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The PASTA domains of *Bacillus subtilis* PBP2B strengthen the interaction of PBP2B with DivIB

Danae Morales Angeles†, Alicia Macia-Valero, Laura C. Bohorquez‡ and Dirk-Jan Scheffers*

**Abstract**

Bacterial cell division is mediated by a protein complex known as the divisome. Many protein–protein interactions in the divisome have been characterized. In this report, we analyse the role of the PASTA (Penicillin-binding protein And Serine Threonine kinase Associated) domains of *Bacillus subtilis* PBP2B. PBP2B itself is essential and cannot be deleted, but removing the PBP2B PASTA domains results in impaired cell division and a heat-sensitive phenotype. This resembles the deletion of *divIB*, a known interaction partner of PBP2B. Bacterial two-hybrid and co-immunoprecipitation analyses show that the interaction between PBP2B and DivIB is weakened when the PBP2B PASTA domains are removed. Combined, our results show that the PBP2B PASTA domains are required to strengthen the interaction between PBP2B and DivIB.

**INTRODUCTION**

The synthesis of peptidoglycan during cell division is essential for the completion of division and in fact considered one of the drivers for constriction itself [1, 2]. Cell division is mediated by a complex of proteins collectively known as the divisome (Fig. 1). In most bacteria, the divisome contains two division-specific peptidoglycan synthesis proteins, FtsW, a protein from the SEDS-family with glycosyl transferase activity, and a division-specific class B penicillin-binding protein (bPBP) with transpeptidase activity [3, 4]. These proteins interact through their transmembrane segments, and the presence of the bPBP is required for the activation of the glycosyl transferase activity of FtsW [3]. A recent co-crystal structure from the homologous RodA–PBP2 elongasome complex from *Thermus thermophilus* revealed the activation of RodA glycosyl transferase activity by the extracytoplasmic ‘pedestal’ domain of PBP2, and large movements of the PBP2 cytoplasmic domain that allow this activation as well as transpeptidase activity [5]. In *Bacillus subtilis*, the divisome SEDS/bPBP proteins are FtsW and PBP2B, which are both essential [6, 7]. Recent work from our lab and work from Daniel and colleagues has shown that it is the presence of PBP2B that is essential, rather than its transpeptidase activity, which fits with the observed activation of FtsW transglycosylase activity [3, 5, 8, 9]. This was similar to a previous report on the *Streptococcus pneumoniae* homologue PBP2x, of which the transpeptidase activity is also not essential [10]. Both PBPs contain two PASTA – for Penicillin-binding protein And Serine Threonine kinase Associated – domains at their C-terminus. PASTA domains are exclusively found in Gram-positive bacteria, in some high molecular mass PBPs and in eukaryotic-like serine/threonine kinases (eSTKs) [11]. These domains contain 60–70 amino acids and have a characteristic secondary structure, which consists of three β strands and an α helix; the first and the second β strands are connected by a loop, but the sequence of the domain is not well conserved. Proteins can contain single or multiple PASTA domains. A truncated version of PBP2b (at residue 632), in which part of the PASTA domain 1 and the entire PASTA domain 2 are deleted, has been reported to grow as filaments [12]. In PBP2x, loss of the PASTA domains abolishes the binding of Bocillin-FL, a fluorescent penicillin derivative [13], and localization of PBP2x to the division site [10], suggesting that PASTA domains mediate the interaction with peptidoglycan. This was nicely illustrated recently by a series of crystal structures that revealed that the PBP2x PASTA domains form an allosteric binding site for a pentapeptide stem in a nascent...
peptidoglycan strand, which positions another stempeptide on the same strand in the active site so that it can be cross-linked [14]. The allosteric binding site is formed at the interface of the two PASTA domains and the transpeptidase domain, and comprises the entire first and part of the second PASTA domain. Binding of the terminal d-Ala-d-Ala of the stempeptide at this side displaces a ‘gatekeeper’ arginine residue on the transpeptidase domain, which subsequently forms salt bridges with an aspartate and a glutamate residue on the first PASTA domain that opens up the active site, so that the donor stempeptide for transpeptidation on the same glycan strand can bind [14]. The PASTA domains of *B. subtilis* PBP2B lack all the residues required for this allosteric activation.

Not all PASTA-domain containing proteins bind peptidoglycan, and various proteins contain multiple PASTA domains out of which one can bind peptidoglycan [15]. Bioinformatics analyses revealed a key difference between PASTA domains that bind peptidoglycan and PASTA domains that do not – in a residue that determines the flexibility of the ‘putative binding pocket’, a conserved region localized at the end of the β strand β′. Binder PASTA domains have an arginine or a glutamate residue at this position, while non-binders have a proline [15]. An example of this is *B. subtilis* PrkC, which induces spore germination upon peptidoglycan binding [16]. Only PrkC PASTA domain 3 contains such an arginine, and mutation of this residue completely abolishes peptidoglycan binding, indicating that PASTA domains 1 and 2 do not bind peptidoglycan [17]. Crystal structures of the three PrkC PASTA domains of *Staphylococcus aureus* [18] and of *Mycobacterium tuberculosis* PknB [19, 20], which has four PASTA domains, showed linear arrangements of the domains, in comparison with the more folded arrangement of the PBP2x two PASTA domains [18]. Other examples of proteins with PASTA domains are *Staphylococcus aureus* Stk1 [21] and *Streptococcus pneumoniae* StkP [22, 23]. PBP2B has prolines at both sites in its PASTA domains – as does PBP2x, but as explained above, PBP2x forms an allosteric binding site.

Thus, PBP2B does not have residues associated with peptidoglycan binding by its individual PASTA domains nor residues associated with an allosteric site formed between the PASTA domain and the transpeptidase domain. Combined with our previous observation that deletion of the PBP2B PASTA domains does not affect localization or binding of Bocillin-FL [8], this suggests that the PASTA domains of PBP2B have a different function than peptidoglycan binding.

Other reported functions for PASTA domains include protein localization and kinase activation [24]. In *Streptococcus pneumoniae* StkP, which contains four PASTA domains, the fourth domain is critical for localization through interaction with the peptidoglycan hydrolase LytB, whereas the first three PASTA domains function as a ruler that positions the fourth domain to control cell-wall thickness [25].

In this study, we have further investigated the role of the PASTA domains of PBP2B, and show that these domains strengthen the interaction between PBP2B and the divisome protein DivIB. This interaction becomes critical when cells are grown at higher temperatures.

**METHODS**

**Strains and media**

Strains used in this study are listed in Table 1. All *B. subtilis* strains were grown in casein hydrolysate (CH) medium at 30 °C with shaking, unless other conditions are specified. When necessary, kanamycin (5 µg ml⁻¹) and spectinomycin (50 µg ml⁻¹) were added. To induce the expression of genes under control of the *P*<sup>xyl</sup> and *P*<sup>spoVD</sup> promoters, either IPTG (0.5 mM) or xylose (0.2 %, w/v) was added to the medium.

**Construction of PBP2B chimeras**

Chimeras (Fig. S1, available with the online version of this article) were constructed using restriction-free cloning [26]. Hybrid primers were used to amplify *prkC* and *spoVD* regions encoding PASTA domains from chromosomal DNA of *B. subtilis*. The hybrid primers were designed using the Restriction Free Cloning website (http://www.rf-cloning.org/), primers...
Table 1. Strains

| Strain   | Genetic features                       | Source                        |
|----------|-----------------------------------------|-------------------------------|
| B. subtilis strains |
| 168      | trpC2                                   | Laboratory collection         |
| 3295     | trpC2 chr::PspB neo                     | [41]                          |
| 4132     | trpC2 chr::PspB neo amyE:pDMA001(sp Psyl-gfpmut-ppbB) | [8]                          |
| 4133     | trpC2 chr::PspB neo amyE:pDMA002(sp Psyl-gfpmut-ppbB) | [8]                          |
| 4137     | trpC2 chr::PspB neo amyE:pDMA006(sp Psyl-ppbB) | [8]                          |
| 4138     | trpC2 chr::PspB neo amyE:pDMA007(sp Psyl-ppbB) | [8]                          |
| 4146     | trpC2 chr::PspB neo amyE:pDMA011(sp Psyl-ppbB) | This work                    |
| 4147     | trpC2 chr::PspB neo amyE:pDMA012(sp Psyl-gfpmut-ppbB) | This work                    |
| 4148     | trpC2 chr::PspB neo amyE:pDMA013(sp Psyl-ppbB) | This work                    |
| 4149     | trpC2 chr::PspB neo amyE:pDMA014(sp Psyl-gfpmut-ppbB) | This work                    |
| 4174     | divIB-3xFLAG ercC amyE:pDMA001(sp Psyl-gfpmut-ppbB) | This work                    |
| 4175     | divIB-3xFLAG ercC amyE:pDMA002(sp Psyl-gfpmut-pbpB1-1991) | This work                    |
| GP2005   | divIB-3xFLAG ercC                       | Gift from Jörn Stülke         |
| ΔdivIB   | ΔdivIB tet                              | Gift from Leendert Hamoen     |
| E. coli strains |
| DH5α     | F− endA1 glvV44 thi-1 recA1 relA1 gyrA96 deoR nupG pur20 y806lacZAM15 Δ(lacZYA-argF)U169 hisD17 (rE - mE -) λ−   | Laboratory collection         |
| BTH101   | F− cya-99 araD139 galE15 gmk16 rpsL1 (Str*) hisD2 mcrA1 mcrB1 | [42]                          |

(Table S1) contained complementary sequences to prkC or spoVD and plasmids pDMA002 or pDMA007. A first PCR was performed using the hybrid primers to create a mega-primer that contains prkC or spoVD PASTA domains flanked by complementary sequences of pDMA002 or pDMA007. The mega-primers were used in a second PCR to replace the ppbB PASTA domains from pDMA002 or pDMA007 with prkC or spoVD PASTA domains. DpnI was added to the products obtained in the second PCR in order to degrade the original plasmid. After digestion, the PCR products were used to transform Escherichia coli DH5α cells. The resulting plasmids (Table 2) were sequenced and cloned into the amyE locus of B. subtilis 3295. Integration into the amyE locus was verified by growing the transformants on starch plates to check for amylase activity by iodine staining. A lack of clear zones around the colonies indicates that the construction was integrated into the amyE locus. Integration was confirmed by PCR.

Table 2. Plasmids

| Plasmid   | Genetic features                           | Source                        |
|-----------|--------------------------------------------|-------------------------------|
| pDMA002   | bla amyE3 spc Pxyl-gfpmut1-ppbB′ amyE5    | [8]                          |
| pDMA007   | bla amyE3 spc Pxyl-ppbB′ amyE5            | [8]                          |
| pDMA011   | bla amyE3 spc Pxyl-ppbB′ prkC′ amyE5      | This work                    |
| pDMA012   | bla amyE3 spc Pxyl-gfpmut1-ppbB′ prkC′ amyE5 | This work                    |
| pDMA013   | bla amyE3 spc Pxyl-ppbB′ spoVD′ amyE5     | This work                    |
| pDMA014   | bla amyE3 spc Pxyl-gfpmut1-ppbB′ spoVD′ amyE5 | This work                    |
| pKT25     | Plasmid encoding T25 fragment of Bordetella pertussis cyaA, Km   | [42]                          |
| pUT18C    | Modified version of pUT18 with the polylinker located on the C-terminal end of T18, Amp8    | [42]                          |
| pKT25-zip | Derivative of pKT25 with a leucine zipper of GCN4 fused to the T25 fragment, Km8     | [42]                          |
| pUT18C- zip | Derivative of pUT18C with leucine zipper of GCN4 fused to the T18 fragment, Amp8  | [42]                          |

Growth curves

Strains were grown overnight in the presence of kanamycin (5 µg ml⁻¹) and spectinomycin (50 µg ml⁻¹) when necessary. IPTG (0.5 mM) was added to the medium to express wild-type ppbB and to ensure the proper growth of all strains before performing the growth curves. The following day, the strains were diluted to an OD600 0.05 and grown until early exponential phase. Next, cells were washed with CH medium to remove the IPTG. Cells were diluted to an OD600 0.001 in CH medium containing 0.2 % (w/v) xylose to express PBP2B, PBP2B-ΔPASTA or PBP2B chimera. A 200 µl aliquot of culture (in triplicate), of each condition under test, was loaded in a 96-well plate. The cultures were grown at 30 or 48 °C with shaking, and OD600 was measured every 10 min and recorded using a microplate spectrophotometer (Powerwave 340; Biotek).

Microscopy

Cells were grown until exponential phase. Nile red (Sigma-Aldrich) (5 µg ml⁻¹) and DAPI (Sigma-Aldrich) (1 µg ml⁻¹) were used to stain membranes and DNA, respectively. Cells were spotted on agarose pads (1 %, w/v, in 1x PBS) and imaged using a Nikon Ti-E microscope (Nikon Instruments) equipped with a Hamamatsu Orca Flash4.0 camera. Image
Formants were plated on LB agar plates containing X-Gal BTH101. To test for protein interactions, the trans-
E. coli mids were sequence verified and were used to co-transform IPTG (0.5 mM), kanamycin (50 µg ml⁻¹) and ampicillin (100 µg ml⁻¹). Plates were incubated at 30 °C for 36 h and scored for blue colour development. The β-galactosidase assay was performed as described elsewhere [28], with some modification. E. coli BTH101 containing the plasmids to be tested were grown as overnight cultures in LB containing IPTG (0.5 mM), kanamycin (50 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) at 30 °C. The next day, 200 µl cells were transferred to a tube containing buffer Z (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 50 mM β-mercaptoethanol, pH 7). To permeabilize the cells, 20 µl (0.01 %, w/v) SDS and 40 µl chloroform were added to each tube. After mixing, the chloroform was allowed to settle down and 150 µl permeabilized cells were transferred to a 96-well plate containing 150 µl buffer Z. Then, 40 µl (4 %, w/v) ONPG was added to start the enzymatic reaction. When the samples were yellow, the reaction time was recorded and reactions were stopped by adding 96 µl 1M Na₂CO₃. The absorbance at 420 nm and 550 nm was measured in a Powerwave 340 (Biotek) microplate reader and β-galactosidase activity was calculated as:

\[ \text{Miller units} = \frac{1000 \times (OD_{550} - 1.75 \times OD_{420})}{T \times V \times OD_{600}} \]

T=time in minutes; V=volume in millilitres

### Co-immunoprecipitation

Overnight (O/N) cultures of strains GP2005 (mock), 4174 (expressing GFP-PP2B) and 4175 (expressing GFP-PBP2B-ΔPASTA) were diluted 1 : 100 in LB, induced with 0.2 % (w/v) xylol and grown to an OD₆₀₀ 0.4. Co-immunoprecipitations were performed essentially as described previously [29]. Cells were harvested, resuspended in buffer I [10 mM Tris-HCl, 150 mM NaCl, pH 7.4, with complete ULTRA tablets (mini EDTA-free, EASYpack) protease inhibitors (Roche)] and disrupted via sonication. Cell debris was removed by low-speed centrifugation and membranes were isolated through ultracentrifugation (100 000 g, 1 h, 4 °C) and solubilized with 1 % (w/v) n-dodecyl-β-D-maltopyranoside (DDM; Anatrace) in buffer I by gentle shaking (4°C, 30 min). Solubilized material was recovered as the supernatant from a second ultracentrifugation step (100 000 g, 30 min, 4 °C). The protein concentration was determined with the DC (detergent compatible) protein assay kit (Bio-Rad Laboratories) and 200 ng total membrane proteins were incubated for 1 h at 4 °C, with gentle shaking on a roller mix with 25 µl GFP-Trap agarose beads (Chromotek) in a final volume of 100 µl 1 % (w/v) DDM in buffer I, according to the manufacturer’s recommendations. Beads had been previously blocked by 1 h incubation with 1 % (w/v) BSA in the corresponding buffer. After incubation, the flow-through fraction was collected (100 µl) using centrifugation (2500 g for 2 min) at 4 °C, washed twice with buffer I with 1 % (w/v) DDM, and resuspended in 40 µl 1× SDS-PAGE sample buffer. Low-binding tubes (Thermo Fisher Scientific) were used during the whole process. The input, flow-through and eluate fractions were analysed by SDS-PAGE and Western blotting. Blots were developed using anti-FLAG M2 mouse mAb (Sigma-Aldrich; 1 : 1000) or anti-GFP rabbit polyclonal antibody (Chromotek;
Results and Discussion

Absence of PBP2B PASTA domains results in a temperature-sensitive phenotype

In a previous study [8], we created a series of strains expressing PBP2B variants from which the PASTA domains were removed (Fig. S1). As pbpB is an essential gene, we generated strains in which the expression of wild-type pbpB is under control of IPTG with an extra copy of the pbpB variant (with/without PASTA, with/without gfp) inserted in the amyE locus under control of the P_

\text{pol}_{30} promoter. This strategy allows cultivation of the strains while expressing wild-type pbpB, followed by depletion of PBP2B (Fig. 4a – P_

\text{pol}_{30} pbpB strain without IPTG) and a switch to PBP2B variant production by the removal of IPTG and the addition of xylose; thus, ensuring that the observed phenotype is not a product of a suppressor mutation. Previously, we showed that PBP2B-ΔPASTA was able to complement PBP2B depletion under standard conditions (CH medium, 30 °C), indicating that PASTA domains are not essential under standard conditions [8]. However, we noted that the cells were slightly elongated, which we have now quantified. The strain producing PBP2B has a mean length of 3.34 µm (n=200 cells), while the strain producing PBP2B-ΔPASTA has a mean length of 4.85 µm (n=200 cells), which is ~1.5 times longer (Mann–Whitney U-test, P=0) (Fig. 2, Tables S2 and S3). In addition, there is more variation in the length distribution of the PBP2B-ΔPASTA-producing strain, as can be observed in the boxplot. When the temperature was increased to 37 °C, the mean length of the strain producing PBP2B-ΔPASTA increased to 5.20 µm (n=200 cells), whereas the strain expressing PBP2B (n=200 cells) was slightly shorter than at 30 °C (Fig. 2, Table S2). In other work, we observed that some phenotypes associated with peptidoglycan synthesis defects at the septum are not observed when cells are grown on minimal medium [30]. Therefore, we also analysed the effect of the deletion of PASTA domains in cells grown in a defined minimal medium (SM medium). This revealed that the elongated phenotype for the strain expressing PBP2B-ΔPASTA is still observed (Fig. S2a–c, Tables S2 and S3). At 37 °C, cells grown on SM medium were shorter overall but, again, the strain expressing PBP2B-ΔPASTA displayed an elongated phenotype (Mann–Whitney U-test, P=0).

The increase in cell length is a characteristic phenotype indicative of a problem in cell division. To discard the possibility that the delay in cell division was a consequence of problems with chromosome segregation, DAPI was used to stain DNA. The PBP2B and PBP2B-ΔPASTA strains grown at 30 and 37 °C presented condensed nucleoids in all cells (Fig. 2), indicating that chromosome segregation was not affected. Finally, strains expressing GFP-fusions to PBP2B and PBP2B-ΔPASTA, grown at 30 °C in LB with 0.5% xylose, were scored for the presence of the GFP-PBP2B variant at the division site (Fig. S2d). GFP-PBP2B was present at the division site in 58.8 % (±4.9 %, n=609) of the cells, whereas GFP-PBP2B-ΔPASTA was present at the division site in 37.8 % (±1.0 %, n=606) of the cells (Chi-square test, P<0.01). This again indicates that cell division is delayed/impaired when the PASTA domains are absent.

As we noticed that the elongation phenotype in CH medium was more severe at 37 °C than at 30 °C, the temperature was increased to 48 °C. Surprisingly, the PBP2B-ΔPASTA strain did not grow at 48 °C (Fig. 3a). This result suggests that the PBP2B-ΔPASTA strain is temperature sensitive. We also noted that after prolonged incubation, the control deletion strain with pbpB controlled by P_

\text{pol}_{30} started growing (Fig. 3a). This also happened at lower temperatures and analysis of several deletion strains revealed that this is due to the appearance of suppressor mutations in P_

\text{pol}_{30}; the promoter used to control pbpB (not shown). To get more insight into the effects of high temperature on the phenotype, the strains were grown under normal conditions (30 °C, CH medium) to make sure that the cells were growing healthy. Then, cultures were shifted to 48 °C and pictures were taken every 20 min. The strain expressing PBP2B showed no drastic changes in the phenotype during the course of the experiment (Fig. 3b). However, after 40 min, the strain expressing PBP2B-ΔPASTA started to display cells with decreased contrast, a characteristic of dying cells (Fig. 3b). After 1 h at 48 °C, we observed that the amount of dying cells in the culture of the strain expressing PBP2B-ΔPASTA increased. These observations confirm that the deletion of the PASTA domains from PBP2B confers a temperature-sensitive phenotype.

PASTA domains of PBP2B can be partially replaced by PASTA domains from other Bacillus proteins

B. subtilis has two other proteins that contain PASTA domains, SpoVD and PrkC. SpoVD is a PBP paralogous to PBP2B. It is crucial for spore cortex synthesis and contains a single PASTA domain [31]. PrkC, which contains three PASTA domains, is a eukaryotic-like serine/threonine kinase that is involved in processes like germination and biofilm formation, WalR activation and that localizes to the septum [16, 32, 33]. In order to test whether the PASTA domains of SpoVD and PrkC were able to replace the function of the PASTA domains of PBP2B, the PBP2B PASTA domains were exchanged for PASTA domains from SpoVD and PrkC (Fig. S1). Growth of the strains expressing the chimera proteins was followed at 30 °C in CH medium (Fig. 4a), and was found to be similar to the background deletion strain. This indicates that the exchange of the PASTA domains did not interfere with the essential function of PBP2B. The cells expressing the chimera proteins were examined by microscopy, which showed that the cells expressing the chimeras were longer (Table S3) than cells expressing wild-type PBP2B [3.34 µm (±0.06)]. The PBP2B-PASTA_{spoVD} chimera [3.57 µm (±0.07)] resulted in mild elongation, whereas the PBP2B-PASTA_{prkC} chimera [4.55 µm (±0.12)] gave a more striking elongation, although
Fig. 2. Phenotype of strains producing PBP2B and PBP2B-ΔPASTA. (a) Phase-contrast microscopy of the strains producing PBP2B (strain 4137) and PBP2B-ΔPASTA (strain 4138). Cultures were grown in CH medium at 30 and 37 °C until exponential phase. Membranes and DNA were labelled with Nile red (v-viii) and DAPI (ix-xii), respectively. (i, v, ix) PBP2B (strain 4137) at 30 °C; (ii, vi, x) PBP2B-ΔPASTA (strain 4138) at 30 °C; (iii, vii, xi) PBP2B at 37 °C; (iv, viii, xii) PBP2B-ΔPASTA at 37 °C. Bar, 5 µm; the same for all panels. (b) Length distribution of cells. Cells were grown in CH medium at 30 or 37 °C until exponential phase. As *B. subtilis* forms chains, cells were labelled with Nile red in order to determine the boundaries of single cells. The lengths of the cells were obtained using ChainTracer [27]. The values obtained (n=200 per strain) are shown as box plots. White circles show the medians (PBP2B 30°C, 3.32 µm; PBP2B-ΔPASTA 30 °C, 4.67 µm; PBP2B 37 °C, 3.10 µm; PBP2B-ΔPASTA 37 °C, 4.97 µm); box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values.
not to the same extent as PBP2B-ΔPASTA cells [4.85 µm (±0.10)] (Fig. 4c, e, Tables S2 and S3).

GFP-fusions to the chimera proteins showed that both chimeras localize to division sites, as expected from the observation that the chimeras do not interfere with the essential function of PBP2B (Fig. 4d). Subsequently, these strains were grown at 48 °C to see whether the chimeric proteins complemented the temperature-sensitive phenotype. Although both chimeric proteins did allow some growth at 48 °C, the lag phase of the cells was longer compared to the PBP2B strain and cells did not reach similar OD 600 values (Fig. 4b). Again, the strain expressing the PBP2B-ΔPASTA PrkC chimera was most affected. This is not wholly surprising, as the structural organization of PrkC PASTA domains seemed different from that of PBP PASTA domains [18], while SpoVD is a sporulation-specific PBP that, like PBP2B, is partnered with a cognate SEDS protein, SpoVE [31, 34]. These results indicate that the PASTA domains from other B. subtilis proteins can partially, but not fully, complement the absence of the PBP2B PASTA domains.

**PBP2B PASTA domains strengthen the interaction with DivIB**

A possible explanation for the temperature-sensitive phenotype of the strain expressing PBP2B-ΔPASTA is that the PBP2B-ΔPASTA protein becomes more labile at increased temperatures. An analysis of PBP2B and PBP2B-ΔPASTA stability in isolated membranes at 30 and 48 °C revealed that although PBP2B is less stable at 48 °C, both PBP2B and PBP2B-ΔPASTA are degraded at similar rates (Fig. S3). This indicates that the in vitro intrinsic stability of these proteins is the same. It could be that in vivo there is a difference, but such a difference would be indistinguishable from increased degradation caused by a secondary effect such as divisome instability resulting in degradation [35]. We noted that the temperature sensitivity of the PBP2B-ΔPASTA strain was similar to the phenotype of a divIB deletion strain [36]. DivIB (in other organisms FtsQ) is a divisome protein that interacts with DivIC (in other organisms FtsB) and FtsL, and that regulates the turnover of FtsL and DivIC [35, 37]. This turnover is regulated by PBP2B and the transpeptidase domain of PBP2B has been shown to interact with the C-terminus of DivIB [37–39]. We hypothesized that the absence of the PASTA domains from PBP2B might influence the interaction with DivIB and/or other proteins. To test this, we performed a BACTH assay, in which we tested the ability of PBP2B and PBP2B-ΔPASTA to interact with DivIB, DivIC, FtsL and itself. On plates, we confirmed the previous result from Daniel and colleagues [37] that PBP2B interacts with DivIB and FtsL, but not with DivIC, and found no apparent difference between PBP2B and PBP2B-ΔPASTA (Fig. 5a). Notably, we did not detect a PBP2B self-interaction. Also, we only found positive results when the PBP2B variants were expressed from the pKT25 plasmid (Fig. S4) – this is probably due to the difference in copy numbers between the two plasmids used in the assay and not uncommon in BACTH screens of interactions between PBPs and other proteins [40]. We also analysed the interactions using a β-galactosidase assay (Fig. 5b), which has the added benefit of providing a quantitative result, which can give a hint about the strength of the interaction. It has to be noted that the ‘strength’ of an interaction does not scale 1 : 1 with β-galactosidase activity and, thus, changes in activity are only indicative of a change in interaction. The β-galactosidase assay confirmed the interactions of PBP2B with DivIB and FtsL, and the observation that the interaction with DivIB ‘appears’ the strongest fit with the observation of Robichon and colleagues who used an artificial targeting assay in E. coli to show that the interaction with DivIB is the strongest...
interaction between PBP2B and other division proteins [39]. In the absence of the PASTA domains, the activity resulting from the interaction with DivIB was roughly halved, whereas the activity resulting from the interaction with FtsL was unchanged (Fig. 5b). This result suggests that the PASTA domains of PBP2B are not required for the interaction with DivIB, but that they do increase the strength of the interaction.

To validate the results from the BACTH experiments, co-immunoprecipitation experiments were performed. GFP-PBP2B and GFP-PBP2B-ΔPASTA were produced in a B. subtilis strain that produces a FLAG-tagged version of DivIB at the native locus under control of the wild-type promoter (GP2005, a kind gift from Jörg Stülke). DivIB-FLAG is functional as the GP2005 strain is not thermosensitive (not shown). Anti-GFP nanobodies coupled to agarose (GFP-Trap) were used to immunoprecipitate GFP-PBP2B and GFP-PBP2B-ΔPASTA, and the immunoprecipitates were analysed by Western blot, with detection using anti-FLAG and anti-GFP antibodies. The amount of DivIB-FLAG immunoprecipitated from cells producing GFP-PBP2B appeared significantly higher than that from cells producing GFP-PBP2B-ΔPASTA, although the overall recovery in both cases was low (Fig. 5c). This was confirmed by quantification of the amount of immunoprecipitated DivIB-FLAG as a fraction of the unbound material in the flowthrough of the sample, in an experiment where strain GP2005, which produces only DivIB-FLAG, was included as a mock control and the blots were developed with anti-FLAG only, to avoid potential cross-reactivity (Fig. 5d and S4). Recovery of DivIB-FLAG by GFP-PBP2B was significantly higher than from the mock and the GFP-PBP2B-ΔPASTA producing strain, where levels were not higher than the background. Combined, the BACTH and co-immunoprecipitation results indicate that the PASTA domains of PBP2B strengthen the DivIB-PBP2B interaction.
Conclusions

In this paper, we show that although the PASTA domains are not absolutely essential for the scaffolding role of PBP2B, they do become essential at elevated temperatures. This phenotype is similar to the phenotype described for a \textit{divIB} deletion [36], suggesting the PASTA domains are involved in the same pathway. We could show that the PASTA domains are involved in the interaction between PBP2B and DivIB using both a BACTH and a co-immunoprecipitation approach. Earlier, King and colleagues identified an interaction between the C-terminal part of DivIB and the transpeptidase domain of PBP2B [38]. In the modelled structure of PBP2B, the transpeptidase domain and the DivIB C-terminus are at similar distance from the membrane, but this is also the distance at which the PASTA domains can be found [38]. It is possible that the PASTA domains strengthen the interaction between
DivIB and the transpeptidase domain, but alternatively the PASTA domains interact with another region of DivIB. Given the broad range of conformations recently reported for the elongasome bpBP bound to its SEDS partner, and the influence on these conformations by interaction partners such as MreC [5], it is tempting to speculate that the PASTA domains may be required to stabilize one of these conformations in PBP2B, to control peptidoglycan synthesis (Fig. 1). Our results show that PASTA domains can have distinct functions in similar proteins – whereas the PASTA domains in *Streptococcus pneumoniae* clearly function to allosterically activate the transpeptidase activity of the protein [14], their function in *B. subtilis* PBP2B seems to be the stabilization of an important protein–protein interaction in the divisome.

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**Author contributions**

Conceptualization – D.M.A., D.-J.S. Methodology – D.M.A., A.M.-V., L.C.B. Validation – D.M.A., A.M.-V., L.C.B. Formal analysis – D.M.A., A.M.-V. Investigation – D.M.A., A.M.-V., L.C.B. Writing – original draft preparation – D.M.A., D.-J.S. Writing – review and editing – D.M.A., A.M.-V., L.C.B. Conceptualization – D.M.A., D.-J.S. Methodology – D.M.A., A.M.-V., L.C.B. Formal analysis – D.M.A., A.M.-V. Investigation – D.M.A., A.M.-V., L.C.B. Writing – original draft preparation – D.M.A., D.-J.S. Writing – review and editing – D.M.A., A.M.-V., L.C.B., D.-J.S. Validation – D.M.A., A.M.-V., L.C.B., D.-J.S. Supervision – D.-J.S. Project administration – D.-J.S. Funding – D.-J.S.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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