Penicillin-Binding Protein 1 (PBP1) of *Staphylococcus aureus* Has Multiple Essential Functions in Cell Division

Katarzyna Wacnik,a,b Vincenzo A. Rao,c Xinyue Chen,b,d Lucia Lafage,a,b Manuel Pazos,e Simon Booth,c Waldemar Vollmer,e Jamie K. Hobbs,b,d Richard J. Lewis,c,* Simon J. Foster,a,b

*aSchool of Biosciences, University of Sheffield, Sheffield, United Kingdom*  
*bThe Florey Institute for Host-Pathogen Interactions, University of Sheffield, Sheffield, United Kingdom*  
*cBiosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom*  
*dDepartment of Physics and Astronomy, University of Sheffield, Sheffield, United Kingdom*  
*eCentre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom*

**ABSTRACT**  
Bacterial cell division is a complex process requiring the coordination of multiple components to allow the appropriate spatial and temporal control of septum formation and cell scission. Peptidoglycan (PG) is the major structural component of the septum, and our recent studies in the human pathogen *Staphylococcus aureus* have revealed a complex, multistage PG architecture that develops during septation. Penicillin-binding proteins (PBPs) are essential for the final steps of PG biosynthesis; their transpeptidase activity links the peptide side chains of nascent glycan strands. PBP1 is required for cell division in *S. aureus*, and here, we demonstrate that it has multiple essential functions associated with its enzymatic activity and as a regulator of division. Loss of PBP1, or just its C-terminal PASTA domains, results in cessation of division at the point of septal plate formation. The PASTA domains can bind PG and thereby potentially coordinate the cell division process. The transpeptidase activity of PBP1 is also essential, but its loss leads to a strikingly different phenotype of thickened and aberrant septa, which is phenocopied by the morphological effects of adding the PBP1-specific β-lactam, meropenem. Together, these results lead to a model for septal PG synthesis where PBP1 enzyme activity is required for the characteristic architecture of the septum and PBP1 protein molecules enable the formation of the septal plate.

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*Staphylococcus aureus*, cell division, penicillin-binding proteins, peptidoglycan

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transpeptidase (TP) activities, polymerize glycan chains and cross-link them into a mesh-like hydrogel (4, 5). Since the cell wall is essential for maintaining bacterial life, PBPs and PG synthesis are a target of some of the most important antibiotics, β-lactams (penicillins) and glycopeptides (vancomycin) (6, 7). The major human pathogen Staphylococcus aureus has a minimalist PBP system, as it encodes only four PBPs, PBP1 to PBP4 (8). Only PBP1 (class B PBP with only TP activity, bPBP) and PBP2 (class A bifunctional PBP with both TG and TP activities, aPBP) are essential and sufficient for septal and peripheral PG synthesis in S. aureus (8, 9). PBP2 is the major PG synthase of S. aureus, and the septum formation activity of PBP2 is mediated by its substrate, lipid II (10). Although PBP2 is essential, loss of its TP activity can be compensated for by a horizontally acquired class B PBP2A in methicillin-resistant S. aureus (MRSA) (11). PBP2A, however, cannot replace PBP1, whose loss is detrimental to the viability of S. aureus (12). PBP1 and PBP3 (bPBP) form cognate pairs with the monofunctional TGs, FtsW and RodA, belonging to the SEDS (shape, elongation, division, and sporulation) family (13) to facilitate septum formation (PBP1-FtsW) and to maintain the prolate cell shape (PBP3-RodA) of S. aureus, respectively (14). Activation of the transglycosylase activity of FtsW requires complex formation with PBP1 (15). PBP4 is a class C PBP with D,D-carboxypeptidase activity (cPBP) and has a TP activity that contributes to the high-level cross-linking of PG and MRSA resistance to β-lactams (16, 17).

The cell wall of Gram-positive bacteria is decorated with wall teichoic acid (WTA) glycolopolymers (18). WTA regulates cell shape, ion homeostasis, autolytic enzymes, growth, and division (19). In S. aureus, WTA plays a crucial role in virulence, MRSA resistance to β-lactam antibiotics, PBP4 localization at the septum, and PG cross-linking (20–23).

Although S. aureus PBPs have been studied over many years, the specific roles of PBP1 in cell division, PG synthesis, and architecture are not well understood. Previous studies have shown that while PBP1 is essential, its TP activity is not, implying another role (12, 14). However, this work was performed in an MRSA background that contains PBP2A, encoded by mecA, which is non-native to S. aureus (24). While PBP2A cannot replace PBP1, how these proteins interact is unknown. We have recently shown that the presence of mecA has a profound effect on cellular physiology (25). Thus, it is important to understand individual and combined roles of S. aureus PBPs in both the presence and absence of the exogenous PBP2A, as the vast majority of S. aureus infections are caused by methicillin-sensitive strains.

RESULTS

S. aureus PBP1 PASTA domains are essential for growth and PBP1 functionality. PBP1 has a short cytoplasmic fragment, a membrane-spanning sequence, an exocytosomic N-terminal pedestal domain, and a C-terminal region consisting of the TP domain and two PASTA domains (for penicillin-binding protein and serine/threonine kinase-associated domain) (26, 27). We created a set of conditional mutants of pbp1 to investigate the role of PBP1 in cell division and PG synthesis. An ectopic copy of pbp1 under the control of the Pspace promoter (Pspace-pbp1) was placed at the lipase locus (geh::Pspace-pbp1) of S. aureus SH1000, and a series of changes were made in this genetic background at the native pbp1 locus: (i) an in-frame deletion of pbp1 (Dpbp1), (ii) a deletion of the region encoding the two PASTA domains (pbp1D PASTA), and (iii) the substitution of the catalytic Ser314 to Ala in the TP domain (pbp1*). We examined the essentiality of PBPI, the PASTA domains, and the active TP domain with these mutants. Depletion of PBP1 via IPTG (isopropyl-β-D-thiogalactopyranoside) removal (Fig. 1C and Fig. S1A and B) resulted in cell death, confirming the essentiality of PBPI (Fig. 1C and D and Fig. S1C and D). Deletion of the PASTA domains also led to growth inhibition and more than 99% cell death within 4 h (Fig. 1D and Fig. S1C and D). Importantly, this phenotype was not associated with PBP1PASTA instability (Fig. 1C and Fig. S1A) or loss of its ability to bind its substrate analogue BocillinFL (Fig. S1B). In contrast, deletion of the PASTA domains of Streptococcus pneumoniae PBP2x, a PBP1 orthologue, resulted in a complete loss of
BocillinFL binding (28). These results indicate that the PASTA domains are essential for *S. aureus* growth and PBP1 functionality but not its stability. During construction of the *pbp1*^STOP^ mutant we obtained, by serendipity, a *pbp1*^STOP^ mutant in which a single-nucleotide polymorphism (SNP) in the codon for Glu292 resulted in its replacement with a premature stop codon and the truncation of the entire TP and PASTAs region of PBP1 (Fig. S1E and F). However, immunoblot analysis using anti-PBP1 sera could not confirm the presence of the PBP1^STOP^ protein in the *pbp1*^STOP^ mutant (Fig. S1G), suggesting that stability of the N-terminal domain of PBP1 is dependent on its C terminus but not the PASTA domains. Although inactivation of PBP1 TP activity (*pbp1*^*) did not affect protein stability (Fig. 1C), it did remove the ability of PBP1 to bind BocillinFL (Fig. S1B). The loss of PBP1 TP activity resulted in severely compromised growth on solid medium (Fig. 1D and Fig. S1C) and reduced cellular viability in liquid culture (Fig. 1E and Fig. S1D). Thus, the TP activity of PBP1 is required for growth in the SH1000 background. Inactivation of the PBP1 TP activity was previously reported not to affect growth in the COL strain background (14). The differences in the necessity for the PBP1 TP activity could result from COL being MRSA, whereas SH1000 is a methicillin-sensitive *S. aureus* (MSSA) strain.

**PBP1 TP activity is crucial in MSSA but not in MRSA.** We have recently developed a set of defined strains where high-level β-lactam resistance of MRSA is mediated by *mecA* encoding PBP2A and a mutation in either *rpoB* or *rpoC* (25). This combination of genetic alterations (*mecA^−^* rpoB) is present in COL (25). To test if the apparent disparity in PBP1’s role is associated with MRSA, we developed a high-level resistant mutant of *pbp1*^*^ in the well-characterized *S. aureus* SH1000 by adding the *mecA* rpoB^H929Q^ to the MSSA *pbp1*^*^ mutant, resulting in SH1000^MSSA* *pbp1*^*^ (Fig. S2A). Inactivating PBP1 TP did not affect the ability of SH1000^MSSA* *pbp1*^*^ to grow in the absence of IPTG, whereas *pbp1* depletion led to growth inhibition in the isogenic Δ*pbp1* MSSA and MRSA strains.

![Figure 1](https://example.com/figure1.png)

**FIG 1** PBP1, its PASTA domains, and transpeptidase activity are essential in MSSA. (A) Schematic representation of genetic constructs used in this study. In *S. aureus* WT (wt) the 5’ end of *pbp1* overlaps with the 3’ end of *ftsL*. The *pbp1* gene encodes a protein containing the short cytoplasmic tail, transmembrane helix (TM), N-terminal pedestal domain (N), transpeptidase (TP) domain, and two PASTA domains (P1 and P2). In the mutants, an ectopic copy of *pbp1* is placed under the control of the PspaB promoter at the lipase (geh) locus, whereas the gene in the native *pbp1* locus is either deleted (Δ*pbp1*), has P1 and P2 domains removed (*pbp1*^ΔPASTA^), or has a point mutation which results in inactivation of the TP domain (*pbp1*^*^). (B) Schematic representation of the domain architecture of PBP1 in *S. aureus* WT (wt) and PBP1 forms produced by Δ*pbp1*, *pbp1*^ΔPASTA^, and *pbp1*^*^ mutants in the absence of inducer. The TP domain inactivation is shown by dotted shading. (C) Immunoblot showing PBP1 levels in SH1000* lacI* (wt) and in Δ*pbp1*, *pbp1*^ΔPASTA^, and *pbp1*^*^ grown with IPTG (+IPTG) and for 0, 1, 2, and 3 h without inducer (–IPTG) analyzed using anti-PBP1 antibody. Expected sizes: PBP1 and PBP1^*^ = 83 kDa (black and gray arrowheads, respectively) and PBP1^ΔPASTA^ = 67 kDa (light blue arrowhead). (D) Plating efficiency of Δ*pbp1*, *pbp1*^ΔPASTA^, and *pbp1*^*^ (MSSA) cells and MRSA *pbp1*^*^ (MRSA) cells upon inducer removal compared to the control groups grown in the presence of inducer. The P value was determined by Mann-Whitney U tests. P = 0.0043 (**, P < 0.01). Data represent the mean ± standard deviation (SD). Error bars that are smaller than the symbols are not shown. Data are representative of three (C and E) and at least four (D) independent experiments.
Thus, the fundamental role of PBP1 in growth and division can only be studied in an MSSA background, as otherwise, the role of PBP1 can be confounded by the presence of the MRSA resistance apparatus.

**PBP1 PASTA domains are required for septum progression.** PG synthesis still occurred in Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} in the absence of IPTG, despite cell growth (Fig. 1D and E and Fig. S2B to D). Thus, the fundamental role of PBP1 in growth and division can only be studied in an MSSA background, as otherwise, the role of PBP1 can be confounded by the presence of the MRSA resistance apparatus.

![Fig 2](image-url)

**FIG 2** Role of PBP1 in cell division and PG synthesis in S. aureus. (A) Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} grown with or without IPTG for 2 h, incubated with HADA for 5 min to show nascent PG, and counterlabeled with NHS-ester Alexa Fluor 555 to image the cell wall. Images are average intensity projections of z stacks. Scale bars = 2 μm. (B) Cell volumes of SH1000 lacI (wt) and Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} grown with (+) or without (-) IPTG for 2 h as measured by fluorescence microscopy after NHS-ester Alexa Fluor 555 labeling. Each dot represents a single cell. The median of each distribution is indicated by a black line. The P value was determined by Mann-Whitney U tests (****, P < 0.0001). From left to right, P = 3.033e-033, 4.670e-049, and 2.206e-022. The number of cells analyzed for each mutant and condition was n ≥ 100. (C) Quantification of cellular phenotypes for SH1000 lacI (wt) and Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} based on HADA incorporation (panel A) after incubation with (+) or without (-) IPTG for 2 h. From left to right, n = 370, 427, 332, 314, 364, 512, and 331. (D) TEM of Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} grown for 2 h in the absence of inducer. Scale bars = 500 nm. (E) Quantification of cellular phenotypes based on TEM data of SH1000 lacI (wt) and Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} grown for 2 h in the presence (+) or absence (-) of IPTG. Examples of cells classified as normal (blue) are shown in Fig. S4B. Cells with abnormal phenotypes (light blue) are shown in panel D and Fig. S4C. From left to right, n = 304, 391, 329, 314, 377, 263, and 302. (F) AFM topographic images of internal surface of purified sacculi from Δpbp1, pbp1\textsubscript{PASTA}, and pbp1\textsuperscript{*} grown in the absence of inducer for 2 h. (Left) Diagram of the inside of the cell before septal plate formation and (right) AFM images of sacculi (top images, scale bars = 500 nm, data scales [z]: 450, 300, and 100 nm from left to right, respectively) and higher magnification images (bottom images, scale bars = 50 nm, data scales [z]: 70, 100, and 50 nm from left to right, respectively) scanned within the boxed areas from the top images. The arrowheads indicate abnormal piecrusts. The boxed areas show details of piecrust features in Δpbp1 and pbp1\textsubscript{PASTA}, and material agglomeration in pbp1\textsuperscript{*}. Data are representative of two (D to F) and (A to C) three independent experiments.
inhibition, as measured by the incorporation of the fluorescent d-amino acid derivative HADA (Fig. 2A). This was not a consequence of the nonsynthesis, exchange reaction carried out by PBP4, as it occurred in pbp4 as well as with the dipeptide ADA-DA (9, 29) (Fig. S3). All variants increased in cell volume upon depletion of pbp1, whereas pbp1<sub>PASTA</sub> was enlarged by almost twice as much as Δpbp1 and pbp1* (Fig. 2A and B and Fig. S4A). Despite differences in cell size, both Δpbp1 and pbp1<sub>PASTA</sub> demonstrated a decrease in the proportion of cells with complete septa compared to the parent (Fig. 2A and C). Transmission electron microscopy (TEM) showed that more than 80% of the population had morphological defects, including cell wall thickening, PG blebs, and misshapen and/or multiple incomplete septa. (Fig. 2D and E and Fig. S4B and C). Such septa had abnormally thick bases and sharply pointed leading edges, suggesting that there is a problem with septal progression after initiation. Atomic force microscopy (AFM) previously revealed that the first step in cell division is the formation of a thick band of PG called the “piecrust” (30). Within this, the septal plate is formed, which has two PG architectures: disordered mesh facing the cell membrane and concentric rings in the septum core (5). Here, lack of PBP1 or the PBP1 PASTA domains led to formation of more than one, and often misplaced, piecrust. These mutations also caused an increase in unfinished septal annuli and alterations in the PG ring architecture (Fig. 2F and Fig. S5A to C, arrowheads), a feature that is revealed immediately after cell scission (5). Thus, depletion of PBP1 did not stop septum initiation, but the loss of the PASTA domains was enough to cause formation of irregular piecrusts, arrest septal plate formation, and lead to an altered septal PG architecture.

**PBP1 TP activity is required for the characteristic septal PG architecture.** The pbp1* mutant gave a novel phenotype quite distinct from loss of entire PBP1 or PASTA domains. Inactivation of PBP1 TP activity did not prevent initiation and closing of the septa but, instead, resulted in accumulation of cells with aberrant septa and separation defects in about 80% of the population (Fig. 2A, C, and E). The septa in such cells had a rounded leading edge, were curved and abnormally thick (Fig. 2D and E and Fig. S4B and C), and had agglomerations of mesh-like material close to the septal center in addition to irregular piecrusts as observed by AFM (Fig. 2F and Fig. S5A and B). The intracellular agglomerations are PG, as they stain heavily with HADA and ADA-DA (Fig. 2A and Fig. S3C and F) and could be observed in purified sacculi (Fig. 2F and Fig. S5B). No ring architecture, only mesh-structured PG, could be observed on the surface of the pbp1* mutant. Importantly, with fluorescence microscopy, the pbp1* pbp3 pbp4 mutant, in which PBP2 is the only active TP, presented a similar phenotype upon IPTG removal as pbp1*, exemplified by misshapen septa and agglomerations of PG material marked by HADA (Fig. S4D). Therefore, aberrant septal synthesis and progression occur in the pbp1* mutant. Associated PG synthesis results from PBP2 transpeptidase activity and potentially the transglycosylase activity of FtsW, acting to produce un-cross-linked glycan strands.

The pbp1* phenotype occurred specifically because of the loss of the TP activity of this essential enzyme. This phenotype is mirrored by the mode of action of β-lactam antibiotics, which bind to and inhibit the TP activity of PBPs (7). We have recently described the morphological effects of methicillin and oxacillin on *S. aureus*, which result in cell swelling and cessation of septal and peripheral cell wall synthesis (31). Our results suggest that PBP1 TP activity has a role in septal plate formation, and without this, the septum is misshapen. The conditional lethal strains made here allow for functional analysis of the genes concerned. However, phenotypes tend to accumulate on depletion of the wild-type protein over time, confusing the precise roles for individual components. To independently corroborate the role of the TP activity of PBP1, we utilized an approach to directly, and selectively, inhibit its activity. Meropenem (MEM) has a higher affinity for PBP1 than PBP2 (32, 33), and therefore, we hypothesized that its effect on *S. aureus* would match that of pbp1*. In a MEM-titration, treatment with 1 × MIC of MEM was sufficient to lead to cell death and a significant increase in SH1000 wild-type (WT) cell volume after 1 h (Fig. 3A and B and Fig. S6A). More than 70% of
MEM-treated cells had growth defects that manifested as aberrantly shaped septa and accumulation of PG as shown by HADA labeling (Fig. 3A, C, and D and Fig. S6C and E), similar to observations made with the \( \text{pbp1}^* \) mutants (Fig. 2A and C to E and Fig. S3C and F). The MEM phenotype of malformed septa was not linked to PBP3 or PBP4, as it was also observed in the corresponding double mutant (Fig. 3C and D and Fig. S6B, D, and F), which corroborated the role of PBP2 in misshapen septal genesis. The MEM phenotype differed from methicillin treatment, which inhibits both PBP1 and PBP2, as this results in a cessation of PG synthesis and apparent plasmolysis (31).

**PASTA domains mediate PBP1 interaction with division-associated components.**

The morphologies of the \( \Delta \text{pbp1} \) and \( \text{pbp1}_{\text{PASTA}} \) mutants resemble \( S. aureus \) depleted of DivIB in which EzrA and FtsZ form multiple rings and the synthesis of the cross wall is blocked, despite the normal recruitment of early cell division proteins and piecrust formation (34). EzrA is a marker of early division protein recruitment but also remains until septal completion (9, 35). In the \( \Delta \text{pbp1} \text{ezrA-gfp} \) strain, EzrA, which here acts as an early cell division marker, was localized at midcell in the majority of cells and formed additional arcs or rings in 33% of the population (Fig. 4A and D). Multiple EzrA rings were observed in 43% of the \( \text{pbp1}_{\text{PASTA}} \text{ezrA-gfp} \) mutant cells (Fig. 4B and D), supporting the requirement for PBP1 PASTA domains for correct selection of the division site and/or

**FIG 3** Effect of meropenem (MEM), an antibiotic with high affinity for PBP1, on \( S. aureus \). (A) Fluorescence images of SH1000 WT treated with 1× MIC of MEM for 1 h, labeled with HADA for 5 min to show nascent PG, and counterlabeled with NHS-ester Alexa Fluor 555 (cell wall). Images are average intensity projections of z stacks. Scale bars = 2 μm. (B) Cell volumes of SH1000 WT and \( \text{pbp3 pbp4} \) treated with 1× MIC of MEM for 1 h as measured by fluorescence microscopy after NHS-ester Alexa Fluor 555 labeling (panel A). Each dot represents a single cell. The median of each distribution is indicated by a black line. The \( P \) value was determined by Mann-Whitney \( U \) tests (****, \( P < 0.0001 \)). From left to right, \( P = 1.276e-042 \) and 1.303e-034. The number of cells analyzed for each condition was \( n > 100 \). (C) TEM of SH1000 WT and \( \text{pbp3 pbp4} \) treated with 1× MIC of MEM for 1 h. Scale bars = 200 nm. (D) Quantification of phenotypes of SH1000 WT and \( \text{pbp3 pbp4} \) treated with MEM (1× MIC) for 1 h based on TEM data (panel C and Fig. S6E and F). Examples of cells classified as normal (blue) are shown in Fig. S6E and F. Cells with abnormal phenotypes (light blue) are shown in panel C and Fig. S6E and F. From left to right, \( n = 343, 287, 403, \) and 365. Data are representative of two independent experiments.
septal progression. Alternatively, the multiple division rings could result from a lack of the septal progression whereby the unproductive division machinery results in futile additional alternative initiation attempts, suggesting that PASTA domains are involved in the progression from piecrust to septal plate formation. While the number of cells with complete septa (EzrA-GFP visible as a line or focus) decreased by at least 6-fold in \( \text{pbp1} \text{ezrA-gfp} \) and \( \text{pbp1} \text{D}\text{PASTA ezrA-gfp} \), only \( \text{pbp1}^* \text{ezrA-gfp} \) grown with or without IPTG, respectively; Fig. 4C and D), confirming that septum progression, although reduced, still occurred when PBP1 TP was inactive, implying that TP activity is necessary for correct septal architecture during cell division.

PBP1 is important for the septal surface PG ring structure (Fig. 2F and Fig. S5B), where it has been proposed that mature WTA is not present throughout the septum (23, 36). Loss of WTA also results in a proportion of cells with aberrant septa (21), suggesting a potential link with PBP1 function. Loss of tarO (leading to a lack of WTA) caused minor cell division defects in SH1000 (Fig. S7A, E, and F). Combining tarO with the mutations in pbp1 exacerbated the observed morphological defects, with the appearance of distinct septal and off-septal PG foci appearing (marked with HADA) in \( \Delta\text{pbp1} \text{tarO} \) and \( \text{pbp1} \text{D}\text{PASTA tarO} \) (Fig. S7B to F), demonstrating that both WTA and PBP1 are involved in cell cycle progression in parallel.

**FIG 4** Role of PBP1, PASTA, and TP domains in EzrA localization in S. aureus. (A to C) Localization of EzrA-GFP in \( \Delta\text{pbp1} \text{ezrA-gfp} \), \( \text{pbp1} \text{D}\text{PASTA ezrA-gfp} \), and \( \text{pbp1}^* \text{ezrA-gfp} \) grown in the presence or absence of IPTG for 2 h and labeled with HADA for 5 min to stain PG. Images are average intensity projections of z stacks. Scale bars = 5 \( \mu \)m. (D) Quantification of EzrA-GFP localizations in \( \Delta\text{pbp1} \text{ezrA-gfp} \), \( \text{pbp1} \text{D}\text{PASTA ezrA-gfp} \), and \( \text{pbp1}^* \text{ezrA-gfp} \) grown with or without IPTG. “Abnormal” includes those cells with multiple and/or misplaced EzrA rings. From left to right, \( n = 395, 499, 481, 438, 360, \) and 382. Data are representative of two independent experiments.
As PBP1 PASTA has a role in the regulation of septal plate formation, this may be determined by interacting with other protein components. In order to examine this hypothesis, we performed a bacterial two-hybrid assay, in which PBP1 has previously been found to have apparent, multiple interactions (35). Truncation of the PASTA domains reduced *S. aureus* PBP1 interaction not only with DivIB but also with FtsW, while recognition of other known interacting partners of PBP1 (EzrA, PBP2, and DivIC) were unaffected by the PASTA truncation (Fig. S8A and B), suggesting that these potential, wider interactions involve the N-terminal domain of PBP1.

**PBP1 PASTA domains bind peptidoglycan.** Impaired interaction with DivIB could be one explanation for why cells depleted of PBP1 PASTA domains initiate irregular piecrusts and septation defects accrue as a consequence. PASTA domains have long been associated with PG binding because of work performed mainly on serine/threonine protein kinases (STPK) (26, 37–39). Very recently, PBP1 PASTA domains have been shown to bind isolated, small fragments of PG (27). Therefore, we assessed whether *S. aureus* PBP1 and its PASTA domains could recognize PG by measuring their affinities for *S. aureus* cell wall PG with or without WTA (±WTA) with a semiquantitative fluorescence-binding assay and *S. aureus* PBP1 derivatives produced in *Escherichia coli* (Fig. 5A and Fig. S8C). Cytochrome c (34) was used as a negative control to rule our nonspecific binding (dissociation constant \(K_d\), 1,126 ± 37 nM [+WTA] and 1,171 ± 363 nM [–WTA]) (Fig. S8D). Both SaPBP1 (\(K_d\) 19 ± 4 nM [+WTA] and 115 ± 21 nM [–WTA]) and its PASTA domains (SaPASTAPBP1; \(K_d\) 198 ± 42 nM [+WTA] and 109 ± 23 nM [–WTA]) bound PG (Fig. 5B). Inactive SaPBP1* was still able to bind PG with a preference for PG with WTA present (\(K_d\) 53 ± 8 nM [+WTA] and 227 ± 46 nM [–WTA]; Fig. 5B), similar to active SaPBP1. Although removal of the PASTA domains did not abolish BocillinFL binding (Fig. S8C), it considerably reduced the ability of SaPASTA1 to bind PG, and binding was abolished in the presence of WTA (\(K_d\) >2,000 nM [+WTA] and 440 ± 57 nM [–WTA]; Fig. 5B). In contrast, the PASTA domains (SaPASTAPBP1) on their own bind to *S. aureus* PG but are incapable of binding BocillinFL (Fig. 5B and Fig. S8C). These results demonstrate that PBP1 is a PG-binding protein, and the PASTA domains have a dominant role in this interaction. Sequence conservation analysis of PASTA domains revealed the presence of either Arg or Glu residues in classifying a PASTA domain as a PG-binder (40). The PASTA domains of *S. aureus* PBP1 each have proline at the equivalent positions (residues Pro603 and Pro661), and thus PBP1 would be predicted as a non-PG-binder. Our data suggest that the predicted significance of conserved Arg or Glu residues with regard to PG binding is either only relevant to PASTA domains found in STPKs, linear arrangements of tandem PASTA repeats, or is not suitable for proteins with multiple and complex functions like PBPs.

To gain a better understanding of the role of the PASTA domains in *S. aureus* PBP1 (SaPASTAPBP1), we determined their structure by X-ray crystallography. Soluble recombinant protein was obtained in high yield from the cytoplasm of *E. coli* cells, and well-ordered crystals were subsequently produced that diffracted to a maximum resolution of 1.78 Å (Table S1). The structure was solved by molecular replacement using the corresponding PASTA domains present in *S. pneumoniae* PBP2x from PDB entry 5OAU (41), which shares 26% sequence identity with SaPASTAPBP1. The asymmetric unit contains two monomers (labeled A and B), each forming a 2-layer sandwich comprising an α-helix and a three-stranded antiparallel β-sheet, distinct from the TP domain (Fig. 5C). Clear and continuous electron density allowed the modeling and unambiguous assignment of both PASTA domains (Fig. 5C). When SaPASTAPBP1 is compared with other structures deposited in the PDB using DALI (42), the top hit identified was *S. pneumoniae* PBP2x (Z-score, 15.7), showing a significant conservation of the PASTA fold despite low sequence identity (Fig. 5C). Unlike the linear arrangement observed for PASTA domains in serine/threonine kinases (43, 44), SaPASTAPBP1 adopts a compact upside-down globular arrangement (Fig. 5C). The arrangement of the two PASTA domains solved here, in isolation from the TP domain in comparison to structural analyses of SpPBP2x, is entirely consistent with a nonlinear PASTA domain arrangement. First, the structures of SaPASTAPBP1 and the PASTA domains

*Staphylococcus aureus* PBP1

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of SpPB2x share a pairwise root mean square deviation (RMSD) of 2.2 Å over 114 Cα, and when SaPASTA_{PBP1} is superimposed on the PASTA domains of SpPB2x, there are no steric clashes with the TP domain. Second, the linker between PASTAs in SaPASTAPBP1 has a sequence of DGDLTMPDMSGW, is neither glycine- nor alanine-rich, is not predicted to be disordered using the IUPred2 or ANCHOR2 web servers, and has a mean B factor of 44 Å² in comparison to a mean B factor of 42 Å² for the entire chain. Third, the interface between the PASTA domains is more reminiscent of the hydrophobic core of a globular
protein than the more polar interface observed between molecules in crystal packing. Finally, the two proline residues that apparently define PBP1 as a nonbinder of PG are found buried from solvent either at the interface of PASTA domain 1 with the TP domain (Pro603) or at the interface between the TP domain and PASTA domains 1 and 2 (Pro661). The latter interface includes the only tryptophan (Trp666) in the sequence of SaPASTAPBP1; tryptophan residues are frequent markers of carbohydrate binding sites in proteins (45), and in the absence of any obvious grooves or surface features associated with conserved sequence distributions and/or electrostatics, it remains unclear how the PASTA domains of SaPBP1 recognize PG.

**DISCUSSION**

*S. aureus* has just two essential PBPs (46) and so forms an apparently simple system to understand cell wall growth and division. Even the transpeptidase activity of these two enzymes can be substituted by a single enzyme in the presence of β-lactam antibiotics via the acquisition of PBP2A, encoded by *mecA*, in MRSA strains. Our recent study has revealed that the presence of *mecA* and associated genetic lesions has a profound effect on *S. aureus*, even in the absence of antibiotics (25), leading to the discovery here that the PG biosynthetic activity of PBP1 is essential in MSSA but not in MRSA (Fig. 1D). This observation has important ramifications for many studies in *S. aureus*, where the use of an MRSA background can complicate phenotype interpretation. To understand the fundamental role of PBP1 activity in basic cell physiology, we have thus used an MSSA strain with a defined genetic background.

The essential function of PBP1 is associated with its crucial role in septal PG synthesis (14, 47). Here, we show that PBP1, in MSSA, has roles in both early and later stages of septum synthesis and can interact with other cell division components and make and bind to PG. PG binding is primarily mediated by the PASTA domains that are essential for cell division. There is clear overall structural similarity between *S. aureus* PBP1 and *S. pneumoniae* PBP2x PASTA domains in the way that the two tandem PASTA domains associate into an antiparallel bundle (Fig. 5C); this is in marked contrast to the head-to-tail linear PASTA domain repeats more typically found in STPKs. The highly hydrophobic interface between the two PASTA domains means it is unlikely to open up like butterfly wings to bind to PG; similarly, an extensive, linear interaction with PG, which is likely to occur with the head-to-tail PASTA domain arrangements seen in STPKs and which may require their dimerization (44), does not occur in SaPBP1.

Despite the successful production of diffracting crystals of SaPASTAPBP1 grown in the presence of PG fragments (including an N-acetylglucosamine:N-acetylmuramic acid disaccharide), none of the structures yielded electron density features consistent with the stable binding of PG fragments. There are several potential explanations, including a lack of affinity of PASTA domains for small PG fragments, unrepresentative of the sacculus of *S. aureus*; our sedimentation assay does not permit the analysis of the binding of PASTA domains to small, soluble PG precursors. Consequently, and in common with all other PASTA domain structural analyses, the molecular details of PG recognition by SaPBP1 remain elusive. During the preparation of the manuscript, Martínez-Caballero et al. (27) published a crystal structure of the two PASTA domains of PBP1, also in the absence of endogenous ligand, which is indistinguishable (RMSD, 0.7 Å over 204 superimposed residues) from the structure that we report here. The same authors also solved structures of SaPBP1 in the presence and absence of β-lactams and pentaglycine (in which the PASTA domains were disordered). The latter structural analysis revealed that the pentaglycine substrate mimetic is not long enough to span between the transpeptidase active site and the PASTA domains, suggesting that the PG feature(s) recognized by the PASTA domain is/are chemically more complex than a simple short polypeptide.

*S. aureus* is a spheroid coccus that can divide successively in orthogonal planes (30, 48). Septation is first observed as the formation of a band of PG known as the piecrust (30). This then transitions to the production of the septal plate itself, an initially V-
shaped structure with a narrower leading edge (9). After closure of the septal annulus, 
the now bowed septum fills out to yield the mature structure prior to septal scission.
The septal plate has two distinct PG architectures with a ring-like pattern at its core, 
which is exposed upon scission, and a subsequently synthesized fine mesh, akin to the 
rest of the peripheral cell wall (5). Loss of the entire PBP1, or just its PASTA domains, 
does not prevent piecrust formation but does result in multi- and/or off-center pie-
crusts without the ability to produce the septal plate (Fig. 2F). Thus, piecrust formation 
does not require PBP1 but is likely the result of the activity of the essential PBP2. PBP1 
may regulate division site selection through PG cell wall recognition via its PASTA 
domains. Alternatively, as the division apparatus is unable to progress effectively to 
septal plate formation due to the lack of PBP1, this may lead to further rounds of initia-
tion and piecrust formation. PBP1 has a clear role in septal plate formation where in 
the absence of PBP1 or the PASTA domains, cells form aberrantly shaped septa that do 
not close their annuli (Fig. 2A to E). In stark contrast, inactivation of PBP1 TP activity 
(pbpb1+) does not stop inward septum progression, as observed with loss of PBP1 or the 
PASTA domains. However, such septa are misshapen, curved, and abnormally thick 
(Fig. 2A to E and Fig. 3). The use of the PBP1-specific antibiotic MEM at 1 × MIC led to 
the similar morphology of thickened and missshapen septa. Two independent avenues 
of research both led to the conclusion that PBP1 TP activity is essential for continued 
division and colony formation, and while septum formation is disturbed, it is not entirely prevented. Therefore, PBP1 retains its regulatory function(s) regardless of activity 
loss. Loss of PBP1 activity may result in futile glycan strand synthesis (49) by its partner 
transglycosylase FtsW (14) and/or the continued activity of PBP2, resulting in the observed aberrant septa and stasis. FtsW in S. aureus is essential and required for sep-
tum progression (14). In Bacillus subtilis the cell division-associated PBP2B is essential, 
but its enzyme activity is not and can be compensated for by PBP3 (50). Here, in the 
pbp3 pbp4 background loss of PBP1 activity did not lead to death of the cells (Fig. S4E 
and F), suggesting that the nonessential enzymes do not support survival in the ab-
sence of PBP1 TP activity, whereas deletion of the PASTA domains leads to rapid death 
of the cells (Fig. 1D) due to loss of protein functionality not observed in the pbpb1+. 
Differences in plating efficiency and rate of loss of cellular viability between Δpbp1 and 
spbpb1const may reflect aberrant function of the truncated protein. As well as binding to 
the cell wall, PBP1 also apparently interacts with multiple protein partners, including 
EzrA, DivIB/C, PBP2, and FtsW (Fig. S8A and B) (14, 35). Recently, the PASTA domains 
from B. subtilis PBP2B were shown to regulate PBP2B interaction with DivIB (51). S. aur-
eus DivIB is a PG-binding protein essential for division, the depletion of which leads to 
septal plate formation loss (34, 35). Here, the PBP1 PASTA domains were found to be 
involved in binding to DivIB and FtsW, alluding to their essential role in cell division. 
This could be a direct interaction, or loss of PBP1 PASTA may cause a conformational change in the remaining protein, as other’s data suggest the FtsW-PBP1 interaction 
occurs via the PBP1 stalk domain (52). FtsW is a SEDS protein whose TG activity 
requires the presence of PBP1. Bifunctional aPBP (including PBP2) and bPBP-SEDS 
(including PBP1-FtsW) pairs share similar activities, but the fact that they coexist in 
many bacterial species implies there is a division of responsibilities between them. 
Indeed, it has been proposed lately that bPBP-SEDS pairs likely lay the primary PG ma-
trix, while aPBP’s support the initial PG by modifying, filling in, and adding PG to it (53, 
54). The S. aureus septal plate PG has two distinct architectures, a disordered mesh 
present on its cytoplasm facing side and a ring structure at its core, which is revealed 
after the cells have split (5, 30) (Fig. 6). Recent AFM analysis from Staphylococcus war-
neri also describes the distinct PG architectures during septation as piecrust and septal 
plate rings/mesh (55). When sacculi are purified from S. warneri, the septum can split 
apart, revealing the rings, even in septa that have not closed their annulus, showing 
that the rings are not a likely result of PG hydrolysis during cell scission. We hypothe-
size (Fig. 6) that once the piecrust has been produced, PBP1 and FtsW use this as a 
foundation to initiate septal plate formation. Together they make the rings of material
that become the core of the developing septum, providing the framework for PBP2 to make the bulk of the septal plate as a tight mesh alongside PBP4 and the insertion of WTA via the tar pathway. Loss of PBP1 TP activity in the presence of active PBP2 leads to the lack of the ring framework and aberrant, unproductive septum formation. The rings that form the center of the developing septum also provide the cleavage plane during scission.

Cell division is a fundamental requirement for life. A central question in bacteria is how is the division septum synthesized and then split to yield two daughter cells while maintaining cellular integrity in the face of internal turgor? Here, we have begun to answer this question by revealing the complex synthesis coordination mechanisms that allow this biological engineering feat to be accomplished.

**MATERIALS AND METHODS**

**Bacterial growth conditions.** The strains used in this study are listed in Table S1A.

All *Staphylococcus aureus* strains were grown in tryptic soy broth (TSB) containing appropriate antibiotics at 37°C, unless otherwise indicated, with aeration. All *Escherichia coli* strains, unless otherwise stated, were grown in Lysogeny broth (LB) containing appropriate antibiotics at temperatures ranging from 20°C to 37°C with aeration. For solid medium, 1.5% (wt/vol) agar was added. When necessary, growth medium was supplemented with kanamycin (50 μg mL⁻¹), tetracycline (1 μg mL⁻¹), chloramphenicol (10 μg mL⁻¹), *S. aureus*; 30 μg mL⁻¹, *E. coli*), erythromycin (5 μg mL⁻¹), spectinomycin (250 μg mL⁻¹), ampicillin (100 μg mL⁻¹), meropenem (0.4 μg mL⁻¹, 1× MIC for SH1000 WT; 0.2 μg mL⁻¹, 1× MIC for *pbp3 pbp4*), 5-bromo-4-chloro-3-indolyl β-β-thiogalactopyranoside (X-Gal; 80 μg mL⁻¹, *S. aureus*; 40 μg mL⁻¹, *E. coli*), or isopropyl β-β-thiogalactopyranoside (IPTG; 50 μM or 1 mM).

**FIG 6** Conceptual model of septum formation in *S. aureus*, where PBP1 is required for septum formation and its characteristic ring-like PG architecture. (A and B) The growing *S. aureus* cell increases in volume (63). (C) Septal synthesis starts by formation of the piecrust (red) (30). This occurs as the result of the activity of PBP2 and forms the foundation for the septal plate. (D and E) The V-shaped septal plate (9) progresses inward by insertion of an initial, concentric ring-like structured PG synthesized by PBP1-FtsW at its core. Without PBP1 or PBP1 PASTA, the septal plate cannot be initiated, but in PBP1÷ it progresses but is aberrant. The PBP1-derived ring structure acts as a framework for the ensuing mesh-structured PG produced by PBP2. (F) The annulus closes, resulting in a bowed septum. (G) The septum is filled out by peptidoglycan insertion executed by PBP2, and this continues until the cross-wall is of uniform thickness (9). (H) The cell wall is hydrolyzed at the plane of septation. (I) Daughter cells separate. The cell wall of the daughter cell (colored insets) is a chimera of the old cell wall with both internally and externally mesh-structured PG and a nascent cell wall with the external ring-structured PG and the mesh-like cytoplasmic facing PG (5).
**Plasmid construction.** The plasmids and oligonucleotides used in this study are listed in Table S1 parts B and C, respectively.

Plasmids were cloned using *E. coli* NEB5a following previously described methods (56, 57).

**pKB-Pspac-pbp1**. A fragment containing the ribosome-binding site (RBS) and coding region of *S. aureus* pbp1 was PCR amplified from the genomic DNA of *S. aureus* SH1000 using pCQ-pbp1-F/-R primers and cloned into pCQ11-Fis-Z-SNAP (9) cut with Nhel and Ascl by Gibson assembly, resulting in pCQ11-Pspac-pbp1. Next, the region containing Pspac, RBS, and pbp1 was PCR amplified from pCQ11-Pspac-pbp1 using pKB-Pspac-pbp1-F/-R primers and cloned into cloned into BamHI and EcoRI cut pKASBAR (34) by Gibson assembly, giving pKB-Pspac-pbp1.

**pMAD-Δpbp1**. Fragments encompassing 1-kb regions flanking upstream (from −980 bp upstream of pbp1) to the first 20 bp of pbp1 and downstream (from 2,214 bp of pbp1 to 970 bp downstream of pbp1) were PCR amplified from *S. aureus* SH1000 genomic DNA using primer pairs pbp1-A/-B and pbp1-C/-D, respectively, and cloned into BamHI and EcoRI cut pMAD by Gibson assembly, creating deletion vector pMAD-Δpbp1.

**pMAD-pbp1**. Fragments encompassing 1.5-kb regions flanking the region encoding pbp1 PASTA domains (upstream, from 286 bp to 1,785 bp of pbp1; downstream, from 2,214 bp of pbp1 to 970 bp downstream of pbp1) were PCR amplified from *S. aureus* SH1000 genomic DNA using pbp1-E/-F and pbp1-G/-H primers and cloned into BamHI and EcoRI cut pMAD by Gibson assembly, resulting in deletion vector pMAD-pbp1.

**pVR plasmids.** Full-length pbp1 (M1-D744) was *E. coli* codon optimized, synthesized with GenScript, PCR amplified using VR7F/R, and cloned into KpnI and HindIII cut pOPINRF using In-Fusion cloning (TaKaRa Bio), resulting in pVR01. Construction of pVR02 (SoPBP1, M37-D744) and pVR06 (SoPASTA_pbp1, S595-D744) was performed using inverse PCR (iPCR) (58), with pVR01 as a template and primer pairs VR49F/VR49R, VR5F/VR5R, and VR3F/VR3R, respectively. PCR products were ligated with pMAD cut with EcoRI and BamHI by Gibson assembly, resulting in pVR-pbp1.

**T25-PBP1**. A fragment carrying *S. aureus* pbp1 without the PASTA domains (M1-S595) was PCR amplified from *S. aureus* SH1000 genomic DNA using T25-pbp1-F and T25-pbp1pasta-R and cloned into BamHI and EcoRI cut pKT25, resulting in T25-PBP1.

**pVR plasmids.** Full-length pbp1 (M1-D744) was *E. coli* codon optimized, synthesized with GenScript, PCR amplified using VR7F/R, and cloned into KpnI and HindIII cut pOPINRF using In-Fusion cloning (TaKaRa Bio), resulting in pVR01. Construction of pVR02 (SoPBP1, M37-D744) and pVR06 (SoPASTA_pbp1, S595-D744) was performed using inverse PCR (iPCR) (58), with pVR01 as a template and primer pairs VR49F/VR49R, VR5F/VR5R, and VR3F/VR3R, respectively. PCR products were ligated with pMAD cut with EcoRI and BamHI by Gibson assembly, resulting in pVR-pbp1.

**Construction of *S. aureus* mutants.** All vectors were passed through a restriction-deficient *S. aureus* RN4220 before being transformed into a final *S. aureus* SH1000 strain. Transformation and phage transduction of *S. aureus* were carried out as described previously (59, 60).

**Δpbp1, pbp1, and pbp1**. For construction of pbp1 mutation strains, first, an ectopic copy of pbp1 under the control of the Pspac promoter was introduced at the lipase (geh) locus. Electrophoretic YT136 was transformed with pKB-Pspac-pbp1. The chromosomal fragment containing the integrated plasmid was moved into *S. aureus* SH1000 by phage transduction, resulting in SJF4588 (*S. aureus* SH1000 geh::Pspac-pbp1). Next, electrocompetent RN4220 was transformed with pMAD-pbp1, pMAD-pbp1_paste, or pMAD-pbp1*, and the plasmids were moved to SJF4588 by phage transduction. Integration at 42°C and excision at 28°C of pMAD-Δpbp1, pMAD-pbp1_paste, or pMAD-pbp1* resulted in strains SJF5116, SJF5275, and SJF4590, respectively. To allow controlled expression of pbp1 from Pspac, pGL485, a multicopy plasmid carrying lacI was introduced, creating strains Δpbp1 (*S. aureus* SH1000 geh::Pspac-pbp1 Δpbp1 lacI), pbp1_paste, (*S. aureus* SH1000 geh::Pspac-pbp1 pbp1_paste lacI), and pbp1* (*S. aureus* SH1000 geh::Pspac-pbp1 pbp1* lacI). On all occasions, consistent colony size and growth kinetics were monitored to prevent the selection of suppressor mutations.

**MRSA Δpbp1 and MRSA pbp1**. In order to construct high-level β-lactam-resistant mutants, Δpbp1 and pbp1* were transformed with a phage lysate from SJF5046 (B. subtilis SH1000 lysA::pmeC A ropB[8203] with selection for erythromycin resistance, resulting in low-level β-lactam-resistant Δpbp1 pmeC A and pbp1* pmeC A. The low-level-resistant mutants were transduced again with the phage lysate from SJF5046 and selected for kanamycin resistance, resulting in MRSA Δpbp1 (S. aureus SH1000 geh::Pspac-pbp1 Δpbp1 lacI lysA::pmeC A ropB[8203]) and MRSA pbp1* pbp1 (S. aureus SH1000 geh::Pspac-pbp1 pbp1* lacI lysA::pmeC A ropB[8203]). MIC values were determined using antibiotic susceptibility tests using Etest MIC evaluator (Oxoid) strips.

**pbp3 pbp4.** SH1000 was transformed with a phage lysate from NE420 (S. aureus JE2 pbp3::Tn), resulting in SH4421 (S. aureus SH1000 pbp3::Tn). To swap the erythromycin resistance cassette to a kanamycin cassette, SH4425 (S. aureus SH1000 pbp4::Tn) was transduced with a phage lysate from NE3004 (S. aureus RN4220 pKAN). Integration at 42°C and excision at 28°C of pKAN resulted in strain SH5115 (*S. aureus
SH1000 ppb4-kan). SH4421 was subsequently transduced with a phage lysate from SH5115 (S. aureus SH1000 ppb4-3Tn, resulting in ppb3 ppb4 (SH5483; S. aureus SH1000 ppb3-3Tn ppb4-kan).

\[ \Delta \text{ppb}1 \text{ ppb}4, \text{ ppb}3 \text{ spc-pbp}1, \text{ and ppb}1^* \text{ were transduced with a phage lysate from SH5115 (S. aureus SH1000 ppb4-kan), resulting in } \Delta \text{ppb}1 \text{ ppb}4, \text{ and ppb}1^* \text{ were transduced with a phage lysate from SH4421 (S. aureus SH1000 ppb3-3Tn ppb4-kan).} \]

ppb1* ppb3 ppb4, ppb1* ppb3, and ppb1* were transduced with a phage lysate from SH4421 (S. aureus SH1000 ppb3-3Tn), resulting in ppb1* ppb3 ppb4 (S. aureus SH1000 geh:Pspac-ppb1 ppb1* lacz ppb4-kan), ppb1* ppb3, and ppb1* were transduced with a phage lysate from SH4421 (S. aureus SH1000 ppb3-3Tn), resulting in ppb1* ppb3 ppb4 (S. aureus SH1000 geh:Pspac-ppb1 ppb1* lacz ppb4-kan), respectively.

\[ \Delta \text{ppb}1 \text{ ppb}4, \text{ ppb}1^* \text{ spc-pbp}1, \text{ and ppb}1^* \text{ were transduced with a phage lysate from JLG227 (S. aureus SH1000 eza-gef+) (35), resulting in } \Delta \text{ppb}1 \text{ eza-gef} (S. aureus SH1000 geh:Pspac-ppb1 } \Delta \text{ppb1 lacz eza-gef}, \text{ ppb1* spc-pbp1 eza-gef} (S. aureus SH1000 geh:Pspac-ppb1 ppb1* spc-pbp1 lacz eza-gef), \text{ and ppb1* eza-gef} (S. aureus SH1000 geh:Pspac-ppb1 ppb1* lacz eza-gef), \text{ respectively.} \]

\[ \Delta \text{ppb}1 \text{ eza-gef}, \text{ ppb1* spc-pbp1, and ppb1* eza-gef, } \Delta \text{ppb}1 \text{ ppb}4, \text{ and ppb1* were transduced with a phage lysate from } \Delta \text{tarO} \text{ (S. aureus SA113 } \Delta \text{tarO} \text{; pUC1-tarO+ (31), resulting in } \Delta \text{ppb}1 \text{ tarO, } \Delta \text{ppb}1 \text{ spc-pbp1, and ppb1* were transduced with a phage lysate from } \Delta \text{tarO} \text{ (S. aureus SH1000 geh:Pspac-ppb1 } \Delta \text{ppb1 lacz } \Delta \text{tarO; ery}, \text{ ppb1* spc-pbp1 tarO (S. aureus SH1000 geh:Pspac-ppb1 ppb1* lacz } \Delta \text{tarO; ery}, \text{ and ppb1* tarO (S. aureus SH1000 geh:Pspac-ppb1 ppb1* lacz } \Delta \text{tarO; ery), respectively.} \]

PPB1 depletion. Pspac-ppb1 strains were grown from an optical density at 600 nm (OD600) of 0.1 to the exponential phase (OD600 ~0.5) in TSB containing 10 μg mL⁻¹ chloramphenicol and 50 μM IPTG. Cells were washed three times by centrifugation and resuspension in TSB. Washed cells were then used to inoculate TSB containing 10 μg mL⁻¹ chloramphenicol. Cultures were inoculated to an OD600 of 0.05 for phenotypic studies and an OD600 of 0.005 for growth studies. For phenotypic analysis, cultures were incubated for 2 h to allow depletion of PPB1 before microscopy imaging. Control samples were grown in TSB supplemented with 10 μg mL⁻¹ chloramphenicol and 1 mM (50 μM, eza-gef mutants) IPTG.

For the plating efficiency test, cells grown in the presence of 10 μg mL⁻¹ chloramphenicol and 50 μM IPTG to the exponential phase (OD600 ~0.5) were washed three times in phosphate-buffered saline (PBS). Serial dilutions of washed cells were plated on TSB containing 10 μg mL⁻¹ chloramphenicol, with or without 1 mM IPTG. Relative plating efficiency (% CFU) is expressed as the number of cells that grow on plates without IPTG (CFUw/IPTG) to cells that grow in the presence of IPTG (CFUw/IPTG) multiplied by 100%:

\[ \% \text{CFU} = \frac{\text{CFU}_{\text{w/IPTG}}}{\text{CFU}_{\text{w/IPTG}}} \times 100\% \]

Meropenem activity assays. S. aureus strains were grown overnight in TSB. The overnight cultures were used to inoculate fresh TSB medium to an OD600 of 0.05. When cells reached an OD600 of 0.2 to 0.4, meropenem was added, and the change of bacterial count was monitored. The CFU per mL of culture measures were normalized to the initial CFU/mL at the time of the antibiotic addition, at time zero (t₀).

Relative CFU/mL = \( \frac{\text{CFU}_{\text{w/IPTG}}}{\text{CFU}_{\text{w/IPTG}}} \)

For phenotypic analysis, cells were treated for 1 h with 1× MIC meropenem before microscopy imaging.

Fractionation of S. aureus membranes. The membrane fraction of S. aureus was prepared as previously described (61) with the following modifications. S. aureus cells grown to the appropriate growth phase were recovered by centrifugation (5,000 × g, 10 min, 4°C) and washed three times by resuspension and centrifugation (5,000 × g, 10 min, 4°C) in PBS. Cells were resuspended in 50 mM Tris, 100 mM NaCl, pH 8.0 containing Complete Protease Inhibitor (Roche) and broken using 1.1-mm silica spheres (lysis matrix B) and FastPrep homogenizer (MP Biomedicals) in 12 cycles of 30 s, at maximum speed (6.5 m s⁻¹), with 5 min of incubation on ice between cycles. Cell lysates were centrifuged (8,000 × g, 10 min, 4°C) to remove unbroken cells. The supernatant was then spun (8,000 × g, 10 min, 4°C) to sediment cell wall material. The membrane fraction was recovered from the supernatant by centrifugation (35,000 × g, 20 min, 4°C), and the pellet (membranes) was resuspended in PBS. The total protein concentration was estimated by Bradford assay.

In vitro labeling of S. aureus PBPs with BocillinFL. This method was adapted from a published protocol (62) with minor modifications. Membrane proteome samples (25 μg in 20 μL PBS) and purified proteins (2.5 μg in 25 μL HEPES pH 7.5 150 mM NaCl) were incubated with 25 μM BocillinFL (Invitrogen) for 20 min at 37°C. Additionally, for the competition assay, purified SdBPP1 was mixed with 2.5 μg (~286 μM final concentration) ampicillin and incubated at 37°C for 10 min prior to the addition of BocillinFL. The reaction was stopped by the addition of 5× SDS-PAGE loading buffer. Membrane proteome was additionally incubated for 10 min at 90°C. The samples were run on a 6 to 20% (wt/vol) SDS-PAGE gradient or 10% (wt/vol) SDS-PAGE gel and visualized using a Bio-Rad ChemiDoc MP imaging system or a GE Typhoon FLA 9500.

Labeling S. aureus d-amino acids. S. aureus cells were incubated with 500 μM (2 mM for ppb4 mutants) HADA or 1 mM ADA-DA at 37°C for 5 min. Cells were then washed by centrifugation and resuspension in PBS.
Click chemistry. ADA-DA containing an azide functional group was fluorescently labeled with Atto 488 alkyne at 5 μg mL⁻¹ via the click reaction [copper (i)-catalyzed alkyne-azide cycloaddition]. This was carried out using the Click-IT cell reaction buffer kit (Thermo Fisher) according to the manufacturer’s protocol.

Labeling *S. aureus* with fluorescent NHS-ester. Fixed cell wells were resuspended in PBS containing 8 μg mL⁻¹ Alexa Fluor 555 NHS-ester (Invitrogen) and incubated at room temperature for 30 min. Cells were washed twice by centrifugation and resuspension in PBS.

Fixing for fluorescence microscopy. Cells were fixed by incubation in 1.6% (wt/vol) paraformaldehyde at room temperature for 30 min.

Fluorescence microscopy. Fixed cells were dried onto a poly-l-lysine-coated slide, mounted in PBS, and imaged on a Nikon Ti inverted microscope fitted with a Lumener Spectra X light engine. Images were taken using a 100× PlanApo (1.4 NA) oil objective using 1.518 RI oil and detected by an Andor Zyla sCMOS camera.

Cell volume estimation. Cell volume calculations were carried out as previously described (63). The long and short axes of cells were measured using Fiji. The volume was then calculated based on a prolate spheroid shape with volume

\[ V = \frac{4}{3} \pi ab^2, \]

where \( a \) and \( b \) are the radii along the long and short axes, respectively.

Transmission electron microscopy. *S. aureus* strains were prepared for electron microscopy as previously described (64).

Preparation of *S. aureus* sacculi. Peptidoglycan from *S. aureus* cells was extracted and, if required, hydrofluoric acid (HF)-treated to remove cell wall accessory polymers as previously described (64).

Sacculi immobilization for AFM imaging. The immobilization surface was prepared by adding the solution mixed with 171 μL of 100 mM NaHCO₃, 3 μL of 1 M NaOH, and 6 μL of Cell-Tak (Corning, 5% [wt/vol] in acetic acid) on freshly cleaved mica. After 30 min of incubation, the surface was washed with 5 × 200 μL high-pressure liquid chromatography (HPLC)-grade water. Sacculi stocks were 10 times diluted in HPLC-grade water and briefly tip-sonicated to resuspend them prior to immobilization. Then, 10 μL of the sacculi suspension was added to 40 μL of HPLC-grade water on the Cell-Tak immobilization surface and incubated for 1 h. The surface was then thoroughly rinsed with HPLC-grade water, blow-dried with nitrogen, and stored in a petri dish at room temperature before AFM imaging.

AFM imaging and image analysis. AFM imaging was carried out on a Nanowizard III ULTRA Speed system (JPK, Germany). Rectangular cantilevers with a nominal spring constant of 0.3 N/m and resonant frequency (in liquid) of ~150 kHz (USC-F0.3-k0.3; NanoWorld, Switzerland) were used. The spring constant and deflection sensitivity of each cantilever were calibrated prior to each measurement (65, 66).

Measurements were carried out in quantitative imaging mode at room temperature in the buffer composed of 200 mM KCl and 10 mM Tris. Scans were driven at a line rate of ~0.78 Hz, with a typical Z length of 300 nm and trigger force of 20 nN. The resultant topographic images were processed using JPK Data Processing. No flattening or surface subtraction was applied. A high-pass filter (scale: 100% to 500%, degree of smoothing: 5 px, horizontal) was applied to the higher magnification images to enhance the contrast without modifying the morphological features. The morphological features of sacculi were summarized from images obtained on abundant technical repeats of 2 biological replicates.

Recombinant protein production and purification. *E. coli* BL21(DE3) cells containing plasmid pSAS0 were grown in LB medium supplemented with 0.1% ampicillin at 37°C to an OD₆₀₀ of 0.5. Protein overproduction was induced by addition of 0.5 mM IPTG to the cell culture and further incubation for 4 h at 30°C. Cells were harvested by centrifugation (6,200 × g, 15 min, 4°C), and the pellet was resuspended in basic buffer (25 mM Tris-HCl, 100 mM NaCl, pH 7.5). After addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), a 1:1,000 dilution of protease inhibitor cocktail (Sigma-Aldrich), and Dnase, the cells were disrupted by sonication (Branson digital sonifier). The cell lysate was centrifuged (130,000 × g, 60 min, 4°C), and the supernatant was recovered. The supernatant was incubated with Ni-NTA Superflow (Qiagen) for 2 h at 4°C with gentle stirring, which had been pre-equilibrated in basic buffer. The resin was poured into a gravity column and washed with 20 volumes of wash buffer (25 mM Tris-HCl, 150 mM NaCl, 10% [vol/vol] glycerol, 10 mM MgCl₂, 600 mM imidazole, pH 7.5). Bound protein was eluted with elution buffer (25 mM Tris-HCl, 150 mM NaCl, 10% [vol/vol] glycerol, 10 mM MgCl₂, 600 mM imidazole, pH 7.5). Next, 10 mM L-threonine (TaKaRa) was added to the Ni-NTA-eluted protein to remove the oligohistidine-GST tag during dialysis against 3 L of dialysis buffer I (25 mM Tris-HCl, 150 mM NaCl, 10 mM EGTA, 10% [vol/vol] glycerol, pH 7.5) for 20 h at 4°C. Digested protein was dialyzed against 3 L of dialysis buffer II (25 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl₂, 10% [vol/vol] glycerol, pH 7.5), for 3 h at 4°C. The protein was incubated in the same Ni-NTA beads (pre-equilibrated in dialysis buffer II) for 2 h at 4°C to remove the contaminants and the His-GST tag from the sample. The flowthrough and the washes (2 volumes of wash buffer) were pooled, dialyzed against storage buffer (25 mM HEPES-NaOH, 150 mM NaCl, 10 mM MgCl₂, 10% [vol/vol] glycerol, pH 7.5), and concentrated using a Vivaspin Turbo 15 column (molecular weight cutoff [MWCO] of 50,000 Da).

All recombinant proteins were produced in *E. coli* Rosetta (DE3) cells at 37°C in Terrific Broth (TB) medium supplemented with 50 μg mL⁻¹ kanamycin and 30 μg mL⁻¹ chloramphenicol. Once cultures had reached an OD₆₀₀ of 0.9, protein expression was induced with 1 mM IPTG for 20 h at 20°C. Cells were harvested by centrifugation (4,000 × g at 4°C for 30 min), and the pellet was resuspended in a buffer of 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole supplemented with one EDTA-free protease inhibitor cocktail tablet (Roche), and Dnase (4 μg mL⁻¹)
Generation of anti-PBP1 antibody. Serum against sPBP1A-BAP was produced from rabbits following a 28-day immunization program at Eurogentec (Belgium), and it was purified as previously described (67).

Immunoblot analysis. S. aureus cultures were washed three times by resuspension and centrifugation (5,000 × g, 10 min, 4°C) in PBS. Cells were resuspended in TBSI (50 mM Tris, 100 mM NaCl, pH 8.0, plus Complete Protease Inhibitor Cocktail [Roche]) and broken using 0.1 mm silica spheres (lysing matrix B) and FastPrep homogenizer (MP Biomedicals) in 12 cycles of 30 s, at maximum speed (6.5 m s⁻¹), with a 5-min incubation on ice between cycles. Cell lysates were centrifuged (5,000 × g, 10 min, 4°C) to remove unbroken cells. Approximately 60 µg of total protein was separated on a 12% (wt/vol) SDS-PAGE gel and electrophoretically transferred onto a nitrocellulose membrane and blocked in 5% (wt/vol) skimmed milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [vol/vol] Tween 20). The membrane was incubated with primary polyclonal anti-PBP1 (1:1,000) overnight with gentle agitation at 4°C. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad) and Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad) reagent according to the manufacturer’s protocol (44). The membrane was incubated against MUG (4-methylumbelliferyl-D-galactopyranoside) using an assay as previously described (35). Immunoblot analysis was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad) and Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad) reagent according to the manufacturer’s protocol (44). The membrane was incubated against MUG (4-methylumbelliferyl-D-galactopyranoside) using an assay as previously described (35).

Cell wall binding assays. Cell wall binding assays of recombinant PBP1 proteins fluorescently labeled with Cy2 bis-reactive dye (GE Healthcare) were performed as previously described (34), except for the binding buffer: 25 mM HEPES (pH 7.5), 150 mM NaCl, and 10 mM MgCl₂. Cy2-labeled cytochrome (Cy2-CytC) was included as a negative control. Binding of Cy2-labeled cytochrome (Cy2-CytC) was included as a negative control. Binding of Cy2-labeled cytochrome (Cy2-CytC) was included as a negative control. Binding of Cy2-labeled cytochrome (Cy2-CytC) was included as a negative control. Binding of Cy2-labeled cytochrome (Cy2-CytC) was included as a negative control.

Bacterial two-hybrid assay. Competent BTH101 was cotransformed with pKT25 and pUT18 derivatives. Transformants were selected on LB agar plates containing 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ kanamycin, and 40 µg mL⁻¹ X-Gal and incubated at 30°C. Single colonies were grown in 150 µL LB with 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ kanamycin, and 0.5 mM IPTG at 30°C. To qualitatively measure for pairwise interactions, 5 µL of each overnight culture was spotted onto LB agar plates containing 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ kanamycin, 0.5 mM IPTG, and 40 µg mL⁻¹ X-Gal. The plates were incubated at 30°C for 24 to 48 h in an environment protected from light and imaged. To quantify interactions, overnight cultures were assayed for β-galactosidase activity against MUG (4-methylumbelliferyl-β-D-galactopyranoside) using an assay as previously described (35).

Data availability. All study data are included in the article and/or supporting information. The data that support the findings of this study are available in the Online Research Data Figshare from the University of Sheffield with the identifier https://figshare.shef.ac.uk/collections/Penicillin-Binding_Protein_1_PBP1_of_Staphylococcus_aureus_Has_Multiple_Essential_Functions_in_Cell_Division/5656339/1. The crystal structure of the S. aureus PBP1 PASTA domains (PDB ID 7O61) can be accessed at https://www.rcsb.org/structure/7O61.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

FIG S1, TIF file, 1.6 MB.

FIG S2, TIF file, 1.3 MB.
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We declare no competing interests.
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