AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Environmental Microbiology | Full-Length Text

Penicillin-binding protein redundancy in *Bacillus subtilis* enables growth during alkaline shock

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ABSTRACT Penicillin-binding proteins (PBPs) play critical roles in cell wall construction, cell shape maintenance, and bacterial replication. Bacteria maintain a diversity of PBPs, indicating that despite their apparent functional redundancy, there is differentiation across the PBP family. Apparently-redundant proteins can be important for enabling an organism to cope with environmental stressors. In this study, we evaluated the consequence of environmental pH on PBP enzymatic activity in *Bacillus subtilis*. Our data show that a subset of PBPs in *B. subtilis* change activity levels during alkaline shock and that one PBP isoform is rapidly modified to generate a smaller protein (i.e., PBP1a to PBP1b). Our results indicate that a subset of the PBPs are favored for growth under alkaline conditions, while others are readily dispensable. Indeed, we found that this phenomenon could also be observed in *Streptococcus pneumoniae*, implying that it may be generalizable across additional bacterial species and further emphasizing the evolutionary benefit of maintaining many, seemingly-redundant periplasmic enzymes.

IMPORTANCE Microbes adapt to ever-changing environments and thrive over a vast range of conditions. While bacterial genomes are relatively small, significant portions encode for "redundant" functions. Apparent redundancy is especially pervasive in bacterial proteins that reside outside of the inner membrane. While conditions within the cytoplasm are carefully controlled, those of the periplasmic space are largely determined by the cell's exterior environment. As a result, proteins within this environmentally exposed region must be capable of functioning under a vast array of conditions, and/or there must be several similar proteins that have evolved to function under a variety of conditions. This study examines the activity of a class of enzymes that is essential in cell wall construction to determine if individual proteins might be adapted for activity under particular growth conditions. Our results indicate that a subset of these proteins are preferred for growth under alkaline conditions, while others are readily dispensable.

KEYWORDS *Bacillus*, cell division, pH regulation, penicillin-binding proteins, activity-based probes

The evolutionary success of bacteria is largely due to their ability to sense and respond to environmental change. Bacteria, therefore, have become ubiquitous and occupy ever-changing and even extreme environmental conditions, including temperature, pH, osmolarity, nutrients, and chemical pressures. The first line of defense against changing external environments is the cellular envelope. In Gram-negative bacteria, this protective layer consists of an inner lipid membrane, a thin layer of peptidoglycan, and an outer lipid membrane. Alternatively, Gram-positive bacteria possess a single, inner lipid bilayer and a much thicker peptidoglycan layer. The microbial cell envelope enables the cell to maintain cytoplasmic homeostasis and protects the internal biochemical processes from changing external conditions. For example, enzyme kinetics are highly dependent on pH, so bacteria have developed several strategies for coping with

Editor Arpita Bose, Washington University in St. Louis, St. Louis, Missouri, USA

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The authors declare no conflict of interest.

See the funding table on p. 15.

Received 3 April 2023
Accepted 13 November 2023
Published 21 December 2023

fluctuations in environmental pH (e.g., proton pumps, other monovalent cation efflux, and regulating stress response) to maintain a neutral cytoplasmic pH and enable optimal enzymatic activity (1, 2). However, both Gram-negative and Gram-positive bacteria possess proteins that are external to the protective inner membrane and are therefore subject to environmental pH variability. Thus, bacteria must employ different strategies to maintain extracellular enzymatic activity, particularly those proteins responsible for the synthesis of the essential cell envelope.

Among these crucial proteins is a group of key cell envelope-associated proteins, the penicillin-binding proteins (PBPs), which perform the final steps of peptidoglycan biosynthesis. Peptidoglycan, a biopolymer composed of glycan chains, is a major structural element in the bacterial envelope and provides protection from physical and chemical threats (3). PBPs are responsible for several key reactions: polymerization of the glycan (transglycosylation) and pentapeptide cross-linking (transglycosylation). The PBPs are divided into three classes. Class A PBPs are designated as high molecular weight (HMW) proteins that contain both transglycosylase and transpeptidase domains. Class B PBPs are also HMW proteins but only perform a transpeptidation reaction. Class C or low molecular weight (LMW) PBPs are carboxypeptidases that catalyze pentapeptide hydrolysis to prevent additional cross-linking or possess endopeptidase activity (3, 4).

PBPs are notable for their redundant activity (5). Bacteria may harbor 4–16 different PBPs, indicating that the evolution and maintenance of proteins with overlapping functions is advantageous. In fact, mutation of one PBP often has little physiological effect, as its altered activity is masked by other isoforms (4, 6). While PBPs of numerous bacterial species have been studied extensively, with particular attention paid to the differential inhibition of PBPs by β-lactam antibiotics (6–9), little is understood about differential PBP function in typical ecological environments. Extracellular enzymes tend to be more redundant than cytoplasmic proteins because of the variations in the external environment (10). It is likely that many PBPs are specialized for particular environmental conditions.

We sought to investigate the specialization of PBPs in *Bacillus subtilis*, a commonly studied Gram-positive, rod-shaped bacterium found in diverse environments (11). The lifecycle of *B. subtilis* includes endospore formation, which enables the bacterium to survive extreme conditions and makes it a particularly interesting model organism for investigating bacterial replication and cell wall synthesis. *B. subtilis* harbors 16 PBPs: four class A, six class B, and six class C PBPs. The activity of only seven of these PBPs can readily be observed during vegetative growth (6) (Table 1). *B. subtilis*, like most neutrophiles, is capable of replication across a wide pH range (pH 6–9) (12). Alkaliphilic *Bacillus* spp. are capable of growth at even more extreme pH values (up to pH 10.8) while still maintaining a cytoplasmic pH conducive to optimal catalysis (1, 13, 14). Given the ability of this genus to thrive under various environmental conditions, we postulated that *B. subtilis* PBPs may be differentially regulated to enable the organism to cope with external changes. As such, we assessed the activity of *B. subtilis* PBPs *in vivo* across a range of pH values.

We focused our work on alkaline pH, as *B. subtilis* is known to grow in conditions up to pH 10 and the *Bacillus* sp. includes several species of alkaliphiles (21). The activity changes of single, purified PBPs have been reported over a range of pH values, but to our knowledge, there are no reports of how pH affects PBP activity *in vivo* (22–24). We found that during alkaline shock, PBPH and PBP4 are inactivated. We also show that base treatment promotes the transition between the two products of *ponA*, PBP1a and PBP1b. These changes in PBP activity did not result in notable differences in cell morphology, indicating the benefit of PBP redundancy. PBP-null mutant *B. subtilis* strains revealed the role of alkaline-active PBPs (2a, 2b, 3, and 5) in maintaining bacterial replication in alkaline media. We also found that *Streptococcus pneumoniae* possesses a base-sensitive PBP, suggesting that redundancy within this protein family is likely important for survival in environments with differing pH values in multiple organisms.

TABLE 1 Summary of *B. subtilis* PBPs resolved in the Bocillin-FL gel assay[a]

Protein	Gene	MW (kDa)	Isoelectric point	Class	Location	Mutant phenotype	Reference	Similarity to other *Bacillus subtilis* and *Streptococcus pneumoniae* PBPs
PBP1a/b	*ponA*	99.56	4.94	A	Septa	Longer, thinner, bent cells and growth rate decrease	(15–17)	2c: 64% coverage, 37% identity 4: 58% coverage, 33% identity 2d: 68% coverage, 32% identity *S. pneumoniae* (PBP1a): 85% coverage, 38% identity *S. pneumoniae* (PBP2a): 75% coverage, 34% identity
PBP2a	*pbpA*	80.14	9.22	B	Even distribution	None	(15, 18)	H: 94% coverage, 43% identity *S. pneumoniae* (PBP2b): 97% coverage, 33% identity
PBPH	*pbpH*	79.35	8.93	B	Even distribution	None, double mutant with *pbpA* is not viable	(19)	2a: 91% coverage, 44% identity
PBP2b	*pbpB*	79.32	8.9	B	Septa	Essential	(5)	spoVD: 83% coverage, 34% identity 3: 70% coverage, 26% identity *S. pneumoniae* (PBP2x): 96% coverage, 32% identity
PBP3	*pbpC*	74.41	6.24	B	Periphery	None	(5, 16)	spoVD: 70% coverage, 27% identity 2b: 62% coverage, 27% identity
PBP4	*pbpD*	70.66	9.12	A	Septa and periphery	Reduced growth rate, morphological changes	(5, 15)	2c: 90% coverage, 34% identity 1: 93% coverage, 32% identity 2d: 91% coverage, 30% identity *S. pneumoniae* (PBP1b): 70% coverage, 28% identity
PBP5	*dacA*	48.64	5.77	C	Septa and periphery	None	(20)	None

[a]The predicted molecular weight (ExPASy), isoelectric point, class, localization, and function of the PBPs were resolved in an activity assay [Bocillin-FL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)], as well as their similarity to other *Bacillus subtilis* vegetative PBPs and *Streptococcus pneumoniae* PBPs (7). These seven PBPs are active during vegetative growth of *B. subtilis*. Although *ponA* produces two possible protein products, only the sequence and molecular weight of PBP1a are known.

RESULTS AND DISCUSSION

Alkaline shock reduces the activity of select PBPs

To assess the activity of *B. subtilis* PBPs, we enlisted an activity-based probe that covalently labels proteins in proportion to their catalytic activity. As their name suggests, PBPs are the binding target of β-lactam antibiotics such as penicillin. These molecules covalently tag the PBPs, which prevents enzymatic action and halts new cell wall synthesis, resulting in cell death. All three classes of PBPs contain a catalytic serine that interacts with the terminal D-Ala-D-Ala of the peptidoglycan pentapeptide, the moiety that β-lactams mimic (Fig. 1). Bocillin-FL, a fluorescent analog of penicillin V, has affinity for all active PBPs and therefore can be used as an activity-based probe to report on the enzymatic activity of the entire protein suite (6, 25).

To determine the effect of alkalinity on PBP activity, we cultured *B. subtilis* cells in Luria-Bertani (LB) to the early exponential phase (OD_{600} 0.4–0.6) and then exposed the cells to alkaline pH in phosphate buffered saline (PBS) for 30 min. Next, the cells were incubated with Bocillin-FL to label the active PBPs. Following lysis, cell membranes were isolated, and the proteome was separated via polyacrylamide gel electrophoresis. Gels were scanned to visualize the fluorescence intensity of the bands, which indicates the degree of activity of individual PBPs. We found that with increasing pH, there was a loss of the catalytic activity of PBPH and PBP4 and a shift in activity from PBP1a to PBP1b, as indicated by changes in Bocillin-FL labeling (Fig. 2; also see band integrations in Table S1 and preliminary titration data in Fig. S1). PBPH (*pbpH*), which is evenly distributed across the bacterial membrane and more active in the later log phase of vegetative *B.*

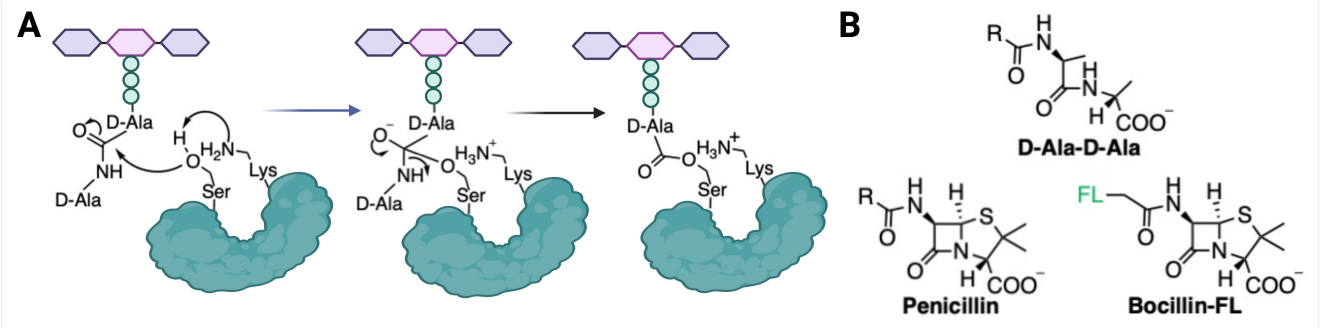

FIG 1 Peptidase activity and substrate mimetics of PBPs. (A) The transpeptidase or carboxypeptidase PBP domains contain a lysine that activates serine for acylation by the D-Ala-D-Ala of the stem peptide. (B) β-Lactam antibiotics, such as penicillin, mimic this natural substrate. A β-lactam conjugated a fluorophore (FL) can be employed to assess the activity and localization of the PBPs. Figure generated with BioRender.

subtilis growth, is fully inactivated at pH 9 (19). PBPH and PBP2a (*pbpA*) have the highest sequence and functional homology among the PBPs in *B. subtilis* (Table 1). *B. subtilis* mutants lacking either of these genes do not display morphological defects, although mutants without both PBPs (*ΔpbpHΔpbpA*) are not viable. Despite sequence similarity with PBPH, PBP2a activity is not affected by increased alkalinity (Fig. 2). This may suggest that the redundancy of PBPH and PBP2a facilitates growth in a wider pH range, as PBP2a appears to have greater activity under alkaline conditions.

Inactivation of PBP4 (*pbpD*) begins at pH ~10 (Fig. 2). PBP4 functions at the septa and periphery of *B. subtilis* during vegetative growth. *B. subtilis* strains lacking PBP4 (*ΔpbpD*) have a reduced growth rate and slight morphological changes (5, 15). When compared to other PBPs in *B. subtilis,* PBP4 is most homologous to other class A PBPs: vegetative PBP1 (*ponA*) and spore-forming PBPs, PBP2c (*pbpF*) and PBP2d (*pbpG*), the latter of which are not observed in this assay as it was performed under vegetative growth conditions (Table 1). Therefore, it is unclear if the activity of the spore-forming PBPs is affected by an alkaline environment. However, we do also see a substantial change in PBP1 activity (see below), which aligns with the predicted similarity between PBP1 and PBP4.

The final observed change in PBP activity was a shift from PBP1a to PBP1b at pH ~10.5 (Fig. 2). PBP1a and PBP1b are two products of different molecular weights encoded by the same gene, *ponA*. The biological process that results in two functional proteins of different sizes from *ponA* is not well understood but is suggested to be due to differential C-terminal processing (16). *B. subtilis* strains lacking *ponA* (Δ*ponA*) result in a loss of both PBP1a and PBP1b isoforms, leading to morphological changes such as a reduction in cell diameter, cell bending, and decreased sporulation efficiency (Table 1) (18). Interestingly, PBP1a is generally dominant at neutral pH, but during alkaline shock, PBP1b is activated, with almost no PBP1a activity detected. Other research has shown the role of PBPs in

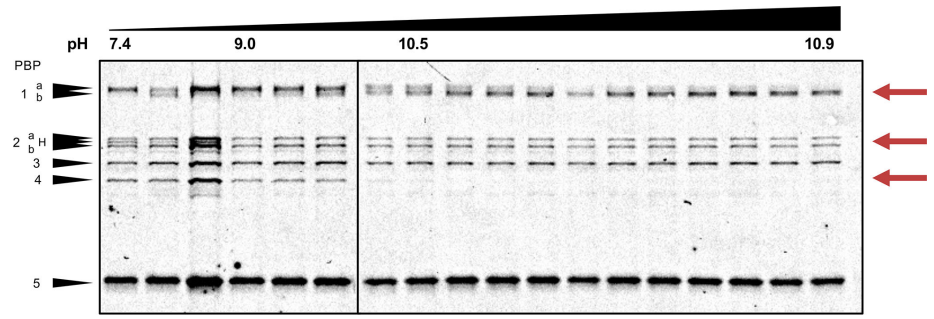

FIG 2 Inactivation of PBPH and PBP4 and activity shift from PBP1a to PBP1b following treatment with NaOH. Representative SDS-PAGE gel image for titration of *B. subtilis* 3610 cells over the indicated pH range. The fluorescent signal is a result of the degree of labeling with Bocillin-FL, an activity-based probe. Red arrows indicate the inactivation of noted PBPs.

enabling cell wall synthesis over a range of environmental pH or the changes in PBP activity across a pH range *in vitro*, but to the best of our knowledge, this work is the first report of *in vivo* PBP activity changes due to pH perturbations (26). We also conducted a preliminary investigation of the sensitivity of *B. subtilis* PBPs to acidic conditions. We found that PBPH was also sensitive to acidic conditions and that unlike under alkaline conditions, PBP2a activity was affected (Fig. S2). However, most of the bacteria died at pH <7, and we observed overall reduced PBP labeling intensity, making it unclear if this result is largely due to cell lysis and/or protein denaturation. Future work will more thoroughly evaluate this parallel result.

To evaluate any differences between *in vivo* and *in vitro* PBP activity and to determine if the base-mediated PBP (de)activation events result from a limitation of the assay instead of being a genuine biological result, we altered the order of the steps (i.e., base treatment, cell lysis, and Bocillin-FL labeling). *In vitro* analysis was performed on *B. subtilis* lysates, which were treated with base, washed, and then labeled with Bocillin-FL. Bocillin-FL labeling of *B. subtilis* lysates does not produce the same PBP profile as labeling on the whole cell, and both PBP2a and PBPH remain unlabeled in lysate control samples, so we are unable to make conclusions about the regulation of PBPH (Fig. 3A). This loss of PBP2a and PBPH activity upon lysis supports our hypothesis that the similar activity loss under acidic conditions may be due to cell death. However, these data do show that PBP4 is inactivated around pH 10.5, indicating that the loss of PBP4 activity does not require the native environment of the cell and is more likely due to biochemical changes such as the ionization state of amino acids or variations in protein folding. We also observed a decrease in the activity of PBP1a but no activation of PBP1b, implying that PBP1b activation requires additional cellular machinery, such as enzymes, to generate PBP1b from PBP1a or pH-sensing proteins that regulate PBP1b activity. This was further supported by experiments performed on cells that were first treated with base, lysed, and then labeled with Bocillin-FL. Again, the activity of PBP4 was lost. However, because base exposure was now performed *in vivo*, we saw a transition of PBP1a activity to PBP1b labeling, indicating that intact cells are required (Fig. 3B). Finally, we investigated the stability of Bocillin-FL-labeled proteins to base by treating the Bocillin-FL-labeled proteome with alkaline conditions. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a dissimilar PBP (de)activation trend, presumably due to base-promoted cleavage of the Bocillin-FL-PBP acyl-enzyme conjugate (Fig. 3B).

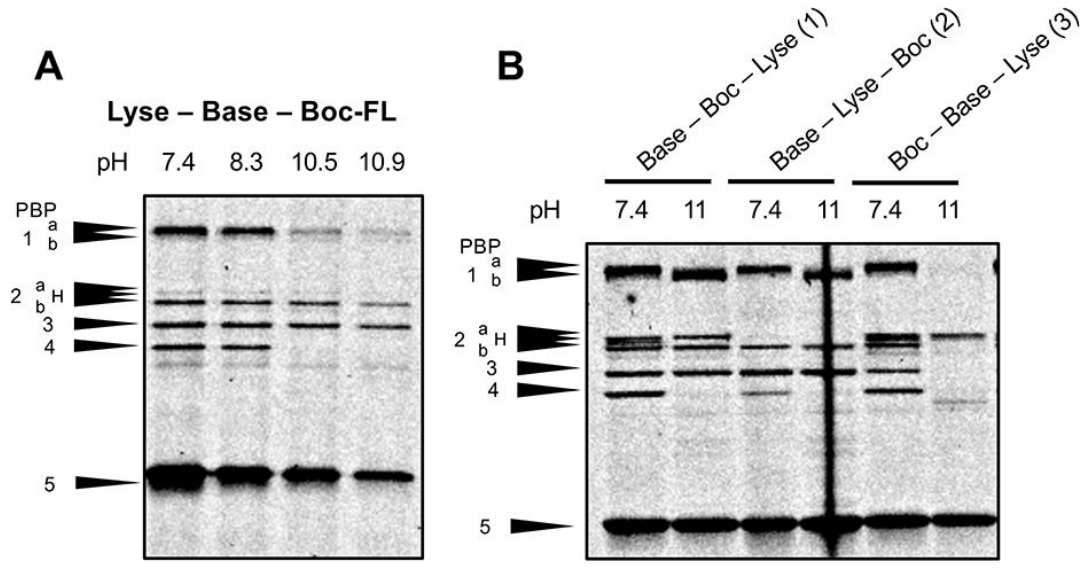

FIG 3 The order of alkaline shock, Bocillin-FL incubation, and cell lysis alters the activity profile of PBPs. (A) PBP profile of *B. subtilis* lysates and the effect of NaOH titration on the PBP activity. (B) Representative gel comparing the order of operations: (1) base treatment, Bocillin-FL, and then lysis (standard); (2) base treatment, lysis, and then Bocillin-Fl labeling; or (3) Bocillin-FL labeling, base treatment, and then lysis.

Together, these data confirm that the observed changes in PBP activity are not due to artifacts of the assay.

To further evaluate the PBP1a to PBP1b transition, we performed a time course assay *in vivo*. These protein isoforms are encoded by the same gene (*ponA*) and are thought to result from differential posttranslational processing. PBP1a and PBP1b are predicted to have an acidic isoelectric point (pI), indicating their potential catalytic activity under basic conditions (Table 1). Time course experiments show that the base-catalyzed transition from PBP1a and PBP1b is longer than PBP4 and PBPH inactivation (10 min vs 5 min), perhaps due to regulation of the responsible posttranslational modification machinery (Fig. S3). Given the relatively short time frame of the assay and that the cells are in a nutrient-poor environment, it is unlikely that protein expression changes are responsible for the loss of activity of PBPH and PBP4. This is further supported by a lack of apparent differences in overall protein levels, as evidenced by Coomassie staining (Fig. S1 to S4, S6, and S9). Like other class A PBPs, PBP1 has both transglycosylase and transpeptidase domains. Very little is understood about the difference between PBP1a and PBP1b, but Popham and Setlow reported that PBP1b is likely generated by truncation of the carboxy terminal region of PBP1a (16). As PBP1a and PBP1b are similar in molecular weight, it is possible that PBP1b may result from a cleavage event of less than 30 amino acids or some other more complex modification of the PBP1a isoform. It has been postulated that the regulation of PBP1a and PBP1b production may be related to the presence of *prfA* (penicillin-binding protein-related factor A), a gene found just upstream of *ponA* on the same operon (16). *B. subtilis* strains lacking *prfA* (Δ*prfA*) display greatly reduced replication rates and abnormal cell shapes (27). It is possible that alkaline shock affects the regulation of *prfA*. However, *prfA* is also found in organisms that do not have a cell wall, and additional investigation has linked *prfA* to chromosomal segregation in *B. subtilis* (27, 28).

Overall, these experiments highlight the importance of investigating protein activity and redundancy in live cells and under environmentally relevant conditions. The apparent increase in protein processing to generate PBP1b from *ponA* would not have been identified in a standard *in vitro* assay. Indeed, these studies provide the first known conditions that favor the activity of PBP1b over PBP1a.

Investigation of chemical-based inhibition mechanisms

We next sought to determine the mechanism by which basic conditions reduce PBP activity *in vivo*, whether by *chemical perturbation* of the enzymatic environment (e.g., perturbed reactivity of the active site nucleophile) or *biological regulation* of PBP activity. PBPs perform three core functions (transglycosylation, transpeptidation, and carboxypeptidase activity), and their active sites are uniquely tailored to each enzymatic reaction (6). The reactivity of these sites can be perturbed by the pH of the local environment, so we further investigated if changes in the catalytic activity were due to the amino acid protonation state or regulatory processes. PBPH and PBP4 (class B and class A, respectively) quickly lose activity upon base incubation (5 min; Fig. S3), so we postulated that chemical inactivation could be responsible for their decreased activity, which agrees with the inactivation of PBP4 during alkaline shock in *B. subtilis* lysates (as above; Fig. 3). PBPs use an active site serine as a nucleophile, which is acylated by the Bocillin-FL β-lactam probe, with at least one lysine required to modulate serine reactivity (29). While it is challenging to determine the pKa values of individual amino acids *in vivo*, changes in enzymatic activity across pH indicate the reactivity of the catalytic residues and suggest that the activity of PBPs is controlled by the protonation state of the active site lysine (Fig. 1) (30). The literature indicates that for optimal catalysis, the proximal lysine must be in its free base form, which should be favored under basic conditions—activating the proteins. Interestingly, our experimental data reveal a more complex scenario wherein alkaline conditions inactivate a subset of PBPs, as was observed with PBP4 and PBPH. In fact, *in vitro* experiments have determined that the pH optima of many PBPs are in the basic range (pH 9–10) with lysine pK_a values of 8–10 (Table S2) (23, 24, 31, 32).

Although increased alkalinity should benefit PBP activity through lysine deprotonation, the stability of a protein is also affected by pH. The pI is the pH at which a protein has a net neutral charge and is often when proteins are least active and most unstable, which is why most proteins do not have a pI near the pH of their environment (33). For example, alkaliphilic *Bacillus* spp. are capable of growth at pH 6.8–10.8, so it follows that their PBPs and other cell wall construction enzymes are functional under extreme alkalinity and would have more acidic pI values (Table S3) (1, 13, 14, 21, 34–36). We expected that the pI values of *B. subtilis* PBPs may contribute to the observed pH-dependent activity and that base-sensitive PBPs would have basic pI values. However, we found no consistent trend in the relationship between predicted pI and *in vivo* PBP activity under alkaline conditions (Table 1). Both PBPH and PBP4 have predicted pI values close to the pH at which we saw reduced activity, 9 and 10.5, respectively. This agrees with our prediction that activity reduction under alkaline conditions for these proteins is of a chemical nature. However, the pI values do not explain the activity shift in PBP1a to PBP1b or the persistent activity of other vegetative PBPs that should be inactivated during alkaline shock (PBP2a and PBP2b). PBP2a and PBPH are the most homologous PBPs and have similar predicted pI values but different base sensitivities. The most notable difference between PBP2a and PBPH is an additional 27-amino acid sequence unique to PBP2a. This short sequence has a predicted pI of 10, which could be partially responsible for the different predicted pI values for the proteins and serves to further stabilize PBP2a.

We would expect that the reported pH optima and predicted pI of PBPs differ, which is true for several surveyed neutrophilic organisms (e.g., *Staphylococcus aureus* PBP2 and *Escherichia coli* PBP6), while some have similar pI values and pH optima (e.g., *Neisseria gonorrhoeae* PBP4) (Table S2). These reported pH optima, however, were all obtained from purified proteins, so they do not necessarily correlate with the *in vivo* activity of the proteins or reflect the pH of the organism's habitat (23).

Next, we overexpressed PBPH and PBP4 from *B. subtilis* as soluble constructs in *E. coli* to investigate differences in enzyme activity that occur in a non-native environment. We did not include PBP1 since the observed transition from PBP1a to PBP1b requires protein processing. *E. coli* strains harboring plasmids of these *B. subtilis* PBPs were lysed, and the proteome was exposed to base before Bocillin-FL labeling. Contrary to what was observed upon treatment of *B. subtilis* cells, we found that overexpressed PBP4 and PBPH were *activated* under basic conditions (Fig. S4). This differential response of PBPH and PBP4 to alkaline treatment indicates a complex interplay of biological regulation, protein stability, and perturbation of active site reactivity, which are likely responsible for the observed PBP activity changes upon bacterial exposure to basic conditions.

Evaluation of reagent-specific effects on base sensitivity

We next explored the generality of the observed PBP activity changes across a variety of basic reagents and buffers. Other work has shown that bicarbonate and triethylamine yield unique PBP5 activity profiles in *E. coli* (23). We found that the identity of base (e.g., lithium hydroxide, potassium hydroxide, and trimethylamine) had no discernable effect on the resulting PBP activity profiles in *B. subtilis* (Fig. S5). Several media were also tested to evaluate if buffering capacity or osmolarity stabilizes PBP activity (Fig. S6). Alkaline shock in PBS, HEPES (2 mM), and Milli-Q water all produced identical PBP activity profiles (pH >10.5). However, no PBP activity changes were observed when NaOH was added to media with higher buffering capacity (i.e., minimal media, 10 mM HEPES, and 10× PBS), indicating that pH and not specific ions or ionic strength is primarily responsible for the observed deactivations. Still, salt concentration and media identity have some effect. We hypothesized that alkaline shock in minimal media (0.25×) should alter the PBP profile as the pH is >10.5 (Table S4); however, we only saw decreased PBPH labeling. The opposite trend was observed when alkaline shock was performed in NaCl solutions. As the concentration of NaCl increased, the PBP profile shift became more extreme, including the loss of PBP2a and PBP5 activity. Salt concentrations are known to influence

base sensitivity in both neutrophiles and alkaliphiles and can compensate for some PBP-null mutants (18, 37, 38).

Regulating pH response: alkaline media

We next sought to determine if differential PBP activity levels under alkaline conditions had direct consequences on *B. subtilis* growth. The morphology of bacteria that underwent alkaline shock was unchanged compared to controls, perhaps due in part to the short duration and lack of nutrients during the exposure (Fig. S7a). We analyzed the viability of the bacteria after a 30-min alkaline shock using a colony-forming unit (CFU) assay. At pH 8.5 (loss of PBPH activity), bacteria are 100% viable, and at pH 10.5 (loss of PBP4 activity and shift from PBP1a to PBP1b), the cells are 40% viable (Fig. S7b).

Next, we determined if chronic exposure to basic pH would enable the organism to adapt and alter the observed PBP labeling profile. We found that *B. subtilis* was unable to survive chronic exposure to pH >9.5, similar to previously reported values (39). Therefore, chronic exposures were performed at pH ≤9.5. Overnight cultures were grown in standard LB (pH 6.65) or with the addition of NaOH. The bacteria were able to partially neutralize the media, as LB is not buffered at basic pH values (e.g., pH 9.4 was neutralized to 8.0 overnight by the PBP4 mutant; Table S5). After overnight growth in different pH media, bacteria were subsequently exposed to a 30-min treatment. The bacteria exposed to neutral media for 30 min did not recover the activity of PBPH, while PBP1a and PBP4 activity remained inactivated (Fig. 4; "overnight media pH" 9.4 and "alkaline shock" 7.4). Interestingly, the PBP4 activity was not lost during alkaline shock (pH 11; 30 min) if the bacteria were previously exposed to basic media (pH 9.4) overnight. This likely indicates either the presence of mechanisms to increase the stability and activity of this PBP under basic environmental conditions or that additional biological processes have been activated at higher pH values to partially neutralize the periplasm and enable maintenance of PBP4.

Alkaline sensitivity in PBP-null mutants of *B. subtilis*

Next, we evaluated the roles of specific PBPs in enabling *B. subtilis* to survive alkaline shock. To do this, we investigated mutant organisms missing each of the vegetative PBPs, except the essential PBP2b. All mutant strains produced the same PBP profile change as the wild-type organism during alkaline shock (30 min): loss of PBPH activity, then PBP4 activity, and finally, transition from PBP1a to PBP1b (Fig. 5). These data also show increased activity of PBP3 in some strains, those lacking PBPH, PBP4, or PBP5, which could indicate compensatory activity. The mutant strains also did not change morphology after alkaline shock (Fig. S8), although some strains have a different morphology than the wild-type strain. The PBP1-null mutant, *ΔponA*, is more filamented than wild-type *B. subtilis* under normal growth conditions, while *ΔpbpH* and *ΔpbpD* have very similar morphology to the wild-type strain.

We next investigated the ability of wild-type and mutant strains to recover and neutralize media during long-term growth (48 h) in alkaline conditions to better understand the roles of individual PBPs (Table S5). In overnight cultures, wild-type and

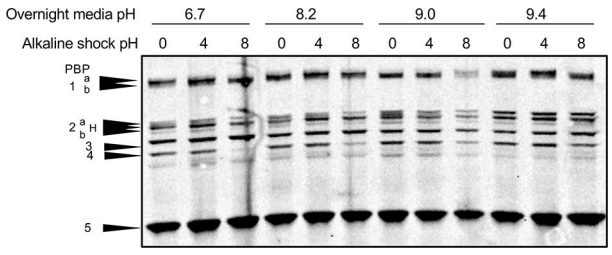

FIG 4 The activity of *B. subtilis* PBPs after overnight growth in basic media. Bacteria were cultured overnight in LB media that started at a pH of 6.7–9.4. *B. subtilis* PBPs were subsequently subjected to alkaline shock at either pH 10.5 or 11.

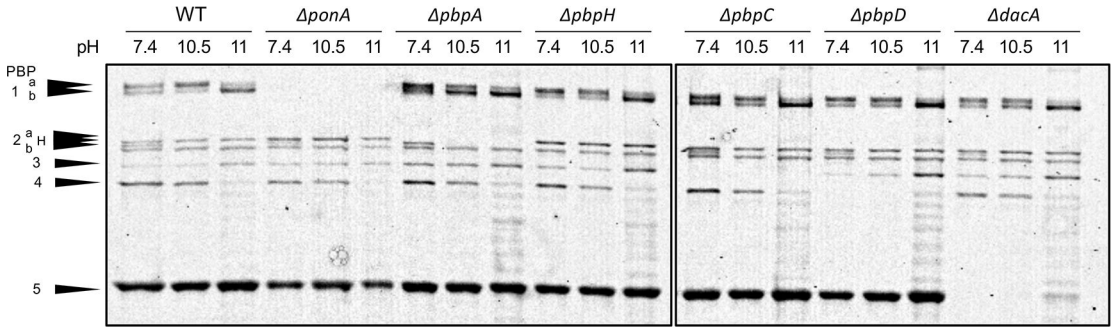

FIG 5 Base-mediated PBP inactivation in mutant strains. Inactivation of PBPH and PBP4 and activity shift from PBP1a to PBP1b in basic media (30 min). Representative SDS-PAGE gel image of Bocillin-FL labeling of *B. subtilis* 3610 cells and PBP-null mutants at pH 7.4, 10.5, or 11. Wild-type *B. subtilis* (strain 3610), PBP1 mutant (*ΔponA*), PBP2a mutant (*ΔpbpA*), PBPH mutant (*ΔpbpH*), PBP3 mutant (*ΔpbpC*), PBP4 mutant (*ΔpbpD*), and PBP5 mutant (*ΔdacA*).

PBP knockout strains neutralize their media under all conditions. As expected, there was no significant difference in the ability of different mutant strains to neutralize the media, as the changes in media pH are likely the result of bacterial metabolism, not the activity of PBPs (1). Growth in basic pH (pH = 9) appeared to condition both wild-type and mutant strains for growth at more extreme pH (pH = 9.5), as others have reported (12). However, *B. subtilis* mutants lacking alkaline-stable PBPs (PBP2a, PBP3, and PBP5) may prevent the bacteria from being able to grow at higher pH values and neutralize the media.

We next explored if the growth rate of the PBP mutants was affected by alkaline conditions. Both wild-type and mutant strains of *B. subtilis* were cultured in LB with increasing alkalinity, and growth curves were analyzed for differences in base sensitivity. This experiment differs from the shock experiment in that we used LB instead of PBS (since growth was required), and the pH of the solutions was not as extreme, as higher pH values killed the cells upon longer incubation. Once again, LB media were not buffered to enable the bacteria to recover from the alkaline shock and neutralize the media. We compared the amount of time required for the different strains to achieve the maximal growth rate and the maximal growth rate value (Fig. 6). We calculated the maximum growth rate (peak exponential phase) by taking the first derivative of the natural log transformation of the OD_{600} measurements. Overall, we noted significant increases in the time to peak exponential phase (3–7 h) in a basic environment, which is consistent with previous reports of *B. subtilis* growth during alkaline shock (5-h growth arrest) (39). Reductions in the growth rate caused by a high-pH environment are likely a result of increasing cytoplasmic pH, which causes an additional burden on the cells (12, 40). Since each mutant strain exhibits different growth rates, we compared the changes in the growth rate between standard growth conditions (i.e., neutral) and alkaline conditions within each strain. Wild-type and *ΔykuA* mutants (encoding PBPH, the most readily dispensable PBP in alkaline conditions) were the least sensitive to increasing pH values, followed by Δ*ponA* and Δ*pbpD* (PBP1 and PBP4). This was expected given that the activity of these three proteins changes during acute alkaline shock, implying that they are not crucial for growth and replication in basic environments. These data also suggest that PBP1b does not provide vital functionality. The Δ*pbpC* and Δ*pbpA* strains (PBP3 and PBP2a, respectively) were more sensitive to basic pH, with Δ*dacA* (PBP5) exhibiting the highest base sensitivity with the greatest increase in time to the maximum growth rate. PBP3, PBP2a, and PBP5 maintain activity during alkaline shock, so it is likely that these PBPs are required for growth in an alkaline environment. As noted previously, Δ*pbpA*Δ*pbpH* double mutants produce a lethal phenotype, so it follows that Δ*pbpA* mutants that lose PBPH activity under alkaline conditions would also have a reduced growth rate. As PBP5 is the only class C PBP active during vegetative growth, it is possible that mutants without an active carboxypeptidase may be more sensitive to alkaline shock.

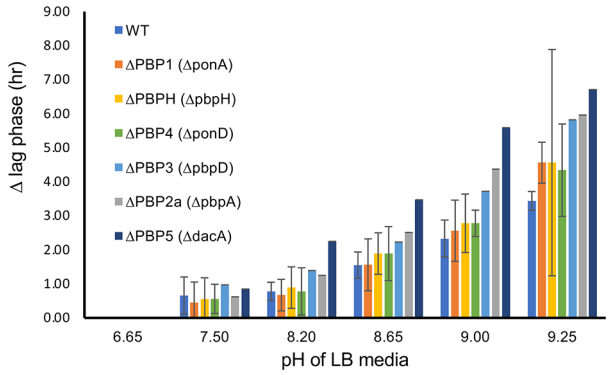

FIG 6 Characterization of the growth of *B. subtilis* PBP mutants. The time for a culture to achieve the maximal growth rate is considered the lag phase. The difference between the duration of the lag phase at each alkaline condition and neutral LB media is plotted for each strain.

Additional organisms

We sought to determine if other organisms have also evolved specialized PBPs to cope with environmental pH shifts and investigated two additional Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus pneumoniae* (41, 42). *S. aureus* and *S. pneumoniae* are both cocci-shaped bacteria that possess fewer PBPs than *B. subtilis,* suggesting less functional redundancy. Alkaline shock had no effect on the activity of the PBPs in *S. aureus* (Fig. 7A). In *S. pneumoniae*, the activity of a class A PBP, PBP1a, decreased following base treatment (Fig. 7B). This suggests that PBP1a in *S. pneumoniae* may not be specialized for growth in alkaline conditions. PBP1a and PBP1b in *S. pneumoniae* are produced from two different genes (*pbp1a* and *pbp1b,* respectively), unlike *B. subtilis*, where PBP1a and PBP1b are two functional products of the same gene (*ponA*). However, of all the *B. subtilis* PBPs, PBP1 (*ponA*) has the highest sequence similarity to PBP1a (*pbp1a*) in *S. pneumoniae* (38% protein sequence identity). The level of similarity between these two PBPs is comparable to that of *B. subtilis* PBPs that are not all affected by alkaline shock, so sequence similarity is likely not a strong predictor of base sensitivity.

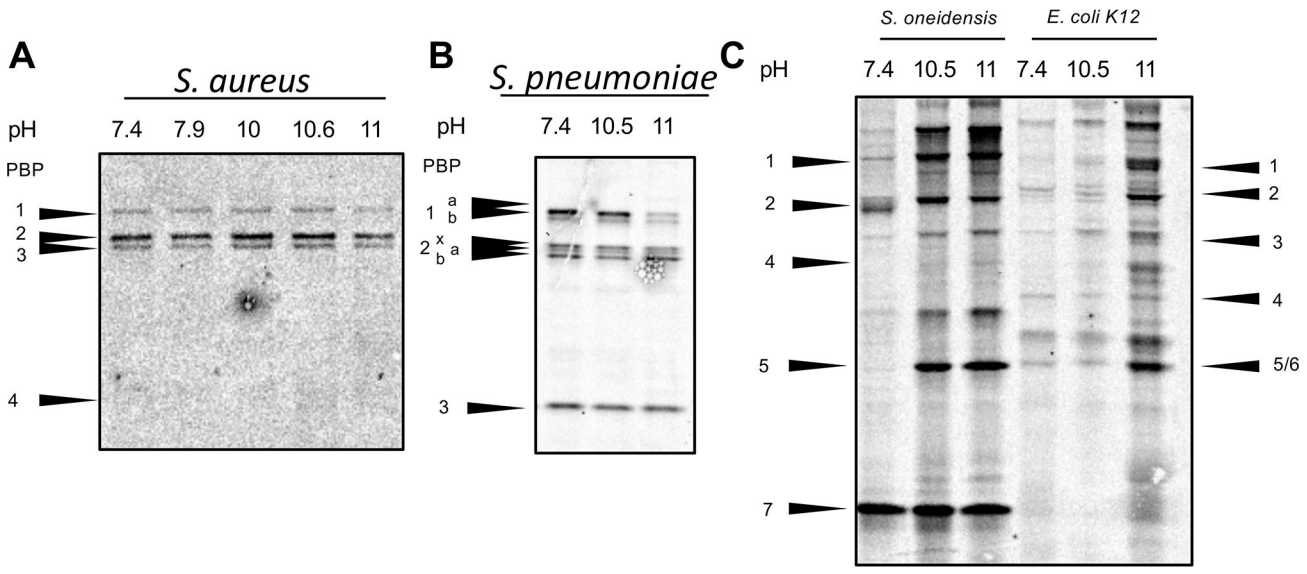

FIG 7 Representative gels of alkaline shock in other bacteria. (A) *S. aureus* PBP4 is not labeled with Bocillin-FL, (B) *S. pneumoniae*, and (C) *S. oneidensis* and *E. coli*. The PBPs of *S. oneidensis* have not been confirmed in the literature. The arrows indicate locations where each PBP would be expected based on the predicted molecular weight.

Gram-negative, rod-shaped bacteria *Shewanella oneidensis* and *Escherichia coli* K12 were also investigated (43). The ability of Gram-negative bacteria to maintain pH homeostasis has been well studied, but the PBP activity in Gram-negative bacteria is difficult to assess with a β-lactam-based probe because of the prevalence of β-lactamases, efflux pumps, and an outer membrane that shields the peptidoglycan layer (44–47). Alkaline shock increased the intensity of all bands labeled by Bocillin-FL, which suggests that the bacterial outer membrane is damaged and that there is not a specific collection of PBPs that enable growth in fluctuating environmental pH in these two microbes (Fig. 7C).

Conclusion

An organism's response to changes in environmental pH is vital for cell viability because the pH gradient across cell membranes generates energy (proton motive force) and the biochemical reactions within a cell are performed at an optimal pH (2). There are however numerous proteins that are not protected within the buffered cytoplasm, such as those used to maintain the bacterial cell envelope (e.g., PBPs). It is likely that the apparent redundancy of the PBPs is due to the importance of specialized enzymes that can withstand environmental fluctuations (26, 48). For example, in *E. coli*, for peptidoglycan synthesis in the periplasmic space, there are around four enzymes available for every biosynthetic reaction, but only 14 enzymes are needed to perform 12 reactions in the cytoplasmic synthesis of peptidoglycan precursors—nearly every protein performing a unique synthetic reaction (49). Mueller et al. reported that the redundancy in PBPs in *E. coli* is likely needed to facilitate growth in a range of environmental pH values (26). Similarly, cytoplasmic peptidoglycan synthesis in *B. subtilis* employs numerous discrete enzymes for unique biosynthetic reactions in contrast to the redundancy in enzymatic action in the periplasmic space (50).

The inactivation of a subset of PBPs by alkaline shock in *B. subtilis* suggests that the apparent redundancy of PBPs may enable peptidoglycan synthesis in diverse environmental conditions. These changes in activity would likely not have been resolved through predictive software or by using purified proteins. The PBP1 isoforms and PBP4 are all bifunctional (class A), but during alkaline stress, PBP1b becomes active, while PBP1a and PBP4 lose activity. Class B PBPs PBP2a, PBPH, PBP2b, and PBP3 all perform transpeptidation, but only PBPH is inactivated during alkaline shock. Environmental stresses, such as salt concentration, osmolarity, and temperature, can be compounded by alkaline stress (1). For example, alkaliphiles require higher Na^+ to grow at neutral pH (37, 38). Additionally, the effect of pH on the bacterial replication machinery has also been linked to antibiotic resistance (2, 12, 26, 51). Finally, this work indicates that the apparent functional redundancy of classes of periplasmic proteins likely indicates differential specialization for dynamic environmental conditions (10).

MATERIALS AND METHODS

Materials and media

Bocillin-FL was purchased from Thermo Fisher Scientific (Invitrogen, cat# B13233) and was stored at −80°C. LB media (Lennox; 20 g/L) were autoclaved for 20 min (Sigma, powder, cat# L3022). Tryptic soy broth was prepared at 30 g/L (BD Bacto). Minimal media were prepared as a buffer (11.6 mM NaCl, 4.0 mM KCl, 1.4 mM $MgCl_2$, 2.8 mM Na_2SO_4, 2.8 mM NH_4Cl, 10 mM HEPES, 88.1 µM Na_2HPO_4, and 50.5 µM $CaCl_2$). Phosphate-buffered saline was prepared as 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH = 7.4.

Bacterial culturing, media, and exposure

Bacillus subtilis 3610 and *B. subtilis* PBP-null mutant strains were cultured in LB at 37°C while shaking at 220 rpm. *E. coli* harboring the *B. subtilis* PBP plasmid constructs was also

cultured in LB with ampicillin (100 µg/mL) at 37°C while shaking at 220 rpm. *Shewanella oneidensis* MR-1 was cultured in LB at 30°C while shaking at 250 rpm. *E. coli* K12 was also cultured in LB at 37°C while shaking at 220 rpm. *Staphylococcus aureus* was cultured in LB at 37°C while shaking at 220 rpm. *Streptococcus pneumoniae* was cultured in Brain Heart Infusion media at 37°C with 5% CO_2 without shaking. All strain identities are shown in Table 2.

pH exposures

B. subtilis cells were grown in an overnight culture and were sub-cultured with a 1:10 dilution in fresh media until reaching an exponential culture with an OD_{600} of 0.4–0.6. A 1.0-mL aliquot was harvested by centrifugation (10,000 × g for 1 min at room temperature (RT); consistent throughout the protocol). Cell pellets were washed with 1 mL of PBS. Pellets were resuspended in 1 mL of PBS unless otherwise indicated at the indicated pH with NaOH, HCl, or other indicated solutions with controls at a pH of 7.4 for 30 min at RT. Cells were once again pelleted, washed with 1 mL of PBS, and resuspended in 100 µL of PBS containing 5 µM Bocillin-FL for 15 min at RT in the dark. Cells were pelleted and washed with 1 mL of PBS.

Gel-based PBP activity assay

Bocillin-FL-labeled cells were resuspended in 100 µL of PBS containing 10 mg/mL lysozyme and incubated at 37°C for 30 min. Samples were lysed on ice with a Hielscher vial tweeter UP200st (70% C, 95% A, and 5% adjustment snap) for 10 min with 30-s intervals with 30-s cooling in between. The membrane proteome was collected by centrifugation at 21,000 × g for 15 min at 4°C. The supernatant was discarded, and the membrane fraction was resuspended in 100 µL with 1% SDS followed by 33 µL of 4× SDS loading buffer. To denature proteins, samples were heated at 95°C for 5 min and then cooled to RT before loading 12 µL into the well of an SDS-polyacrylamide gel. Gels (10%) were prepared with acrylamide:bis-acrylamide 29:1 (Bio-Rad) in 1.5-mm cassettes (Thermo Fisher, NC2015). Proteins were resolved by gel electrophoresis at 180 V, 400 mA, and 60 W for 1 h. Gels were rinsed with de-ionized water and scanned on a Typhoon 9210 gel scanner (Amersham Biosciences, Pittsburgh, PA, USA) with a 526-nm filter at 50-µm resolution. Gels were background subtracted, adjusted uniformly, and analyzed using ImageJ software (NIH). Coomassie staining was used to visualize total protein loading in each lane (Fig. S9).

TABLE 2 Bacterial strains used in this publication

Species	Strain	Notes	Reference
B. subtilis	3610	Wild-type strain	
	DK653	*pbpA::kan comI*^{Q12L}	This study
	DK654	*dacA::kan comI*^{Q12L}	(7)
	DK740	*pbpD::kan comI*^{Q12L}	This study
	DK694	*pbpH::kan comI*^{Q12L}	(7)
	DK695	*pbpC::kan comI*^{Q12L}	(7)
	DK1042	*comI*^{Q12L}	(52)
	DS9744	ponA::tet	This study
E. coli (BL21)	563	pDP563 P_{tac}-*GST-pbpD amp*	This study
	564	pDP564 P_{tac}-*GST-pbpH amp*	This study
S. pneumoniae	IU1945	Unencapsulated derivative of the D39	(41, 42)
S. aureus	MW2	MRSA	BAA 1707
E. coli	K12		(43)
S. oneidensis	MR-1		BAA 1096

Microscopy

Cells were grown and exposed as described above. Cells were incubated with 3% paraformaldehyde in PBS for 25 min and then washed with PBS. Fixed cells (5 µL) were pipetted onto slides (SuperFrost, FisherBrand) to air-dry and covered with a 1.5-mm coverslip. Microscopy was performed with an Olympus IX73 microscope. Images were acquired with an ORCA-FLASH4.0LT+ SCMOS camera and were processed with ImageJ.

Colony-forming units

B. subtilis cells were grown and exposed as described above. Cells were diluted and dropped onto a plate (10 µL) in triplicate, and the resultant colonies were counted after overnight growth.

Plate reader

B. subtilis cells (200 µL) were cultured overnight in a Tecan Spark plate reader in LB (37°C, shaking at 220 rpm) with 20-min time points.

Marker replacement mutants

The kanamycin resistance cassette insertion-deletion constructs in *pbpA*, *pbpC*, *pbpD*, *pbpH*, and *dacA* were generated by isothermal "Gibson" assembly (ITA) (53). The regions upstream and downstream of each gene were amplified using DK1042 chromosomal DNA as a template, and the primer pairs are indicated in parentheses: *pbpA* (3534/3535; 3536/3537), *pbpC* (3538/3539; 3583/3584), *pbpD* (3598/3599; 3600/3601), *pbpH* (3579/3580; 3581/3582), and *dacA* (3542/3543; 3544/3545) (Table 3). Next, the kanamycin resistance gene was PCR amplified using the plasmid pDG780 (54) as a template and primer pair 3250/3251. For each gene, the upstream amplicon, the downstream amplicon, and the kanamycin resistance gene amplicon were mixed in an ITA reaction, column cleaned, and transformed into *B. subtilis* strain DK1042 (52), selecting for resistance to kanamycin. Each insertion-deletion mutant was verified using PCR length polymorphism using the far upstream and downstream primers.

Isothermal assembly reaction buffer (5×) [500 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT (Bio-Rad), 31.25 mM PEG-8000 (Fisher Scientific), 5.02 mM NAD (Sigma Aldrich), and 1 mM of each dNTP (New England BioLabs)] was aliquoted and stored at −80°C. An assembly master mixture was made by combining prepared 5× isothermal assembly reaction buffer (131 mM Tris-HCl, 13.1 mM $MgCl_2$, 13.1 mM DTT, 8.21 mM PEG-8000, 1.32 mM NAD, and 0.26 mM each dNTP) with Phusion DNA polymerase (New England BioLabs) (0.033 units/µL), T5 exonuclease diluted 1:5 with 5× reaction buffer (New England BioLabs) (0.01 units/µL), Taq DNA ligase (New England BioLabs) (5,328 units/µL), and additional dNTPs (267 µM). The master mix was aliquoted as 15 µL and stored at −80°C.

To generate the *ponA::tet* allele, the region upstream and downstream of *ponA* was PCR amplified using 3610 chromosomal DNA as a template and primer pairs 3053/3054 and 3055/3056. The tetracycline resistance gene was PCR amplified from pDG1515 (54) using the primer pair 2973/2974. Next, the upstream fragment was digested with BamHI-PstI, the tetracycline resistance cassette was digested with PstI-EcoRI, the downstream fragment was digested with EcoRI-XhoI, and all three fragments were simultaneously ligated into the BamHI-XhoI sites of plasmid pUC19 to make pRC18. The plasmid pRC18 was transformed into DS2569 (52), selecting for tetracycline resistance, and transduced into strain 3610 using the generalized transducing phage SPP1 (55).

E. coli expression plasmids

To express the *B. subtilis* GST-PBP fusion proteins in *E. coli*, the *pbpD* gene was amplified from DK1042 chromosomal DNA using the primer pair 7520/7521 and the *pbpH* gene

TABLE 3 Primer pairs used in mutant generation and expression plasmid construction

Primer	Sequence
2973	aggaggaattcgtgtacgtaaaaagattaaattattgct
2974	ctcctctgcagctgttataaaaaaaggatcaattttgaac
3053	aggagggatccaccatcaaatcgagcatatgaagca
3054	ctcctctgcagatctcttttccatttttatcataaatcgt
3055	aggaggaattcaaactcatcatccattgaaaaaacaaat
3056	ctcctctcgagagcttcagcaggatattaaatcaatc
3250	acgactcactatagggcgaattg
3251	ctcactaaagggaacaaaagctgg
3534	ccaagattctccgctttcag
3535	caattcgccctatagtgagtcgtcagttccacaataatccagg
3536	ccagcttttgttccctttagtgaggagctgaaatcgaagcagga
3537	gtaaactcttcggaagagaag
3538	tacagatttcaagaaaatagga
3539	caattcgccctatagtgagtcgtgaatctgttttggagcatccg
3542	gtaccagcaaagtctgagg
3543	caattcgccctatagtgagtcgtacacaaccaatgacatcagt
3544	ccagcttttgttccctttagtgagggaagcattgttgatacggta
3545	ctacaatacgaccattatgc
3579	cggaggctggtcattgtcaa
3580	caattcgccctatagtgagtcgtgagcttaaaaattaatgcagtgaa
3581	ccagcttttgttccctttagtgagacgggaacagcggaaacattt
3582	atgtcccgaagaatgacctg
3583	ccagcttttgttccctttagtgaggtatcagcctgtcgtgaaag
3584	cctgagactctaataacgaac
3598	ctaacatagctagaaaacgtc
3599	caattcgccctatagtgagtcgtggaagcgataactgtaaatgc
3600	ccagcttttgttccctttagtgaggatacaccgaccagcgttg
3601	ccatgacgacaacaaattcc
7520	gtcgtgcatctgttggatccccttccggaaaagaagtaaaacaaatg
7521	cagtcagtcacgatgaattcccttaataagccgcttgcagcgttc
7522	gtcgtgcatctgttggatcccctgaaggcgaacagcatgaagaag
7523	cagtcagtcacgatgaattcccttatttttactgtgttttttttcgagc

was PCR amplified using the primer pair 7522/7523 (Table 3). Next, the plasmid pGEX-2TK (Sigma-Aldrich) was digested with SmaI, and the digested plasmid was mixed with each pbp gene fragment separately in an ITA reaction. The reactions were transformed into *E. coli* to generate plasmids pDP563 (P_{tac}-*GST-pbpD amp*) and pDP564 (P_{tac}-*GST-pbpH amp*). Plasmid isolates were confirmed to have the predicted insertion by digestion with EcoRI and BamHI.

Plasmids were transformed into *E. coli* from New England Biolabs (C2566) following the included protocol. Transformations were confirmed by diagnostic EcoR1 digestion (NEB). PBP constructs were expressed in *E. coli* followed by experimental alkaline shock. PBP constructs were extracted from *E. coli* cells by incubation with 10 mg/mL lysozyme at 37°C for 30 min followed by lysis on ice with a Hielscher vial tweeter UP200st (70% C, 95% A, and 5% adjustment snap) for 10 min with 30-s intervals with 30-s cooling in between. Lysates were treated with NaOH and then labeled with Bocillin-FL. Samples were prepared for SDS-PAGE analysis as described above.

Software

ExPASy ProtParam was used to predict the molecular weight and isoelectric point of the PBPs (56). NIH Protein Blast was used to compare sequence coverage and identity

similarities between PBPs (57). *B. subtilis* 168 was used in Protein Blast and UniProt comparisons, as this strain is the closest relative of *B. subtilis* 3610 (58).

ACKNOWLEDGMENTS

We thank Rebecca Calvo for the materials (*ponA::tet* construct). This material is based upon work supported by the National Science Foundation under Grant No. CHE-2001611, the NSF Center for Sustainable Nanotechnology. The Center for Sustainable Nanotechnology is part of the Centers for Chemical Innovation Program. S.L.M. gratefully acknowledges the support of the University of Minnesota's Doctoral Dissertation Fellowship during the development of this work. D.B.K. was funded by NIH R35 GM131783.

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FUNDING

Funder	Grant(s)	Author(s)
National Science Foundation (NSF)	CHE-2001611	Erin E. Carlson
HHS | National Institutes of Health (NIH)	R35 GM131783	Daniel B. Kearns

AUTHOR CONTRIBUTIONS

Stephanie L. Mitchell, Conceptualization, Investigation, Methodology, Writing – original draft | Daniel B. Kearns, Funding acquisition, Methodology, Writing – review and editing | Erin E. Carlson, Funding acquisition, Project administration, Supervision, Writing – review and editing

DIRECT CONTRIBUTION

This article contains supporting information (Fig. S1 to S9; Tables S1 to S5) (17, 59, 60)., including references (22, 23, 30–32, 59–61).

DATA AVAILABILITY

All the data produced for this work are contained within the article and the supporting information.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplementary document (AEM00548-23-s0001.pdf). Supplementary figures and tables.

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