The cell cycle regulator GpsB functions as cytosolic adaptor for multiple cell wall enzymes

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Bacterial growth and cell division requires precise spatiotemporal regulation of the synthesis and remodelling of the peptidoglycan layer that surrounds the cytoplasmic membrane. GpsB is a cytosolic protein that affects cell wall synthesis by binding cytoplasmic mini-domains of peptidoglycan synthases to ensure their correct subcellular localisation. Here, we describe critical structural features for the interaction of GpsB with peptidoglycan synthases from three bacterial species (Bacillus subtilis, Listeria monocytogenes and Streptococcus pneumoniae) and suggest their importance for cell wall growth and viability in L. monocytogenes and S. pneumoniae. We use these structural motifs to identify novel partners of GpsB in B. subtilis and extend the members of the GpsB interactome in all three bacterial species. Our results support that GpsB functions as an adaptor protein that mediates the interaction between membrane proteins, scaffolding proteins, signalling proteins and enzymes to generate larger protein complexes at specific sites in a bacterial cell cycle-dependent manner.
Scaffolding proteins including DivIVA, EzrA and GpsB, also recruits downstream proteins, including PBPs, to coordinate bacterial cell growth and division. The Gram-negative paradigm Escherichia coli and Staphylococcus aureus maintaining cell wall integrity during antibiotic stress. The hyphal growth and branching and GPSB low G dynamics and membrane anchoring of the FtsZ Z-ring, the ftsA gene is essential in the pneumococcal cell division, the septum of dividing cells, the same localisation pattern as that caused marked growth and division defects at 37 °C and results in Listeria monocytogenes and S. pneumoniae clinical isolates revealed that the presence of GpsB variants was correlated significantly with β-lactam and fosfomycin antibiotics, reduced virulence in an insect infection model, and caused alterations to PG structure. Mutations in GpsB that affected binding to the PG synthase PBP1 also showed a lethal phenotype in L. monocytogenes at 42 °C. The gpsB gene is essential in the Streptococcus pneumoniae D39 progenitor strain as well as in some of its laboratory derivatives and its inactivation resulted in elongated cells unable to divide. In addition, a genome-wide association study of S. pneumoniae clinical isolates revealed that the presence of gpsB variants was correlated significantly with β-lactam resistance, suggesting that GpsB may have fitness and pleiotropic roles in maintaining cell wall integrity during antibiotic stress. The gpsB gene has also been described as essential in the spherical bacterium Staphylococcus aureus, but the biochemical properties and physiological functions of S. aureus GpsB in this recent report differ to what has been described in B. subtilis and S. pneumoniae.

In both B. subtilis and L. monocytogenes, the cytosolic GpsB localises to the lateral side walls of newborn, growing cells and to the septum of dividing cells, the same localisation pattern that as that of B. subtilis PBP1. In S. pneumoniae, GpsB localises to mid-cell, the only region of active PG synthesis for both peripheral (side-wall) elongation and cell division in this bacterium. The localisation of GpsB at regions of active PG synthesis allows for the interaction of GpsB with the poorly characterised cytoplasmic mini-domains of PG synthases. GpsB (SpGpsB) has been found to co-immunoprecipitate with SpPBP2a, SpPBP2b and SpMrec, suggesting these proteins form a complex that is regulated by SpGpsB.

To gain molecular understanding of GpsB function, we report three crystal structures of PBP cytoplasmic mini-domains in complex with GpsB, the first structures of a PG synthase in complex with a cytoplasmic cell cycle regulator. Despite a marked absence of sequence and structural homology, we find that the PBP domains interact with equivalent surfaces in GpsB using an arginine that is conserved in the respective orthologues of the PBPs. The visualisation of each complex permits a comprehensive mutagenesis strategy and functional study to rationalise the role of each interfacial amino acid in the PBP-GpsB pairs. We uncover a sequence motif used by the B. subtilis PG synthase to interact with GpsB. We identify two new members of the GpsB interactome in this organism by querying the B. subtilis proteome with this motif for potential new partners of GpsB, and provide evidence for their interaction to other, established proteins in growth and division. We also identify extensive GpsB interactomes in B. subtilis, L. monocytogenes and S. pneumoniae by bacterial two-hybrid assays (BACTH). The role of GpsB in the bacterial cell cycle is that of an adaptor, docking PG synthases to other cell wall enzymes, scaffolds and shape determinants into protein complexes for division (the divisome) and peripheral growth (the elongosome).

Results

The first 16 residues of BgsBp1 dictate the BgsBp1 interaction. GpsB is an influential cell cycle regulator in low G+ Gram-positive bacteria and we set out to establish the common rules by which GpsB interacts with major PG synthases in three important bacteria—one model species (B. subtilis) and two pathogens (L. monocytogenes and S. pneumoniae). It has been determined previously by us by SPR that the first 31 amino acids of the cytoplasmic mini-domain of B. subtilis PBP1 (BgsBp1) were critical for binding of BgsBp1 using the same technique the binding site was further mapped to the first 16 amino acids of BgsBp1 by comparing the interaction of BgsBp1 with SPR chips coated with full-length BgsBp1 and a PBP1 deletion mutant, BgsBp1Δ1-17, where the codons for the first 16 amino acids were removed genetically. While submicromolar concentrations of BgsBp1 bound to chips coated with the wild-type BgsBp1, there was no interaction even when 25 μM BgsBp1 was injected over equivalent chips coated with BgsBp1Δ1-17 (Fig. 1a).

We subsequently solved the crystal structures of the complex between the N-terminal domain of BgsBp1 whose termini were truncated slightly to expedite crystallisation, BgsBp1Δ1-49, with the first 17 amino acids of BgsBp1, BgsBp1Δ1-17 (Fig. 1b, c). The BgsBp1Δ1-17 α-helix is stabilised by an intramolecular salt bridge between BgsBp1Δ1-17Glu9 and BgsBp1Δ1-17Arg12 and by a hydrogen bond between the sidechain of BgsBp1Δ1-17Ser7 and the backbone amide of BgsBp1Δ1-17Ala10. A prominent feature of the complex is the deep penetration of the sidechain of BgsBp1Arg8 into the groove between BgsBp1Δ1-49 α-helices 1 and 2, contacting the mainchain carbonyl oxygens of BgsBp1Asp14, BgsBp1Asp15 and BgsBp1Arg18 and forming a salt bridge with BgsBp1Glu31 (Fig. 1c), which in turn is tethered in place by hydrogen bonds to the hydroxyl of BgsBp1Tyr25. The backbone amides of BgsBp1Arg8 and BgsBp1Glu9 interact with BgsBp1Asp35 mimicking the mainchain interactions in successive turns in an α-helix. In a longer α-helix, the backbone amides of BgsBp1Arg8 and BgsBp1Glu9 would not be able to interact with BgsBp1Asp35 because of intra-helical hydrogen bonds with the mainchain carbonyls of BgsBp1Asp5 and BgsBp1Asn6. The sidechain of BgsBp1Arg11 forms hydrogen bonds with the carbonyl oxygen of BgsBp1Leu14 and a salt bridge with BgsBp1Glu17. Van der Waals’ interactions connect BgsBp1Arg8 to BgsBp1Leu34 (Fig. 1c) and BgsBp1Glu9 with BgsBp1Lys32. The BgsBp1Δ1-17 peptide binds to a groove between α-helices 1 and 2 in only one molecule of BgsBp1Δ44 in the crystallographic asymmetric unit as the second

Pepitidoglycan (PG), a network of glycan strands connected by short peptides, forms the essential cell envelope that maintains cell shape and protects bacteria from osmotic stresses. High molecular weight (HMW) bi-functional penicillin binding proteins (class A PBPs) are PG synthases that catalyse glycan strand polymerisation and peptide crosslinking, whereas HMW class B mono-functional PBPs only have transpeptidase functions. The PG layer needs remodelling to enable normal cell growth and division and thus the bacterial cell cycle requires the extracellular activities of PBPs and PG hydrolases to be coordinated. The outer membrane-anchored LpoA/B lipoproteins activate their cognate PBP1A/1B PG synthases in the synthesis of the thin, periplasmic PG layer in the Gram-negative paradigm B. subtilis, whereas dedicated cell cycle scaffolding proteins including DivIVA, EzrA and GpsB, also recruits downstream proteins, including PBPs, to coordinate bacterial cell growth and division. The Gram-negative paradigm E. coli and S. aureus maintaining cell wall integrity during antibiotic stress. The hyphal growth and branching and GpsB low G dynamics and membrane anchoring of the FtsZ Z-ring, the ftsA gene is essential in the pneumococcal cell division, the septum of dividing cells, the same localisation pattern as that caused marked growth and division defects at 37 °C and was lethal at 42 °C. Moreover, gpsB deletion in L. monocytogenes also resulted in enhanced susceptibility to β-lactam and fosfomycin antibiotics, reduced virulence in an insect infection model, and caused alterations to PG structure. Mutations in GpsB that affected binding to the PG synthase PBP1 also showed a lethal phenotype in L. monocytogenes at 42 °C. The gpsB gene is essential in the Streptococcus pneumoniae D39 progenitor strain as well as in some of its laboratory derivatives and its inactivation resulted in elongated cells unable to divide. In addition, a genome-wide association study of S. pneumoniae clinical isolates revealed that the presence of gpsB variants was correlated significantly with β-lactam resistance, suggesting that GpsB may have fitness and pleiotropic roles in maintaining cell wall integrity during antibiotic stress. The gpsB gene has also been described as essential in the spherical bacterium Staphylococcus aureus, but the biochemical properties and physiological functions of S. aureus GpsB in this recent report differ to what has been described in B. subtilis and S. pneumoniae.

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BsGpsB-binding site is blocked by crystal contacts. The key interactions are mapped onto GpsB and PBP1 sequences in Supplementary Figure 1A.

To rationalise the features of PBP cytoplasmic domains important for determining recognition by GpsB, the interactions described above were analysed further by fluorescence polarisation (FP) and circular dichroism (CD). The BsGpsB\textsubscript{1-68}\textsubscript{Glu17Ala}, BsGpsB\textsubscript{Tyr25Phe}, BsGpsB\textsubscript{1-68}\textsubscript{Asp31Ala} and BsGpsB\textsubscript{1-68}\textsubscript{Asp35Ala} mutations had little impact on protein stability (Supplementary Figure 1B and Supplementary Note 1) and each reduced the affinity for BsPBP1\textsubscript{1-32} by more than 8-fold (Supplementary Figure 1C, Supplementary Table 1). BsPBP1\textsubscript{Arg8Lys}
BsPBPA1Arg8Ala and BsPBPA1Arg11Ala mutations each resulted in reduced affinities for BsGpsB1-68 by at least 5-fold (Fig. 1d, Supplementary Table 1). BsPBPA1-32Arg8Ala had no effect on binding (Fig. 1d, Supplementary Table 1), suggesting that the overall positive charge of the peptide is not the primary determinant of BsGpsB:BsPBPA1 interactions, rather the unique physicochemical characteristics of an arginine at position 8 in BsPBPA1 is essential. The BsPBPA1-32Ser7 and BsPBPA1-32Ala10Pro mutations each reduced the affinity for BsGpsB1-68 by at least 6-fold by affecting the α-helix of BsPBPA1-32 (Fig. 1d, Supplementary Figure 1D, Supplementary Table 1 and Supplementary Note 1), and the reduction in affinity was the same irrespective of the position of the fluorophore in the peptide (Supplementary Figure 1E, Supplementary Table 1). BsPBPA1-32Ser7 acts as the helix N-cap, a role that is performed preferentially by Ser, Asn and Thr12, and alanine and proline substitutions in helical positions equivalent to BsPBPA1-32 Ser7 and BsPBPA1-32 Ala10, respectively, destabilise model peptides32,33. Finally, the importance of BsPBPA1-32Ser7, BsPBPA1-32Arg8 and BsPBPA1-32Arg11 to GpsB binding is highlighted because these are the most well conserved residues in an alignment of the cytoplasmic mini-domains of Bacillaceae PBPA1 PG synthases (Supplementary Figure 1F).

*L. monocytogenes* GpsB interacts with PBPA1 by a TRxxYR motif. The deletion of gpsB alone in *B. subtilis* has no readily apparent phenotype until combined with deletions in errA11 or ftsZ12, by contrast, the deletion of gpsB in *L. monocytogenes* is lethal when grown at 42 °C19. Since GpsB in both species interact with class A PG synthases, we next determined whether the rules established above for the BsGpsB:BsPBPA1 interaction could be applied directly to *Lm*GpsB:LmPBPA1. The cytoplasmic mini-domain of LmPBPA1, LmPBPA1-20, has an abundance of positively charged residues, but lacks an exact copy of the SRxxR(K/R) motif of Bacillaceae PBPA1 (Supplementary Figure 1F, 2A); the closest equivalent is TRxxYR. In FP, the first 20 amino acids of LmPBPA1, LmPBPA1-20, bound to the N-terminal domain of LmGpsB, LmPBPA1-73, with an affinity similar to that of BsPBPA1-32 for BsPBPA1-68 (Supplementary Figure 2B, Supplementary Table 1), but we were unable to co-crystallise LmGpsB constructs with LmPBPA1 peptides to visualise these interactions and to compare them to BsPBPA1-68:BsPBPA1-17. Consequently, and to expedite crystallisation, we solved the structure of the first 15 amino acids of LmPBPA1, LmPBPA1-15, bound to BsPBPA1-64Lys32Glu, which is a surrogate for LmPBPA1-73 because (i) all the residues within 8 Å of the BsPBPA1-64:BsPBPA1-17 interface are conserved in LmPBPA1 except for Lys32, which is glutamate in LmPBPA1, and thus all peptide-contacting residues are maintained—as well as bystander residues that help indirectly to shape the PBP binding site; (ii) LmGpsB and BsGpsB use overlapping PBP binding sites19; (iii) the Kα of BsPBPA1-68Lys32Glu and LmPBPA1-73, for the first 20 amino acids of LmPBPA1, LmPBPA1-20, are almost identical (Supplementary Figure 2B, Supplementary Table 1). Therefore, the K32E variant of the N-terminal domain of BsGpsB is as close a surrogate for the equivalent LmPBPA1 domain that could be obtained.

The subsequent structure of the BsPBPA1-64Lys32Glu:LmPBPA1-15 complex revealed that, in contrast to the BsPBPA1-64:BsPBPA1-17 complex, the majority of the LmPBPA1 peptide was disordered except for LmPBPA1-15Arg8 and the immediately-adjacent main-chain atoms. LmPBPA1-15Arg8 adopts the same orientation and makes the same interactions as described above for BsPBPA1-17Arg8 in the BsPBPA1-64:BsPBPA1-17 complex (Figs. 1c, 2a). The bidentate interaction of Glu35 of BsGpsB5-64Lys32Glu with the backbone amides of LmPBPA1Arg8 and LmPBPA1Ser9 are maintained just as in the BsGpsB5-64:BsPBPA1-17 complex and, as the backbone torsion angles of LmPBPA1Arg8 are also α-helical, it suggests that the role of the conserved LmPBPA1Thr17 is to N-cap this helix19. The interaction of BsPBPA1-64Lys32Glu with LmPBPA1-15 seemingly centres almost entirely on a single arginine. How LmGpsB discerns LmPBPA1Arg8 over other positively charged residues in the arginine-lysine-rich cytoplasmic domain of LmPBPA1 (Supplementary Figure 2A) was determined by FP and BACTH. LmPBPA1-20Arg8Ala and LmPBPA1-20Arg12Ala mutations reduced the affinity for BsGpsB1-68Lys32Glu by >15- and >4-fold, respectively (Supplementary Figure 2B, Supplementary Table 1), and had significant negative impact on the LmGpsB:LmPBPA1 interaction by BACTH (Fig. 2b). Alanine substitution of LmPBPA1Thr17 has a comparatively milder impact in BACTH (Fig. 2b) and in FP (Supplementary Figure 2B, Supplementary Table 1). Introducing positive charge into LmPBPA1Arg8Arg11 did not restore wild-type binding affinity in FP as LmPBPA1-20Arg8AlaSer16Arg8 still bound to BsPBPA1-64Lys32Glu with an affinity at least 10-fold weaker than wild-type (Supplementary Figure 2B, Supplementary Table 1). BACTH also supports the central importance of LmPBPA1Thr17, which presumably plays a structural role in the LmPBPA1-15 peptide possibly by acting as an N-cap and positioning LmPBPA1Arg8 at the start of an α-helix. At the alanine substitution of the other positively charged residues downstream of residue 14 had no impact in BACTH (Fig. 2b), consistent with the particularly stringent specificity of LmGpsB for LmPBPA1Arg8 in comparison to other positively charged residues.

LmPBPA1Arg8 and BsPBPA1Arg8 are equivalent in their interactions with GpsB. Of the other GpsB-binding determinants of BsPBPA1, LmPBPA1 lacks an analogous BsPBPA1Arg11. The sequential equivalent is LmPBPA1Thr11, but this residue is completely disordered and its mutation to alanine reduced the affinity for BsGpsB1-68Lys32Glu only by 2-fold (Supplementary Figure 2B). To further decipher the reason for the specificity for LmPBPA1Arg8, the importance of α-helix formation in LmPBPA1-15 for GpsB binding was analysed with Q10P-mutated LmPBPA1 peptides. A Q10P mutation caused a >7-fold reduction in binding affinity (Supplementary Figure 2B, Supplementary Table 1) and CD confirmed a significant impact of the Q10P mutation on the α-helicity of LmPBPA1-15 peptides (Supplementary Figure 2C). Finally, the effects of mutations to the crucial LmGpsB-interacting residues in LmPBPA1 were also probed in *Listeria* using fosfomycin sensitivity as a reporter since GpsB mutants are more susceptible to this antibiotic at 37 °C than wild-type *L. monocytogenes*. Fosfomycin inhibits the final enzyme in the biosynthetic pathway of PG, MurA, and the ΔgpsB mutant is hypersensitive to it probably because of unproductive consumption of PG precursors due to mis-regulated LmPBPA1. By contrast, removal of pppA1 reduces substrate turnover in PG biosynthesis and thus the cells become more resistant to fosfomycin, and a *pppA1 pppA2* double mutant is not viable. Effects on fosfomycin sensitivity were apparent in mutants carrying the *pppA1*Arg8AlaArg12Ala and *pppA1*Gln10Pro alleles but only when LmPBPA2, the LmPBPA1 parologue, was also absent (Fig. 2c). Synthetic lethality with *pppA2* and a growth defect at 42 °C is characteristic of the *L. monocytogenes* null gspB mutant19, suggesting that the observed effects partially phenocopy ΔgpsB. However, no *pppA1* mutation phenocopied the ΔgpsB mutant completely (Supplementary Figure 2D).

Taken together, our data highlight the importance of a conserved arginine and α-helicity in class A PG synthases for interacting with GpsB in two species. In both cases the arginine is adjacent to a conserved residue with high propensity to act as a
helix N-cap, implying that positioning of the arginine at the start of the helix is critical. Furthermore, since \textit{pbpA1} does not phenocopy \textit{gpsB} in \textit{L. monocytogenes}, and \textit{gpsB} deletion on its own in \textit{B. subtilis} has no clear phenotype, GpsB must have additional functions in both bacteria.

**Extending the \textit{B. subtilis} and \textit{L. monocytogenes} GpsB interactomes.** The data presented above describe features critical for interactions involving \textit{BsGpsB}, which include a helical SRxxR(R/K) motif in close proximity to the membrane. To identify hitherto unidentified \textit{BsGpsB}-interacting proteins, the \textit{B. subtilis} proteome was queried with the SRxxR(R/K) motif. Two previously uncharacterised ORFs, \textit{BsYpbE} and \textit{BsYrrS}, conform to all the features described above. \textit{BsYpbE} is a membrane protein with a 56-residue cytoplasmic domain that encodes a SRVERR motif. The extracellular region, residues 80–240, contains a LysM (lysin motif) domain between residues 189 and 235; LysM domains are ~40-residue, degenerate PG- and chitin-binding modules widespread in bacteria and eukaryotes. \textit{yrrS} is found in a bicistronic operon widely conserved in the \textit{Bacillaceae} with the gene (\textit{yrrR})
encoding a class B PBP, PBP4b35, suggesting these genes have a linked function in cell wall homoeostasis36. B. subtilis YrrS comprises an 18-residue cytoplasmic domain with two potential, overlapping BspGs-binding motifs SRYENR and NRDKRR and an extracellular domain that belongs to the widespread and currently uncharacterised DUF1510 family.

LysM domains are frequently found as tandem repeats within bacterial proteins37 and the individual domains can act cooperatively to bind PG38,39. BspYbE contains one LysM domain hence oligomerization of BspYbE may enhance PG binding, with the oligomerisation of the extracellular LysM domain of BspYbE presumably controlled by cytoplasmic, hexameric B. subtilis YrrS (32), the essential form of the protein in bacteria19. In the absence of purified, full-length BspYbE to test this hypothesis directly, monomeric and dimeric forms of BspYbE130-240, which encompasses the sole extracellular LysM domain, were generated instead. Dimeric BspYbE130-240 was prepared by mutation of Ser132 to a GpsB Tyr25 in de GpsBAsp31Ala and GpsB variants (Supplementary Figure 3A). Therefore, the binding of YpbE to PG in B. subtilis will be stimulated by YpbE multimerisation, induced by hexameric GpsB.

The interaction of BspBs1-68 with B. subtilis YrrS and BspYbE was assessed by FP and BACTH. BspBs1-68 bound to a 21-residue fragment of the cytoplasmic domain of BspYbE, BspYbE1-21, that encompasses the SRVERR motif, and the entire cytoplasmic mini-domain of B. subtilis YrrS, BspYbE1-21, with K_d values of 13 μM (Fig. 3a) and 430 μM (Fig. 3b), respectively. The modest affinity of the BspYbE1-21 to BspBs1-68 (Supplementary Table 1) probably translates to a substantially tighter affinity in bacterial cells, because of an avidity effect resulting from BspBs1-68 and BspYbE1-21 associating with membranes, effectively increasing the local concentration of each significantly. The specificity of these interactions measured by FP was consistent with the impact of BspBsAasp31Ala and BspBsGly32Ser mutations, each of which reduced the affinities for BspYbE1-21, and BspYbE1-21 by 7- and ~40-fold, respectively (Fig. 3a, b), and in-line with the roles of BspBsAasp31 and BspBsGly32 in defining the BspBP1 binding site. Interactions of BspBs1-68 with B. subtilis YrrS and BspYbE were also detected by BACTH, with the interactions mapping to the N-terminal domain of BspBs1-68 in both cases (Fig. 3c). BspBs1-68 is only conditionally essential in B. subtilis11,12, and perhaps it is not surprising that no obvious cell growth or division phenotypes were identified in our hands or previously40 on deleting the genes encoding BspYbE, BspBP4b or BspYbE. BACTH was used to confirm that BspYbE interacted with BspBs1-68, BspBP1 and BspRodZ; the latter two proteins have established roles in cell division, growth and morphogenesis41,42. The interaction between BspBP1 and a fragment of B. subtilis YrrS lacking residues 13-16, BspYbE13-16 (which reduced non-specific binding to the BspBP1-immobilised SPR chip and did not affect the SRYENR motif) was quantified by SPR, and BspYbE13-16 bound to BspBP1 with a K_d of 20 nM (Supplementary Figure 3B). Therefore, these gene products are capable of forming a network of interactions (Fig. 3d) that may be nucelated by the formation of a BspBP1: BspYbE complex.

Homologues of YpbE do not exist in L. monocytogenes and the YrrS homologue (Lmo1495) does not contain a signature BspGs-binding motif and neither protein is found in S. pneumoniae. No strong potential GpsB-interacting candidates were identified when the L. monocytogenes proteome was searched with either TRXXYR or SRRXXR(R/K) as the query. BACTH was thus used to uncover additional potential LmGsB functions in L. monocytogenes using a bank of 27 listerial components from the known elongation and division machineries in bacteria (Supplementary Note 2). Twelve proteins were shown to interact with LmGsB and only these are shown in Supplementary Figure 3C. There is no consensus motif shared by these proteins, though all have at least one arginine present in their cytoplasmic regions that is conserved in their respective orthologues.

Two arginines dictate S. pneumoniae GpsB::PBP2a molecular recognition. S. pneumoniae, more distantly related to either Bacillus or Listeria, is an ovoid-shaped Gram-positive coccus in which GpsB is essential22-24. SpGpsB co-immunoprecipitated with SpPBP2a (one of three pneumococcal class A PBPs), SpMreC and other proteins, suggesting they interact at some point in the pneumococcal cell cycle24. Synthetic lethality studies in pneumococcal AgsB suppressor mutants revealed that pbp1a, and not pbp2a, became essential in the absence of gpsB indicating that SpPBP2a is the class A PBP regulated by SpGpsB in S. pneumoniae24. We found that the cytoplasmic mini-domain of SpGpsB and many of its orthologues contain the conserved sequence (S/R)RS(R/G)(K/S)XRR (Supplementary Figure 4A) that resembles the Bacillaceae PBP1 SRXXR(R/K) motif (Supplementary Figure 1F). A 22-residue peptide of SpPBP2a that encompasses this region, SpPBP2a13-16, was found by FP to bind to the N-terminal domain of SpGpsB, SpGpsB1-63, with a K_d of 80 μM whereas SpGpsB1-63Aasp35Ala (equivalent to SpGpsBAsp35Ala) had a ~40-fold reduced affinity for SpPBP2a13-16 (Fig. 4a, Supplementary Table 1). The crystal structure of a slightly truncated form of the N-terminal domain of SpGpsB, SpGpsB1-63 (to expedite crystallisation), was solved in the presence of a 14-residue peptide of SpPBP2a, SpPBP2a14-27, which includes the (S/R)RS(R/G)(K/S)XRR motif. In this instance, each subunit of the SpGpsB dimer is peptide-bound (Fig. 4b). Peptide binding principally involves two arginines but each SpGpsB subunit recognises the peptide differently. In SpGpsB4-63 molecule 1, SpPBP2a27-40 recognition centres on SpPBP2aArg31 and SpPBP2aArg36 (Fig. 4c, whereas molecule 2 involves SpPBP2aArg33 and SpPBP2aArg36 (Fig. 4d). The arginine pairs occupy the same positions as BspBP1Arg11 and BspBP1Arg8 in the BspBsB1-64:BspBP11-17 complex (Fig. 1c); SpPBP2aArg36 is equivalent to BspBP1Arg11 whereas SpPBP2aArg31 and SpPBP2aArg33 are equivalent to BspBP1Arg8.

The SpGpsB::SpPBP2a interaction was confirmed by BACTH (Supplementary Figure 4B). The interaction was lost completely with SpGpsBAsp35Ala, SpGpsBVal32Ala, SpGpsBAsp29Ala, SpGpsBLeu32Ala and SpGpsBAsp33Ala mutated proteins and reduced with SpGpsBAsp36Ala (Fig. 5a). All the SpGpsB variants retained the ability to self-interact and to interact with wild-type SpGpsB (Fig. 5a, Supplementary Figure 4C); the impact of the mutations on interactions with SpPBP2a thus does not reflect impaired expression of the relevant fusion proteins. Moreover, all
the SpGpsB variants, except SpGpsBAsp29Ala, retained some ability to interact with SpMreC, which was also confirmed to interact with SpGpsB by BACTH (Fig. 5a, Supplementary Figure 4B).

Despite differences in the secondary structures of the two independent SpPBP2a peptides bound to the SpGpsB1-63 dimer (Fig. 4b), the two arginines form a similar network of interactions with SpGpsB as described for BspGpsB and LmGpsB (Figs. 1c, 2a) with additional sidechain contacts in molecule 1 between SpPBP2aArg31 and SpGpsBAsp33 (Fig. 4c), and SpPBP2aArg31 and SpGpsBTyr23. The importance of SpPBP2aArg31 and SpPBP2aArg33 is further supported by their sequence conservation (Supplementary Figure 4A) and FP (Fig. 4a, Supplementary Table 1). Although SpPBP2aArg31Lys had only a 2-fold reduced affinity, which probably reflects the ability of SpPBP2aArg33 to compensate for the loss of SpPBP2aArg31, the binding affinity of SpPBP2aArg31Lys,Arg33Lys was reduced >25-fold relative to wild type. The importance of the SpGpsB residues involved in the interactions with SpPBP2a is also consistent with the phenotype because of the severe growth (Fig. 5b) and morphological defects (Fig. 5c) of S. pneumoniae strains harbouring the SpGpsB Tyr23Ala, SpGpsBVal28Ala, SpGpsBAsp29Ala, SpGpsBLeu32Ala and SpGpsBAsp33Ala alleles even though the mutated proteins were still capable

**Fig. 3** The SRxxR(R/K) motif identifies BsypbE and BsyrrS as new BspGpsB binding partners. a, b BsyypbE1-18 and BsyrrS1-21 bind to BspGpsB1-68 at the same site as BspPBP1. Fluorescence polarisation of BspGpsB1-68 binding to fluorescein-labelled BsyypbE1-18 (a) and fluorescein-labelled BsyrrS1-21 (b). The interaction of wild-type proteins is depicted by black curves, whereas the red curves and dashed black lines correspond to BspGpsBAsp31Ala and BspGpsBAsp29AlaTyr25Phe mutants, respectively. c BACTH reveals a new BspGpsB interaction network involving proteins encoding the SRxxR(R/K) motif. The panel shows pairwise combinations of proteins expressed as N-terminal fusions to both halves of adenylate cyclase in the BACTH host strain. Their presence in complexes containing BspRodZ, BspPBP4b and BspPBP1 imply roles for BsyrrS and BsyypbE in sidewall synthesis during cell growth. The validity of the observed interactions is supported by the behaviour of the T18-BspGpsB4-63 fusion, which does not interact with any other partner except T25-BspG, and therefore acts as an internal control. The T18-BspGpsB4-63/T25-BspGpsB interaction is consistent with the hexameric nature of BspGpsB [19,26]. The absence of interactions between BspGpsB4-63 and BspGpsB or BsyypbE is consistent with data in b that shows that the N-terminal domain of GpsB interacts with the SRxxR(R/K) motif of YpbE and YrrS. d A model recapitulates interactions between BspGpsB and partners. Surface representations of the SAXS structure of LmGpsB [26] (coloured teal) and the closest homologues in the PDB by sequence (BspPBP1, yellow, PDBid Z0LV; BspPBP4b, pale blue, 4L0I; BspRodZ, pink, 2WUS; BspMreC, dark grey, 2JSU; BspYpbE, salmon, 2MKX and the linker represents the disordered region, residues 131-188) are used as models for the B. subtilis proteins. Amorphous blobs, the surface area of which are scaled proportional to molecular weight, are used for BsyrrS (green) and the extracellular domain of BspRodZ (pink) where there is no structural information. The N-terminal domain of each membrane protein is cytoplasmic and 22-αmino acid model helices represent each TM helix. The TMPred-predicted TM boundaries are: BspPBP1 (38-60); BspPBP4b (9-31); BsyrrS (19-41); BsyypbE (57-79); BspRodZ (89-111) and BspMreC (7-24). The GpsB-interacting domains of BsyypbE, BsyrrS and BspPBP1 are based on the BspPBP1-7 structure.
Fig. 4 The SpPBP2a minidomain is not α-helical but still interacts with SpGpsB through conserved arginines. a Arginine residues of SpPBP2a play a key role in binding to SpGpsB. Unless otherwise indicated, the fluorescence polarisation binding curves represent the interaction of TAMRA-labelled SpPBP2a peptides with wildtype SpGpsB4–63. The relevant dissociation constants are listed in Supplementary Table 1. b The structure of the SpGpsB4–63:SpPBP2a27–40 complex reveals the critical role of SpPBP2a arginines for the interaction with SpGpsB. In this cartoon, SpGpsB4–63 is coloured cyan, and the SpPBP2a27–40 peptide is coloured yellow (molecule 1) and green (molecule 2). The sidechains of Arg8 and Arg11 from the 8SpGpsB5–64:8SpBP11–17 complex are shown as red sticks after a global superimposition of equivalent GpsB atoms. In molecule 1, SpPBP2aArg31 and SpPBP2aArg36 superimpose with 8SpGpsB5–64Arg8 and 8SpGpsB5–64Arg11 whereas molecule 2 accommodates SpPBP2aArg33 and SpPBP2aArg36. c, d Close-up view of the interactions of SpPBP2a from molecule 1 (c) and 2 (d) with SpGpsB4–63. Key interfacial sidechains and backbone atoms are represented in stick format; SpGpsB4–63 is coloured cyan and SpPBP2a27–40 is coloured green. The van der Waals’ interactions between SpGpsBLeu32 and SpPBP1Arg31 (molecule 1) and SpPBP1Arg33 (molecule 2) are in yellow. The carbonyl oxygens of SpGpsBIle11, SpGpsBPh12, SpGpsBGlu13, and SpGpsB Gin14 are denoted by respective red numerals.
of self-interactions (Supplementary Figure 4C) and were expressed at wild-type levels (Supplementary Figure 4D). However, no obvious phenotype was observed in S. pneumoniae strains carrying the corresponding SpPBP2aArg31Ala, SpPBP2aArg33Ala or SpPBP2aArg31Ala,Ser32Ala,Arg36Ala alleles, even when pbp1a was deleted to decouple the effects of mutations in SpPBP2a from SpPBP1a activity (Supplementary Table 2). Nevertheless, SpPBP2a mutants in which amino acids 32–37 or 27–38 or 26–45 were deleted in a Δpbp1a background showed progressively reduced growth rates in the three deletion strains and pronounced morphological defects in the two strains with larger deletions (Supplementary Figure 5A, 5B), despite wild-type levels of protein expression at the respective expected molecular masses (Supplementary Figure 5C). At least eight residues, including two...
arginines and one lysine, were retained in all the deletion constructs before the predicted start of the SpPBP2a TM helix (residue 54), providing the necessary charge to satisfy the inside-positive rule\cite{33}. Consistent with the \textit{pbr2a} deletion mutant phenotypes, BACTH results for the correspondent truncated SpPBP2a variants showed reduced interactions with SpGpsB in comparison to the wildtype but not with SpMreC (Supplementary Figure 5D, 5E).

Together, these results support a critical role of the (S/R)RS(R/G)(K/S)X\textit{R} motif between SpPBP2a residues 30 and 36 for mediating protein–protein interactions with SpGpsB and the importance of its reduction or loss in \textit{S. pneumoniae}. However, the observation that all three \textit{Δpbr1a pbr2a} deletion mutants were viable and both growth and morphology phenotypes were different between \textit{S. pneumoniae Δpbr1a} strains depleted for \textit{gpsB} (Fig. 5d) and \textit{Δpbr1a} strains depleted for \textit{pbr2a} (Fig. 5e) implies that SpPBP2a binding is also just one function for SpGpsB. It is possible that the SpPBP2a:GpsB interaction is only part of the \textit{S. pneumoniae ΔgpsB} phenotype and this interaction per se is not essential, unless the interaction of GpsB with an additional partner is also lost. Alternatively, since deleting the GpsB binding motif of \textit{SpPBP2a} did not abolish the interaction with GpsB completely, the deletion might reveal, or even generate, an unpredicted GpsB binding site in the juxta-membrane region untouched by the deletions.

Since the \textit{pbr2a} mutant did not phenocopy \textit{gpsB} depletion, and given the complex network of GpsB interactions in \textit{B. subtilis} and \textit{L. monocytogenes}, we also sought to extend the pneumococcal GpsB interactorome starting from those proteins that were reported to form a complex with SpGpsB by co-immunoprecipitation (co-IP)\cite{24}. Besides the SpGpsB interactions detected with SpPBP2a and SpGpsB with MreC by BACTH, and as observed with the reciprocal hybrid pairs, they were unaffected by any of the \textit{SpGpsB} alleles mentioned above (Supplementary Figure 6A). The interactions of GpsB in the pneumococcus, as detected by BACTH, FP and co-IP, are summarised in Supplementary Figure 6D.

Taken as a whole, our data on three important bacterial systems agree that GpsB is an adaptor protein\cite{29-31} that connects a major class A PG synthase with other cell wall and cell cycle proteins, and to cell shape determinants such as MreC. The identity and mode of interaction of the GpsB-binding partners varies from species to species and may reflect the different physiologies of each bacterium and their modes of growth and division.

**Discussion**

Bacterial cell growth and division necessitates tight co-ordination between the replication and segregation of the chromosome, the fission of the cell membrane and the remodelling of the PG. Consequently proteins and their complexes with major functions on either side of the membrane must co-ordinate their activities. One potential mechanism involves the interactions of major PG synthases with their intracellular regulators. Herein we present the first structures of the cell cycle adaptor, GpsB, in complex with the cytoplasmic mini-domains of PG synthases from three different bacteria, the rod-shaped \textit{B. subtilis} and \textit{L. monocytogenes}, in which \textit{gpsB} is conditionally essential\cite{11,12,19} and the ovococcal \textit{S. pneumoniae} in which \textit{gpsB} is essential\cite{22-24}. In common with mammalian adaptors GGA\cite{31} and 14-3-3\cite{34} proteins, the primary binding surface of GpsB is restricted to a conserved groove between α-helices. The cytoplasmic mini-domains of the three PG synthases in the three organisms have little in common except that each utilises a conserved arginine in their respective sequences to interact with the cognate GpsB. The PG synthase arginine finger pores into a negatively charged cavity
situated between α-helices 1 and 2 of GpsB and is fixed in the same orientation in all structures, just as the phosphoryl group defines the binding orientation of peptides to 14-3-3\(^2\). The arginine complements the cavity best when the mainchain amide protons of it and its downstream residue are accessible to form hydrogen bonds with BsGpsB\(^{A35}\), LmGpsB\(^{A37}\) or SpGpsB\(^{A33}\). This scenario can occur when the arginine is either at the start of an α-helix, such as BspPB1\(^{A38}\), or at the i + 1 position in a type I β turn, such as SpPBPA2\(^{A31}\), which explains why free λ-arginine does not displace pre-bound PBP peptides from GpsB even when present at 100-fold molar excess (Supplementary Figure 2E). Similarly, contact to the backbone amide at the i + 2 position in 14-3-3 ligands is essential for binding\(^2\). Despite a lack of strong sequence and structural homology in the PG synthase cytoplasmic mini-domains, their binding is dependent upon an identical subset of GpsB residues including BsGpsB\(^{Tyr25}\), BsGpsB\(^{Asp31}\), BspPBPA1\(^{Arg8, Arg10}\) (Fig. 1c) and their structural equivalents LmGpsB\(^{Tyr22}\), LmGpsB\(^{Asp33}\), LmGpsB\(^{Asp37}\) (Fig. 2a), and SpGpsB\(^{Tyr23}\), SpGpsB\(^{Asp29}\) and SpGpsB\(^{Asp33}\) (Fig. 4c, d, Supplementary Figure 1A). Mutations at these positions in GpsB from S. pneumoniae and L. monocytogenes are lethal or have marked growth defects (Fig. 5b, c)\(^19\) and do not interact with their cognate peptidoglycan synthase by FP (Supplementary Table 1)\(^9\) or by BACHT (Fig. 5a)\(^19\). A phenotype analysis of equivalent mutations in B. subtilis to assess their importance is not straightforward to determine for two reasons. First, a phenotype for the gpsB null mutant is only observed synthetically, when gpsB is mutated alongside either ftsZ\(^12\) or ezrA\(^11\). Second, a B. subtilis mutant lacking all class A PBPs is viable\(^46\), precluding an unequivocal assessment of the precise importance of the BsGpsB:BspPB1 interaction. Nonetheless, the convergence of all our experimental data on three bacterial systems suggests strongly that the tyrosine and aspartate dyad also have important roles under normal growth conditions in S. pneumoniae. In every Firmicute (and Actinobacterium) tested thus far, apart from S. aureus\(^26\), GpsB (or the homologous DivIVA/Wag31/antigen 84) acts as an adaptor to coordinate PG synthase activity with other processes depending on the physiology of the cell. GpsB hexamerisation can bridge the interaction of multiple binding partners, a function GpsB shares with 14-3-3 proteins that can form ternary complexes with BCR and Raf-1 by 14-3-3 dimerisation\(^50\). In bacilli, BsGpsB plays a role in shutting between the side wall during elongation and the septum during division\(^11\) and, given that BsGpsB\(^{D47}\) is regulated by α factors E\([35]\) and F\([51]\), complexes of BspPB4 and BspYrrS, bridged by BsGpsB (Fig. 3c, d), presumably play a role in the asymmetric cell division characteristic of endospore-forming bacilli. In listeria, which is closely related to bacilli and shares with them a rod-like morphology, GpsB appears to connect several PBPs with proteins with known roles in cytokinesis, including Z-ring polymerisation modulators (ZapA, EzrA, SepF), late division proteins (DivIB, DivIC) and the elongasome (MreC, MreBH) (Supplementary Figure 3C,D), all of which except SepF and MreBH have also been tested in B. subtilis and found not to interact with BsGpsB\(^{B11}\). By contrast, S. aureus GpsB appears to modulate Z-ring assembly\(^26\), however, no interaction between FtsZ and GpsB has been identified in B. subtilis\(^11\), S. pneumoniae\(^23,24\) or L. monocytogenes (Supplementary Figure 3C).

The pneumococci have an ovoid cell shape and lack key components such as the MinCD system for cell division site selection\(^52\), and MreB-like proteins required for side wall synthesis\(^33\). Presumably SpGpsB interacts with one or more pneumococcal-specific proteins, the loss of which may be related to the lethal phenotype. Furthermore, SpGpsB affects both StkP autophosphorylation\(^23,24\) and the StkP-catalysed phosphorylation of SpDivIVA\(^23,24\), SpMapZ/LocZ\(^24,54\), SpJag/EloR/KhpP\(^55-57\) and SpMacP\(^58\). It is not yet clear how the complexes formed by these
proteins are affected by their phosphorylation, except that SpBPB2a activity is dependent upon phosphorylated SpMac\(^{25}\), at least in the presence of functional SpStkP, or what the impact is of potential cross-talk to two-component signalling systems\(^{29}\). Finally, the different phenotypic outcomes associated with gpsB deletion or depletion in the three systems studied herein may reflect the presence of redundant systems in the large genome (4.2 Mbps) of the bacilli, partial redundancy in listeria (2.9 Mbps), and a relative absence of redundancy in the striped-down genome (2.1 Mbps) of the pneumococci. The relative affinities and cellular concentrations of GpsB partners probably dictate which protein is likely to lead to an increase in avidity of GpsB\(^{25}\) as commonly found in antibody-antigen interactions. However, the intricate networks involving GpsB will only be uncovered by validating the full GpsB interactor.

**Methods**

**Bacterial strains and growth conditions.** Supplementary Table 3 lists the bacterial strains used in this study. *L. monocytogenes* EGD-e-derived strains were routinely cultivated in brain heart infusion (BHI) broth or on BHI agar plates at 37°C in 7% CO\(_2\). If required, erythromycin (5 μg mL\(^{-1}\)) and X-Gal (40–100 μg mL\(^{-1}\)) were added. All *L. monocytogenes* growth experiments were repeated three times and average values and standard deviations are shown. All *S. pneumoniae* strains were derived from unencapsulated serotype 2 D39 strains IU1824 (D39 ΔdivC psrL1) or IU1945 (D39 Δpsr)\(^{39}\). *S. pneumoniae* strains were grown on Petri dishes containing the appropriate antibiotic, modified tryptophase soy agar II (Becton-Dickinson) and 5% (vol per vol) defibrinated sheep blood (TSAI-BA), and the plates were incubated at 37°C in 5% CO\(_2\). Bacteria were cultured statically in BHI (Becton-Dickinson) broth at 37°C in an atmosphere of 5% CO\(_2\), and growth was monitored by OD\(_{620}\).

**General methods, manipulation of DNA and oligonucleotide primers.** E. coli transformation and isolation of plasmid DNA (the plasmids used are listed in Supplementary Table 4) was performed according to standard protocols\(^{2,32}\). All *L. monocytogenes* strains were transformed by electroporation. Enzymatic modification of plasmid DNA was carried out as described by the instructions given by the manufacturers. Quikchange mutagenesis was employed for restriction-free modification of plasmids\(^{32}\). DNA sequences of oligonucleotide primers are listed in Supplementary Tables 5–7. All deletions and insertions were confirmed by PCR, and all constructs and clones were verified by Sanger DNA sequencing.

**Constitution of *L. monocytogenes* mutant strains.** Plasmid plS1497 was first constructed to facilitate mutagenesis of the chromosomal copy of *pbpA1*. A DNA fragment comprising recU and the first 2063 bp of *pbpA1* was amplified from chromosomal DNA using primer pair SHW737/SHW747 and cloned into pMAD using BamHI/NcoI. An unwanted mutation in front of recU resulted in a correction of this plasmid by Quikchange with primer pair SHW777/SHW778. The corrected plS1497 was used as template in further Quikchange reactions to introduce Thr7Ala (SHW744/SHW745, psH504), Arg8Ala (SHW746/SHW747, psH505), Tyr11Ala (SHW755/SHW756, psH506), Gln10Pro (SHW787/SHW788, psH509) and Arg12Ala mutations (SHW748/SHW749, psH507) into *pbpA1*, or to remove *pbpA1* nucleotides 4-1250 (SHW775/SHW776, psH503). psH508 (pbpA1ΔArg12AlaΔArg12ΔArg12) was obtained by Quikchange with primers SHW750/SHW751 and plasmid psH505 as the template. An N-terminal fragment of *pbpA1* was excised from the *L. monocytogenes* chromosome using plasmid psH503 and the insertion/ excision protocol for construction of clean deletions, resulting in strain LMS211. The mutated *pbpA1* alleles present on psH504/psH509 were then reintroduced into LMS211 following the same protocol.

**L. monocytogenes fosfomycin susceptibility assays.** Fosfomycin susceptibility was recorded using filter discs (Ø 6 mm) soaked with 10 μL of a 10 mg mL\(^{-1}\) fosfomycin solution. *L. monocytogenes* colonies, grown on BHI agar plates, were resuspended in BHI broth and used to swab-inoculate BHI agar plates. Filter discs soaked with fosfomycin were placed on top of the agar surface and the plates were incubated at 37°C overnight. Diameter of growth inhibition zones was measured and corrected for the filter disc diameter. All experiments were performed three times and average values and standard deviations were calculated. Significance was determined using the t-test and differences were considered to be significant when *P* < 0.01.

**L. monocytogenes and *B. subtilis* BACTH assay.** The BACTH system\(^{25}\) was used for analysis of protein-protein interactions. First, LmPBPa was screened against B. subtilis strain 168\(_{R}^R\) variants lacking LmPBPa residues in LmPBPa. This is important for binding LmPBPa. To facilitate screening, we used a T25 fragment of Bordetella pertussis adenylate cyclase that had been fused to the N-terminal 91 amino acids of LmPBPa1 to create a T25-LmPBPa1 fusion containing the N-terminal cytoplasmic mini-domain and the transmembrane helix, but lacking the extracellular globular glycosyltransferase (T25-ΔGT-TP) in plasmid psH437\(^{21}\). LmPBPa1 residues that corresponded to the BgpB binding motif of BgpBP1 (Thr7, Arg8, Tyr11, Arg12) and additionally positively charged amino acids (Lys14, Lys20, Lys21, Lys22, Lys25, Arg26, Lys28 and Arg29) were deleted with alanine prototropism. T25-ΔGT-TP was deleted in the vicinity of the LmPBPa1-encoding region (IU12361, IU12440, IU12612, IU12615, IU13121), or with pbpA1Δ mutation (IU13141, IU13364, IU13366, IU13370, IU13372 and IU13374) were performed in the presence of 0.5 mM ZnCl\(_2\) + 0.05 mM MnSO\(_4\). Transformation of *B. subtilis* 168 was performed using calcium chloride-mediated transformation method.
P-erm into IU11388 (AgsbA::aadB//bgA::tet-fl-zef-A::erm-gpsB) and IU13465 (Δpfl dependent markerless bgA::kan-fl-zef-A::erm-gpsB) was performed in the presence of 0.4 mM ZnCl₂ + 0.04 mM MnSO₄. Strains containing markerless pfl² alleles in the native chromosomal locus were constructed using Janus cassette allele replacement via transformation of a Δpfl²::erm amplicon into S. pneumoniae strains. IU11945 (wild-type parent) and IU11286 (gpsB+//A::erm-gpsB) were inoculated from frozen glycerol stocks into BHI broth, serially diluted, and incubated for <13h statically at 37 °C in an atmosphere of 5% CO₂. The numbers of colonies were normalised to 1 mL of transformation mixture.

**Growth and microscopy of S. pneumoniae strains.** Strains IU11945 (wild-type parent) and IU11286 (gpsB+//A::erm-gpsB) were inoculated from frozen glycerol stocks into BHI broth, serially diluted, and incubated for <13h statically at 37 °C in an atmosphere of 5% CO₂. For IU11388 (AgsbA::aadB//bgA::tet-fl-zef-A::erm-gpsB) and all gpsB alleles expressed in S. pneumoniae strains, cultured duplicates were incubated at 37 °C in an atmosphere of 5% CO₂. For IU11286 (AgsbA::aadB//bgA::tet-fl-zef-A::erm-gpsB) and all gpsB alleles expressed in S. pneumoniae strains, cultured duplicates were incubated at 37 °C in an atmosphere of 5% CO₂. The mean doubling time and the standard error of the mean (±) was calculated. OD₆₂₀ was performed at OD₆₂₀ of 0.1-0.4 were diluted to OD₆₂₀ of 0.003 in BHI broth lacking (-Zn) or containing (+Zn) 0.5 mM ZnCl₂ + 0.05 mM MnSO₄ and cultured under the same conditions. Growth was monitored turbidimetrically every 45 min to 1 h with a GeneSys 2 spectrophotometer (Thermo Scientific). At 3.75 h after dilution into BHI broth with or without Mn/Zn supplement, cell pellets were obtained from 1 mL of culture, and resuspended in 100 µL of 4% paraformaldehyde. Cells were fixed for 15 min at RT and the tubes with fixed cells were left on ice until microscopy. For microscopic analyses, samples (1.5 µL) were taken and examined using a Nikon E-400 fluorescence phase-contrast microscope. Growth and microscopy of strains containing pfl alleles were performed as above, but with no Mn/Zn addition to the BHI broth. Microscopic results were presented as the mean ± standard deviation (p < 0.05).

**Co-immunoprecipitation (Co-IP) of S. pneumoniae strains.** Co-IP experiments of S. pneumoniae FLAG-tagged strains were performed with the use of anti-FLAG (M2) monoclonal antibody (4µg). lysates were obtained from cultures grown exponentially at 37 °C in an atmosphere of 5% CO₂ in 400 mL of BHI to OD₆₆₀ ≈ 0.25–0.4. Cell pellets were washed once with 30 mL of 1X PBS (4 °C) and resuspended in 19.8 mL 1X PBS (4 °C). About 200 µL of 10% (vol per vol) paraformaldehyde solution (EMS) were added for crosslinking to a final concentration of 1% (vol per vol). Mixtures were incubated at 37 °C for an incubator for 1 h. Cross-linking reactions were quenched by the addition of 4 mL 1.0 M glycine followed by incubation at 25 °C for 10 min. Cells were collected by centrifugation (16,500 × g for 5 min at 4 °C). Pellets were washed twice with 20 mL cold 1X PBS (4 °C) and resuspended in 2 mL cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100 (vol per vol)) with 1 tablet of protease inhibitor (ThermoFisher Scientific, 78429) freshly added per 10 mL of lysis buffer. The suspension was transferred into two lysing matrix B tubes (Biometra) with 1 mL in each tube. The tubes were shaken ten times in a FastPrep homogenizer (4×, 5 min on ice, 3×, 5 min on ice and 3×) with 6.0 M s⁻¹ for 40 s each at 4 °C. Cell debris and lysing matrix from tubes were removed by centrifugation at 16,000 x g for 5 min at 4 °C. The protein concentration of each sample was determined by Bio-Rad DC™ protein assay. About 1 mL of lysis of similar amounts of total protein (5–7 mg·mL⁻¹) was added to tubes with 30 µL of anti-FLAG magnetic beads (Sigma, M8823). The same amount of protein was loaded onto the beads for strains expressing FLAG-tagged proteins and the corresponding control strains lacking FLAG-tagged proteins in each experiment. The tubes were rotated for 2 h at 4 °C. The beads were washed three times with 1 mL of lysis buffer (4 °C) with 10 min incubation at 4 °C each time. FLAG-tagged proteins were eluted from the beads by incubation with 100 µL of FLAG elution solution (150 mg 3X FLAG peptide per mL) (Sigma). In a volume of 100 µL, 1 µL of the original lysate added to magnetic beads (input) were separately mixed with 100 µL 2× Laemmli sample buffer (BioRad) containing 5% (vol per vol) β-mercaptoethanol (Sigma) and heated at 95 °C for 1 h to break the cross-links. A volume of 40 µL of each elution sample mixed with 2× sample buffer was loaded on each lane of a 13% precast SDS-PAGE gel or submitted to western blotting using affinity-purified SpBP2Px polyclonal antibodies (dilution 1:10,000) or an anti-GpsB antibody as the primary antibodies.

**S. pneumoniae BACTH assay.** The target genes were amplified by PCR from S. pneumoniae D39 chromosomal DNA. PCR fragments for pbp2a, mreC, pbp1a, pbp2x and pbp2b were purified, digested with appropriate restriction enzymes and cloned downstream of the pKT25/pUT18 expression plasmids encoding the corresponding hybrid proteins fused at the C-terminal ends of the T25 and T18 fragments, respectively. Plasmids pKNT25-gpsB/pUT18-gpsB, pKNT25-stk/pUT18-stkP and pKNT25-ecz/pUT18-eczA were already constructed [46]. The mutated and truncated gspB and pbp2a alleles were amplified from their respective DNA templates and the corresponding PCR products cloned into the corresponding sites of the BACTH vectors as described above for the wild-type alleles. E. coli DH5α transformants were selected on LB agar plates containing ampicillin (100 µg·mL⁻¹) or kanamycin (50 µg·mL⁻¹) and 0.4% glucose to repress leaky expression [47]. For BACTH experiments, each pair of plasmids was co-transformed into the E. coli cyo strain BTH101 and co-transformation mixtures were spotted onto LB agar plates supplemented with ampicillin (100 mg·mL⁻¹), kanamycin (50 mg·mL⁻¹) and X-Gal (40 µg·mL⁻¹), followed by incubation at 30 °C. Plates were inspected and photographed after 24 and 48 h. Plasmid pairs pKNT25/pUT18 and pKT25-pBP2x were excised from agar plates using appropriate positive and negative controls, respectively. All experiments were repeated at least twice.

**Plasmid construction for recombinant protein and peptide work.** (i) GpsB: All mutations were undertaken by the Quickchange protocol where mutagenesis reactions failed to generate a DNA fragment of the expected size, a modification of the Quikchange protocol with two separate PCR steps was used instead [59]. Gln17Ala, Asp31Ala and Asp35Ala mutations were introduced to the SpBP2x construct using the Quikchange protocol with two separate PCR steps.[90] The codon for Pro64 was mutated to the codon for Glu17Ala, Asp31Ala and Asp35Ala mutations were introduced to the SpBP2x construct using the Quikchange protocol with two separate PCR steps.[90]

(ii) PBP peptides: Ser16Cys, Ser7Aha, Arg8Aha, Ala10Pro, Arg11Aha and Arg28Aha mutations in SpBP1132 fused to maltose binding protein (MBP) [96] were
introduced by Quikchange mutagenesis. Plasmids expressing LmPBP1A1-20 and SpPBPA2x13 peptides fused to MBP were prepared by PCR amplifying the relevant ORFs from L. monocytogenes strain EGD-e and S. pneumiae ribosomal RNA with primers LmPBP1A1ncoI and SpPBPA2xhoI respectively. The subsequent PCR fragments were digested with NcoI and XhoI for ligating into similarly restricted pMAT11, a modified version of pHTAI48, Ser19 and Lys11 were mutated in LmPBP1A1 to create a double mutant. Quikchange to cysteine and stop codons, respectively; similarly Quikchange was used to change SpPBPA2x Gly43 and Arg46 to cysteine and stop codons, respectively. Further mutations in LmPBP1A1 were also made by Quikchange; the Arg8AlaSer16Arg double mutant was prepared in two successive steps with the first ArgAla mutation, followed by the ArgSer mutation. The SpPBPA2x13 construct was prepared in several steps. First, nucleotides 1–1165 of pbp2x were PCR amplified in two steps: a initial fragment generated from S. pneumiae ribosomal RNA with primers SpPBPA2xhoI/SpPBPA2x31ncoI was used as a template for further PCR amplification with SpPBPA2x31xhoI/SxB20 and SpPBPA2x31xhoI/SxB22BsrI primers: this fragment was then ligated into (Met11-30)3 of pMAT11. Second, cysteine, serine and stop codons were introduced by Quikchange in place of residues 30–302 with primers SpPBPA2x30S3132S32STOP5 and SpPBPA2x30S3132S32STOP3. A second Quikchange step (primers SpPBPA2x30S3132S32STOP5, SpPBPA2x30S3132S32STOP3) introduced extra codons encoding a GSG sequence after the TEV cleavage to improve cleavage of the MBP-PBP2x–20 fusion protein.

Recombinant protein purification. (i) GpsB. BsGpsB1-28 proteins for use in assays were purified by Ni-NTA affinity chromatography followed by protelolytic removal of the His6-tag by thrombin and subsequent size exclusion chromatography of the His6-tag released fraction. (ii) GpsB1. The same protocol was used for SpPBPA1-20 except that an ammonium sulphate precipitation step was added after thrombin removal of the His6-tag. Ammonium sulphate was added to the thrombin-cleaved protein to a final concentration of 2.8 M (60% saturation at 0 °C) by adding a stock solution of 100% saturated ammonium sulphate that was prepared by adding solid ammonium sulphate directly into the thrombin lane of the N-terminal fragment. The mixture was stirred at 4 °C for 1 h and the membrane pellet was resuspended in 15 mL 50 mM Tris.HCl (pH 8), 300 mM NaCl, 50 mM Tris.HCl (pH 8), 250 mM NaCl supplemented with 0.5 mg•mL−1 lysozyme, 2 μg•mL−1 DNAase and Roche Complete protease inhibitor cocktail.

Crystallisation and structure determination. Successful crystallisation of the N-terminal domains of GpsB proteins was improved by truncation of a few amino acids from both N- and C-terminals, which are too far from the PBP binding site (25 and 45 Å, respectively) to affect binding and correspond to the minimum ordered segment of GpsB5-64. The BsGpsB5-64Lys32Glu:ΔΔyrrS crystal was transferred to 100% ammonium sulphate (pH 8), 250 mM NaCl, 3 mM MgCl2, 0.3 mM DTT supplemented with Roche complete EDTA-free protease inhibitor cocktail, 0.5 mM β-mercaptoethanol. After 2 h stirring at 4 °C the sample was then centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was supplemented with imidazole to 5 mM then incubated for 2 h at 4 °C with NiNTA-agarose Select resin. This mixture was then passed through a gravity column and the resin washed with resuspension buffer supplemented with 5 mM imidazole and then BsPBPA1-20 proteins were eluted with resuspension buffer containing 250 mM imidazole. The His6-tag was removed by thrombin treatment, followed by the digest was purified by Superdex 2500 size exclusion chromatography in a buffer of 25 mM NaCl, 50 mM Tris.HCl (pH 8) and 15% glycerol, 0.2 mM ABEFS, 0.5 mM DTT and Roche complete EDTA-free protease inhibitor cocktail. The recrystallised mother liquors were then supplemented with 15% glycerol, 0.2 mM AEBSF, 0.5 mM DTT and Roche complete EDTA-free protease inhibitor cocktail and incubated at −80 °C.

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ph 8, 0.2 M zinc acetate, 20% PEG 3000 were transferred to a cryoprotectant
liquid supplemented with 20% PEG400 before mounting and freezing as above.
20% PEG 8000 and were cryoprotected by direct transfer to a solution of the mother
liquor containing 2.5 mg of PBP1 and 2.5 mg−1 mL−1 of the LmPBPA1-15 peptide; after 3 min the crystals were mounted
in rayon loops and frozen in liquid nitrogen. The SpGsBs4-63:SpPBPA1-15 complex crystallised in 0.1 M Tris.HCl pH 8.5, 0.2 M magnesium chloride,
250 mM NaCl, 0.1% reduced Triton X-100. The excitation wavelength was 540
nm and fluorescence emission was recorded above 590 nm for TAMRA-labelled
peptides. The N-terminal domains of GpsB proteins BsGpsB4-63, LmGpsB1-13,
SpGpsB4-63 were used in all experiments in preference to the full-length GpsB
proteins due to the high solubility of the former, which facilitated achieving the high
protein concentrations necessary to saturate peptide binding. The validity of the N-
terminal domain as a substitute for full-length GpsB proteins in these experiments is
supported by the comparable affinity of BsGpsB and SpGpsB4-63 for a labelled
BsPBP1A1-13 peptide in FP experiments (Supplementary Table 1). 

SPR. All SPR experiments used a running buffer of 10 mM Tris.HCl (pH 8.0), 250
mM NaCl, 0.1% reduced Triton X-100. BsPBP1 and BsPBP1A1-13 were immobilised
on the surface of a CM5 chip (GE Healthcare). For the BsPBP1/YrrS4-63
titration 800 RU of BsPBP1 were immobilised on the chip surface; for the BsPBP1/
LmGpsB titration 1200 RU of BsPBP1 were immobilised. YrrS4-63, which lacks residues
13–16 in the cytoplasmic domain, was used in these experiments to minimise non-specific interactions with the chip matrix; binding of YrrS4-63 to the
reference surface was negligible and the deletion of residues 13–16 leaves the GpsB-binding motif intact. The YrrS4-63 titration was carried out in single-cycle
mode, without regenerating the surface between successive injections of protein,
because harsh reagents were necessary for full surface regeneration. The fit of the
single-cycle mode data to a 1:1 binding model was achieved with a χ-value of 2 and
a corresponding χ2 of 46.1.

CD analysis. CD spectra were recorded on a JASCO J-810 spectropolarimeter with a
P-4235 Peltier temperature controller using 1 mm path length quartz cuvettes. For
full wavelength scans a scan speed of 100 nm−1 and a response time of 4 s was
used, the final spectra were the average of at least four measurements. BsPBP1A1-13
spectra were recorded at 4 °C in a buffer of 20 mM sodium phosphate, pH 7.3 at a
peptide concentration of 25 μM. Peptide helical contents were estimated from molar
eclipticities at 222 nm using the equationhelix = [(θ)222 nm] / (39,500(1–2θ/180°)19) where

maps for the peptide-bound structures are displayed in stereographic mode in
Supplementary Figure 8.

FP assays. FP experiments were undertaken in a buffer of 10 mM Tris.HCl (pH 8.0), 250 mM NaCl, 0.1% reduced Triton X-100. The excitation wavelength was 540
nm and fluorescence emission was recorded above 590 nm for TAMRA-labelled
peptides. The N-terminal domains of GpsB proteins BsGpsB4-63, LmGpsB1-13,
SpGpsB4-63 were used in all experiments in preference to the full-length GpsB
proteins due to the high solubility of the former, which facilitated achieving the high
protein concentrations necessary to saturate peptide binding. The validity of the N-
terminal domain as a substitute for full-length GpsB proteins in these experiments is
supported by the comparable affinity of BsGpsB and SpGpsB4-63 for a labelled
BsPBP1A1-13 peptide in FP experiments (Supplementary Table 1). Km values ± the
standard error, generated after simultaneously fitting all the binding data to a 1:1
interaction model are reported and there are three measurements at each data point in
a titration.

Table 1 Data collection and refinement statistics

| Data collection | BsGpsB4-63:BsPBP1A1-15 | BsGpsB4-63:Lys32Glu:LmPBPA1-15 | Unbound SpGsBs4-63 | SpGsBs4-63:PBP2a27-40 |
|-----------------|------------------------|-------------------------------|--------------------|----------------------|
| Space group     | P 2_1, 2 1             | P 2_1, 1                      | C 1 2 1            |                      |
| a, b, c (Å)     | 31.5, 53.8, 85.9       | 26.6, 31.4, 81.0              | 83.1, 26.4, 65.9   |                      |
| α, β, γ (°)     | 90, 90, 90             | 90, 92.4, 90                  | 90, 106.4, 90      |                      |
| Resolution (Å)  | 45.57 (1.95) (2.00-1.95) | 31.42 (1.63-1.60)           | 27.22-1.90 (1.94-1.90) | 39.85-1.80 (1.84-1.80) |
| Rmerge          | 0.059 (0.473)          | 0.044 (0.386)                 | 0.041 (0.311)      | 0.051 (0.269)        |
| CC (1/2) (%)    | 99.7 (72.0)            | 99.7 (74.3)                   | 99.8 (78.0)        | 97.6 (88.3)          |
| I/εl            | 8.2 (1.1)              | 8.4 (1.8)                     | 13.9 (4.0)         | 9.0 (2.2)            |
| Completeness (%)| 100.0 (100.0)          | 99.8 (99.9)                   | 100 (100)          | 99.8 (99.9)          |
| No. of observations | 78,385 (5459)    | 63,406 (3019)                 | 497,944 (28,050)   | 46,904 (2454)        |
| No. of unique reflections | 11,206 (768) | 17,944 (902)                  | 19,776 (1353)      | 13,052 (763)         |
| Redundancy      | 7.0 (7.1)              | 3.5 (3.3)                     | 25.2 (20.7)        | 3.6 (3.2)            |
| Refinement      |                        |                               |                    |
| Resolution (Å)  | 45.57-1.95             | 29.29-1.60                    | 26.01-1.90         | 39.85-1.80           |
| No. of reflections | 11,162              | 17,912                        | 19,738             | 13,007               |
| Rwork/Rfree    | 0.187/0.214           | 0.173/0.204                   | 0.188/0.249        | 0.178/0.225          |
| No. of atoms    | Protein 951            | 986                           | 1919               | 999                  |
|                | Peptide 94             | 20                             | 220                |                      |
|                | Ligand/ion 1           | 5/8                           | 30/1               |                      |
|                | Water 48               | 106                           | 260                | 87                   |
| B-factors (Å2) | Protein 34.0           | 25.2                          | 24.8               | 18.7                 |
|                | Peptide 39.3           | 36.0                          | 32.5               |                      |
|                | Ligand/ion 23.7        | 30.9/36.5                     | 57.9/12.1          |                      |
|                | Water 36.4             | 30.6                          | 28.1               |                      |
| R.m.s. deviations | Bonds (Å) 0.006       | 0.016                         | 0.017              | 0.015                |
|                | Angles (%) 0.673       | 1.372                         | 1.399              | 1.393                |

*Values in parentheses refer to the highest resolution shell.
n is the number of residues in the peptide. 

LmrPBPA1 is a lipoprotein cofactor located in the outer membrane of B. subtilis PG (SigmaAldrich) was prepared as a 10 mg/mL stock solution in PBS containing 0.1% sodium azide. 25 μg of protein were added to a 100 μL sample containing 66 μg of PG and the mixture incubated with gentle agitation at room temperature for 30 min. The mixture was centrifuged at 16,000 × g for 7 min, the supernatant removed and the pellet resuspended in 1 mL of ice-cold PBS and immediately centrifuged for 2 min at 16,000 × g. The pellet was resuspended in 1 mL of the appropriate buffer and centrifuged for 2 min at 16,000 × g. Finally the pellet was boiled for 3 min in 60 μL SDS-PAGE loading buffer before analysis by SDS-PAGE; the SDS-PAGE loading buffer lacking reducing agents for YphE10-24 context was used.

Pseudotulip pulldown assay. PG pulldown assays were carried out in PBS buffer. B. subtilis PG (SigmaAldrich) was prepared as a 10 mg/mL stock solution in PBS containing 0.1% sodium azide. 25 μg of protein were added to a 100 μL sample containing 66 μg of PG and the mixture incubated with gentle agitation at room temperature for 30 min. The mixture was centrifuged at 16,000 × g for 7 min, the supernatant removed and the pellet resuspended in 1 mL of ice-cold PBS and immediately centrifuged for 2 min at 16,000 × g. The pellet was resuspended in 1 mL of the appropriate buffer and centrifuged for 2 min at 16,000 × g. Finally the pellet was boiled for 3 min in 60 μL SDS-PAGE loading buffer before analysis by SDS-PAGE; the SDS-PAGE loading buffer lacking reducing agents for YphE10-24 context was used.

Data availability

The crystallographic data that support the findings of this study are available in the PDB with the identifiers: 6GGP for BsGpsA-44; 6GZP for BsGpsA-44; 6JGQ for SpGpsA-45; 6PB2A-40; and 6GQA for unperturbed BsGpsA-44. All the other data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
R.M.C. and Z.R. conducted the biochemical and X-ray crystallography experiments and R.M.C., Z.R. and R.J.L. analysed these data. R.M.C. performed bioinformatics analyses. J.R. and S.H. conducted the Listeria BACTH screen and the fosfomycin resistance assays and analysed both these data. F.A.A. conducted the Bacillus BACTH screen, and F.A.A. and R.A.D. analysed these data. F.C. conducted the Streptococcus BACTH screen and F. C. and O.M. analysed these data. H.-C.T.T. conducted the Streptococcus phenotype and growth analyses and performed the Western experiments and H.-C.T.T., O.M. and M.W. analysed these data. R.M.C., H.-C.T.T., S.H., O.M. and R.J.L. wrote the first draft of the paper and all authors contributed to the final submitted manuscript.

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