mutant strains produce essentially a normal

level of heat resistant spores (Table 2, strains LUL10 and LUL23, respectively)(9, 94), showing that SpoVD is active in these cells. Hence, StoA is required only in the presence of BdbD. The fact that *B. subtilis* produces a specific protein to break an undesired disulfide bond in SpoVD raises two possibilities: i) SpoVD is meant to be activated only at a specific time point or in a specific cell compartment during sporulation, and this is achieved through the redox switch; or ii) the cysteine residues in SpoVD must be kept reduced, because either one or both are important for function, for example interaction with substrate or association with other proteins. The first possibility seems unlikely because, as mentioned above, sporulation in a BdbD deficient strain, in which SpoVD is presumably active all the time, proceeds normally. Mature spores of such mutants are heat resistant indicating fully developed cortex layer. In BdbD deficient strains, SpoVD is presumably active also in the mother cell membrane, but apparently this does not affect the mother cell envelope.

Nevertheless, we have tested whether production of SpoVD in vegetative *B. subtilis* cells has an effect on growth and/or sporulation. For production of SpoVD during vegetative growth, we constructed a *B. subtilis* strain that carries the *spoVD-mCherry* gene fusion in the chromosome under a xylose-inducible promoter (strain LMD110). To avoid oxidation of SpoVD by BdbD, we subsequently inactivated the *bdbD* gene in strain LMD110, yielding strain LMD118. The growth of LMD110 and LMD118 in NSMP or on TBAB agar plates was not affected by the production of SpoVD-mCherry fusion protein (unpublished data). Also, both strains yielded near wild type levels of heat resistant spores (Table 3). We then wondered whether a combined effect of BdbD deficiency and production of SpoVD-mCherry together with StoA in vegetative cells would give a detectable phenotype. Strain LMD118/pLLE83 (Table 3) was therefore constructed and tested. We observed that growth was normal (unpublished data) and the sporulation efficiency was similar to the wild type strain (Table 3). Altogether, it seems that presence of SpoVD does not interfere with the synthesis of vegetative cell wall peptidoglycan and apparently there is no specific reason to keep the protein inactive in the mother cell membrane during sporulation.

**Table 3.** Sporulation efficiency of *B. subtilis* strains.

| Strain | Relevant genotype | Yield (%) of heat resistant spores [a] |
|---|---|---|
| 1A1 | Wild type | 76 |
| LMD101 | *ΔspoVD* | $< 6 \times 10^{-7}$ |
| LMD110[b] | *ΔspoVD lacA::$P_{xyl}$-spoVD-mCherry* | 48 |
| LMD118[b] | *ΔspoVD lacA::$P_{xyl}$-spoVD-mCherry ΔbdbD::Tn10* | 68 |
| LMD118/pLLE83[b,c] | *ΔspoVD lacA::$P_{xyl}$-spoVD-mCherry ΔbdbD::Tn10, pLLE83($P_{spac}$-stoA)* | 75 |

[a] Cells were sporulated by growth in NSMP for 2 days at 37°C. Yield of heat resistant spores was assayed by incubation of the culture at 80°C for 10 min. Percentage of heat resistant spores was calculated as colony-forming units (CFU) after heating divided by CFU of not heated culture.
[b] The growth medium was supplemented with 0.2 % (w/v) xylose.
[c] The growth medium was supplemented with 1mM IPTG.

## 8.5. Is there redox communication between SpoVD and SpoVE?

SpoVD is presumably part of a multi-protein complex that synthesizes cortical peptidoglycan. The cysteine residues in SpoVD might there have a role in protein-protein interaction. The only known functional partner protein for SpoVD is SpoVE (56). SpoVE is a sporulation-specific SEDS protein with a putative function in lipid II translocation (flipping) (59, 63). Spores of SpoVE deficient strains are heat sensitive, indicating lack of the cortex layer (63)(9)(Paper V). Interaction between SpoVD and SpoVE was demonstrated by using co-affinity purification as well as FRET (Förster Resonance Energy Transfer) (56). Remarkably, SpoVE in *Bacillus* species has two conserved cysteine residues, Cys82 and Cys174 (in *B. subtilis*) positioned in the third and the sixth transmembrane segment, respectively (Figure 13) (9, 63)(Paper V). These cysteines are not found in other SEDS proteins that function in vegetative cells, so we wondered whether they are functionally interrelated to cysteine residues of SpoVD. Substitution of each of the two cysteine residues in SpoVE for alanine did not have any effect on cortex synthesis in sporulating cells (Paper V). This finding suggests that the conserved cysteine residues are not related to the function of SpoVE in cortex synthesis.

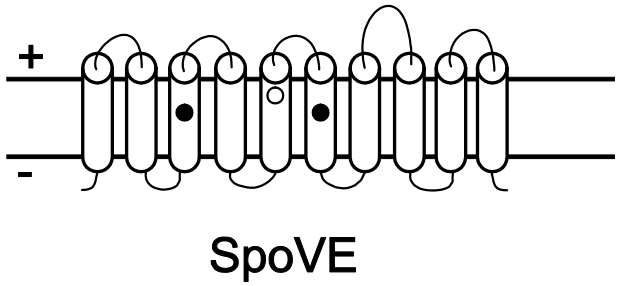


**Figure 13.** Topology model for *B. subtilis* SpoVE in the outer forespore membrane. Position of cysteine residues is indicated by circles; filled circles indicate cysteine residues conserved in the genus *Bacillus*, an open circle indicates a variable cysteine residue. The plus symbol (+) indicates the side of membrane that faces the intermembrane compartment of the forespore and the minus symbol (-) indicates the side facing the mother cell cytoplasm.

## 8.6. Are the two cysteine residues in SpoVD equally important?

The observation that one of the cysteine residues is always positioned within the SXN motif (Cys351 in SpoVD) and that the position of the upstream cysteine residue varies between different PBPs raises the question whether the former cysteine residue is more important for the protein's function. As shown by *in vivo* experiments using different single-cysteine mutant variants of SpoVD, the SpoVD(Cys351Ser) variant is not functional, while the SpoVD(Cys332Asp) variant performs almost as well as the wild type protein (Table 2, strains LMD24 and LMD21, respectively)(9). These data suggest that it is the cysteine residue in the SXN motif that is of major importance for the function of SpoVD. Although SpoVD(Cys332Asp) was found functional, substitution of Cys332 with a serine residue leads to an about 100-fold decrease in the yield of heat resistant spores (Table 2, strain LMD22) (9). This suggests that a negatively charged residue is required at position 332 in *B. subtilis* SpoVD. The functionality of the SpoVD(Cys332Asp) variant is independent of StoA and CcdA (Table 2, strains LMD32, LMD48 and LMD52)(9), which supports the proposed model that StoA functions to break the disulfide bond in SpoVD.

Importantly, the non-functional SpoVD(Cys351Ser) variant can still form an acyl-enzyme complex. Purified soluble SpoVD(Cys351Ser) was found to covalently bind bocillin, a fluorescent β-lactam derivative (Paper III). However, as mentioned before, bocillin is much smaller than the native substrate and the possibility that *in vivo* interaction of the mutant protein with the peptidoglycan side chains is affected cannot be ruled out. Another aspect to consider is that the bocillin-binding assay tests only the first step of the transpeptidation reaction, i.e. formation of an acyl-enzyme complex (Figure 5A). It is therefore possible that *in vivo* a SpoVD(Cys351Ser) variant

can form an acyl-enzyme complex with the native substrate, but is blocked in the second step of the reaction, i.e. deacylation of the acyl-enzyme complex. This property would be consistent with the recently suggested role for the SXN motif in deacylation (96).

## 8.7. Interaction of an amino acid residue in the β2a-β2d loop with residue X of the SXN motif

By comparison of several structures of different class B PBPs, an apparently conserved interaction between a residue in the β2a-β2d loop and the middle residue of the SXN motif has been revealed (97). Multiple sequence alignments distinguished three major groups of the conserved interacting residues: i) in the first and largest group, a central serine of the SSN motif interacts with an aspartate in the β2a-β2d loop, ii) in the second group, a cysteine residue of the SCN motif interacts with a cysteine residue in the β2a-β2d loop, iii) in the third and smallest group, an aspartate residue of the SDN motif interacts with either an arginine or a threonine residue in the β2a-β2d loop. It has been suggested that disruption of this conserved interaction leads to increased flexibility of the β2a-β2d loop and that this in turn negatively affects the *in vivo* transpeptidase function of the protein, but has little effect on the covalent binding of penicillin (97). These findings suggest that the interaction between the two conserved cysteine residues in SpoVD is actually desired for normal function of the protein, contradictory to what was concluded by Liu *et al.*, 2010 (9). I provide a possible explanation to this discrepancy in the next subsection.

The increased flexibility of the β2a-β2d loop in *Neisseria gonorrhoeae* PBP2 results in an increased susceptibility of the protein to proteolytic degradation (97). This can explain why purified *B. subtilis* SpoVD(Cys351Ser) is less stable than the wild type variant, which has been demonstrated by increased trypsin sensitivity of the sSpoVD(Cys351Ser) as compared to the wild type protein (Paper III). The *in vivo* observation that the SpoVD(Cys332Asp) is functional (Table 2) could be a result of maintained interaction between Cys351 and the β2a-β2d loop. This is supported by the fact that purified sSpoVD(Cys332Asp) has similar sensitivity to trypsin as the wild type protein (Paper III).

As originally suggested by Tomberg *et al.* (97), the β2a-β2d loop may be critical for binding and orienting the natural peptide substrate. PBP variants with increased flexibility of the loop might not be able to accommodate substrate properly.

## 8.8. Does oxidation and reduction of SpoVD occur repeatedly in *B. subtilis* cells?

A disulfide bond in SpoVD is unwanted, but interaction between the two cysteine residues seems important for the function of the protein. One possible explanation to this puzzle could be that the two cysteine residues in SpoVD functionally interact through hydrogen-bonding. This would stabilize the protein and allow dynamics in the β2a-β2d loop region (Figure 8). In this scenario, the role of BdbD and StoA would be as follows (Figure 14). SpoVD synthesized in the mother cell cytoplasm is secreted and inserted into the outer forespore membrane as a predominantly unfolded polypeptide. The two cysteine residues are then readily oxidized by the action of BdbD before SpoVD is completely folded. Disulfide-bonded SpoVD polypeptide is a substrate for StoA which breaks the bond. Next, the maturation of reduced SpoVD can be completed and a hydrogen bond between Cys332 and Cys351 is formed preventing the oxidation of the cysteines by BdbD. Alternatively, StoA might act on folded oxidized SpoVD. In this proposed scenario, oxidation of SpoVD by BdbD and its reduction by StoA would be essentially a single event per each newly synthesized SpoVD molecule. This can explain the need for the only low amounts of StoA which are apparently present in sporulating cells (98). Moreover, the function of StoA would be analogous to that of ResA, which is a structural paralogue of StoA. ResA functions in cytochrome *c* assembly to break disulfide bonds in unfolded apocytochrome *c* polypeptide (99, 100). It is not known how folding of SpoVD proceeds in the intermembrane space of the forespore. The presence of *B. subtilis* PrsA, a chaperone that catalyses post-translocational folding of exported proteins, is essential for folding of PBP2a, PBP2b, PBP3 and PBP4 (101). Since SpoVD is a paralogue of PBP2b, it is likely that folding of SpoVD is also PrsA-dependent.

Intrapeptide hydrogen bonds can stabilize the folded state of proteins. Hydrogen bonds between two cysteine residues are not commonly seen, but can be found in some proteins (102, 103). Hydrogen bonds also modulate the $pK_a$ of cysteine residues. For example, the $pK_a$ of $Cys_1$ in the conserved $Cys_1XXCys_2$ motif (where X is any amino acid residue) in the Trx superfamily is influenced by the number of hydrogen bonds to the acceptor sulphur of $Cys_1$. In general, the more hydrogen bonds, the lower the $pK_a$ of the thiol. These hydrogen bonds are also suggested to stabilize the thiolate form (reactive form) of the cysteine (103, 104).

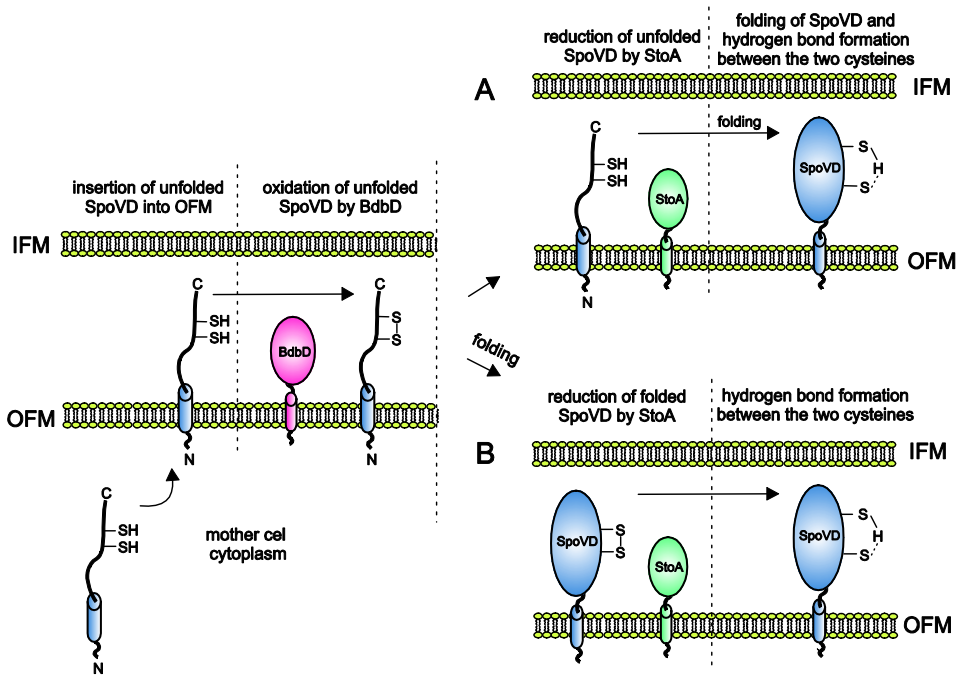


**Figure 14.** New model for the function of BdbD and StoA in modulating the redox state of SpoVD (for a detailed description see text). IFM – inner forespore membrane, OFM – outer forespore membrane.

# 9. What is exactly the role of SpoVD in cortex synthesis?

Apart from SpoVD, several other HMW PBPs are present in the forespore membrane(s) (Table 1). We have demonstrated that cortex synthesis depends on the transpeptidase activity of SpoVD specifically (Paper I). Why cannot other PBPs with transpeptidase activity compensate for the lack of SpoVD activity? The structural feature that distinguishes SpoVD from other sporulation-specific PBPs is the presence of a C-terminal PASTA domain. The PASTA domain of SpoVD is, however, not essential for cortex synthesis (Paper II). As discussed in detail in the previous subsections, the two conserved cysteine residues in the transpeptidase domain seem important for the function of SpoVD. Interestingly, PBP4b which also contains the conserved cysteine residues in the transpeptidase domain and is present in the forespore at the same time as SpoVD (49) (Paper IV), apparently cannot substitute for SpoVD in a SpoVD-deficient or SpoVD active-site mutant strain. It is possible that SpoVD is the only class B PBP that can interact with SpoVE (56) and this interaction may be crucial for assembly of cortex. As suggested by Fay *et al.* (56), SpoVD could be involved in binding of lipid II delivered across the membrane by the SpoVE and then, in cooperation with a class A PBP, incorporate the disaccharide-peptide unit into nascent peptidoglycan. Since a SpoVE-SpoVD fusion protein is functional (56), it would be interesting to test whether a SpoVE-Pbp4b fusion protein can complement a *ΔspoVD ΔspoVE* strain.

The germ cell wall peptidoglycan has a slightly different structure as compared to cortical peptidoglycan (Figure 4). Presumably, the germ cell wall serves as a template for the synthesis of the cortex (30). SpoVD could be the only PBP that can form peptide bridges between the two types of peptidoglycan. If so, SpoVD is only needed for the synthesis of cortex adjacent to the germ cell wall layer.

# 10. Conclusions

The disulfide bond in SpoVD was originally assumed to function as an "on" and "off" switch for enzyme activity. Additionally, this feature was attributed only to sporulation-specific PBPs. Over the time of my PhD project, we have learned that two conserved cysteine residues in the transpeptidase domain can also be found in PBPs of non-sporulating bacteria. The data published by other research groups, which unravelled the importance of interaction between the SXN motif and the β2a-β2d loop of the transpeptidase domain, helped us to understand that interaction between reduced Cys332 and Cys351 in SpoVD stabilizes the β2a-β2d loop. The role of this stabilization remains unclear. The fact that interaction between the two conserved cysteines is necessary, but the formation of the disulfide bond between them blocks activity, led us to hypothesize that the two cysteine residues interact through hydrogen bonding. We also speculate that substrate binding and/or the transpeptidase reaction involves local dynamics of the active-site region, especially the β2a-β2d loop, and that would be restricted in the presence of a disulfide bond.

It seems, however, that the role of two cysteine residues in SpoVD is not limited to apparent stabilization of the β2a-β2d loop. It is observed in other PBPs that similar stabilization can be achieved through the interaction between, for example, serine and aspartate residues. Why the two cysteine residues in SpoVD have been kept throughout evolution, enforcing *B. subtilis* cells to produce a specific protein (StoA) to break the disulfide bond between them, remains an open question. Perhaps, structural studies on the transpeptidation mechanism of PBPs can resolve the function of the two conserved cysteine residues in SpoVD.

# 11. Future perspectives

Until very recently, biochemical studies of *B. subtilis* PBPs with natural substrates have been impossible to carry out due to the lack of synthetic lipid II that is relevant for *B. subtilis*. The synthesis of this peptidoglycan precursor recently reported by Lebar *et al.* (105), provides a valuable experimental tool. Attempts can now be made to carry out *in vitro* transpeptidase assays using for example reduced and oxidized variants of SpoVD. Also, crystallization of SpoVD in complex with native peptidoglycan precursor would provide information about interactions of the substrate with the active site region of the transpeptidase domain. NMR (Nuclear Magnetic Resonance) spectroscopy could be applied to study differences in the dynamics of the active site region in purified water soluble oxidized and reduced SpoVD upon substrate binding.

Testing the deacylation rate of wild type and cysteine mutant variants of SpoVD can provide information whether the mutant variants are affected in this step. This could be done using labelled penicillin.

To date, heat resistance assay and transmission electron microscopy (TEM) of spores are the commonly used techniques to assess the presence of cortex in *B. subtilis* spores. The heat resistance is however an indirect assay and it is unclear whether, for example, a thinner, thicker or somewhat disordered cortex in a certain mutant will be reflected in altered heat resistance. Similarly, absence or the presence of a regular cortex in the majority of spores can be observed by TEM but in the case of strains that produce a very low yield of heat resistant spores it is essentially impossible to identify these spores among a large majority of heat sensitive spores in the preparation. Some more direct techniques are required for detailed studies of individual spores in heterogeneous spores sample. A real-time tracking of peptidoglycan synthesis in the developing spore using fluorescent derivatives of D-amino acids (FDAAs) (106) is impossible due to limited access to the forespore intermembrane space in intact cells. However, a recently published method that incorporates fluorescently labelled D-amino acids into cytoplasmic precursors of peptidoglycan (107) can probably be used in *B. subtilis* sporulating cells to visualise cortex synthesis. It would be interesting to investigate whether any peptidoglycan precursors are incorporated into cortex in different SpoVD mutant strains.

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