Transpeptidase activity of penicillin-binding protein SpoVD in peptidoglycan synthesis conditionally depends on the disulfide reductase StoA

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Summary
Endospore cortex peptidoglycan synthesis is not required for bacterial growth but essential for endospore heat resistance. It therefore constitutes an amenable system for research on peptidoglycan biogenesis. The *Bacillus subtilis* sporulation-specific class B penicillin-binding protein (PBP) SpoVD and many homologous PBPs contain two conserved cysteine residues of unknown function in the transpeptidase domain – one as residue x in the SxN catalytic site motif and the other in a flexible loop near the catalytic site. A disulfide bond between these residues blocks the function of SpoVD in cortex synthesis. With a combination of experiments with purified proteins and *B. subtilis* mutant cells, it was shown that in active SpoVD the two cysteine residues most probably interact by hydrogen bonding and that this is important for peptidoglycan synthesis *in vivo*. It was furthermore demonstrated that the sporulation-specific thiol-disulfide oxidoreductase StoA reduces SpoVD and that requirement of StoA for cortex synthesis can be suppressed by two completely different types of structural alterations in SpoVD. It is concluded that StoA plays a critical role mainly during maturation of SpoVD in the forespore outer membrane. The findings advance our understanding of essential PBPs and redox control of extracytoplasmic protein disulfides in bacterial cells.

Introduction
Peptidoglycan in the cell wall of bacteria determines shape and confers mechanical stability to the cell. Its synthesis is the target of many antibiotics in clinical use, for example, beta-lactams and vancomycin. During cell growth and division, peptidoglycan disaccharide-pentapeptide precursors are synthesized in the cytoplasm and in the form of Lipid II molecules brought to the outer surface of the cytoplasmic membrane by flippase transport proteins. There, the disaccharide-pentapeptide units are incorporated into nascent peptidoglycan polymer by the catalytic action of membrane-bound enzymes with glucosyl transferase and transpeptidase activity. Enzymes with the latter activity are called penicillin-binding proteins (PBPs) (for review see Typas et al., 2012). The essentiality of the cell wall for normal growth and division of bacterial cells is often a major obstacle for *in vivo* studies aiming to increase understanding of peptidoglycan synthesis. The cortex protective layer of *Bacillus subtilis* endospores is of interest on its own and is an excellent experimental system because this layer consists largely of modified peptidoglycan (Popham, 2002; Popham and Bernhards, 2015). In cortex peptidoglycan approximately every second N-acetyl-muramic acid moiety is modified to delta-lactam muramic acid and the extent of cross-linking is low compared with peptidoglycan in the cell wall of vegetative bacteria (Warth and Strominger, 1972; Popham, 2002). Endospores deficient in cortex are heat sensitive. Hence, presence of an assembled cortex layer can be assayed by the heat resistance of sporulated cells. For a recent review on endospore formation in *B. subtilis* see cf., (Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014).

The three sporulation-specific proteins SpoVB, SpoVD, and SpoVE are required for endospore cortex synthesis in *B. subtilis* (Stragier, 1991; Daniel et al., 1994; Popham and Vasudevan et al., 2007; Real et al., 2008; Bukowska-Faniband and Hederstedt, 2013). SpoVB is a putative Lipid II flippase (or a protein associated with this function), SpoVD is a class B high
molecular weight (HMW) PBP with transpeptidase activity, and SpoVE is a SEDS family protein probably with glucosyl transferase activity, as shown recently (Cho et al., 2016; Meeske et al., 2016). The properties of vegetative cells of mutants defective in any of these three membrane proteins do not differ from those of wild type cells, but the mutant sporulating cells form heat-sensitive spores devoid of cortex (Vasudevan et al., 2007). SpoVD and SpoVE are known to form a complex in the outer forespore membrane (Fay et al., 2010) and together they apparently confer most of the transpeptidase and glucosyl transferase activity needed for cortex synthesis (Fig. 1). B. subtilis SpoVD (71 kDa) is anchored to the membrane by a single transmembrane segment residues 12–31 and has three protein domains exposed to the forespore intermembrane space where cortex peptidoglycan is assembled. A domain of unknown function residues 54–206 is followed by the transpeptidase domain residues 246–557 and one PASTA domain residues 584–638 at the C-terminal end (Finn et al., 2014). The transpeptidase domain of SpoVD and other PBPs contains three signature sequences that contribute to the catalytic site SxXK (contains the serine that forms an intermediate covalent bond to peptide substrate during the transpeptidase reaction), SxN, and KTG (Goffin and Ghuysen, 1998; Zapun et al., 2009). SpoVD orthologs contain two conserved cysteine residues in the transpeptidase domain (Liu et al., 2010). One is residue x in the SxN motif (residue Cys351 in B. subtilis SpoVD). The other (Cys332 in SpoVD) is positioned in a flexible polypeptide loop adjacent to the transpeptidase catalytic site (Liu et al., 2010; Fedarovich et al., 2012).

By sequence comparisons, Tomberg et al. (2012) revealed that the residue x in the SxN motif and a residue in the loop in HMW PBPs occur as a Cys–Cys or Asp–Ser pair, and sometimes as an Arg–Asp pair. They showed, by in vitro and in vivo studies with Neisseria gonorrhoeae PBPs (has an Asp–Ser pair), that the two residues are critical for function and seemingly interact by hydrogen bonding, as supported by crystal structure data for several HMW class B PBPs of clinical importance, for example, Staphylococcus aureus PBPs and Streptococcus pneumoniae PBPs (Tomberg et al., 2012). Some results from these in vivo studies were negative findings (absence of growth), because active PBP2 is essential for cell growth. In this article, we address the functional role of the conserved residue pair in HMW PBPs using B. subtilis SpoVD as an experimental system.

Cys332 and Cys 351 in B. subtilis SpoVD can form a disulfide bond, as demonstrated by studies with isolated protein and structure modeling (Liu et al., 2010). There is furthermore a disulfide bond between the corresponding Cys residues in the crystal structures of the transpeptidase domain of the homologous Mycobacterium tuberculosis PBPs (Fedarovich et al., 2010, 2012). Previously, we showed that oxidized SpoVD containing this disulfide bond is inactive in cortex synthesis and that the sporulation-specific, membrane anchored, small thiol-disulfide oxidoreductase StoA (18 kDa) (Crow et al., 2009b) is required for a high yield of heat-resistant, cortex-containing endospores (Liu et al., 2010). Deficiency in StoA and SpoVD, respectively, results in a similar defective spore cortex phenotype (Erlandsson et al., 2004; Imamura et al., 2004). These findings, combined with the properties of SpoVD mutant strains, suggested that the function of StoA is to break the disulfide bond in oxidized SpoVD (Fig. 1). The experimental results also showed that a disulfide bond in SpoVD is not essential and that oxidation of SpoVD is mediated by the thiol-disulfide oxidoreductase BbdD (Liu et al., 2010), a membrane bound enzyme that is not required for endospore maturation and which has the same general function as the well-known DsbA protein in the periplasm of Escherichia coli (Erlandsson and Hederstedt, 2002; Möller and Hederstedt, 2006). At the time, we proposed that SpoVD is regulated by a redox switch of unknown physiological significance (Liu et al., 2010).

In the present study, using experiments with purified proteins (Supporting Information Fig. S1) and mutant strains (Table 1), we demonstrate that the oxidized SpoVD protein is the direct substrate for StoA, and that the requirement for StoA is conditional. In addition, we
The spoVD and StoA would result in short-lived mixed disulfides, proteins. The reaction between isolated wild type SpoVD and oxidized StoA, that is, SpoVD(C332D), SpoVD(C351S), one of the two cysteine residues present in each of these proteins, that is, SpoVD(C332D), SpoVD(C351S), StoA(C65A) and StoA(C68A).

Both SpoVD and StoA are in the B. subtilis cell membrane anchored by an N-terminal single transmembrane peptide that also functions as a signal sequence for secretion. The principal function of the much larger membrane-extrinsic domain of each protein is not dependent on the membrane anchor (Imamura et al., 2004; Crow et al., 2009b; Liu et al., 2010). To facilitate purification and experiments with isolated protein, SpoVD and StoA were produced as water-soluble proteins without the respective membrane anchor and are denoted sSpoVD (residues 33–646) and sStoA (residues 21–165). Protein production in E. coli cells and purification was carried out as described in the Supporting Information. Purity of used protein preparations is shown in Supporting Information Fig. S1.

To screen for pairs of single cysteine sSpoVD and sStoA variants that can form a mixed disulfide we provide supportive evidence for a functional hydrogen bond interaction between the two cysteine residues in the reduced SpoVD protein.

## Results

**SpoVD forms a mixed disulfide with StoA**

Molecular interaction between oxidized (disulfidebonded) SpoVD and reduced StoA is suggested from in vivo studies with B. subtilis mutant strains, but has not been demonstrated (Liu et al., 2010). In the forespore intermembrane compartment (Fig. 1), one of the two cysteine residues in reduced StoA can make a nucleophilic attack on the disulfide bond in the oxidized SpoVD polypeptide, resulting in an intermolecular disulfide bond between the two proteins. This mixed disulfide bond would subsequently be attacked by the second cysteine residue in StoA, leading to the release of reduced SpoVD and oxidized StoA. To investigate physical interaction between StoA and SpoVD we carried out in vitro mixed disulfide trapping experiments with isolated proteins. The reaction between isolated wild type SpoVD and StoA would result in short-lived mixed disulfides, due to the action of the resolving thiol group (Fig. 2A). The mixed disulfide state can, however, be trapped if the resolving thiol group is missing (Fig. 2B). We, therefore, made use of SpoVD and StoA variants that lacked one of the two cysteine residues present in each of these proteins, that is, SpoVD(C352D), SpoVD(C351S), StoA(C65A) and StoA(C68A).

Table 1. B. subtilis strains used in this work.

| Strain | Description | Reference |
|--------|-------------|-----------|
| 1A1    | trpC2       | BGSC      |
| 46a    | trpC2 spoVD Δ(ykvU-stoA)::tet Δccda::ble              | This work |
| LAM15  | trpC2 spoVD amyE::spoVD29; SpR | This work |
| LMD101 | trpC2 ΔspoVD | (Bukowska-Faniband and Hederstedt, 2013) |
| LMD21  | trpC amyE::ery spoVD21; EmR | (Liu et al., 2010) |
| LMD24  | trpC amyE::ery spoVD24; EmR | (Liu et al., 2010) |
| LMD52  | trpC amyE::ery Δ(ykvU-stoA)::tet Δccda::Tn10 spoVD21; EmR; SpR; TcR | (Liu et al., 2010) |
| LMD132B| trpC2 ΔspoVD amyE::Pxyfg-spoVD-StrepII; SpR | (Bukowska-Faniband and Hederstedt 2015) |
| LMD136 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet; TcR | This work |
| LMD137 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble; PrR; TcR | This work |
| LMD138 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble amyE::spoVD; PrR; TcR; SpR | This work |
| LMD145 | trpC amyE::ery spoVD45; EmR | This work |
| LMD148 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble amyE::spoVD47; PrR; TcR; SpR | This work |
| LMD149 | trpC2 ΔspoVD amyE::Pxyfg-spoVD47, SpR | This work |
| LMD150 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble amyE::Pxyfg-spoVD47; PmR, TcR; SpR | This work |
| LMD151 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble amyE::Pxyfg-spoVD-StrepII; PmR, TcR; SpR | This work |
| LMD152 | trpC2 ΔspoVD amyE::spoVD32; SpR | This work |
| LMD153 | trpC2 ΔspoVD amyE::spoVD33; SpR | This work |
| LMD154 | trpC2 ΔspoVD amyE::spoVD34; SpR | This work |
| LMD155 | trpC2 ΔspoVD amyE::spoVD36; SpR | This work |
| LMD171 | trpC2 ΔspoVD amyE::Pxyfg-spoVD48, SpR | This work |
| LMD176 | trpC2 ΔspoVD Δ(ykvU-stoA)::tet Δccda::ble amyE::Pxyfg-spoVD48; PmR, TcR; SpR | This work |
| LUL30  | trpC2 Δ(ykvU-stoA)::tet; TcR | (Erlandsson et al., 2004) |

a. EmR, PrR, SpR, TcR indicate resistance to erythromycin, phleomycin, spectinomycin and tetracycline respectively.

The spoVD alleles encode the following SpoVD variants: spoVD21–SpoVD(C332D), spoVD24–SpoVD(C351S), spoVD26–SpoVD(C332A), spoVD29–SpoVD(C332G), spoVD32–SpoVD(C332N), spoVD33–SpoVD(C323E), spoVD34–SpoVD(C332T), spoVD45–SpoVD(C332D C351S), spoVD46–SpoVD46, spoVD47–SpoVD47, spoVD48–SpoVD48. The spoVD gene of strains has its normal promoter region if nothing else is indicated. BGSC: Bacillus Genetic Stock Center, Columbus, Ohio.

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incubated the proteins in all possible combinations (Fig. 2C). The thiol group of the target protein was activated by reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The reaction product can be regarded as a mimic of the oxidized (disulfide-bonded) form of the protein. This thio-2-nitrobenzoic acid (TNB)-activated target was mixed with a reduced partner protein (Fig. 2B and C). After incubation, the formation of mixed disulfide between sSpoVD and sStoA was determined by SDS-PAGE under non-reducing conditions. The expected size of the sSpoVD-sStoA heterodimeric complex is ~ 83 kDa. As shown in Fig. 3A (samples without dithiothreitol (DTT)), such a product was obtained in some combinations. Immuno-blot analysis using anti-SpoVD and anti-StoA polyclonal antisera verified that the complexes contained both proteins (Fig. 3B and C). Addition of DTT to the samples before electrophoresis led to disappearance of the ~ 83 kDa band (Fig. 3A, samples with DTT) confirming that the two proteins were held together by a disulfide bond. The highest relative yield of sSpoVD–sStoA mixed disulfide complexes was obtained when sSpoVD(C351S) was incubated with sStoA(C68A) (Fig. 3A–C; lanes 4 and 8). This result is expected since Cys65 in StoA is surface-exposed (Crow et al., 2009b;}

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Liu et al., 2010) and Cys332 in SpoVD is in the flexible loop and therefore supposedly more exposed than Cys351 of the SCN motif (Liu et al., 2010). The analyses also showed formation of homo-dimers of sStoA and sSpoVD, especially in the case of the sStoA(C68A) and sSpoVD(C351A) variants (Fig. 3).

**Fig. 3.** Screen for mixed disulfide complex with different sSpoVD and sStoA single-cysteine mutant proteins. Variants of sSpoVD and sStoA in molar excess were incubated together (see Fig. 2C) at pH 7.4 for 30 min at 25°C. The proteins mixed are indicated above the lanes [C332D–sSpoVD(C332D), C351S–sSpoVD(C351S), C65A–sStoA(C65A), C68A–sStoA(C68A)].

A. Coomassie blue-stained SDS-PAGE gels run under non-reducing (−DTT) and reducing (+DTT) conditions. B and C. Immuno-blots of non-reduced samples with anti-SpoVD and anti-StoA sera respectively. Loading of samples were the same as for the Coomassie blue-stained gel as indicated by lane numbers. The identity of proteins is indicated on the right-hand side of the panels. Molecular mass markers, in kDa, are indicated on the left-hand side of the panels; sStoA (16 kDa), sSpoVD (67 kDa) and sSpoVD-sStoA complex (83 kDa). Polypeptide bands in the 40–60 kDa range are fragments of SpoVD present in some protein preparations (Supporting Information Fig. S1).

**Specificity of interaction between SpoVD and StoA**

To investigate the specificity of interaction between SpoVD and StoA we determined whether SpoVD can form a mixed disulfide with a homologous, but physiologically unrelated, thiol-disulfide oxidoreductase. *B. subtilis* ResA is similar to StoA in terms of three-dimensional structure and both ResA and StoA are reduced by CcdA in the membrane (Lewin et al., 2006; Crow et al., 2009b). ResA functions in cytochrome *c* biogenesis (Erlendsson et al., 2003) and cannot replace StoA *in vivo* (Erlendsson et al., 2004). Since the sStoA(C68A) variant yielded the highest amount of trapped mixed disulfide with sSpoVD, we used the corresponding sResA variant to test reactivity with sSpoVD,

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that is, hexahistidyl-tagged \(s\text{ResA(C77A)}\) (hereafter called \(s\text{ResA(C77A)}\)-His6) in which the single remaining cysteine (Cys74) is surface-exposed in the protein structure (Lewin et al., 2006). In two parallel incubations, \(s\text{SpoVD(C351S)}\)-TNB was incubated with \(s\text{StoA(C68A)}\) and \(s\text{ResA(C77A)}\)-His6 respectively. \(s\text{SpoVD(C351S)}\) preferentially formed trapped mixed disulfide with \(s\text{StoA(C68A)}\) (Supporting Information Fig. S2).

The difference between the \(pK_a\) value of 7.0 for Cys65 in \(s\text{StoA(C68A)}\) (Crow et al., 2009b), and 8.5 for Cys74 in \(s\text{ResA(C77A)}\) (Lewin et al., 2006), however, needs to be considered. The experiment shown in Supporting Information Fig. S2 was carried out at pH 7.4 at which the majority of the \(s\text{ResA(C77A)}\) molecules would have their cysteine residue protonated and consequently non-reactive. Poor reactivity of \(s\text{SpoVD}\) with \(s\text{ResA}\) can therefore be a result of weak interactions between the ResA and SpoVD proteins or unfavorable buffer conditions. To investigate this, we repeated the above experiment, but at pH 8.5. At this pH, more than 90% of \(s\text{StoA(C68A)}\) and around 50% of \(s\text{ResA(C77A)}\)-His6 proteins would have their cysteine in the thiolate state. To compensate for concentrations of reactive cysteine residues, we incubated \(s\text{SpoVD(C351S)}\) with twice the molar amount of \(s\text{ResA(C77A)}\)-His6 as compared with that of \(s\text{StoA(C68A)}\). As shown in Fig. 4, conditions favoring interactions between \(s\text{SpoVD(C351S)}\) and \(s\text{ResA(C77A)}\)-His6 did not significantly improve reaction between the two proteins. Also at pH 8.5 \(s\text{SpoVD(C351S)}\) formed a mixed disulfide with \(s\text{StoA(C68A)}\) more efficiently than with \(s\text{ResA(C77A)}\). In conclusion, the StoA protein appears to interact with SpoVD specifically.

**Isolation of StoA-independent SpoVD mutant with altered C-terminal sequence**

Transpeptidase activity of SpoVD in wild type *B. subtilis* sporulating cells depends on the presence of reduced StoA (Liu et al., 2010). StoA is kept reduced by CcdA (Fig. 1), which mediates transmembrane electron transfer from cytoplasmic thioredoxin (Möller and Hederstedt, 2006, 2008; Williamson et al., 2015). In the absence of StoA, only 0.04 ± 0.02% of the formed spores are heat resistant (Erlandersson et al., 2004; Liu et al., 2010) (Fig. 5B, strain LUL30). When StoA and CcdA are both missing 0.04% ± 0.01% of the obtained spores are heat resistant (Fig. 5B, strain LMD138).

The C332D mutation in SpoVD is known to effectively suppress the effect on spore cortex synthesis caused by CcdA- and StoA-deficiency (Liu et al., 2010) (Table 2, strain LMD52). We asked whether other types of mutation in *spoVD* can overcome StoA CcdA double deficiency. To find such mutations, plasmid pLEB3 (contains *B. subtilis spoVD*) (Supporting Information Table S1) was subjected to random mutagenesis in the *E. coli* mutator strain XL1-Red. Obtained libraries of mutated plasmids were used to transform the StoA CcdA SpoVD triple null mutant LMD137 (Table 1) to spectinomycin resistance, which selects for clones with the *spoVD* gene of the plasmid inserted by double homologous recombination into the amyE locus. Pools of transformants were screened for clones that produced more than 0.4% heat-resistant spores. After several independent rounds of mutagenesis and selection, one clone, named 46a, was found producing 1.3% ± 0.4% heat-resistant spores (Fig. 5B). Linkage of the suppressor mutation to *spoVD* was assessed by transformation of the LMD137 strain with a limiting amount of chromosomal DNA extracted from clone 46a and selecting for spectinomycin resistance. The transformants formed approximately 1% heat-resistant spores, indicating that the mutation of interest in isolate 46a is in *spoVD*. Transformation of a *spoVD* deletion mutant (LMD101), containing both StoA and CcdA, with clone 46a DNA to spectinomycin resistance, yielded transformants with wild type efficiency (75%–85%) in production of heat-resistant spores. The findings showed that strain 46a contains a mutation in *spoVD* that partially suppresses StoA- and CcdA-deficiency.

Clone 46a was found to have the insertion of an adenine in an adenine-rich region (nucleotides 1719–1724) of the *spoVD* gene. This mutation, named *spoVD*46, is predicted to cause truncation of the SpoVD polypeptide by 49 residues and, from residue 576, an altered amino acid sequence (Fig. 5A). To verify the effect of the identified *spoVD*46 frameshift mutation, we constructed strain LMD148 (Table 1) that carries the site-directed mutation *spoVD*47, which is the insertion of a guanine after nucleotide position 1724 in the *spoVD* gene. The *spoVD*47 mutation causes the same frameshift as *spoVD*46 (Fig. 5A). Strain LMD148 yielded 1.7% ± 0.1% heat-resistant spores, which is similar to that of clone 46a (Fig. 5B).

Immuno-blot analysis of whole-cell lysates of sporulating LMD148 cells harvested 5 hours after induction of sporulation (T5) revealed the presence of a weak SpoVD antigen band migrating slightly faster than the full length protein of wild type strain 1A1 (Supporting Information Fig. S3), suggesting that the frame shift mutation affects the cellular level of the protein. However, the polyclonal SpoVD antiserum used for immuno-blot probably does not recognize truncated proteins efficiently, which leads to an under-estimation of the amount of mutant SpoVD in the sporulating cells. To facilitate detection and quantitation of full length and truncated proteins in cell extracts, wild type SpoVD and SpoVD47 were tagged at the
N-terminal end with green fluorescent protein (GFP). The gene for the respective fusion protein, under control of a xylose-inducible promoter, was inserted into the amyE locus of strains LMD101 (ΔspoVD) and LMD137 (ΔstoA, ΔccdA, ΔspoVD). The B. subtilis strains obtained with wild type GFP-SpoVD yielded 91% ± 0.5% and 0.003% ± 0.001% heat-resistant spores in the presence (LMD132B) and absence of StoA and CcdA (LMD151) respectively (Fig. 5B). Strains containing GFP-SpoVD47 yielded 35% ± 4% heat-resistant spores in the presence (LMD149) and 0.15% ± 0.01% in the absence of StoA and CcdA (LMD150) (Fig. 5B). Use of GFP antibodies in immuno-blot analysis confirmed that the spoVD47 mutation causes truncation of the SpoVD polypeptide, and showed that the amount of GFP-SpoVD47 protein was about half compared with that of the wild type control (Fig. 5C). The combined results show that the modification of the C-terminal end of SpoVD has little effect on the function of the enzyme and partly (30- to 50-fold increased yield of heat-resistant spores) suppresses the defect in production of heat-resistant spores caused by StoA and CcdA deficiency.
The C-terminal truncation in the altered SpoVD comprises the entire PASTA domain (residues 584–638), which is not important for cortex synthesis (Bukowska-Faniband and Hederstedt, 2015). To rule out that the lack of the PASTA domain in SpoVD causes the StoA CcdA suppressor phenotype we expressed the SpoVD(D582–646) variant in the DstoA, DccdA, DspoVD background. The strain was deficient in forming heat resistant spores (data not shown) suggesting that it is other features in the novel C-terminal which confer the suppression phenotype. Remarkably, the most C-terminal part of the SpoVD46/47 polypeptide contains a novel cluster of three cysteine and four arginine residues (Fig. 5A). To test the importance of the cysteine triplet we changed these residues into alanines and the resulting polypeptide was named SpoVD48 (Fig. 5A). The gene encoding the GFP-SpoVD48 fusion protein, under control of a xylose inducible promoter, was inserted into the amyE locus of strains LMD101 (DspoVD) and LMD137 (DstoA, DccdA, DspoVD), resulting in strains LMD171 and LMD176 respectively. Strain LMD171 yielded 15% ± 7% heat resistant spores. Strain LMD176 was found to produce 10-fold less heat resistant spores (0.013% ± 0.0025%) than strain LMD150 containing the GFP-SpoVD47 variant (Fig. 5B). The level of GFP-SpoVD47 in LMD150 and GFP-SpoVD48
in LMD176 was about half relative to wild type SpoVD-GFP in LMD132B (Fig. 5C). Thus, the cysteine triplet in the C-terminal end of the SpoVD46/47 protein contributes to the StoA CcdA-independent phenotype.

A cys residue in position 351 is important

As detailed in the Introduction, Tomberg et al. have revealed a functionally important conserved interaction between two residues in the transpeptidase domain of HMW PBPs. The apparent hydrogen bond interaction is between residue x of the catalytic site SxN motif and a residue positioned in the flexible loop that resides close to the motif (Tomberg et al., 2012). In B. subtilis SpoVD, the postulated interacting residues are Cys332 and Cys351. An aspartate residue in the loop and an SSN catalytic site sequence seem the most common arrangement among the class B PBPs (Tomberg et al., 2012). In B. subtilis, this can, for example, be found in PBP2b, which is a SpoVD paralog essential for septal peptidoglycan synthesis in dividing vegetative cells (Yanouri et al., 1993). We constructed strain LMD145 with a Cys332Asp Cys351Ser double substitution in SpoVD, and it was found to produce only heat-sensitive spores (Table 2). Immuno-blot analysis of whole cell lysates of sporulating cells showed about half the amount of SpoVD protein in LMD145 as compared with the parental strain (Supporting Information Fig. S3). This marginal reduction in the amount of SpoVD does not explain the apparent complete block in cortex synthesis of strain LMD145 (Bukowska-Faniband and Hederstedt, 2015).

To find mutations that suppress the severe functional defect of SpoVD(C332D C351S), LMD145 cells were mutagenized using ethyl methanesulfonate (EMS) as described in the Experimental Procedures. Pools of survivors grown for sporulation were screened for clones that form heat-resistant spores. Several independent clones were obtained, and two were analyzed and found to produce a wild type level of heat-resistant spores. Sequence analysis of the spoVD gene revealed that in both isolates, Ser351 had reverted to Cys (TCC to TGC codon), while Asp332 (GAC codon) was retained. This result is in line with previously presented in vivo properties of SpoVD(C332D) (strain LMD21), which is functional, and the SpoVD(C351S) variant (strain LMD24), which is defective (Liu et al., 2010) (Table 2). The findings reinforce the importance of residue Cys351 in the SCN motif for the function of B. subtilis SpoVD in peptidoglycan synthesis.

Single cysteine SpoVD mutant proteins bind penicillin but show decreased stability

As described in the Supporting Information (under Protein production and purification of proteins), E. coli DnaK co-purified with sSpoVD(C351S). DnaK is an abundant cytoplasmic chaperone that assists folding of newly synthesized proteins (Calloni et al., 2012) and is commonly found as a contaminant of proteins produced in E. coli (Rial and Ceccarelli, 2002). The presence of DnaK in only the sSpoVD(C351S) preparation, as well as the propensity of the protein to be degraded during purification, indicated that the mutant protein is less

Table 2. Ability of various B. subtilis strains to form heat-resistant endospores.

| Strain          | Relevant phenotype        | Yield (%) of heat resistant spores |
|-----------------|---------------------------|-----------------------------------|
| 1A1             | Wild type                 | 67 ± 4                            |
| LMD101          | SpoVD                     | 0 ± 0                            |
| LAM15           | SpoVD(C332G)              | 0.0006 ± 0.0004                 |
| LMD21           | SpoVD(C332D)              | 0.4 ± 0.4                       |
| LMD22           | SpoVD(C332S)              | 0 ± 0                            |
| LMD24           | SpoVD(C351S)              | 0.4 ± 0.4                       |
| LMD25           | SpoVD(C332D) CcdA StoA'   | 61                               |
| LMD145          | SpoVD(C332D C351S)        | 0 ± 0                            |
| LMD152          | SpoVD(C332N)              | 0.00007 ± 0.00003               |
| LMD153          | SpoVD(C332E)              | 0.17 ± 0.03                     |
| LMD154          | SpoVD(C332T)              | 0 ± 0                            |
| LMD155          | SpoVD(C332A)              | 0.0002 ± 0.0001                 |
| LUL10           | BbbD                      | 82 ± 8                           |
| LUL23           | BbbD StoA'                | 98 ± 2                           |
| LUL30           | StoA'                     | 0.04 ± 0.02                     |

a. Cells were sporulated by growth in NSMP for 2 days at 37°C. Heat resistance of spores was assayed by incubation at 80°C for 10 min and the yield was calculated as colony-forming units (CFU) after heating the culture divided by CFU of not heated culture. The results represent an average from three independent experiments ± SEM.
b. Data from references (Liu et al., 2010; Erlendsson et al., 2004; Erlendsson and Hederstedt, 2002).
c. No survivors were found in 0.1 ml of undiluted culture (≥10⁶ colony forming units) heat-shocked and plated on TBAB.

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stable than wild type sSpoVD. Also purified sSpoVD(C332D) appeared less stable than wild type sSpoVD, as determined from the presence of a degradation product in samples (Fig. 3 and Supporting Information Fig. S1). These findings suggest that the single cysteine mutant proteins are disordered to some extent or are more flexible than the wild type protein. To analyze this, purified reduced wild type sSpoVD and the two mutant variants were incubated with a small amount of trypsin, and degradation of the polypeptides over time was monitored by SDS-PAGE followed by staining for protein. Importantly, the studied sSpoVD variants have the same number of predicted trypsin cleavage sites. As shown in Fig. 6, sSpoVD(C332D) and sSpoVD(C351S) showed different patterns of digestion products compared with the wild type protein. Moreover, sSpoVD(C351S) was degraded faster than wild type sSpoVD and sSpoVD(C332D). The three variants also showed different thermal stability, as measured using intrinsic tryptophan fluorescence. The estimated thermal unfolding transition mid-point melting temperature ($T_m$) for the sSpoVD(C332D) and SpoVD(C351S) variants (56°C and 48°C respectively) were lower than that for the wild type variant ($T_m = 66°C$), confirming the relative instability of the sSpoVD variants. We interpret these results as an indication that substitution of one of the two cysteine residues in SpoVD affects the dynamics of the transpeptidase domain.

To assess whether the two purified single cysteine sSpoVD mutant proteins were properly folded, we analyzed them for transpeptidase activity by their ability to catalyze the covalent binding of penicillin to the catalytic site Ser of the SxxK motif. As shown in Fig. 7, both mutant variants bound Bocillin FL, a fluorescent derivative of penicillin known to react with wild type SpoVD (Bukowska-Faniband and Hederstedt, 2013). This showed that the isolated proteins, or at least their transpeptidase domain, were functional. Notably, the activity of SpoVD(C351S) with the native substrate in vivo, that is, peptide side chains of nascent peptidoglycan (which is much larger than Bocillin FL), is clearly impaired, as shown by the inability of strain LMD24 to produce heat-resistant endospores (Table 2).

A residue with a side chain capable of hydrogen bonding seems required at position 332 in SpoVD

Finally, to test the hypothesis that Cys332 in B. subtilis SpoVD functionally interacts with Cys351 by hydrogen bonding, we constructed and analyzed additional variants of SpoVD where Cys332 was changed to Ala, Asn, Glu, Gly, and Thr. Strain LMD153 with SpoVD(C332E) yielded 0.17% ± 0.03% heat-resistant spores, whereas the previously constructed LMD22 strain (Liu et al., 2010) with the SpoVD(C332S) variant yielded 0.4% heat-resistant spores, indicating that these enzymes are functional, but not at the wild type level (Table 2). All other substitutions severely decreased the yield of heat-resistant spores (Table 2, strains LMD152, LMD154, LMD155 and LAM15). Immuno-blot analysis of sporulating cells showed the presence of SpoVD in all strains (Supporting Information Fig. S5), confirming that a low yield of heat-resistant spores is mainly due to defective function rather than lack of enzyme protein.

The SpoVD mutant proteins in membranes isolated from sporulating cells all showed increased sensitivity to proteolytic degradation as determined by quantitative immuno-blot. The half-life of the wild type SpoVD protein was at least fourfold longer than for the mutants (data not shown). These results with membrane-bound proteins...
E. Bukowska-Faniband and L. Hederstedt

between them is avoided or broken in cysteine residues in SpoVD and how a disulfide bond present study, we have investigated the role of the two (Bukowska-Faniband and Hederstedt, 2013). In the peptidase activity of SpoVD cross-links the strands 2016) that polymerizes glycan strands while the trans-

BdbD, together with its partner menaquinone reductase

agree with those obtained for isolated wild type sSpoVD, sSpoVD(C332D) and sSpoVD(C351S) proteins.

Overall, the results demonstrate that Cys332 in the flexible loop close to the catalytic site in SpoVD can in vivo be replaced by an amino acid residue having a side chain capable of hydrogen bonding, that is, by Asp, Glu or Ser but not by Ala, Asn, Gly or Thr, without severe loss of function. We conclude that hydrogen bond interaction between residues Cys332 and Cys351 stabilizes the SpoVD protein and is important for catalytic function of the enzyme in cortex synthesis.

Discussion

Detailed molecular knowledge about peptidoglycan structure and synthesis is important for understanding the action of antibiotics targeting cell wall synthesis, mechanisms of antibiotic resistance and bacterial cell growth and division. SpoVD and SpoVE are main players in the assembly of the thick protective peptidoglycan cortex layer of bacterial endospores. SpoVE is probably a glycosyl transferase (Cho et al., 2016; Meeske et al., 2016) that polymerizes glycan strands while the transpeptidase activity of SpoVD cross-links the strands (Bukowska-Faniband and Hederstedt, 2013). In the present study, we have investigated the role of the two cysteine residues in SpoVD and how a disulfide bond between them is avoided or broken in B. subtilis cells to render production of durable endospores.

The membrane-bound thiol-disulfide oxidoreductase BdbD, together with its partner menaquinone reductase BdbC, introduces essential disulfide bonds in Com proteins in vegetative cells developing competence for DNA acquisition, but is dispensable for spore formation (Erlendsson and Hederstedt, 2002; Möller and Hederstedt, 2006; Kouwen and van Dijl, 2009). BdbD is constitutively produced and seemingly distributed to all membranes in sporulating cells (Crow et al., 2009a; Zheng et al., 2016), also causing non-specific oxidation of some secreted proteins, including formation of an intramolecular disulfide bond between Cys332 and Cys351 in B. subtilis SpoVD polypeptide (Fig. 1). Disulfide-bonded SpoVD is inactive in cortex synthesis (Liu et al., 2010). The sporulation-specific StoA protein breaks the disulfide bond in SpoVD, and this was originally discovered on the basis of in vivo experiments using mutant B. subtilis strains (Liu et al., 2010). These studies suggested, but did not show, direct interaction between StoA and SpoVD. In this work, we demonstrate, by in vitro mixed disulfide-trapping experiments, that the two proteins physically interact. The available data suggest that SpoVD is the only target for StoA (Liu et al., 2010). However, normal sporulation efficiency of BdbD and BdbD StoA double deficient strains (Table 2; strains LUL10 and LUL23), together with the fact that the SpoVD(C332D) variant is fully functional (Table 2; strain LMD21), indicate that a disulfide bond in SpoVD has no purpose in the cell. It is therefore intriguing that the transpeptidase domain of SpoVD and homologous PBPs in a variety of bacteria contains the two cysteine residues enforcing the need for a specific protein (StoA) to break an undesired disulfide bond.

After random mutagenesis of the spoVD gene and screening for suppressor-containing B. subtilis mutants that produce heat-resistant spores in the absence of StoA and CcdA, we found a truncated SpoVD variant lacking the PASTA domain and with a novel sequence of 22 residues at the C-terminal end. Notably, SpoVD without the PASTA domain (SpoVD<sup>Δ582–646</sup>) is active in cortex synthesis (Bukowska-Faniband and Hederstedt, 2015) and depends on StoA for activity (our unpublished data). It is not evident how StoA deficiency is mechanistically suppressed by this truncated SpoVD variant, but the novel C-terminal region and especially the triplet of cysteine residues apparently makes Cys332 and Cys351 less amenable to oxidation by BdbD. For example, BdbD might be more prone to oxidize the three clustered cysteine residues at the C-terminal end of the protein (Fig. 5A), leaving the two cysteine residues in the transpeptidase domain in the reduced state.

Findings reported by Tomberg et al. (2012) suggested that hydrogen bonding between the two cysteine residues in SpoVD is critical for cortex synthesis. We show that the Cys332–Cys351 pair in SpoVD cannot be substituted by an Asp-Ser pair, which is otherwise

Fig. 7. Cysteine mutants of SpoVD bind penicillin. The indicated purified sSpoVD variants were incubated with an excess of Bocillin FL and then analyzed by SDS-PAGE followed by fluorometry of the gel. About 1.35 µg of protein was loaded in each lane. Top panel: fluorogram. Bottom panel: Coomassie blue-stained gel. Molecular mass markers, in kDa, are indicated on the left-hand side of the panels.
commonly found in HMW class B PBPs, for example, in *B. subtilis* PBP2b and *N. gonorrhoeae* PBP2a. In the case of *N. gonorrhoeae* PBP2a, the central serine in the SSN motif can be replaced by cysteine with retained enzyme function (Tomberg et al., 2012). The results of our mutant studies indicate that Cys351 in the SCN catalytic site motif is critical for SpoVD enzyme function. The XxN motif in PBPs is suggested to play a role in deacetylation of the acyl-enzyme catalytic intermediate and this action is thought to be modulated by the neighboring loop (Nicholas et al., 2003). Therefore, Cys351 might play a role in the second step of the transpeptidation mechanism. The ability of the SpoVD(C351S) variant to bind penicillin covalently *in vitro* (i.e. catalyze the first step of transpeptidation) indicate that the deacetylation step could be defective in this mutant enzyme (Table 2; strain LMD24).

Residue Cys332, which resides in the flexible loop next to the catalytic site, seems, in contrast to Cys351, less important for transpeptidase activity of SpoVD. We found that Cys332 can be substituted by several other amino acids without loss of function *in vivo* as long as they are negatively charged or neutral and capable of hydrogen bonding (i.e., Asp, Glu, Ser). The loop may be critical for binding and orienting the natural peptide substrate in nascent peptidoglycan (Tomberg et al., 2012), or play an interactive role with the N-terminal domain of SpoVD or with other proteins involved in peptidoglycan synthesis, such as SpoVE.

We originally assumed that oxidation and reduction of SpoVD occur repeatedly in the cell, that is, that the activity of SpoVD is switched off and on by disulfide bond formation and breakage (Liu et al., 2010). Incorporating the new findings presented in this work, we propose a revised model for the role of BdbD and StoA in modulating the redox state of SpoVD. SpoVD, synthesized in the cytoplasm of the mother cell, is translocated across the forespore outer membrane to be anchored to this membrane most likely initially with the extrinsic domains largely in the unfolded state. The two cysteine residues are in concert with translocation fortuitously oxidized by the action of BdbD. The resulting disulfide-bonded SpoVD polypeptide (and possibly the SpoVD–BdbD mixed disulfide) might fold inefficiently and is the substrate for StoA, which breaks the disulfide bond. Maturation of reduced folded SpoVD is thereby achieved. In this proposed scenario (Fig. 8), oxidation of SpoVD by BdbD and subsequent reduction by StoA would essentially be single events for each newly secreted SpoVD molecule. This can explain the need for only low amounts of StoA in sporulating cells (Crow et al., 2009b). Moreover, the function of StoA would be analogous to that of ResA, which is a paralog of StoA, also kept reduced by CcdA. ResA functions specifically in cytochrome c assembly by breaking BdbD-catalyzed disulfide bonds in unfolded apocytochrome c polypeptides to allow covalent binding of protoheme IX (Erlandsen et al., 2003; Simon and Hederstedt, 2011). Based on the available crystal structure of StoA (Crow et al., 2009b) and modeled structure of SpoVD (Liu et al., 2010) the thiol-disulfide oxidoreductase does not have access to disulfide-bonded Cys332 and Cys351 in fully folded SpoVD, due to steric hindrance (our unpublished data).

The suggested function of StoA in reduction of incompletely folded oxidized SpoVD is consistent with the timing of expression of StoA and SpoVD and the subcellular distribution of these two proteins (Fig. 1). BdbD and CcdA are constitutively produced (expression of the genes depend on sigma-A) (Schiott and Hederstedt, 2000; Crow et al., 2009a). Expression of the stoA gene is under both sigma-G and sigma-E dependence (Imamura et al., 2004). In the sporulating cell, StoA is therefore produced simultaneously with SpoVD and both proteins are inserted into the outer forespore membrane. Later, StoA is also inserted into the inner forespore membrane (Fig. 1). This later sigma-G dependent expression of stoA is known to be sufficient for production of heat-resistant spores (Imamura et al., 2004). It suggests that StoA in the inner forespore membrane can reach oxidized SpoVD polypeptides in the outer membrane despite the presence of the germ cell wall peptidoglycan layer assembled between the two membranes (Meador-Parton and Popham, 2000). Similar functional contact between cell wall synthesizing proteins in separate but adjacent membranes is known, for example, for PBP1a and LpoA in the periplasm of *E. coli* (Egan et al., 2014; Jean et al., 2014). The germ cell wall is believed to prime cortex peptidoglycan synthesis, performed by SpoVB, SpoVE, SpoVD and other proteins, and is additionally required for peptidoglycan synthesis of the vegetative cell wall after germination of the endospore.

The need for StoA in sporulating cells is conditional, at least in *B. subtilis*. Lack of StoA (and CcdA) activity can be suppressed in several ways: (i) by BdbD-deficiency (Liu et al., 2010), (ii) by substitution of Cys332 in SpoVD (Liu et al., 2010, and this work) and (iii) by alteration of the C-terminal part of SpoVD (this work). Despite these alternatives, two cysteine residues corresponding to Cys332 and Cys351 in *B. subtilis* SpoVD are conserved in the transpeptidase domain of many HMW PBPs (Tomberg et al., 2012 and our unpublished bioinformatics data). They are found, for example, in PBPA of *M. tuberculosis* (Fedarovich et al., 2010; Tomberg et al., 2012). In this pathogen, membrane bound thiol-disulfide oxidoreductases, such as the *B. subtilis* BdbD homolog Rv2969c, are most likely involved in oxidation and reduction of this PBP (Chim et al., 2013; Premkumar et al., 2013; Wang...
et al., 2013; Davey et al., 2016). Clearly, the occurrence of the two cysteine residues extends beyond the sporulation-specific HMW PBPs, and seems important for modulation of transpeptidase activity in certain situations of peptidoglycan synthesis in a number of bacteria.

**Experimental procedures**

**Bacterial strains and growth media**

*B. subtilis* strains used in this work are listed in Table 1. Construction of plasmids and *B. subtilis* strains is described in the Supporting Information. *E. coli* TOP10 (Invitrogen) or XL1-Blue (Stratagen) were used for plasmid propagation, *E. coli* TUNER (DE3) (Novagen) or BL21 (Novagen) were used for protein production, and *E. coli* XL1-Red (Stratagen) was exploited for random mutagenesis. *E. coli* strains were grown at 37°C in LB or on LB agar plates (Sambrook and Russell, 2001). *B. subtilis* strains were grown at 37°C in LB, nutrient sporulation medium with phosphate (NSMP) (Fortnagel and Freese, 1968), growth medium and resuspension medium for induction of sporulation (Nicholson and Setlow, 1990), Spizizen's minimal medium (Harwood and Archibald, 1990), or on tryptose blood agar base (TBAB) plates (Difco). Antibiotics were added to the medium when required at the following concentrations: ampicillin 100 µg ml⁻¹ and spectinomycin 150 µg ml⁻¹ for *E. coli*, and spectinomycin 100 µg ml⁻¹, erythromycin 0.5 µg ml⁻¹ combined with lincomycin 12.5 µg ml⁻¹, tetracycline 15 µg ml⁻¹, phleomycin 1.2 µg ml⁻¹ for *B. subtilis*. TBAB plates supplemented with 1% (w/v) soluble starch was used to test for amylase production by *B. subtilis* colonies.

**DNA techniques**

Standard molecular genetic techniques were used (Sambrook and Russell, 2001). Plasmid DNA from *E. coli* was isolated using the Quantum Miniprep Kit (BioRad). *B. subtilis* chromosomal DNA was isolated according to the procedure described in (Marmur, 1963). PCR was carried out using Phusion high-fidelity DNA polymerase (Finnzymes). DNA ligation was performed using T4 DNA ligase (New England Biolabs) at 14°C overnight. All DNA fragments cloned in plasmids were verified by sequence analysis. Plasmids and oligonucleotides used in this work are listed in Supporting Information Tables S1 and S2 respectively.

*E. coli* strains were transformed with plasmid DNA using electroporation or by chemical transformation (Hanahan et al., 1991). *B. subtilis* was grown to natural competence and transformed essentially as described in (Hoch, 1991).

**Mutagenesis of spoVD and selection for StoA-independent SpoVD mutant**

To obtain a library of randomly mutated spoVD, chemically competent *E. coli* XL1 Red cells were transformed with pLEB3 to ampicillin resistance. Transformed cells were plated on 12 LA-ampicillin plates and incubated at 37°C for 24h. Colonies from the respective plates were pooled and grown in 4 ml LB-ampicillin at 37°C for 24h, and plasmid

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*Fig. 8.* Revised model for the function of BdbD and StoA in modulating the redox state of SpoVD in the forespore intermembrane compartment (for further details see text). IFM, inner forespore membrane, OFM, outer forespore membrane.
DNA was isolated from each culture. To estimate the mutation frequency in the obtained libraries, *E. coli* XL-1 Blue was transformed with two of them and the obtained transformants were scored for the loss of spectinomycin resistance encoded by pLEB3. Spectinomycin resistance was lost in 0.2% and 0.8% cases, respectively, showing that one can expect mutations within the spoVD gene in the plasmids. The twelve plasmid pools were used individually to transform *B. subtilis* LMD137 to spectinomycin resistance. In each case 0.5 ml of transformed *B. subtilis* LMD137 was spread on 4 TBAB-spectinomycin plates. Between 2200 and 5000 colonies were obtained in total for each plasmid pool. Colonies on each TBAB-spectinomycin plate were suspended in NSMP and used to inoculate 25 ml of NSMP in a 250 ml E-flask with indentations to an OD<sub>600</sub> of 0.1. Cultures were grown for sporulation (37°C, 200 rpm for 2 days). Five ml of each culture were heated at 80°C for 10 min, and 0.1 ml of 10<sup>2</sup> dilutions (in NSMP) was plated on TBAB-spectinomycin plates and incubated at 37°C overnight. Four colonies (survivors) from each plate were picked and tested for antibiotic markers, that is, resistance to tetracycline and spectinomycin, as well as sporulation efficiency. Strain LMD138 (produces wild type SpoVD in StoA CcdA double deficient background) was used as a reference. Clones with a sporulation efficiency higher than that of LMD138 (i.e., more than 0.04%) were kept and analyzed in more detail.

**EMS mutagenesis**

Bacterial cells in suspension were spread evenly on the surface of a TBAB plate. A paper filter disc containing 10 μl of pure ethyl methanesulfonate (EMS) was then placed in the center and the plate was incubated at 37°C overnight. Bacteria growing at different locations in the border of the inhibition zone of bacterial growth around the disc were picked and used to inoculate 5 ml of NSMP and grown for sporulation (37°C, 200 rpm, for 2 days). The cultures were heat-treated (10 min at 80°C) and 0.1 ml was then spread on TBAB plates followed by incubation at 37°C overnight to select clones able to form heat resistant spores. Chromosomal DNA was isolated from selected such clones and the spoVD gene was amplified by PCR and sequenced.

**Limited proteolysis of SpoVD protein variants**

The protocol used is based on the procedure described in (Tomberg et al., 2012). Samples of 60 μl of pure ethyl methanesulfonate (EMS) was then placed in the center and the plate was incubated at 37°C overnight. Bacteria growing at different locations in the border of the inhibition zone of bacterial growth around the disc were picked and used to inoculate 0.5 ml of NSMP and grown for sporulation (37°C, 200 rpm, for 2 days). The cultures were heat-treated (10 min at 80°C) and 0.1 ml was then spread on TBAB plates followed by incubation at 37°C overnight to select clones able to form heat resistant spores. Chromosomal DNA was isolated from selected such clones and the spoVD gene was amplified by PCR and sequenced.

**Bocillin™-FL binding assay**

Purified wild type or mutant sSpoVD at 1μM in PBS (pH 7.4) was incubated with 10 μM Bocillin™-FL (Innitogen) for 30 min at 35°C. The reaction was stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer followed by incubation at 80°C for 5 min and SDS-PAGE. Bocillin™ FL-labeled proteins were detected in gels using a ChemiDoc™ MP Imaging System (BioRad) (excitation light 455–485 nm, emission light filter 532AE28). Subsequently the gel was stained for protein with Coomassie brilliant blue.

**Modification of protein thiolate groups using DTNB**

An aliquot of sSpoVD(C332D), sSpoVD(C351S), sStoA(C65A), sStoA(C68A) or sResA(C77A)-His6 was reduced by incubation with a 25-fold molar excess of Tris(carboxymethyl)-phosphine (TCEP) for 30 min, at room temperature. After TCEP removal using a spin desalting column the sample was reacted with an 80-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for 30 min, at room temperature. Excess DTNB and released thio-2-nitrobenzoic acid (TNB) were removed using a spin desalting column. Desalting columns (Zeba™, Thermo Scientific) were equilibrated with buffer suitable for the experiment to follow.

**Preparation of mixed disulfide of single-cysteine mutant proteins**

The experimental protocol used is based on the procedure described in (Wang et al., 1996). Purified sSpoVD, sStoA or sResA single cysteine mutant proteins were reduced by incubation with a 25-fold molar excess of TCEP for 30 min, at room temperature. After removal of TCEP using a spin desalting column (Zeba™, Thermo Scientific), the reduced protein was reacted with TNB-modified substrate protein (prepared as described above). The buffers used were PBS, 7.4, or PCTC (potassium phosphate, sodium citrate, Tris and Chess, all at 50 mM), pH 8.5. Reaction mixtures were incubated at 25°C for 180 min, unless otherwise stated. Control experiments ensured that the reactions were completed in less than 30 min, that is, incubation for longer than 30 min did not increase the yield of trapped mixed disulfides. Reaction was stopped by the addition of 3 volumes of 10% trichloroacetic acid, followed by flash freezing in liquid nitrogen. After thawing, samples were mixed with an equal volume of 2× SDS-PAGE sample buffer (with or without DTT as reducing agent) and incubated at 95°C for 5 min. Proteins were then separated by SDS-PAGE.

**Immuno-blot analysis**

After SDS-PAGE the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore) using wet blot. Transfer buffer was 20 mM Tris, 150 mM glycine, 20% (v/v) methanol. Immuno-detection was carried out using rabbit anti-SpoVD (Liu et al., 2010), anti-StoA (Crow et al., 2009b), anti-BdbD (Crow et al., 2009a), anti-ResA (Erlendsson et al., 2003) or anti-GFP (GenScript) polyclonal antibodies.
antibodies, horseradish-peroxidase-conjugated anti-rabbit secondary antibodies from donkey (GE Healthcare), the Super Signal® West Pico Chemiluminescence substrate (Pierce Chem. Co.) and a ChemiDoc™ MP Imaging System (BioRad).

Other methods

*B. subtilis* cells were induced to sporulate either by the resuspension method (Nicholson and Setlow, 1990) or by nutrient exhaustion in NSMP. Spore heat resistance assay was carried out as described in (Bukowska-Faniband and Hederstedt, 2015). Total cell lysates from sporulating cells were prepared as described in (Bukowska-Faniband and Hederstedt, 2015). Deficiency in cytochrome *c* synthesis in *B. subtilis* mutant strains was assayed by the soft agar overlay method as described in (Green and Gennis, 1983). Protein concentrations were determined by using the BCA Protein Assay Kit (Thermo Scientific) or a NanoDrop™ spectrophotometer (Thermo Scientific). Samples were separated using NuPage® 10% Bis-Tris polyacrylamide gels in MES-SDS running buffer (Life Technologies) or by Tricine SDS-PAGE with 4% stacking gel and 8% separating gel (Schägger and von Jagow, 1987). Identities of purified proteins were confirmed by MALDI-TOF mass spectrometry. Thermal stability of isolated sSpoVD proteins was carried out as described in (Bukowska-Faniband and Hederstedt, 2015). Total cell lysates from sporulating cells was assayed using a differential scanning fluorimetry instrument (Prometheus NT.48 nanoDSF, NanoTemper Technologies).

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