The PASTA domain of penicillin-binding protein SpoVD is dispensable for endospore cortex peptidoglycan assembly in *Bacillus subtilis*

Ewa Bukowska-Faniband and Lars Hederstedt

Peptidoglycan is the major structural component of the bacterial cell wall. Penicillin-binding proteins (PBPs), located at the exterior of the cytoplasmic membrane, play a major role in peptidoglycan synthesis and remodelling. A PASTA domain (penicillin-binding protein and serine/threonine kinase associated domain) of about 65 residues is found at the C-terminal end of some PBPs and eukaryotic-like protein serine/threonine kinases in a variety of bacteria. The function of PASTA domains is not understood, but some of them are thought to bind uncross linked peptidoglycan. *Bacillus subtilis* has 16 different PBPs, but only 2 of them, Pbp2b and SpoVD, contain a PASTA domain. SpoVD is specific for sporulation and essential for endospore cortex peptidoglycan synthesis. We have studied the role of the PASTA domain in SpoVD by deleting this domain and analysing the effects on endospore formation and subcellular localization of SpoVD. Our results demonstrate that the PASTA domain in SpoVD is not essential for cortex synthesis and not important for targeting SpoVD to the forespore outer membrane during sporulation.

INTRODUCTION

The bacterial cell wall is a rigid layer located outside the cytoplasmic membrane. It provides structural support and protection to the cell. This cellular sacculus consists mainly of long chains of peptidoglycan that are cross-linked via flexible peptide bridges. Peptidoglycan assembly depends on the activity of penicillin-binding proteins (PBPs) that are located at the exterior of the cell membrane. PBPs catalyse transglycosylation, transpeptidation and carboxypeptidation reactions. Some PBPs are bifunctional, having both transglycosylase and transpeptidase activity (Typas et al., 2012). As essential components of the peptidoglycan synthesis machinery, PBPs constitute an effective target for many antibiotics in clinical use (Bugg et al., 2011).

The PASTA domain (penicillin-binding protein and serine/threonine kinase associated domain) is found at the C-terminus of some PBPs and eukaryotic-like protein serine/threonine kinases (PSTKs) in a variety of bacteria. The domain has 60–70 amino acid residues and occurs singly or as a few consecutive copies. Although PASTA domains show low amino acid sequence similarity, they share strong structural conservation. Each domain has a globular fold formed by three \( \beta \)-strands and one \( \alpha \)-helix (Yeats et al., 2002). Several and inconsistent functions have been ascribed to PASTA domains. Since the time of discovery of a cefuroxime molecule non-covalently bound between the transpeptidase domain and one of the two PASTA domains in *Streptococcus pneumoniae* Pbp2x (Gordon et al., 2000), the PASTA domain has been classified as a \( \beta \)-lactam-binding motif. Structural similarity of the \( \beta \)-lactam ring to the \( \delta \)-alanyl-\( \delta \)-alanine residues of the stem peptidoglycan precursors implied that the PASTA domain is likely to bind un-cross linked peptidoglycan. Several studies have demonstrated interaction of PASTA domains of some PSTKs, e.g. *Bacillus subtilis* PrkC, *S. pneumoniae* StkP and *Mycobacterium tuberculosis* PknB, with crude peptidoglycan or synthetic muropeptides (Maestro et al., 2011; Mir et al., 2011; Shah et al., 2008; Squeglia et al., 2011). To our knowledge, there is only one published study that characterizes the binding properties of the PASTA domain of a PBP. In this recent report the authors showed that the PASTA domain of *M. tuberculosis* PBP PonA2 does not bind muropeptides, \( \beta \)-lactams or \( \beta \)-lactamase (Calvanese et al., 2014). Thus, the question whether the ability to interact with peptidoglycan is a general property of PASTA domains remains open. What would be the importance of peptidoglycan interaction with a PASTA domain? So far, two different roles have been suggested for such interaction: (i) localization of the protein to the site of active peptidoglycan synthesis...

Abbreviations: PBP, penicillin-binding protein; PSTK, protein serine/threonine kinase.

One supplementary table and one supplementary figure are available with the online Supplementary Material.
B. subtilis (strain 168) has three PASTA domain-containing proteins. Two of them, Pbp2b and SpoVD, are PBPs, and the third, PrkC, is a PSTK. Pbp2b (two PASTA domains) and SpoVD (one PASTA domain) are class B high molecular mass PBPs. Pbp2b is the only known essential PBP in B. subtilis and is involved in the formation of septal peptidoglycan at cell division (Daniel et al., 2000). SpoVD is a sporulation-specific protein (Daniel et al., 1994) and its transpeptidase activity is crucial for endospore cortex peptidoglycan synthesis (Bukowska-Faniband & Hederstedt, 2013). The fact that both proteins are necessary for peptidoglycan synthesis, but under different physiological circumstances, and are the only PASTA-containing PBPs in B. subtilis, prompted us to study the functional importance of the PASTA domain in SpoVD.

B. subtilis can form endospores in response to nutrient starvation. At the outset, cells destined to sporulate divide asymmetrically, resulting in two differently sized cells: the larger mother cell and the smaller forespore, which will eventually become a dormant spore. In the subsequent development, the forespore becomes engulfed by the mother cell, which results in the formation of a double-membrane-enclosed forespore inside the mother-cell cytoplasm. In the final stage, the mother cell lyases, releasing the mature endospore into the environment (Eichenberger, 2012; Piggot & Hilbert, 2004).

The endospore cortex is a thick layer of modified peptidoglycan surrounding the spore core and assembled in the intermembrane compartment of the forespore. The cortex layer is required for spore core dehydration and heat resistance (Warth, 1978; Warth & Strominger, 1972). Unlike vegetative cell wall peptidoglycan, endospore cortex is not essential for growth of B. subtilis; thus it constitutes a convenient model for studies on peptidoglycan synthesis. Deficiency in the cortex layer can easily be monitored by the resulting heat sensitivity of spores (Todd et al., 1986).

SpoVD is synthesized in the mother-cell cytoplasm and targeted to the forespore, where it is inserted into the outer membrane with the transpeptidase catalytic domain and the PASTA domain facing the intermembrane compartment. In this paper, we have studied the effects of deletion of the PASTA domain of SpoVD on endospore cortex synthesis and subcellular localization of SpoVD in B. subtilis. Our results demonstrate that the PASTA domain is not necessary for cortex assembly.

METHODS

Bacterial strains and growth media. The bacterial strains used in this work are listed in Table 1. Escherichia coli TOP10 was used for plasmid DNA propagation. E. coli strains were grown at 37 °C in LB medium or on LB agar plates (Sambrook & Russell, 2001). B. subtilis strains were grown at 30 or 37 °C in LB medium, nutrient sporulation medium with phosphate (NSMP) (Fortnagel & Freese, 1968), growth medium and resuspension medium for induction of sporulation (Nicholson & Setlow, 1990), Spizizen’s minimal medium (SMM) (Harwood & Archibald, 1990) or on tryptose blood agar base (TBAB) plates (Difco). Antibiotics were used when appropriate at the following concentrations: 100 µg ampicillin ml⁻¹, 12.5 µg chloramphenicol ml⁻¹, for E. coli; and 100 µg spectinomycin ml⁻¹, 0.5 µg erythromycin ml⁻¹ combined with 12.5 µg lincomycin ml⁻¹, 5 µg chloramphenicol ml⁻¹, for B. subtilis. TBAB medium supplemented with 1% (w/v) starch was used to test the amylase activity of B. subtilis colonies.

DNA techniques. DNA manipulation was performed by standard methods (Sambrook & Russell, 2001). Plasmid DNA from E. coli and B. subtilis was isolated using a Quantum miniprep kit (Bio-Rad). In the case of B. subtilis, the plasmid isolation procedure was slightly modified by the addition of lysozyme at the final concentration of 4 mg ml⁻¹ to the resuspension solution. B. subtilis chromosomal DNA was isolated according to the procedure described by Marmur (1963). PCR was carried out using Phusion high-fidelity DNA polymerase (Finnzymes) and either B. subtilis chromosomal DNA or plasmid DNA as the template. The sequences of the oligonucleotides used in the PCRs are listed in Table S1 (available in the online Supplemental Material). DNA ligation was performed using T4 DNA ligase (New England Biolabs) at 14 °C overnight. Ligates were used to transform E. coli TOP10 either by electroporation or by chemical transformation (Hanahan et al., 1991). B. subtilis was grown to natural competence as described by Hoch (1991) and 0.5 µg plasmid DNA was added to 0.5 ml competent cells. Transformation with chromosomal DNA was carried out at a limiting amount of DNA to restrict recombination to a single locus per cell. DNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific). All DNA fragments cloned in plasmids were verified by sequencing. The plasmids used in this study are listed in Table 1.

Construction of pLEB4. Primers Ewa5 and Ewa6 were used to amplify by PCR a 2125 bp fragment of the B. subtilis 1A1 chromosome comprising the promoter region and the coding sequence of spoVD. After digestion with EcoRI and BamHI, the PCR product was cloned into pHPSK, yielding plasmid pLEB4. This plasmid encodes full-length SpoVD.

Construction of pLEB7. The spoVD-mCherry in-frame gene fusion including the native spoVD promoter region was amplified from pLEB5 using primers Ewa5 and Ewa13, generating a 2800 bp fragment. After digestion with EcoRI and BamHI, the PCR product was cloned into pHPSK, yielding plasmid pLEB7. This plasmid encodes full-length SpoVD fused to mCherry at the C-terminus.

Construction of pLEB34. Primers Ewa52 and Ewa55 were used to amplify a 1793 bp fragment of the B. subtilis 1A1 chromosome comprising the spoVD ORF without the start and stop codons. Primer Ewa52 generated a restriction site for BamHI, and primer Ewa53 generated an EcoRI restriction site as well as the strepII-tag sequence and the translational stop codon. The PCR fragment was digested with BamHI and EcoRI and cloned into pSG1729, resulting in plasmid pLEB34. This plasmid encodes the full-length SpoVD fused to GFP at the N-terminus and with the StrepII-Tag at the C-terminus.

Construction of pLEB35. Primers Ewa52 and Ewa55 were used to amplify a 1793 bp fragment of the B. subtilis 1A1 chromosome comprising the spoVD ORF without the start codon, the sequence for the PASTA domain and the stop codon. Primer Ewa52 generated a restriction site for BamHI, and primer Ewa55 generated an EcoRI restriction site as well as the strepII-tag sequence and the translational stop codon. The PCR fragment was cloned into pSG1729, resulting in plasmid pLEB35. This plasmid encodes a truncated version of SpoVD.
Construction of pLEB34, pLEB38-Stop and pLEB42. Deletion of the sequence encoding the PASTA domain of SpovD in PlEB7 and PlEB8 was achieved by site-directed mutagenesis (Phusion site-directed mutagenesis kit; Finzymes) using primers Ewa58 and Ewa62, resulting in plasmids pLEB38 and pLEB42, respectively. These two plasmids encode a truncated version of SpovD without the PASTA domain, fused to GFP at the N-terminal and the StrepII-Tag at the C-terminal.

Construction of pLEB41. A 1035 bp synthetic DNA fragment was designed, containing a part of the spoVD ORF (encoding residues 599–716 and a destroyed EcoRI restriction site), a strepII-tag sequence and a translational stop codon followed by an EcoRI site at the 3’ end. Gene synthesis was carried out by GenScript. The synthetic fragment was digested with HindIII and EcoRI and cloned in-frame into the corresponding sites of pLEB34, resulting in plasmid pLEB41. This plasmid encodes a truncated version of SpovD without the PASTA domain fused to the twin PASTA domains of Fpb2b and the StrepII-Tag at the C-terminal end and to GFP at the N-terminal end.

**Construction of B. subtilis strains.** B. subtilis strains used in this work are derivatives of strain 1A1 and are listed in Table 1. B. subtilis LMD101 was transformed with pLEB34, pLEB35, pLEB41 and pLEB42 resulting in strains LMD132B, LMD133, LMD141 and LMD142, respectively. Chromosomal DNA from LMD133 was used to transform strain LMD101, giving rise to strain LMD134. Spectinomycin-resistant clones were confirmed for integration of the gene cassette by a double cross-over event at the *amy* locus by the loss of amylase activity, i.e. inability to degrade starch on TBAB plates supplemented with 1% (w/v) starch. Plasmids pLEB7, pLEB38 and pLEB38-Stop were used to transform B. subtilis LMD101 and the strains obtained were named LMD101/pLEB7, LMD101/pLEB38 and LMD101/pLEB38-Stop, respectively.

**Light microscopy.** A 100 μl sample was taken from the culture of sporingulating cells. The cells were collected by centrifugation at 14,000 g for 30 s and suspended in such a volume of PBS that the density of cells was appropriate for microscopy. A total of 5 μl of the cell suspension was mounted on an agarose-coated microscopy glass slide.

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**Table 1. Strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Origin/reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td>E. coli</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr–hisRMS–mcrBC) φ80lacZAM15 ΔlacX74 nupG recA araD139 Δ(ara–leu)7697 galE15 galK16 rpmL (Str&lt;sup&gt;R&lt;/sup&gt;) endA1 Δ; Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>B. subtilis</td>
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<tr>
<td>1A1</td>
<td>trpC2</td>
<td>BGSC*</td>
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<tr>
<td>LMD101</td>
<td>trpC2 ΔspoVD</td>
<td>Bukowska-Faniband &amp; Hederstedt (2013)</td>
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<tr>
<td>LMD104</td>
<td>trpC2 ΔspoVD amyE::P&lt;sub&gt;ae&lt;/sub&gt;-spoVD-mCherry, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>LMD132B</td>
<td>trpC2 ΔspoVD amyE::P&lt;sub&gt;ae&lt;/sub&gt;-gfp-spoVD, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>LMD134</td>
<td>trpC2 ΔspoVD amyE::P&lt;sub&gt;ae&lt;/sub&gt;-gfp-spoVD&lt;sup&gt;582–646&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>LMD141</td>
<td>trpC2 ΔspoVD amyE::P&lt;sub&gt;ae&lt;/sub&gt;-spoVD&lt;sup&gt;582–646–ppbB&lt;sup&gt;999–716&lt;/sup&gt;&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>LMD142</td>
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<td><strong>Plasmids</strong></td>
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<td>pSG1729</td>
<td>amyE integration vector designed to generate N-terminal GFP fusions under the xylose-inducible promoter; Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Johansson &amp; Hederstedt (1999)</td>
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<td>pHPSK</td>
<td>E. coli/B. subtilis shuttle vector; Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB4</td>
<td>pHPSK with a 2.1 kb fragment containing P&lt;sub&gt;ae&lt;/sub&gt;-spoVD; Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bukowska-Faniband &amp; Hederstedt (2013)</td>
</tr>
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<td>pLEB5</td>
<td>pKS-mCherry-E-T3 with a 2.0 kb fragment containing P&lt;sub&gt;ae&lt;/sub&gt;-spoVD, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bukowska-Faniband &amp; Hederstedt (2013)</td>
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<td>pLEB6</td>
<td>pDG1730 with a 2.8 kb fragment containing P&lt;sub&gt;ae&lt;/sub&gt;-spoVD-mCherry gene fusion; Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pLEB7</td>
<td>pHPSK with a 2.8 kb fragment containing P&lt;sub&gt;ae&lt;/sub&gt;-spoVD-mCherry gene fusion; Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB34</td>
<td>pSG1729 with a 2.0 kb fragment containing spoVD-strepII-tag, Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB35</td>
<td>pSG1729 with a 1.8 kb fragment containing spoVD&lt;sup&gt;582–646–strepII-tag&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB38</td>
<td>pLEB7 with a deletion of 0.2 kb fragment encoding the PASTA domain of SpoVD; Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB38-Stop</td>
<td>pLEB38 with a single nucleotide deletion at the junction of spoVD and mCherry sequences; resulting in a stop codon after the spoVD sequence; Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pLEB41</td>
<td>pSG1729 with a 2.1 kb fragment containing spoVD&lt;sup&gt;582–646–ppbB&lt;sup&gt;999–716&lt;/sup&gt;–strepII-tag&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pLEB42</td>
<td>pLEB6 with a deletion of a 0.2 kb fragment encoding the PASTA domain of SpoVD; Ap&lt;sup&gt;R&lt;/sup&gt; Spc&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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* Bacillus Genetic Stock Center, Columbus, OH, USA.
Role of the PASTA domain in SpoVD

(1 % agarose in PBS) and covered with a coverslip. Phase-contrast and fluorescent images were acquired using a Zeiss Axio Imager.Z2 microscope with X-Cite 120 Illumination (EXFO Photonic Solutions), an ORCA-flash 4.0 LT sCMOS camera (Hamamatsu Photonics) and Volocity software (PerkinElmer). Typical exposure times used to obtain fluorescence images of cells were 2 s for mCherry and 1 s for GFP. Images were processed and analysed with Volocity software (PerkinElmer). The background fluorescence was subtracted from each image using the autofluorescence of the WT strain (1A1) as a reference. Background-subtracted images were further used to calculate mean fluorescence intensity within the cells.

Sporulation and heat-resistance assay. Cells were induced to sporulate either by nutrient exhaustion in NSMP or by using the resuspension method (Nicholson & Setlow, 1990). For strains LMD132B, LMD134 and LMD141, all media were supplemented with 0.2 % (w/v) xylose from the start of the cultures. The spor heat-resistance assay was carried out on 2-day-old cultures in the case of the nutrient exhaustion method and 24 h after resuspension in the case of the resuspension method. The heat resistance of spores was analysed by heating 5 ml culture at 80 °C for 10 min. Serial dilutions of heated and unheated samples were spread on TBAB plates. The number of colonies was counted after incubation of the plates at 37 °C overnight and the yield of heat-resistant spores was calculated.

Total cell lysate preparation. Five hours after the induction of sporulation by resuspension, 50 ml cell culture was collected by centrifugation at 5000 g and 4 °C for 15 min. The cell pellet was washed with 20 ml ice-cold 50 mM potassium phosphate buffer, pH 8.0, and centrifuged. The pellet was resuspended in 1 ml ice-cold 20 mM Na-MOPS buffer, pH 7.4, containing 0.5 mM EDTA and complete protease inhibitor cocktail (Roche). The cell suspension was transferred into a pre-chilled screw-cap microcentrifuge tube containing approximately 500 mg of 0.1 mm diameter Zirconia-glass beads. PMSF was added to the cell suspension at the final concentration of 1 mM. Cells were disrupted using a FastPrep-24 (MP Biomedicals) bead beating system: setting 6.5 m s⁻¹, three cycles of 45 s. Samples were kept for 5 min on ice between each cycle. Finally, the glass beads were sedimented by brief centrifugation and the cell lysate was transferred to a fresh tube.

Immunoblot analysis. Proteins in extracts were separated by Tricine SDS-PAGE with 4 % stacking gel and 8 % separating gel (Schägger & von Jagow, 1987). After SDS-PAGE, the proteins were transferred to a PVDF membrane (Immobilon P, Millipore) using a wet blot. The transfer buffer was 20 mM Tris, 150 mM glycine containing 20 % (v/v) methanol. The antibodies used were rabbit anti-dsRed (Clontech) (1:1000), anti-GFP (GenScript) (1:5000), anti-SpoVD (Liu et al., 2010) (1:3000) and anti-BdB (Crow et al., 2009) (1:3000). Immunodetection was carried out by chemiluminescence using anti-rabbit secondary antibodies conjugated to horseradish peroxidase (GE Healthcare) in 1:3000 dilution and SuperSignal West Pico chemiluminescence substrate (Pierce Chemical).

RESULTS

Effect of deletion of the PASTA domain in a GFP-SpoVD fusion protein

To analyse the role of the PASTA domain in SpoVD, a gene encoding a truncated variant of SpoVD lacking the PASTA domain (residues Lys582–Asp646) was constructed. Full-length and PASTA-truncated SpoVD were fused to GFP at their N-terminal end to make it possible to also study the subcellular localization of the SpoVD variants. The genes for GFP-SpoVD and GFP-SpoVDΔ582–646 were put under a xylose-inducible promoter and inserted into the amyE locus of B. subtilis LMD101 (deleted for spoVD), resulting in strains LMD132B and LMD134, respectively (Fig. 1). The strains were grown in NSMP in the presence of 0.2 % xylose for 2 days to complete sporulation and the spores obtained were tested for heat resistance. Strain LMD132B produced a WT (i.e. similar to parental strain 1A1) level of heat-resistant spores (Table 2), indicating that the GFP-SpoVD fusion is functional and that addition of 0.2 % xylose to the medium is sufficient to complement the mutant. In contrast, strain LMD134 carrying GFP-SpoVDΔ582–646 was defective in producing heat-resistant spores (Table 2), showing that most of the spores produced lack the cortex layer. Induction of sporulation by using the resuspension method and growth for 24 h confirmed the production of defective spores for strain LMD134 (0.04 ± 0.02 % heat-resistant spores) and a normal level of heat-resistant spores for the control strain LMD132B (64 ± 6 %). Immunoblot analysis of cell lysates of sporulating cells harvested 5 h after resuspension [the cellular level of SpoVD protein expressed from the native promoter peaks at this time point, as shown previously by Liu et al. (2010) and Bukowska-Faniband & Hedrestedt (2013)] revealed a low amount of GFP-SpoVDΔ582–646 (~94 kDa) as compared to that of full-length GFP-SpoVD (~101 kDa) (Fig 2a). As expected, both protein variants were found to be membrane bound (data not shown). Considering that production of GFP-SpoVDΔ582–646 in LMD134 is dependent on the presence of xylose in the growth medium, we asked whether an increased concentration of xylose leads to an increased yield of heat-resistant spores. Addition of 0.5 % xylose to NSMP did not result in a higher yield of heat-resistant spores (data not shown).

To confirm the difference in the cellular level of SpoVD fusion protein in strains LMD132B and LMD134, we carried out microscopy of sporulating cells and compared the strength of the GFP fluorescence signal from the two strains. In WT B. subtilis cells, expression of spoVD depends on the mother-cell-specific sigma factor E (σE). During sporulation, SpoV is synthesized in the cytoplasm of the mother cell and localized to the forespore outer membrane (Bukowska-Faniband & Hedrestedt, 2013; Fay et al., 2010). We took advantage of the xylose-inducible promoter in our constructs, which makes it possible to produce SpoV during exponential growth (and independently of sporulation) when it becomes inserted into the cytoplasmic membrane. The localization of SpoVD fusion proteins during spore development was examined by fluorescence microscopy, starting with the pre-sporulating cell, through asymmetrical septation, engulfed spore, up to the stage of the phase-grey spore (this late time point corresponds to when the SpoV-dependent cortex formation takes place). In sporulating strain LMD134, GFP-SpoVDΔ582–646 was enriched at the asymmetrical septum and at the forespore, resembling the subcellular localization of full-length GFP-SpoVD in LMD132B (Fig 2b, c). However, the fluorescence...
signal from GFP-SpoVD<sup>582–646</sup> in strain LMD134 was weaker than that of GFP-SpoVD in LMD132B in all observed developmental stages (Fig. 3).

The results of the immunoblot and fluorescence microscopy showed that SpoVD with the PASTA domain deleted was present in the cells at lower amount as compared to the

Table 2. Sporulation efficiencies of <i>B. subtilis</i> strains

Cells were sporulated by growth in NSMP for 2 days at 37 °C. The yield of heat-resistant spores was assayed by incubation at 80 °C for 10 min. The percentage of heat-resistant spores was calculated as c.f.u. after heating divided by c.f.u. of not-heated culture. The results represent the mean from three independent experiments ± SEM.

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Yield (%) of heat-resistant spores</th>
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<tr>
<td>1A1</td>
<td>WT</td>
<td>67 ± 4</td>
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<tr>
<td>LMD101</td>
<td>ΔspoVD</td>
<td>0*</td>
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<tr>
<td>LMD132B†</td>
<td>ΔspoVD amyE::P&lt;sub&gt;xyl&lt;/sub&gt;-gfp-spoVD</td>
<td>91 ± 5</td>
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<td>LMD134†</td>
<td>ΔspoVD amyE::P&lt;sub&gt;xyl&lt;/sub&gt;-gfp-spoVD&lt;sup&gt;582–646&lt;/sup&gt;</td>
<td>0.00007 ± 0.0001</td>
</tr>
<tr>
<td>LMD141†</td>
<td>ΔspoVD amyE::P&lt;sub&gt;xyl&lt;/sub&gt;-spoVD&lt;sup&gt;582–646&lt;/sup&gt;—pbpB&lt;sup&gt;599–716&lt;/sup&gt;</td>
<td>26 ± 6</td>
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<td>LMD104</td>
<td>ΔspoVD amyE::P&lt;sub&gt;ε&lt;/sub&gt;-spoVD—mCherry</td>
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<td>LMD142</td>
<td>ΔspoVD amyE::P&lt;sub&gt;ε&lt;/sub&gt;-spoVD&lt;sup&gt;582–646&lt;/sup&gt;—mCherry</td>
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<td>LMD101/pLEB7</td>
<td>ΔspoVD, pLEB7 (P&lt;sub&gt;ε&lt;/sub&gt;-spoVD—mCherry)</td>
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<td>LMD101/pLEB38</td>
<td>ΔspoVD, pLEB38 (P&lt;sub&gt;ε&lt;/sub&gt;-spoVD&lt;sup&gt;582–646&lt;/sup&gt;—mCherry)</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>LMD101/pLEB4</td>
<td>ΔspoVD, pLEB4 (P&lt;sub&gt;ε&lt;/sub&gt;-spoVD)</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>LMD101/pLEB38-Stop</td>
<td>ΔspoVD, pLEB38-Stop (P&lt;sub&gt;ε&lt;/sub&gt;-spoVD&lt;sup&gt;582–646&lt;/sup&gt;)</td>
<td>74 ± 6</td>
</tr>
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*<6 × 10<sup>-7</sup>.
†The growth medium was supplemented with 0.2% (w/v) xylose.
Fig. 2. Relative cellular amounts and subcellular localization of full-length, truncated and chimeric GFP-SpoVD (strains LMD132B, LMD134 and LMD141, respectively). Cells were sporulated by resuspension at 37 °C in the presence of 0.2% xylose. (a) Immunoblot of cell lysates of the indicated strains. Samples for analysis were taken at 5 h after resuspension. Cell amounts were normalized according to the OD$_{600}$ before lysates were prepared. An equal amount of sample was loaded in each lane. In the upper panel samples were probed with anti-GFP serum. The position of each GFP fusion protein is indicated by an arrow. A, GFP-SpoVD$_{582-646}$–Pbp2b$_{599-716}$; B, GFP-SpoVD; C, GFP-SpoVD$_{582-646}$. A putative degradation product of GFP-SpoVD$_{582-646}$–Pbp2b$_{599-716}$ is indicated by an asterisk. In the lower panel samples were probed with anti-BdbD serum (as a control for equal loading of samples). Molecular mass marker sizes, in kDa, are indicated on the left. (b–d) GFP fluorescence and phase-contrast images of cells of the indicated strains were taken at hourly intervals after induction of sporulation. Different developmental stages, i.e. pre-sporulating cell (~1 h after resuspension), asymmetric division (~2–3 h after resuspension), engulfed forespore (~4 h after resuspension) and phase-grey spore (~5 h after resuspension), were distinguished based on the fluorescence and phase-contrast images. Bars, 2 μm.
full-length protein. The subcellular localization of GFP-SpoVD \textsuperscript{582–646} seemed normal, but it has to be noted that due to the weak fluorescence signal from strain LMD134, possible signal from the fusion protein in the mother-cell membrane would not be detected. Therefore, we could not rule out that lack of cortex, i.e. deficiency in production of heat-resistant spores, was to some degree a result of aberrant localization of GFP-SpoVD \textsuperscript{582–646}.

**SpoVD \textsuperscript{582–646}-mCherry partially complements SpoVD deficiency**

The low amount of GFP-SpoVD \textsuperscript{582–646} in strain LMD134 could be an effect of the truncation or altered sequence at the C-terminal end of SpoVD. In an attempt to compensate the SpoVD protein for the deleted PASTA domain, we added the mCherry domain to the C-terminal end of SpoVD \textsuperscript{582–646}. From previous work we know that a full-length SpoVD-mCherry fusion protein is functional (Bukowska-Faniband & Hederstedt, 2013). mCherry was used instead of GFP because SpoVD is inserted in the outer forespore membrane in such a way that the C-terminus faces the intermembrane space and mCherry, in contrast to GFP, folds correctly in the periplasm (Chen et al., 2005).

Genes encoding SpoVD-mCherry and SpoVD \textsuperscript{582–646}-mCherry under the control of the native spoVD promoter (\(\sigma^D\)-dependent promoter) were inserted at the amyE locus of strain LMD101 yielding strains LMD104 and LMD142, respectively (Fig. 1). Strain LMD104 produced a near WT level of heat-resistant spores, i.e. 55 \%, while strain LMD142, with the PASTA domain deleted, yielded about 3 \% heat-resistant spores (Table 2). This result showed that many LMD142 developing spores were able to assemble cortex and thereby acquire heat resistance. These findings indicated that the PASTA domain is not essential for endospore cortex synthesis.

As determined by immunoblot using mCherry antibodies, LMD142 cells contained SpoVD \textsuperscript{582–646}-mCherry fusion protein (91 kDa), but at a low amount compared to full-length SpoVD-mCherry (99 kDa) in LMD104 (Fig. 4a). Thus, fusion of mCherry to the C-terminal end of PASTA-truncated SpoVD did not restore the WT level of SpoVD protein. Due to the very low intracellular level of mCherry in LMD142 sporulating cells we did not carry out fluorescence microscopy examination with this strain.

**Overproduction of SpoVD \textsuperscript{582–646}-mCherry effectively complements SpoVD deficiency**

The partial complementation of SpoVD deficiency observed with strain LMD142 prompted us to increase the amount of SpoVD \textsuperscript{582–646}-mCherry in AspoVD cells. We placed the spoVD-mCherry and spoVD \textsuperscript{582–646}-mCherry fusions under the native spoVD promoter in the B. subtilis plasmid pHPSK yielding plasmids pLEB7 and pLEB38, respectively. B. subtilis LMD101 was transformed with both plasmids, yielding LMD101/pLEB7 and LMD101/pLEB38 (Fig. 1). As expected, strain LMD101/pLEB7 produced a WT level of heat-resistant spores (Table 2). Strain LMD101/pLEB38 also produced a relatively high level of heat-resistant spores, 42 \% (Table 2). Immunoblot analysis of lysates of sporulating LMD101/pLEB38 cells confirmed that SpoVD \textsuperscript{582–646}-mCherry was overproduced compared to SpoVD-mCherry in strain LMD104 (Fig. 4a). These data confirmed that the PASTA domain of SpoVD is not required for cortex synthesis.

The lower yield of heat-resistant spores obtained with strain LMD101/pLEB38 (42 \%) compared to LMD101/pLEB7 (76 \%) is probably due to the mCherry fusion. This is supported by the observation that overproduced SpoVD \textsuperscript{582–646}, without fusion to mCherry, fully restored the yield of heat-resistant spores to a WT level. During construction of pLEB38 we obtained by serendipity one clone, pLEB38-Stop, with a single nucleotide deletion at the junction of the spoVD \textsuperscript{582–646} and mCherry sequences, causing a stop codon immediately after the spoVD \textsuperscript{582–646} sequence (Fig. 1). B. subtilis LMD101/pLEB38-Stop yielded 74 \% heat-resistant spores, which is the same level as that for B. subtilis LMD101/pLEB4 containing WT SpoVD (Fig. 1, Table 2). Consistent with our findings with the GFP- and mCherry-fusion proteins, the cellular amount of overproduced SpoVD \textsuperscript{582–646} (~64 kDa) was lower than that of overproduced WT SpoVD (~71 kDa) (Fig. 4b).

**Subcellular localization of SpoVD \textsuperscript{582–646}-mCherry**

To determine whether the subcellular localization of SpoVD \textsuperscript{582–646}-mCherry is the same as for the full-length
variant, we examined sporulating cells by fluorescence microscopy. The mCherry fluorescence signal of LMD101/pLEB38 cells was weak early in sporulation, preventing conclusions about localization of SpoVD\(^{D582–646}\)-mCherry at this developmental stage. We could determine localization of the full-length SpoVD-mCherry and its truncated variant at 7 h after induction of sporulation by resuspension at 30 °C. As shown in Fig. 4(c), SpoVD\(^{D582–646}\)-mCherry was enriched at the forespore and its subcellular localization resembled that of full-length SpoVD-mCherry. Thus, the PASTA domain is not important for targeting SpoVD to the forespore.

Properties of a GFP-SpoVD\(^{582–646–Pbp2b599–716}\) chimera

As shown above, C-terminal truncation of GFP-SpoVD affected the cellular level of the fusion protein and this complicated the interpretation of results. mCherry attached to the C-terminal end of PASTA-truncated SpoVD did not restore the WT level of the protein. Therefore, we decided to attach the twin PASTA domains of \(B.\)\textit{subtilis} Pbp2b (residues Lys599–Asp716) to the C-terminal end of GFP-SpoVD\(^{582–646}\) (Fig. 1). Pbp2b is a parologue of SpoVD and essential for septal peptidoglycan synthesis during vegetative growth. Both PASTA domains of Pbp2b show sequence similarity to the PASTA domain of SpoVD (Fig. S1). For this reason, and since it is possible that folding and/or activity of these two PASTA domains are interdependent, we fused both PASTA domains to the C-terminal end of GFP-SpoVD\(^{582–646}\).

The constructed gene encoding the GFP-SpoVD\(^{582–646–Pbp2b599–716}\) chimera under the control of a xylose-inducible promoter was inserted at the \textit{amyE} locus of \(B.\)\textit{subtilis} LMD101. The strain obtained, LMD141, produced near WT levels (26%) of heat-resistant spores (Table 2). Immunoblot analysis of total lysates of LMD141 cells showed that the chimera (~107 kDa) was present at higher amounts than truncated GFP-SpoVD\(^{582–646}\), but apparently at lower amounts than full-length GFP-SpoVD (Fig. 2a). This explains the relatively efficient production of heat-resistant spores by strain LMD141. Fluorescence microscopy of LMD141 cells showed a slightly stronger GFP signal compared to strain LMD134 (Figs 2d and 3), consistent with the immunoblot data. The fluorescence signal of GFP-SpoVD\(^{582–646–Pbp2b599–716}\) seemed enriched at the asymmetrical septum and at the forespore, similar to the localization of the WT reference protein (GFP-SpoVD), suggesting normal subcellular localization of the chimeric SpoVD protein. However, as noted above for GFP-SpoVD\(^{582–646}\), we could not exclude that a small amount of GFP-SpoVD\(^{582–646–Pbp2b599–716}\) remained in the mother-cell membrane.

DISCUSSION

SpoVD is the only sporulation-specific PASTA-domain-containing PBP in \(B.\)\textit{subtilis} and its transpeptidase activity is essential for synthesis of the endospore cortex peptidoglycan
layer (Bukowska-Faniband & Hederstedt, 2013). We took advantage of the non-essential process of peptidoglycan synthesis during sporulation to study the effect of deletion of the PASTA domain in SpoVD. We demonstrated that SpoVD deleted for the PASTA domain is still able to carry out its essential role in the cortex synthesis.

SpoVD is a parologue of Pbp2b, another class B PBP involved in cell division in B. subtilis. The genes for these two proteins (spoVD and pbpB) have probably originated as the result of a tandem gene duplication in the chromosome (Yanouri et al., 1993). Pbp2b contains two PASTA domains at the C-terminal end. Although no systematic analysis of the PASTA domains in Pbp2b has been carried out, a truncation of 84 amino acid residues at the C-terminal end led to the formation of relatively short cell filaments. This indicated that the second PASTA domain of Pbp2b is not essential for protein function but the mutant cells divided at reduced frequency. Carboxy-truncated Pbp2b seemed either degraded or post-translationally processed in vegetative cells and the resulting product was present in the cells at much lower amounts than the full-length protein (Yanouri et al., 1993). It was recently reported that S. pneumoniae Pbp2x deleted for both PASTA domains is subject to proteolytic degradation in the cell (Peters et al., 2014). Our finding that PASTA-truncated SpoVD is present in low amounts in the cell is, therefore, in agreement with the data available for B. subtilis Pbp2b and S. pneumoniae Pbp2x. Altogether, it appears that PASTA domains of PBPs, apart from some hitherto unknown function, are important for protein stability.

In vitro studies on a series of C-terminal-truncated derivatives of S. pneumoniae Pbp2x showed that the presence of the first PASTA domain plus the α-helix of the second PASTA domain appears to be crucial for the binding of β-lactam to the transpeptidase domain. Deletion of both PASTA domains dramatically decreased β-lactam binding to below 10% (Maurer et al., 2012). It is therefore possible that not only the cellular amount of the protein, but also substrate binding, is affected when the PASTA domain of SpoVD is missing. However, our in vivo data unambiguously prove that the PASTA-truncated SpoVD can bind substrate and catalyse peptide cross-linking.

Several studies indicate a role of PASTA domains in protein subcellular localization (Beilharz et al., 2012; Fleurie et al., 2012; Mir et al., 2011; Peters et al., 2014). To study this we used an N-terminal and a C-terminal fusion of GFP and mCherry, respectively, to truncated SpoVD. In both cases the fluorescence signal, although weak, was enriched at the forespore in a pattern resembling localization of full-length protein. It was difficult to compare fluorescence images of cells with different signal strength, i.e. of cells containing full-length SpoVD versus those with truncated and chimeric versions. For this reason we cannot unambiguously conclude that all the truncated proteins in the studied strains are properly targeted and incorporated into the forespore outer membrane.

Localization of SpoVD to the forespore depends on the presence of SpoVE, a putative lipid II flippase (Fay et al., 2010). It has been shown that these two membrane proteins directly interact with each other (Fay et al., 2010), which suggests that subcellular localization of SpoVD depends on the interaction with SpoVE specifically rather than on the availability of peptidoglycan precursors transported by SpoVE (which could be sensed by the PASTA domain). Although specific interaction sites for SpoVD and SpoVE are unknown, it is unlikely that the PASTA domain is involved in this interaction. SpoVE has ten membrane-spanning segments and it probably interacts with SpoVD through the membrane-spanning domain. Such interaction has been shown for PBP3 and FtsW in E. coli (Fraipont et al., 2011). Notably, several bacterial species encode a single large protein that corresponds to a fusion of a class B PBP with a putative flippase.

We observed with all tested strains containing GFP-SpoVD that GFP fluorescence of cells decreased in the late phases of sporulation (Fig. 3). However, mCherry fluorescence of cells containing SpoVD-mCherry was visible only at late stages of sporulation. One possible explanation for this phenomenon could be a stage-specific fluorescence intensity of GFP and mCherry during sporulation in B. subtilis. It was previously shown that the mCherry and GFP fluorescence intensity is sensitive to subcellular environmental changes during spore development. Doherty and co-workers reported (Doherty et al., 2010) that during sporulation stages II and III (which correspond to formation of asymmetrical septum and engulfment of the spore by the mother cell) the mCherry fluorescence is diminished, while the GFP signal is clearly visible. This fluorescence pattern reverses when sporulation reaches stages V and VI (i.e. assembly of the coat layer and spore maturation). Stage V can be distinguished by phase-contrast microscopy, since the spores turn from phase grey to phase bright. At this stage the GFP fluorescence signal becomes weak, while mCherry becomes brighter (Doherty et al., 2010). Another, or additional, explanation for weak mCherry fluorescence at the early stages of sporulation could be a long maturation time for the mCherry fluorophore in B. subtilis sporulating cells, as has been suggested by de Jong et al. (2010). Finally, one has to consider the amount of fusion protein present in the cell at different stages of sporulation. We found that the level of GFP-SpoVD fusion proteins was slightly lower in the final stages of sporulation (immunoblot data not shown). In the case of SpoVD-mCherry fusions, once σE became activated in the mother-cell (it takes place after asymmetrical septation), the proteins were produced and their level was increasing (immunoblot data not shown). σE becomes inactivated upon completion of engulfment and therefore synthesis of SpoVD would decrease. We expect that the mCherry fusion proteins expressed from our constructs should peak when engulfment is completed. This did not match the time point of the strongest fluorescence intensity, which suggests a long maturation time or a stage-specific fluorescence intensity of mCherry.
In summary, the role of the single PASTA domain in SpoVD remains enigmatic. It is essential neither for the catalytic activity of the transpeptidase domain nor for the correct protein localization as demonstrated in this work. In light of results with other PBPs of class B, the PASTA domain of SpoVD seems to play a role in protein stability, and could possibly be of some importance for efficient binding of the substrate to the active site of the transpeptidase domain.

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mid-cell localization requires the C-terminal PASTA domains and is essential for cell shape maintenance. *Mol Microbiol* 92, 733–755.


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