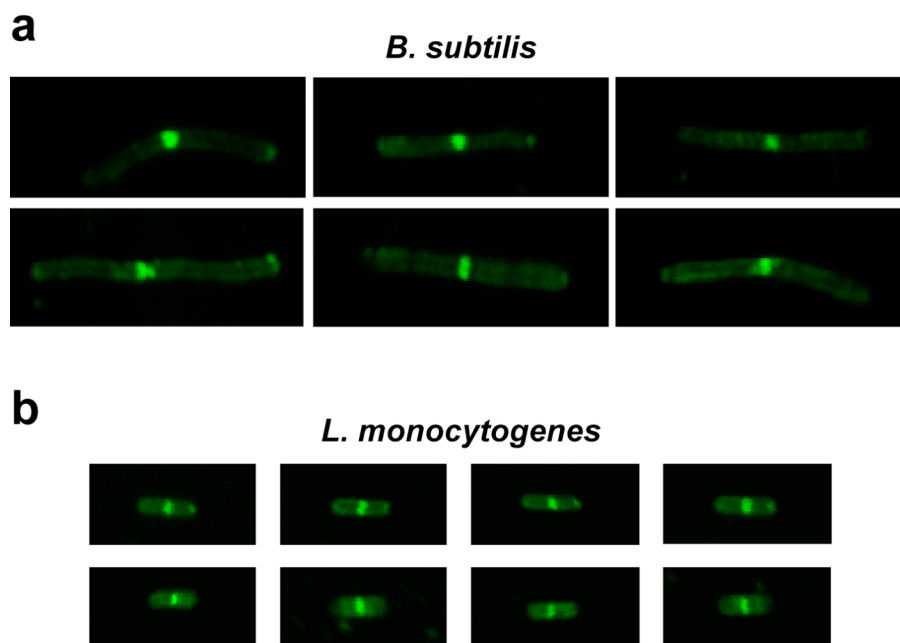


## SUPPLEMENTARY INFORMATION

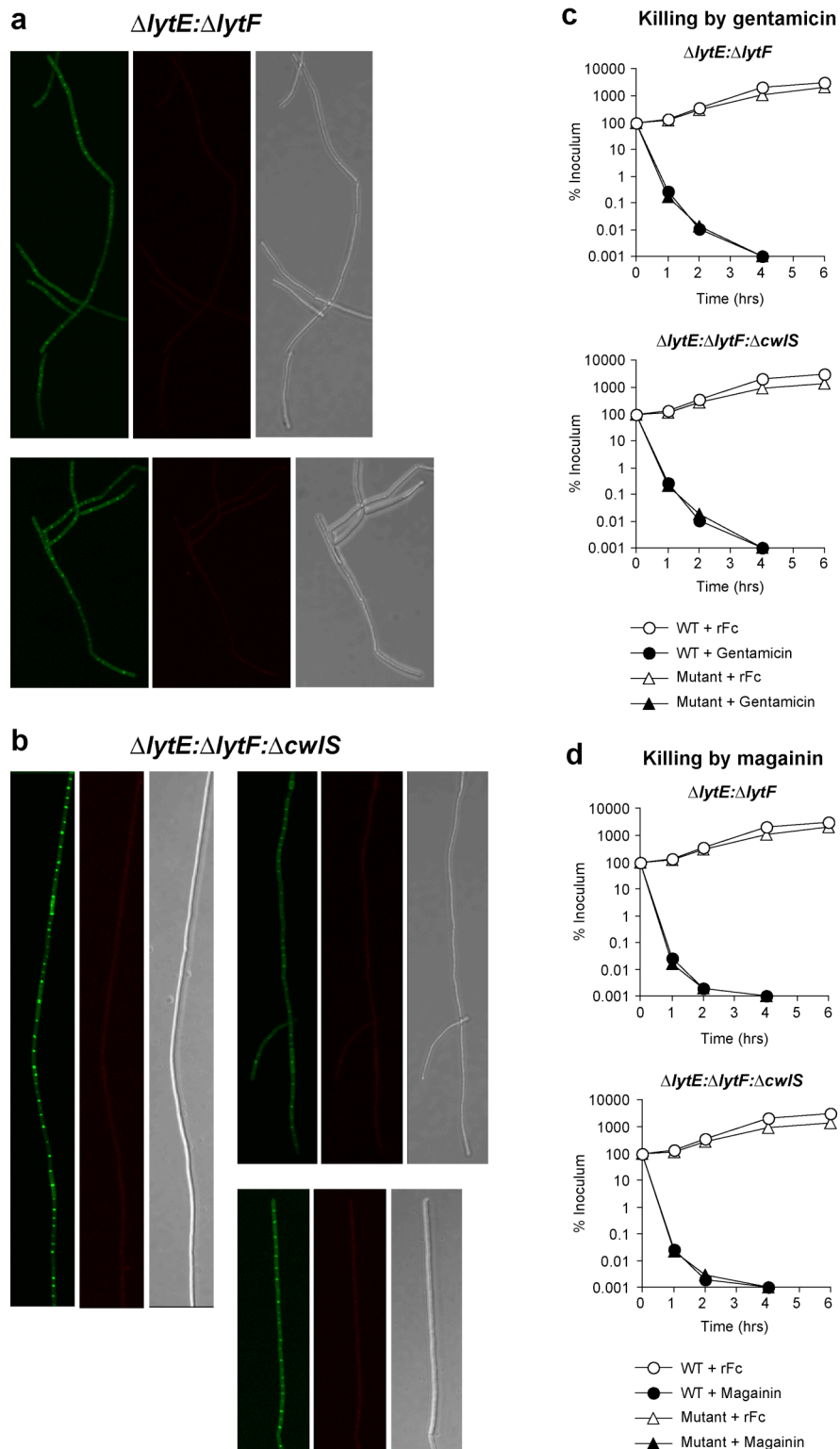
### Peptidoglycan Recognition Proteins kill bacteria by inducing suicide through protein-sensing two-component systems

Des Raj Kashyap, Minhui Wang, Li-Hui Liu, Geert-Jan Boons, Dipika Gupta, & Roman Dziarski

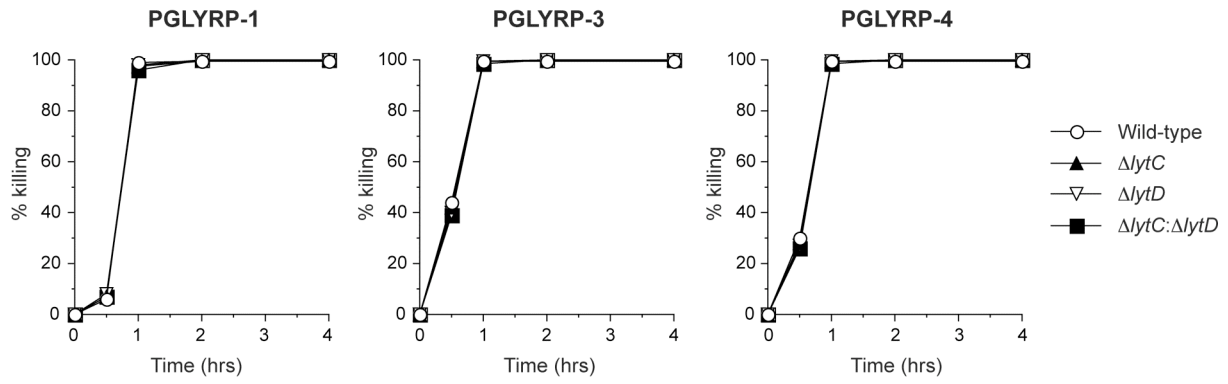
#### SUPPLEMENTARY FIGURES



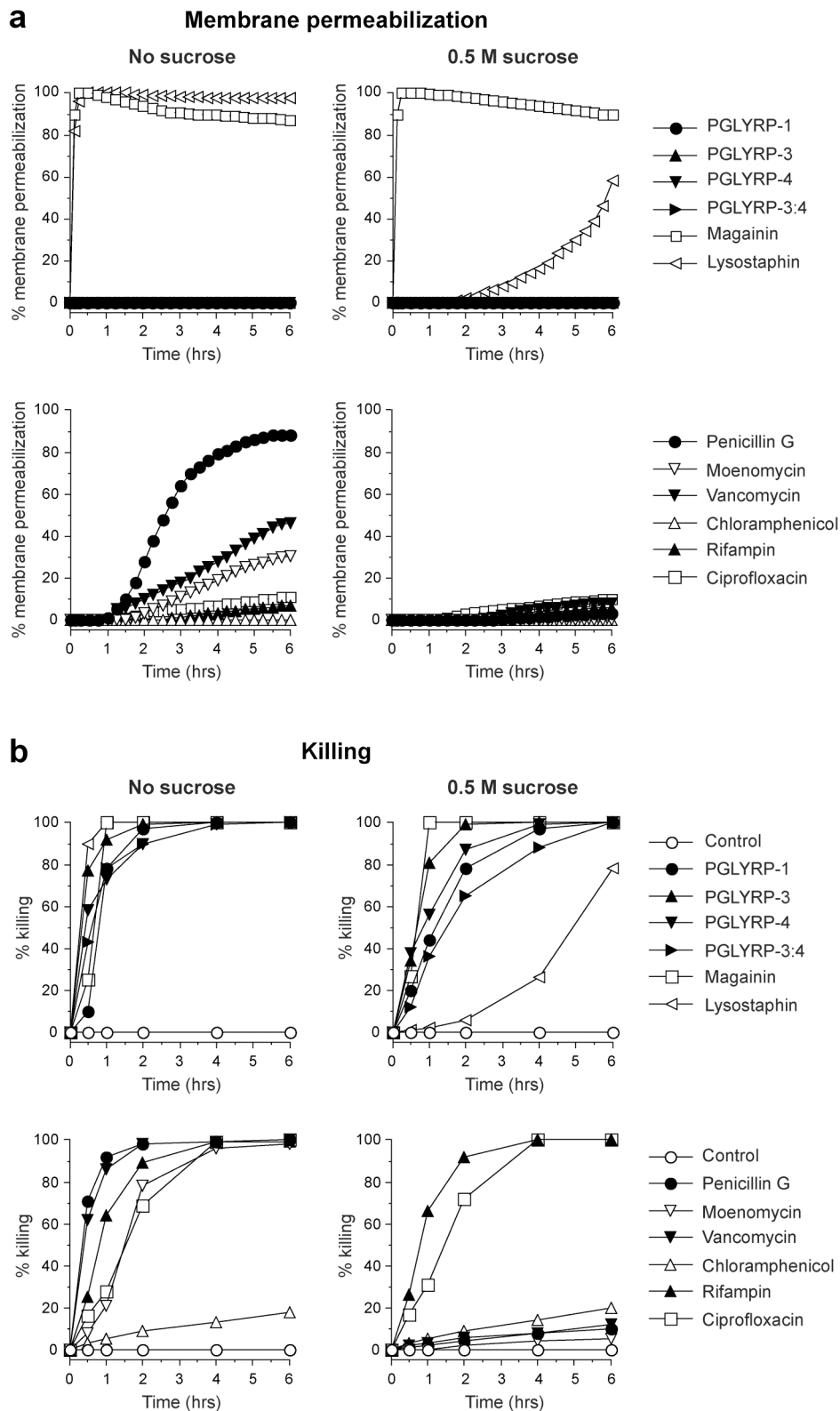
**Supplementary Figure 1. PGRPs bind to cell separation sites in Gram-positive bacteria.** Preferential binding of PGLYRP-1 to cell separation sites in *B. subtilis* (**a**) and *L. monocytogenes* (**b**) detected by confocal microscopy with anti-V5-FITC mAbs. Merged stacks are shown; there was no cytoplasmic staining when individual scans were analyzed (not shown). Similar results were obtained with PGLYRP-4, whereas no staining was obtained with anti-V5-FITC mAbs and recombinant mouse albumin as a control (not shown). Indirect immunofluorescence employed here is more sensitive than the direct staining used in Fig.1d, and thus, in addition to very prominent staining of the cell separation sites with PGRP, faint staining of one cell pole (which is the site of the previous cell division) is also seen, because this pole still has not been completely repaired. The PGRP staining is identical to the localization of LytE and LytF cell-separating hydrolases (Fig. 1e), which also have major presence in the cell separation site and minor presence at the pole that represents the previous division site<sup>19,37</sup>. The results are representative of 3 experiments.



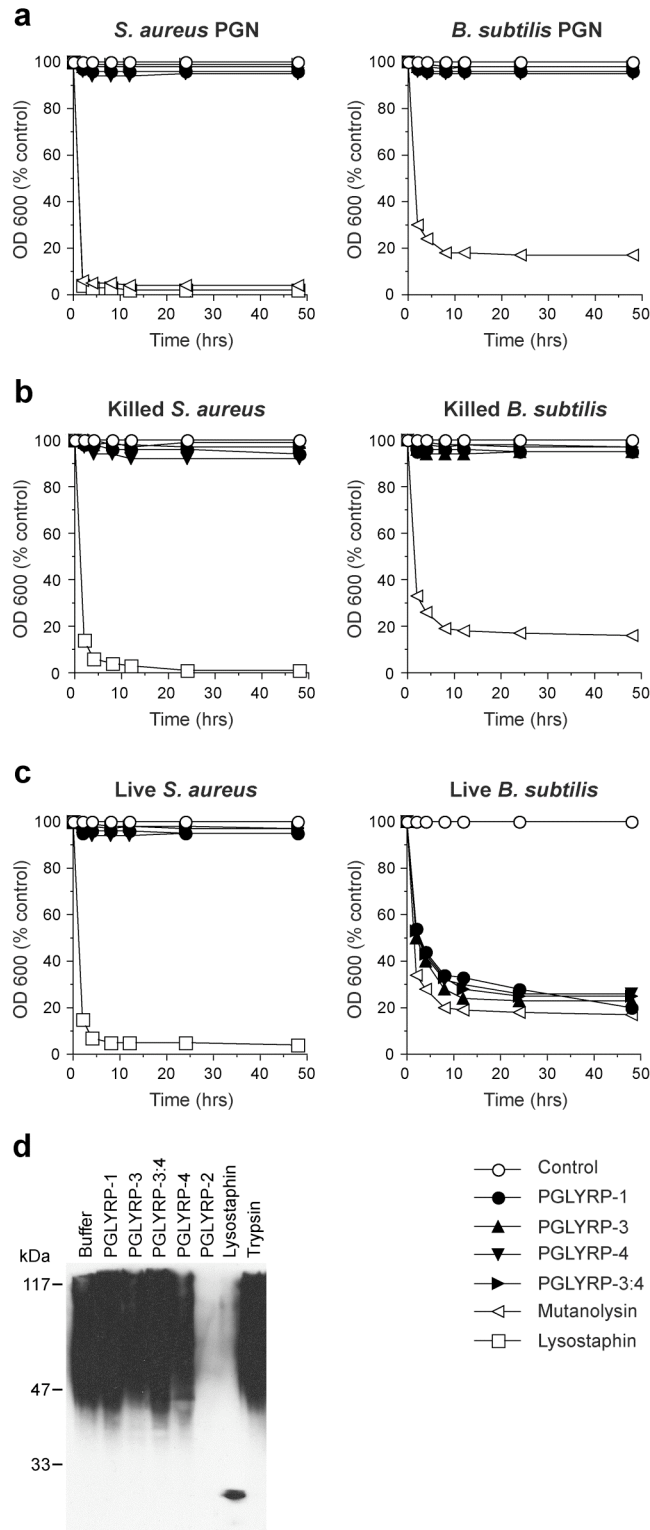
**Supplementary Figure 2. LytE- and LytF-generated cell separation sites are required for efficient binding of PGRPs to *B. subtilis*, but not for killing by gentamicin and magainin.** (a, b) Dual staining of *B. subtilis*  $\Delta\text{lytE}:\Delta\text{lytF}$  or *B. subtilis*  $\Delta\text{lytE}:\Delta\text{lytF}:\Delta\text{cwIS}$  with BODIPY-FL-labeled vancomycin (green, left panels) and with Alexa Fluor 594-labeled PGLYRP-4 (red, middle panels) reveals localization of vancomycin to the sites of new peptidoglycan synthesis (primarily in the new septa), but no labeling with PGLYRP-4 due to the lack of cell separation sites in both mutants. Dark field view is shown in the right panels. The results are representative of 3 similar experiments. Parallel experiments with WT *B. subtilis* with PGLYRP-4 (not shown) to the staining pictured in Fig. 1d in the main paper. (c, d) Similar killing of WT *B. subtilis* and  $\Delta\text{lytE}:\Delta\text{lytF}$  or  $\Delta\text{lytE}:\Delta\text{lytF}:\Delta\text{cwIS}$  mutants by 5  $\mu\text{g/ml}$  gentamicin and 100  $\mu\text{g/ml}$  magainin (means of 3 experiments, SEM were smaller than the symbols).



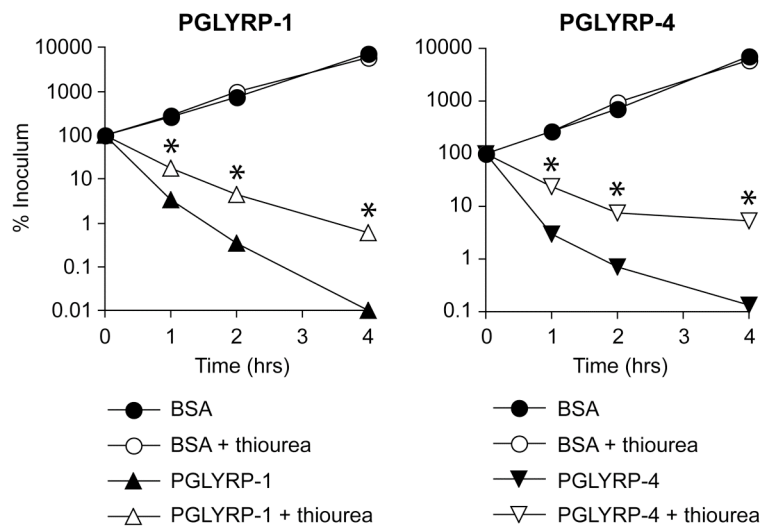
**Supplementary Figure 3. Similar killing of WT and autolysin-deficient *B. subtilis* mutants  $\Delta lytC$ ,  $\Delta lytD$ , and  $\Delta lytC:\Delta lytD$  by PGRPs.** Killing of the indicated *B. subtilis* strains incubated with PGRPs (50  $\mu\text{g}/\text{ml}$ ) added at time 0. The results are expressed as % of killed bacteria and are means of 3 experiments.



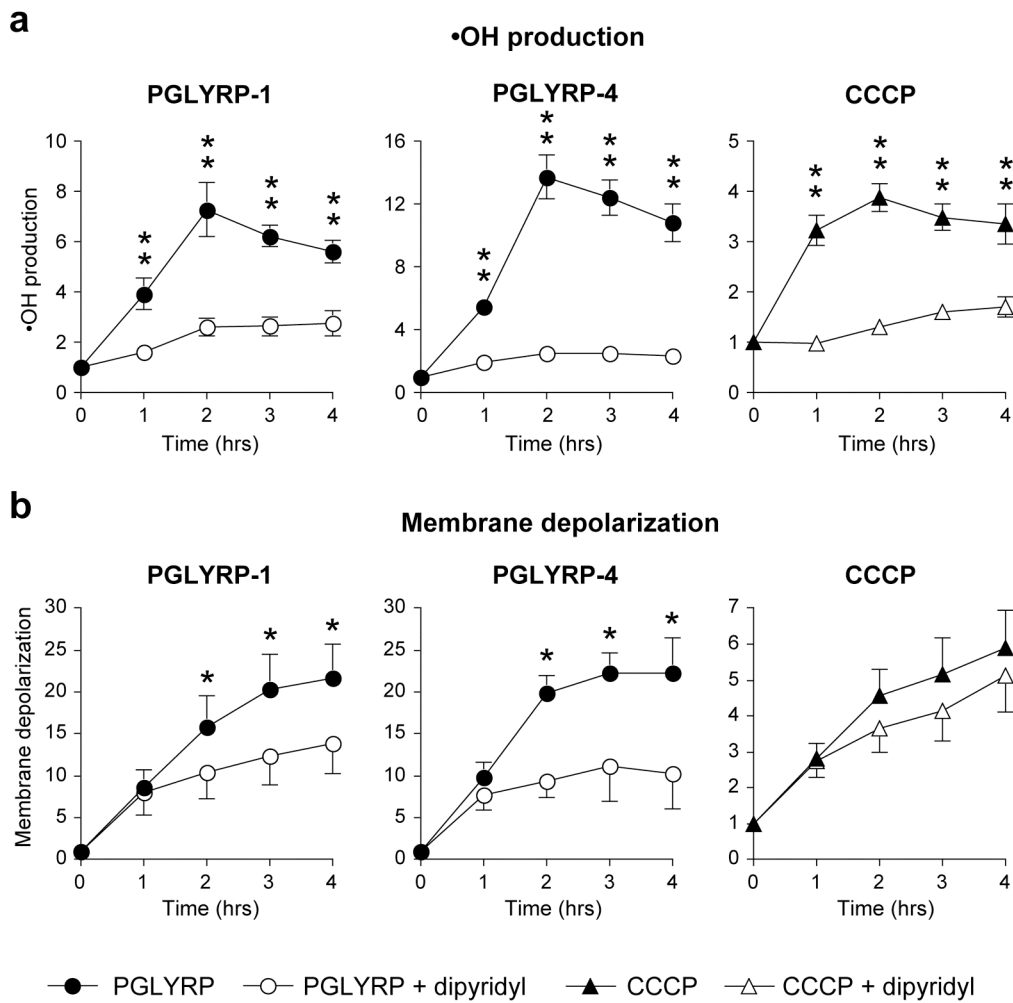
**Supplementary Figure 4. PGRPs kill bacteria but do not permeabilize their cell membranes.** (a) Membrane permeabilization of *S. aureus* incubated with PGRPs (200  $\mu\text{g/ml}$ ), magainin, lysostaphin, or antibiotics (added at time 0) was measured in medium without or with 0.5 M sucrose (which prevents osmotic lysis). The results are expressed as % of maximal membrane permeabilization and are means of 3 experiments. (b) Killing of *S. aureus* incubated with PGRPs, magainin, lysostaphin, antibiotics, or BSA (control) (added at time 0) was measured under identical conditions as in (a) in medium without or with 0.5 M sucrose. The results are expressed as % of killed bacteria and are means of 3-5 experiments.



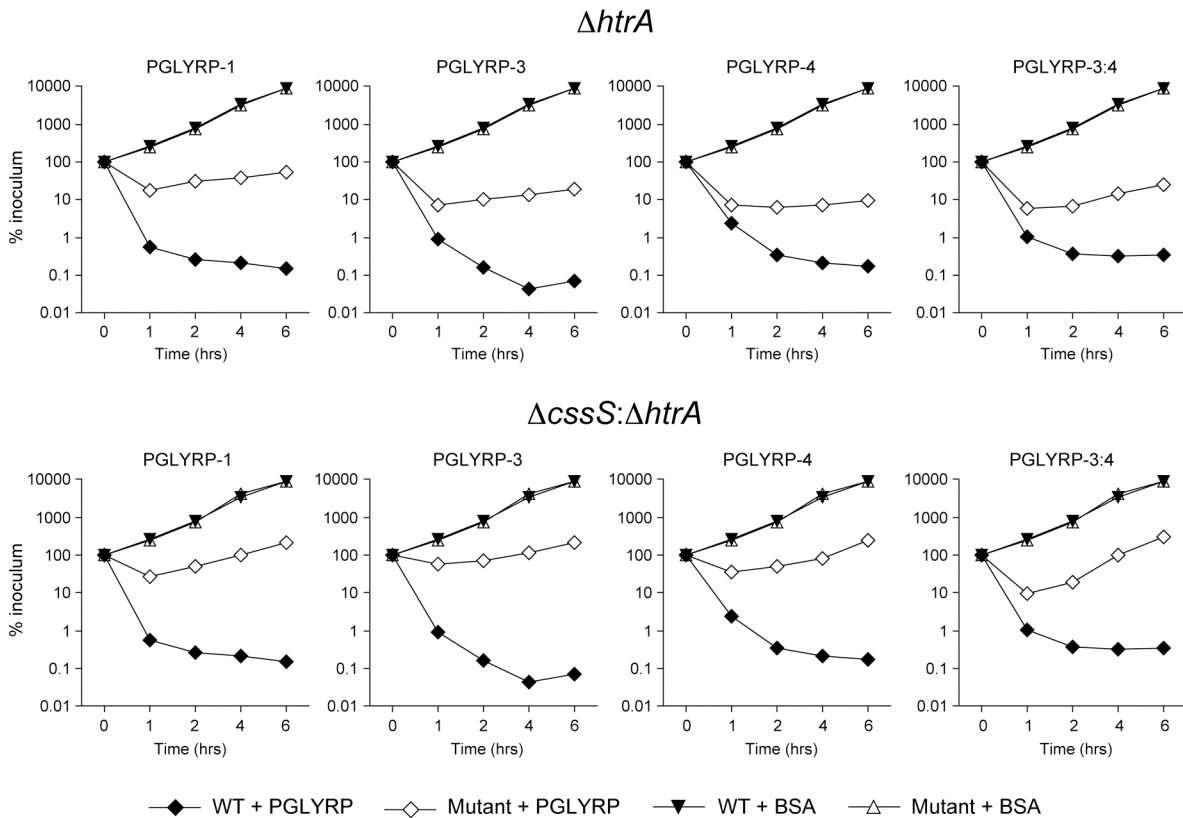
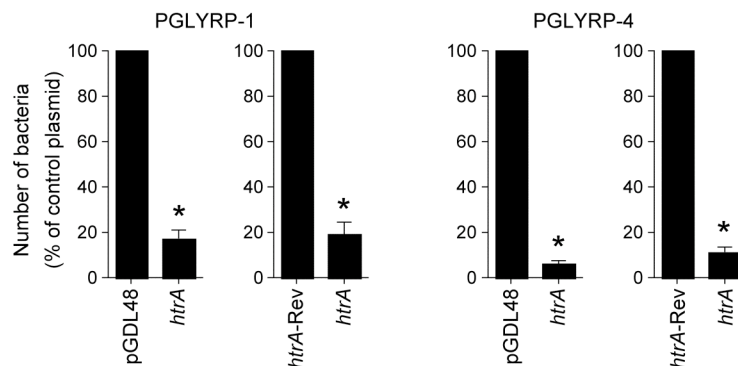
**Supplementary Figure 5. Bactericidal PGRPs do not hydrolyze peptidoglycan or bacteria.** *S. aureus* or *B. subtilis* insoluble peptidoglycan (a) or heat-killed (b) or live (c) bacteria were incubated with PGRPs (200  $\mu\text{g}/\text{ml}$ ), lysostaphin (10  $\mu\text{g}/\text{ml}$ ), or mutanolysin (100  $\mu\text{g}/\text{ml}$ ) added at time 0, and optical density at 600 nm was measured. The results are expressed as % of medium control and are means of 2 experiments. (d) *S. aureus* soluble polymeric uncrosslinked peptidoglycan, labeled with biotin on terminal Gly, was incubated with the indicated bactericidal PGRPs or amidase (PGLYRP-2) or lysostaphin (positive controls), or with buffer alone or trypsin (negative controls) for 4 days. High molecular weight polymeric biotin-peptidoglycan was detected on an immunoblot with streptavidin-peroxidase; the results are from one of two similar experiments.



**Supplementary Figure 6. •OH scavenger thiourea inhibits PGRP-induced killing.** WT *B. subtilis* was incubated with the indicated PGRPs (100-150  $\mu\text{g/ml}$ ) or BSA as a control without or with 150 mM thiourea and the numbers of bacteria were determined at the indicated times by colony counts. The results are means of 3 experiments, SEM were smaller than the size of the symbols; \*,  $P \leq 0.05$  (*t*-test) no thiourea versus with thiourea.



**Supplementary Figure 7. Dipyrindyl inhibits PGRP-induced •OH formation and partially inhibits PGRP-induced but not CCCP-induced membrane depolarization.** WT *B. subtilis* was incubated with the indicated PGRPs (100-200  $\mu\text{g/ml}$ ), or 20  $\mu\text{M}$  CCCP, or BSA without or with 300  $\mu\text{M}$  dipyrindyl, and •OH production (a) or membrane depolarization (b) were measured by flow cytometry with fluorescent probes HPF (a) or DiBAC<sub>4</sub>(3) (b). The results are expressed as ratios of mean fluorescence intensity of PGRP- or CCCP-treated to BSA-treated bacteria. The results are means of 3 experiments  $\pm$  SEM; \*\*, P < 0.01; \*, P < 0.05 (*t*-test) no dipyrindyl versus with dipyrindyl.

**a*****B. subtilis* killing****b****Complementation** **$\Delta htrA$** 

**Supplementary Figure 8. HtrA is required for PGRP-induced killing of *B. subtilis*.** (a) WT *B. subtilis* (closed symbols) or  $\Delta htrA$  or  $\Delta cssS:\Delta htrA$  mutants (open symbols) were incubated with the indicated PGRPs (100  $\mu\text{g/ml}$ ) or BSA as a control and the numbers of bacteria were determined at the indicated times by colony counts. The results are means of 3 experiments; the average SEM ranged from 5 to 19% and are not shown; both mutants show significantly reduced sensitivity to killing by all PGRPs ( $P < 0.05$  versus WT, *t*-test). (b) Complementation of PGRP-induced bacterial killing by *htrA*:  $\Delta htrA$  mutant was transfected with an empty vector (pGDL48) or pGDL48 containing *htrA* in forward orientation (*htrA*) or *htrA* in reverse orientation (*htrA-Rev*) and incubated with PGLYRP-1 or PGLYRP-4 (100  $\mu\text{g/ml}$ ). The numbers of surviving bacteria were determined at 4 h by colony counts. The results are presented as % of surviving bacteria in *htrA*-transfected groups compared to groups transfected with control plasmids (empty vector or reverse sequence); means of 3 experiments  $\pm$  SEM; \*,  $P < 0.05$  versus control plasmid (*t*-test). For further details see Supplementary Results and Discussion.



## SUPPLEMENTARY RESULTS AND DISCUSSION

### Peptidoglycan synthesis and possible mechanisms of inhibition by PGRPs

Peptidoglycan is synthesized in five major steps (Scheme 1). *First*, peptidoglycan precursors are assembled intracellularly into UDP-MurNAc-peptide. *Second*, UDP-MurNAc-peptide binds to the undecaprenyl-phosphate lipid carrier in the membrane to form lipid I. *Third*, lipid I acquires GlcNAc, to form undecaprenyl-phosphate-GlcNAc-MurNAc-peptide (lipid II). This complex then flips from the cytoplasmic side to the extracellular side of the cell membrane. In the *fourth* step, GlcNAc-MurNAc-peptides are assembled into a glycan chain by transglycosylation (this step is inhibited by vancomycin, mersacidin, actagardine, and moenomycin)<sup>38-41</sup>. In the *fifth* final step, peptides of the linear polymeric (GlcNAc-MurNAc-peptide)<sub>n</sub> are crosslinked to the existing peptidoglycan in the cell wall by transpeptidation (this step is inhibited by  $\beta$ -lactam antibiotics).

#### Scheme 1. Steps in peptidoglycan synthesis and their location

UDP-MurNAc-peptide	INTRACELLULAR
↓	
undecaprenyl-phosphate-MurNAc-peptide (Lipid I)	MEMBRANE-BOUND INTRACELLULAR
↓ $\neq$ blocked by ramoplanin	
undecaprenyl-phosphate-GlcNAc-MurNAc-peptide (Lipid II)	MEMBRANE-BOUND EXTRACELLULAR
↓ $\neq$ transglycosylation blocked by vancomycin and moenomycin	
(GlcNAc-MurNAc-peptide) <sub>n</sub>	EXTRACELLULAR
↓ $\neq$ transpeptidation blocked by $\beta$ -lactams	
peptide crosslinked (GlcNAc-MurNAc-peptide) <sub>n</sub> - (GlcNAc-MurNAc-peptide) <sub>n</sub>	EXTRACELLULAR

Antibiotics inhibit peptidoglycan synthesis by two basic mechanisms: (a) they either block the active site of peptidoglycan-synthesizing enzymes (e.g.,  $\beta$ -lactams or moenomycin); or (b) they bind to metabolic precursors of peptidoglycan and prevent their use in peptidoglycan synthesis (e.g., vancomycin, mersacidin, and actagardine).

To study the effects of PGRPs on peptidoglycan synthesis we used Gram-positive bacteria, because their peptidoglycan is abundant and its synthesis is readily accessible to extracellular compounds. We selected assays in intact cells (rather than permeabilized cells or reconstituted enzyme systems) to ensure that the inhibition happens in live growing bacteria, because our goal was to determine which mechanism is responsible for killing bacteria. We showed in the main paper that PGRPs inhibited peptidoglycan synthesis (Fig. 1a). The selectivity of our assay was confirmed by showing no inhibition of <sup>3</sup>H-GlcNAc incorporation into peptidoglycan by chloramphenicol, rifampin, and ciprofloxacin (Fig. 1a) at bactericidal concentrations (bacteriostatic for chloramphenicol) that fully inhibit protein, RNA, and DNA synthesis, respectively (Fig. 2).

PGRPs could inhibit peptidoglycan synthesis through several mechanisms:

*First*, because PGRPs avidly bind to peptidoglycan or its fragments (e.g., GlcNAc-MurNAc-peptide), they could inhibit peptidoglycan synthesis through binding to peptidoglycan biosynthetic precursors and preventing their incorporation into the growing peptidoglycan chain (i.e., inhibit transglycosylation or transpeptidation) as suggested by the structural analysis<sup>15</sup>. This mechanism would be similar to the action of antibiotics vancomycin, mersacidin, and actagardine.

*Second*, PGRPs could hydrolyze peptidoglycan precursor, GlcNAc-MurNAc-peptide, before or after transglycosylation and make it unusable in the subsequent biosynthetic steps. This would manifest as inhibition of transglycosylation or transpeptidation.

*Third*, PGRPs binding to the peptidoglycan that is being newly synthesized or to the existing peptidoglycan near the site of peptidoglycan synthesis could inhibit incorporation of newly synthesized peptidoglycan into the existing cell wall (i.e., PGRPs would inhibit transpeptidation by blocking the newly growing peptidoglycan polymer or by blocking access to the acceptor peptidoglycan in the cell wall).

*Fourth*, PGRPs could bind to peptidoglycan in the cell wall near the site of its synthesis, and could block the access of autolytic enzymes to peptidoglycan, which would also eventually inhibit peptidoglycan synthesis and cell wall growth, because limited digestion of the existing peptidoglycan at the site of new peptidoglycan synthesis is required for the growth of the cell wall (i.e., insertion of the new peptidoglycan into the existing cell wall). This mechanism would manifest as a delayed inhibition of transpeptidation.

Thus, we tested whether PGRPs inhibit the two extracellular steps of peptidoglycan synthesis, transglycosylation and transpeptidation. Such an inhibition would be consistent with the large size of PGRPs (44-115 kDa dimeric proteins), which are unlikely to enter the cytoplasm (because they do not permeabilize cytoplasmic membrane), and also with the structural analysis that suggested inhibition of transpeptidation<sup>15</sup>.

When Gram-positive bacteria are grown in the presence of <sup>3</sup>H-GlcNAc and inhibitors of transpeptidation, such as  $\beta$ -lactam antibiotics, the newly-synthesized polymeric uncrosslinked peptidoglycan is labeled with <sup>3</sup>H-GlcNAc and is secreted into the medium, rather than being crosslinked to the insoluble polymeric peptidoglycan in the cell wall<sup>42</sup>. Therefore, the extent of inhibition of transpeptidation is proportional to the extent of <sup>3</sup>H-GlcNAc incorporation into secreted polymeric uncrosslinked peptidoglycan. As reported in the main paper, penicillin G, as expected, inhibited transpeptidation and induced high incorporation of <sup>3</sup>H-GlcNAc into secreted polymeric uncrosslinked peptidoglycan, whereas PGRPs (at concentrations that were bactericidal and inhibited peptidoglycan synthesis), as well as other antibiotics that have other mechanisms of action, and also magainin and lysostaphin, did not (Fig. 1b). These results suggested that PGRPs do not inhibit transpeptidation. We further confirmed these results as follows.

If PGRPs inhibited transpeptidation by binding to the newly synthesized soluble polymeric peptidoglycan and did not retain it in the cell wall, soluble complexes of newly synthesized peptidoglycan with PGRPs would have been still found in the supernatant and detected in our assay, which was not the case. If PGRPs formed complexes with newly synthesized peptidoglycan and these complexes were retained in the cell wall and not secreted, we would have detected them in the insoluble cell wall fraction, and, thus, we would have seen an increase in <sup>3</sup>H-GlcNAc incorporation into the cell wall, which we did not observe (Fig. 1a). We confirmed these results using an alternative method of polymer precipitation with 10% TCA and we obtained the same results (data not shown), thus confirming no incorporation of <sup>3</sup>H-GlcNAc into polymeric peptidoglycan and excluding the possibility of it being retained in the cell wall by PGRPs. These results indicate that PGRPs do not inhibit transpeptidation, and thus must inhibit an earlier step in peptidoglycan synthesis.

We then tested whether PGRPs inhibit transglycosylation, the step in peptidoglycan synthesis that precedes transpeptidation. In transglycosylation, glycosidically-linked [GlcNAc-MurNAc-peptide]<sub>n</sub> is synthesized from membrane-bound undecaprenyl-phosphate-GlcNAc-MurNAc-peptide (lipid II) on the extracellular side of the cytoplasmic membrane (Scheme 1). This transglycosylation reaction is inhibited by vancomycin, mersacidin, actagardine, and moenomycin, which results in accumulation of lipid II in the cytoplasmic membrane<sup>31,32</sup>. We used vancomycin and moenomycin as positive controls, because they inhibit transglycosylation by two different mechanisms, binding to the substrate (D-Ala-D-Ala, vancomycin)<sup>40</sup>, or binding to the enzyme glycosyltransferase (moenomycin)<sup>43</sup>. As reported in the main paper, as expected, incubation of *S. aureus* with vancomycin or moenomycin in the presence of <sup>3</sup>H-GlcNAc resulted in increased accumulation of <sup>3</sup>H-GlcNAc in the butanol-soluble cell membrane lipid fraction containing lipid II (Fig. 1c). However, PGRPs (at concentrations that were bactericidal and inhibited peptidoglycan synthesis), as well as other antibiotics that have other mechanisms of action, and also magainin and lysostaphin, did not induce accumulation of lipid II in the cell membrane (Fig. 1c). These results indicate that PGRPs do not inhibit transglycosylation, and thus must inhibit an earlier intracellular step in peptidoglycan synthesis.

#### **PGRPs do not permeabilize cell membranes**

Our previous results showed that PGRPs did not permeabilize the cytoplasmic membranes over the period of 45 to 120 min<sup>10</sup>. Because PGRPs induced rapid and simultaneous inhibition of peptidoglycan, protein, RNA, and DNA synthesis that was not prevented by hyperosmotic medium (and thus resembled the effect of membrane-permeabilizing peptides, such as magainin), we next re-evaluated whether PGRPs permeabilized bacterial cell membranes, which would explain their rapid and simultaneous inhibition of all biosynthetic reactions.

PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 did not permeabilize bacterial cell membranes over the period of 6 h, despite rapid killing that exceeded 95% in 2 to 4 h and was not prevented by 0.5 M sucrose (Supplementary Fig. 4). By contrast, both magainin and lysostaphin induced immediate permeabilization of the cell membrane, which correlated with bacterial killing. Magainin directly permeabilized the cell membrane because its effect was not prevented in 0.5 M sucrose, whereas permeabilization by lysostaphin was indirect due to hydrolysis of peptidoglycan and osmotic lysis, because it was largely prevented in 0.5 M sucrose (Supplementary Fig. 4a). Antibiotics that inhibit peptidoglycan synthesis (penicillin G, vancomycin, and moenomycin) caused delayed partial permeabilization of cell membranes, which was indirect due to osmotic lysis that resulted from weakened peptidoglycan, because permeabilization was prevented in 0.5 M sucrose. Killing by these antibiotics was completely prevented by 0.5 M sucrose, confirming that inhibition of peptidoglycan synthesis and the loss of cell wall integrity are primarily responsible for their bactericidal effect. Antibiotics that inhibit protein, RNA, and DNA synthesis (chloramphenicol, rifampin, and ciprofloxacin) did not cause membrane permeabilization, and killing by these antibiotics was not affected by the presence of 0.5 M sucrose (Supplementary Fig. 4).

These results demonstrate that the mechanism of bactericidal activity of PGRPs is different from the mechanism of bactericidal activity of antibiotics that inhibit peptidoglycan, protein, RNA, or DNA synthesis, and is also different from membrane-permeabilizing peptides and from enzymes that rapidly

hydrolyze bacterial cell wall.

### **Bactericidal PGRPs do not hydrolyze peptidoglycan**

We considered enzymatic digestion of peptidoglycan by PGRPs as another possible mechanism responsible for bactericidal effect of PGRPs. This is a less likely mechanism because, as shown in this paper, PGRPs do not cause early indirect permeabilization of cytoplasmic membranes. This contrasts peptidoglycan-lytic enzymes (e.g., lysostaphin), which kill bacteria by rapidly destroying physical integrity of peptidoglycan, which causes immediate osmotic lysis of bacteria, manifested as permeabilization of cytoplasmic membranes that correlates with bacterial killing. Both membrane permeabilization and killing by lysostaphin are prevented by 0.5 M sucrose. However, it was still possible that peptidoglycan hydrolytic activity could be responsible for the bactericidal activity of PGRPs, if the binding of PGRPs to peptidoglycan was of high affinity (essentially irreversible), but the rate of hydrolysis was very slow. Moreover, bovine PGLYRP-1 is bacteriolytic, although this activity does not correlate with its bactericidal activity, because the former is heat-labile and the latter is heat-stable<sup>11</sup>. Therefore, we next tested whether bactericidal PGRPs hydrolyzed peptidoglycan or entire bacteria over a prolonged period of time.

PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 did not significantly hydrolyze insoluble *S. aureus* or *B. subtilis* peptidoglycan over a period of 48 h, in contrast to lysostaphin and mutanolysin, which rapidly hydrolyzed these peptidoglycans (Supplementary Fig. 5a). We tested peptidoglycans from both *S. aureus* and *B. subtilis*, to exclude the possibility that possible hydrolytic activity of any of these bactericidal PGRPs has a preference for Lys- or DAP-type peptidoglycan, as is the case with some insect PGRPs<sup>4</sup>. Our bactericidal PGRPs also did not significantly hydrolyze heat-killed *S. aureus* and *B. subtilis* bacteria, in contrast to lysostaphin or mutanolysin, which rapidly hydrolyzed these bacteria (Supplementary Fig. 5b). We used heat-killed bacteria to test direct digestion of bacterial cells by PGRPs in the absence of autolytic enzymes.

Because soluble uncrosslinked polymeric peptidoglycan and synthetic peptidoglycan fragments are better substrates for PGLYRP-2 amidase<sup>13,14</sup>, we next tested whether bactericidal PGRPs hydrolyze soluble uncrosslinked peptidoglycan and synthetic peptidoglycan fragments. Although our previous results showed that PGLYRP-1, PGLYRP-3, and PGLYRP-4, do not hydrolyze uncrosslinked peptidoglycan<sup>14</sup>, these preparations were expressed in different cells and purified by a different method that does not yield bactericidal preparations and were used at lower concentrations. Therefore, it was important to re-test bactericidal preparations at concentrations that kill bacteria. Bactericidal PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 did not hydrolyze soluble uncrosslinked polymeric peptidoglycan, in contrast to PGLYRP-2, which is a known amidase<sup>14</sup>, and lysostaphin, which we used as positive controls (Supplementary Fig. 5d).

We next tested whether bactericidal PGRPs hydrolyze synthetic peptidoglycan fragments using sensitive mass spectrometry analysis. In addition to testing amidase activity, we also tested whether these bactericidal PGRPs have any other hydrolytic activities, such a carboxypeptidase activity, because insect PGRP-SA is a D-Ala-carboxypeptidase with slow rate of hydrolytic activity, and which, similarly to mammalian PGLYRP-1, PGLYRP-3, and PGLYRP-4, does not have the conserved Cys<sup>44</sup> that corresponds to Cys530 in human PGLYRP-2 that is required for Zn<sup>2+</sup> binding and amidase activity<sup>14</sup>. Such a carboxypeptidase activity of PGRPs would not be bacteriolytic, but it would convert the

peptidoglycan biosynthetic precursor, GlcNAc-MurNAc-pentapeptide, to GlcNAc-MurNAc-tetrapeptide, and make it unsuitable for further steps in the biosynthetic pathway, such as transpeptidation.

PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 did not hydrolyze any bonds in the synthetic peptidoglycan fragments, MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala and MurNAc-L-Ala-D-isoGln-mDAP-D-Ala-D-Ala, and even after 4 days of incubation at 37°C only the parent compounds and no digestion products were detected by mass spectrometry (data not shown). By contrast, our positive control, PGLYRP-2, which is a known amidase<sup>14</sup>, hydrolyzed the lactyl-amide bond between MurNAc and L-Ala (data not shown), as reported by us previously<sup>14</sup>. These results demonstrate that PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 do not have the amidase, carboxypeptidase, or any other peptidoglycan-hydrolytic activity, and, therefore, these activities cannot be responsible for their bactericidal activity and their inhibition of peptidoglycan synthesis.

### **Autolytic enzymes are not responsible for bactericidal effect of PGRPs**

We next used live bacteria to test the possibility that bactericidal PGRPs could induce activation of autolytic enzymes or could synergize with autolytic enzymes, which could be responsible for killing bacteria. Live *S. aureus* bacteria were not hydrolyzed by any of the bactericidal PGRPs (Supplementary Fig. 5c). However, live *B. subtilis* bacteria exposed to bactericidal PGRPs underwent rapid hydrolysis (Supplementary Fig. 5c), which was likely due to activation of autolytic enzymes, because such hydrolysis was not observed with heat-killed *B. subtilis* (Supplementary Fig. 5b). These results suggest that with some bacteria (e.g., *S. aureus*) activation of autolytic enzymes is an unlikely primary mechanism responsible for their killing by bactericidal PGRPs. These results, however, also opened up a possibility that with some other bacteria (e.g., *B. subtilis*), activation of autolytic enzymes contributes to the bactericidal activity of PGRPs.

To test this latter possibility, we compared the killing by bactericidal PGRPs of wild-type *B. subtilis* and its isogenic mutants deficient in one or two major autolytic enzymes, LytC and LytD<sup>20</sup>. If these enzymes were required or significantly contributed to bacterial killing by PGRPs, one would expect a diminished killing of these mutants by PGRPs. However, the killing of wild-type *B. subtilis* 168 and its mutants,  $\Delta$ lytC,  $\Delta$ lytD, and  $\Delta$ lytC: $\Delta$ lytD was similar (Supplementary Fig. 3), thus indicating no requirement for or no significant contribution of these autolytic enzymes to *B. subtilis* killing by PGRPs. Thus lysis of live *B. subtilis* following exposure to PGRPs is likely a secondary event or a consequence of killing, as it is well known that *B. subtilis* easily undergoes autolysis.

### **Bactericidal mechanism of PGRPs is different from bactericidal mechanisms of antibiotics, cell wall-lytic enzymes, and membrane-permeabilizing antibacterial peptides**

In conclusion, the bactericidal mechanism of PGRPs is clearly different from the bactericidal mechanisms of antibiotics, cell wall-lytic enzymes, and membrane-permeabilizing antibacterial peptides. Antibiotics that inhibit protein or nucleic acid synthesis first enter the cell cytoplasm and selectively inhibit one biosynthetic reaction, and as a consequence of this inhibition a stress response ensues, which contributes to bacterial killing<sup>21,23,35</sup>. PGRPs do not enter the cytoplasm and act from an extracellular site, from where they induce membrane depolarization and stress response that results in inhibition of all biosynthetic reactions (see Results in the main paper). Thus, even though PGRPs inhibit peptidoglycan

synthesis, they only indirectly inhibit intracellular assembly of peptidoglycan precursors together with the inhibition of other biosynthetic reactions, likely due to depletion membrane potential-driven generation of energy. PGRPs do not inhibit transglycosylation or transpeptidation (two extracellular steps of peptidoglycan synthesis), which contrasts the effect of the majority of antibiotics that inhibit peptidoglycan synthesis, such as  $\beta$ -lactams, vancomycin, and moenomycin. These antibiotics inhibit transpeptidation or transglycosylation, which eventually causes secondary membrane permeabilization due to osmotic lysis and thus both the killing and membrane permeabilization by these antibiotics can be prevented by hyperosmotic medium. Because PGRPs do not inhibit extracellular assembly of peptidoglycan, they do not induce osmotic lysis, and thus killing by PGRPs is not prevented by hyperosmotic medium.

PGRPs also behave differently from peptidoglycan-lytic enzymes (such as lysostaphin or muramidases). Peptidoglycan-lytic enzymes kill bacteria by causing osmotic cell lysis that immediately results in membrane permeabilization and stops all metabolic reactions, all of which are prevented in hyperosmotic medium. By contrast, bactericidal PGRPs have no peptidoglycan-hydrolytic activity, do not cause membrane permeabilization, and their effects are not prevented in hyperosmotic medium.

PGRPs also behave differently from membrane-permeabilizing antibacterial peptides (e.g., magainin), because although both PGRPs and antibacterial peptides cause rapid and simultaneous inhibition of intracellular synthesis of all macromolecules, the effect of antimicrobial peptides is due to immediate and complete permeabilization of the cytoplasmic membrane. By contrast, PGRPs do not permeabilize the cytoplasmic membrane and their effect is secondary due to the loss of membrane potential and  $\bullet\text{OH}$  production, as shown in the main paper.

Thus, the mechanism of bacterial killing by PGRPs is unique and different from other antibacterial compounds (antibiotics, cell-wall-lytic enzymes, and membrane-permeabilizing peptides), although one aspect of killing by PGRPs resembles killing induced by bactericidal antibiotics, especially inhibitors of protein synthesis<sup>21,23,35</sup>: both involve sensing by a two-component system that induces membrane depolarization and production of  $\bullet\text{OH}$ . However, for antibiotics this is a secondary event: antibiotics first enter the cells and selectively inhibit one metabolic reaction (protein, RNA, DNA, or peptidoglycan synthesis), which then induces stress and synthesis of misfolded proteins that are exported out of the cells and then sensed by the two-component system<sup>21,23,35</sup>. By contrast, activation of protein-sensing two-component system is the primary mechanism of PGRP-induced killing (see Results in the main paper). PGRPs do not enter the cytoplasm and do not selectively inhibit one biosynthetic reaction, but rather bind to the cell wall and are sensed by the two-component system, because they likely mimic misfolded or aggregated self proteins. Persistent activation of the two-component system induces membrane depolarization, stress response, and production of toxic  $\bullet\text{OH}$ , which results in cessation of metabolic activity of the cell and simultaneous (rather than selective) inhibition of all biosynthetic reactions.

### **Effect of membrane depolarization on killing and $\bullet\text{OH}$ production**

We show in the main paper that bacterial killing induced by membrane depolarizer, CCCP, was independent of  $\bullet\text{OH}$  production, because it was not inhibited by dipyrindyl (Fig. 3c).  $\bullet\text{OH}$ -independent killing by CCCP could be due to irreversible inhibition of cytochrome C by this cyanide derivative in aerobic *B. subtilis*. Nevertheless CCCP induced  $\bullet\text{OH}$  production (Supplementary Fig. 7a), thus showing

that •OH production can be induced by membrane depolarization. PGRP-induced membrane depolarization was likely the primary event induced by PGRPs that preceded and was independent of •OH production because during the first hour of PGRP exposure membrane depolarization was not inhibited by dipyriddy (Supplementary Fig. 7b). However, during the second through the fourth hour of PGRP exposure, PGRP-induced •OH further enhanced PGRP-induced membrane depolarization, because at this time PGRP-induced membrane depolarization was inhibited by dipyriddy (Supplementary Fig. 7b). These results suggest an enhancing feedback effect of •OH on membrane depolarization.

### **HtrA is required for PGRP-induced killing of *B. subtilis***

HtrA is a membrane-bound protease directly regulated by the CssR-CssS system<sup>22</sup>. HtrA, similar to CssR-CssS, is also required for bactericidal effect of PGRPs, because  $\Delta htrA$  mutant is less sensitive to PGRP-mediated killing than WT *B. subtilis* (Supplementary Fig. 8a). To confirm the role of HtrA in PGRP killing we performed complementation experiments. We cloned *htrA* from WT *B. subtilis* 168 and expressed it in pGDL48 vector.  $\Delta htrA$  mutant complemented with *htrA*-expressing plasmid was significantly more sensitive to killing by PGRP proteins than the same mutant transfected with an empty vector or with *htrA* inserted into the vector in a reverse orientation (Supplementary Fig. 8b).

It would appear that a mutant with a deleted *htrA* should be more sensitive to killing by PGRPs and not less (as reported here), because the mutant does not have the protease that functions to remove misfolded proteins. One explanation for our observation could be that the CssR-CssS two-component system in *B. subtilis* is negatively regulated by a protease-sensitive protein, similar to CpxP in *E. coli*<sup>23</sup>, and thus lack of the protease would result in an excess of the negative regulator and down-regulation of the two-component system, which would have a similar effect to the *cssR* or *cssS* deletion. With this caveat, these results further support the role of the CssR-CssS two-component system in bactericidal effect of PGRPs.

### **How PGRPs access CssR-CssS in the cytoplasmic membrane**

Because Gram-positive bacteria have a thick cell wall, to activate the two-component system in the cytoplasmic membrane PGRPs need to traverse the cell wall and bind to peptidoglycan near the cell membrane. This can be easiest accomplished at the sites of separation of newly formed daughter cells. After completion of formation of a new septum, cell separation involves limited digestion of peptidoglycan at the new septum between two daughter cells to separate the cells. This digestion is performed by several dedicated peptidoglycan hydrolases (LytE, LytF, and CwIS in *B. subtilis*<sup>18,19</sup>) that temporarily open up the cell wall structure, which can be seen by scanning electron microscopy initially as discrete patches of digested cell wall near the new septum<sup>30</sup>. These patches then coalesce into a groove-like ring surrounding the new septum that deepens and eventually results in cell separation, followed by inactivation of these hydrolases and repair of these hydrolysis sites. The PGRP's binding site observed here closely matches the location of the limited cell wall digestion seen during daughter cell separation<sup>30,31</sup>. PGRPs preferentially bind to the site of daughter cell separation around the newly formed septum and this binding occurs after the synthesis of the new septum is completed and starts in a punctate pattern and progresses to a broader band-like and then ring-like pattern surrounding the newly formed septum. PGRPs do not bind to other parts of the cell (old cell wall, new septum during its

synthesis, and old cell poles after cell separation) and do not bind to  $\Delta\text{lytE}:\Delta\text{lytF}$  and  $\Delta\text{lytE}:\Delta\text{lytF}:\Delta\text{cwIS}$  mutants.

PGRPs have either one (PGLYRP-1) or two (PGLYRP-3 and -4) PGRP domains that have a peptidoglycan-binding groove specific for MurNAc-pentapeptide, which determines their specificity for peptidoglycan<sup>3</sup>. Availability of MurNAc-pentapeptide for PGRP binding in the mature cell wall may be limited because of peptide crosslinking of peptidoglycan, and thus MurNAc-peptides become available at the cell separation sites. Therefore, PGRPs preferentially bind to the sites where peptidoglycan is hydrolyzed by LytE and LytF endopeptidases during separation of the new daughter cells that correspond to the troughs in the cell wall previously observed by electron microscopy<sup>30,31</sup>, which is confirmed here by co-localization of PGRPs with LytE and LytF. Thus, in Gram-positive bacteria these cell-separating enzymes open up the peptidoglycan structure and allow access of PGRPs to deep layers of peptidoglycan near the cell membrane and expose higher affinity binding sites for PGRPs (Fig. 6).

## SUPPLEMENTARY METHODS

### Materials

All antibacterial compounds were used at or above minimum bactericidal concentrations (or bacteriostatic for chloramphenicol), indicated below or in Results. Human PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 (PGLYRP-3:PGLYRP-4 heterodimer) were expressed in S2 cells and purified as previously described<sup>10,12</sup> in a buffer containing 10 mM TRIS (pH 7.6), with 150 mM NaCl, 10  $\mu\text{M}$  ZnSO<sub>4</sub>, and 10% glycerol. Magainin (Ala<sup>8,13,18</sup>-magainin II amide, 75  $\mu\text{g/ml}$ ), lysostaphin (recombinant glycyl-glycine endopeptidase from *Staphylococcus simulans*, 10  $\mu\text{g/ml}$ ), mutanolysin (N-acetylmuramidase from *Streptomyces globisporus*, 100  $\mu\text{g/ml}$ ), penicillin G (80  $\mu\text{g/ml}$ ), vancomycin (10  $\mu\text{g/ml}$ ), chloramphenicol (20  $\mu\text{g/ml}$ ), gentamicin (5 or 15  $\mu\text{g/ml}$ ), rifampin (0.125  $\mu\text{g/ml}$ ), ciprofloxacin (10  $\mu\text{g/ml}$ ), bovine serum albumin (BSA), and all other reagents were from Sigma-Aldrich, unless otherwise indicated. Moenomycin (bambermycin, 20  $\mu\text{g/ml}$ ) was from Huvepharma, Inc. *Staphylococcus aureus* Rb and *Bacillus subtilis* ATCC 6633 were the same as described previously<sup>12</sup> and used for most of the work, except for the experiments involving mutants; *B. subtilis* 168 and its isogenic mutants, ESFKD ( $\Delta\text{lytE}:\Delta\text{lytF}$ ) and EFKYOJLp ( $\Delta\text{lytE}:\Delta\text{lytF}:\Delta\text{cwIS}$ ) were obtained from Junichi Sekiguchi<sup>18</sup>; *B. subtilis* 168-1A304 and its isogenic mutants, SH115 ( $\Delta\text{lytC}$ ), SH119 ( $\Delta\text{lytD}$ ), and SH128 ( $\Delta\text{lytC}:\Delta\text{lytD}$ ) were obtained from Simon Foster<sup>20</sup>; *B. subtilis* 168 and its isogenic mutants, BV2006 ( $\Delta\text{cssS}$ ), BFA2461 ( $\Delta\text{cssR}$ ), BV2003 ( $\Delta\text{htrA}$ ), and BV2002 ( $\Delta\text{cssS}:\Delta\text{htrA}$ ) were obtained from Jan Maarten van Dijk, Oscar Kuipers, Vesa Kontinen and their associates<sup>22</sup>; *E. coli* K-12 and its isogenic mutants, MAK004 ( $\Delta\text{cpxA}$ ) and MAK005 ( $\Delta\text{cpxR}$ ) were obtained from James Collins and Michael Kohanski<sup>23</sup> (Supplementary Table 1). pGDL48 plasmid and its sequence were obtained from Jan Maarten van Dijk and his associates<sup>36</sup>.

Mouse albumin was cloned, inserted into the insect vector pMT/BiP/V5-His, expressed in S2 cells, and purified from culture supernatants by Ni-NTA affinity chromatography as previously described<sup>10,45</sup>. This recombinant mouse albumin (cloned, expressed, and purified by the same methods as PGRPs) and recombinant human IgG1 Fc fragment, obtained as previously described<sup>10</sup>, were used as additional negative controls in some experiments (killing, membrane permeabilization, immunofluorescent



localization, membrane depolarization, •OH induction, activation of gene expression) and gave similar results to BSA (data not shown).

### Peptidoglycan synthesis

The rate of peptidoglycan biosynthesis in *S. aureus* was measured by following the incorporation of  $^3\text{H}$ -GlcNAc (*N*-Acetyl-D-[1- $^3\text{H}$ ]glucosamine, 296 GBq/mmol, Amersham) into peptidoglycan in bacteria exponentially growing in 5-10% LB in saline or in 0.086 M TRIS/HCl pH 7.2 with 50 mg/L  $\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$ , 5 mg/L  $\text{FeSO}_4$ , 50 mg/L  $\text{CaCl}_2$ , 5 mg/L  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 2 g/L glucose and 5% LB (at OD<sub>660</sub> nm = 0.02 to 0.1), without or with 0.525 M sucrose (which prevents osmotic lysis). GlcNAc is the main component of the glycan backbone of peptidoglycan, and given a choice of GlcNAc and glucose, *S. aureus* incorporates GlcNAc almost exclusively into its peptidoglycan<sup>46</sup>. After 10 min of incubation with  $^3\text{H}$ -GlcNAc at 37°C with shaking, PGLYRP-1, PGLYRP-3, PGLYRP-4, or PGLYRP-3:4, or a control protein (BSA, recombinant human IgG1 Fc fragment, or recombinant mouse albumin, at 100–200 µg/ml), or antibiotics, lysostaphin, or magainin (all in the same buffer as PGRPs) were added. The bacteria were incubated with proteins or antibiotics with vigorous shaking at 37°C, and aliquots were removed at the times indicated in Results, immediately chilled on ice, and centrifuged at 20,000xg at 4°C.

We have used a three-step procedure that in the same aliquot measures total inhibition of peptidoglycan synthesis and determines whether peptidoglycan synthesis is inhibited at the transpeptidation step, transglycosylation step, or any of the earlier cytoplasmic steps<sup>40,47</sup>. Total inhibition of peptidoglycan synthesis (at any step) was manifested by a decrease in  $^3\text{H}$ -GlcNAc incorporation into insoluble cell wall peptidoglycan. Inhibition of transpeptidation was manifested as inhibition of  $^3\text{H}$ -GlcNAc incorporation into insoluble cell wall peptidoglycan and simultaneous secretion into the medium of newly synthesized ( $^3\text{H}$ -GlcNAc-labeled) polymeric uncrosslinked peptidoglycan<sup>42,47,48</sup>. Inhibition of transglycosylation was manifested as inhibition of  $^3\text{H}$ -GlcNAc incorporation into insoluble cell wall peptidoglycan and simultaneous accumulation of  $^3\text{H}$ -GlcNAc-labeled undecaprenyl-phosphate-GlcNAc-MurNAc-peptide (lipid II) in the cell membrane that could be extracted with butanol<sup>40</sup>.

To measure  $^3\text{H}$ -GlcNAc-labeled butanol-soluble lipid II, bacterial sediments were treated with 2 M pyridinium acetate, pH 4.2, and extracted twice with butanol<sup>40</sup>. The increase in  $^3\text{H}$ -GlcNAc content in butanol-soluble lipid fraction was determined by scintillation counting and was a reflection of accumulation of lipid II in the cell membrane, and was used as a measure of inhibition of transglycosylation<sup>40</sup>.

To recover the cell wall containing newly synthesized  $^3\text{H}$ -GlcNAc-labeled insoluble crosslinked polymeric peptidoglycan, the butanol-insoluble fraction was suspended in DMSO, sonicated, filtered through Durapore 0.65 µm PVDF filters (Millipore), and washed with 0.4 M ammonium acetate in methanol and then methanol. A decrease in  $^3\text{H}$ -GlcNAc content in this fraction reflects total inhibition of peptidoglycan synthesis at any of its biosynthetic steps (transpeptidation, transglycosylation, formation of lipid II, or any earlier cytoplasmic steps)<sup>40</sup>.

To determine inhibition of transpeptidation,  $^3\text{H}$ -GlcNAc incorporation into soluble uncrosslinked polymeric peptidoglycan secreted into the medium was measured<sup>42,47</sup>. The supernatant from the 20,000xg centrifugation (above) was centrifuged at 750xg for 2 min through Illustra Microspin-G-25 Sephadex column (GE Healthcare), which retains low molecular weight  $^3\text{H}$ -GlcNAc. The amount of  $^3\text{H}$ -GlcNAc in the flow-through, containing uncrosslinked polymeric peptidoglycan, was used as a measure of

inhibition of transpeptidation, because inhibition of transpeptidation in Gram-positive bacteria causes secretion into the medium of newly synthesized soluble polymeric peptidoglycan due to lack of its crosslinking to the existing insoluble polymeric peptidoglycan in the cell wall<sup>42,47</sup>.

We have confirmed that virtually all <sup>3</sup>H-GlcNAc recovered on the PVDF filters is incorporated into polymeric peptidoglycan, because (a) digestion of *S. aureus* with lysostaphin and mutanolysin (muramidase) after <sup>3</sup>H-GlcNAc labeling completely removed <sup>3</sup>H-GlcNAc from the insoluble cell wall fraction; and (b) <sup>3</sup>H-GlcNAc incorporation is immediately and completely inhibited by specific inhibitors of peptidoglycan synthesis (penicillin G, vancomycin and moenomycin), but not by chloramphenicol, rifampin, and ciprofloxacin (specific inhibitors of protein, RNA and DNA synthesis, respectively), all used at bactericidal concentrations that fully inhibit growth of bacteria and selectively inhibit protein, RNA, or DNA synthesis, respectively.

We have confirmed that all <sup>3</sup>H-GlcNAc recovered from the flow-through from the Sephadex G-25 column is incorporated into soluble polymeric uncrosslinked peptidoglycan, because (a) digestion of the supernatant with mutanolysin (muramidase) before loading onto the column completely removed <sup>3</sup>H-GlcNAc from the flow-through fraction; and (b) this <sup>3</sup>H-GlcNAc incorporation is only caused by a specific inhibitor of transpeptidation (penicillin G), but not by inhibitors of other (earlier) steps of peptidoglycan synthesis (vancomycin and moenomycin), or by chloramphenicol, rifampin, and ciprofloxacin (specific inhibitors of protein, RNA and DNA synthesis, respectively), all used at bactericidal concentrations that fully and selectively inhibit peptidoglycan, protein, RNA, or DNA synthesis, respectively. We have also previously shown that the labeled peptidoglycan fragments secreted into the medium in the presence of penicillin G are newly synthesized polymeric uncrosslinked peptidoglycan, and do not contain other peptidoglycan precursors and, within the time-frame used here (1 hr), do not contain degradation products of the existing cell wall, and thus truly represent products of inhibition of transpeptidation<sup>42</sup>.

### **Protein, RNA, and DNA synthesis**

The rate of protein, RNA, and DNA biosynthesis in *S. aureus* was measured by following the incorporation of <sup>35</sup>S-methionine + <sup>35</sup>S-cysteine (PRO-MIX containing 70% L-[<sup>35</sup>S]methionine + 30% L-[<sup>35</sup>S]cysteine, 37 TBq/mmol, Amersham) into proteins, or of <sup>3</sup>H-uridine ([5,6-<sup>3</sup>H]uridine, 1.37 TBq/mmol, Amersham) into RNA, or of <sup>3</sup>H-thymidine ([6-<sup>3</sup>H]thymidine, 888 GBq/mmol, Amersham) into DNA, respectively. *S. aureus* was grown as described above for measuring peptidoglycan synthesis, with one of the above compounds added at time 0 instead of <sup>3</sup>H-GlcNAc. After 10 min of incubation at 37°C with vigorous shaking, PGLYRP-1, PGLYRP-3, PGLYRP-4, or PGLYRP-3:4, or a control protein (BSA, recombinant human IgG1 Fc fragment, or recombinant mouse albumin, at 100–200 µg/ml), or antibiotics, or lysostaphin, or magainin (all in the same buffer as PGRPs) were added, incubation was continued, aliquots were removed into tubes with 10% trichloroacetic acid with 1 M NaCl, the samples were filtered through PVDF filters, the filters were washed with 10% trichloroacetic acid with 1 M NaCl and then with methanol, dried, and <sup>35</sup>S or <sup>3</sup>H was counted in a scintillation counter. The selectivity of incorporation of <sup>35</sup>S or <sup>3</sup>H into proteins, RNA, or DNA was confirmed using antibiotics that selectively inhibit protein, RNA, or DNA synthesis: only chloramphenicol and rifampin inhibited protein synthesis (rifampin inhibits protein synthesis because RNA and protein synthesis are coupled in bacteria), only rifampin inhibited RNA synthesis, and only ciprofloxacin inhibited DNA synthesis (Fig. 2a-c).

## Localization of PGRPs, vancomycin, and LytE and LytF in bacterial cells

PGLYRP-4 or BSA or recombinant mouse albumin (as negative controls) were labeled with Alexa Fluor 594 using the Microscale Protein Labeling Kit (Molecular Probes). Bactericidal activity of Alexa Fluor 594-labeled PGLYRP-4 was confirmed with *B. subtilis* and *S. aureus*. Vancomycin labeled with BODIPY-FL (Molecular Probes) was also used to visualize the sites of peptidoglycan synthesis. Exponentially growing bacteria were used. *B. subtilis*, *L. monocytogenes*, and *E. coli* were cultured as previously described<sup>12</sup>, whereas *S. aureus* was grown with 0.125 M D-serine, which incorporates as the terminal amino acid residue in the peptidoglycan pentapeptide and reduces binding of vancomycin to the “old” peptidoglycan (vancomycin binds to terminal D-Ala-D-Ala, but not to D-Ala-D-Ser). Then for the final 10 min *S. aureus* was grown without D-serine, which allows it to synthesize new peptidoglycan with terminal D-Ala-D-Ala, which is then labeled with vancomycin<sup>49</sup>. Using D-serine is not required for *B. subtilis*, because, in contrast to *S. aureus*, *B. subtilis* efficiently cleaves the terminal D-Ala in the peptidoglycan after its incorporation into the cell wall, and therefore, in *B. subtilis*, vancomycin only labels actively synthesized peptidoglycan, which contains D-Ala-D-Ala<sup>50</sup>. Alexa Fluor 594-PGLYRP-4 was added at 60 µg/ml and after 10 min 8 µg/ml of BODIPY-FL-vancomycin (Molecular Probes) was added for another 5 min of incubation at 37°C with vigorous shaking. In some experiments, single staining with PGRPs was performed and vancomycin was omitted. Bacteria were washed, fixed with 2.4% paraformaldehyde plus 0.1% glutaraldehyde in PBS, pH 7.2, for 15 min at 20°C, washed, and observed in Olympus Fluoview FV300 confocal microscope. The excitation and emission spectra were set such that there was no “cross-bleeding” of the signal between the two dyes used. Individual slices were examined and the pictures shown represent merged stacks. Localization of PGLYRP-1 (and also of PGLYRP-4 in some experiments) was visualized by a similar method, except that unlabeled PGLYRP-1 or PGLYRP-4 was used and it was detected with anti-V5 epitope mAbs (Invitrogen) labeled with Alexa Fluor 594 or with FITC as described above for PGLYRP-4 labeling. No staining of bacteria was obtained with anti-V5-FITC mAbs when recombinant mouse albumin was used as a control (not shown in figures).

For co-localization of PGRPs with LytE and LytF, we obtained antibodies reactive with the conserved C-terminal of both LytE and LytF (also 70% conserved in CwIS, the third cell-separating endopeptidase, but not conserved in other hydrolases, such as LytC and LytD), by immunizing rabbits with Ac-CSYWKPRLGAKRF-OH peptide coupled to KLH, followed by affinity purification on SulfoLink gel (Pierce) with the same peptide linked through the N-terminal Cys, elution with Tris-glycine buffer, pH 2.5, and dialysis against PBS, pH 7.2. Exponentially growing *B. subtilis* was incubated for 15 min at 37°C with 60 µg/ml of PGLYRP-1 or PGLYRP-4, fixed with 2.4% paraformaldehyde plus 0.1% glutaraldehyde as above, washed, and incubated for 1 hr at 20°C with a mixture of 10 µg/ml of mouse anti-V5 mAb and 10 µg/ml of rabbit anti-LytE/LytF antibodies, washed, incubated for 45 min at 20°C with rabbit-anti mouse IgG-TRITC antibodies (not reactive with rabbit IgG) and mouse monoclonal anti-rabbit IgG-FITC antibodies (not reactive with mouse IgG), washed, and observed in Olympus Fluoview FV300 confocal microscope. The excitation and emission spectra were set such that there was no “cross-bleeding” of the signal between the two dyes used. Individual slices were examined and the pictures shown represent merged stacks. The specificity of PGRP detection was confirmed by the loss of red fluorescence when PGRP was omitted from the staining procedure. The specificity of LytE/LytF detection was confirmed by

the loss of green fluorescence when anti-LytE/LytF antibodies were pre-incubated with 200 µg/ml of LytE/LytF peptide (CSYWKPRYLGAKRF) (Fig. 1e), but not following pre-incubation with an unrelated control peptide (CSQRLRELQAHHVHNSG).

### **Hydrolysis of peptidoglycan and bacteriolysis**

Insoluble peptidoglycan was prepared from the cell walls of *S. aureus* (Lys-type) or *B. subtilis* (DAP-type) as previously described<sup>42</sup>, and 2 mg/ml was incubated with PGRPs or peptidoglycan-lytic enzymes (lysostaphin or mutanolysin, positive controls) or trypsin or BSA (negative controls) in 5 mM TRIS (pH 7.6), with 150 mM NaCl, 5 µM ZnSO<sub>4</sub>, and 5% glycerol at 37°C for 48 h, and peptidoglycan degradation was followed by measuring optical density at 600 nm.

Hydrolysis of soluble uncrosslinked peptidoglycan, purified by vancomycin-affinity chromatography from *S. aureus* Rb, and labeled with biotin on the N-terminal glycine of its peptide, was measured as described previously<sup>14</sup>. Peptidoglycan-biotin (750 ng) was incubated for 4 days at 37°C in 10 µl with 2 µg of recombinant bactericidal PGRPs or amidase (PGLYRP-2) or lysostaphin (positive controls), or buffer or trypsin (negative controls). Enzyme-digested peptidoglycan-biotin was subjected to SDS-PAGE and blotted on Immobilon-P, and high Mr peptidoglycan-biotin was detected with streptavidin-peroxidase and enhanced chemiluminescence as described previously<sup>14</sup>. This assay detects amidase or endopeptidases activity, because hydrolysis of the peptide from the glycan chain removes the biotin-labeled peptide, or muramidase activity, because hydrolysis of the glycan backbone converts high Mr peptidoglycan into low Mr fragments that migrate with the buffer front.

Hydrolysis of synthetic peptidoglycan fragments was studied by measuring the release of digestion products by mass spectrometry. 10 µg of MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala or MurNAc-L-Ala-D-isoGln-mDAP-D-Ala-D-Ala, synthesized as described previously<sup>51</sup>, was incubated for 4 days with 2 µg of bactericidal PGRPs or amidase (PGLYRP-2, positive control) or BSA (negative control) in 5 mM TRIS (pH 7.6), with 60 mM NaCl, 4 µM ZnSO<sub>4</sub>, and 4% glycerol. The samples were analyzed by MALDI-TOF mass spectrometry for the presence of undigested compounds and all possible hydrolysis products as described previously<sup>14</sup>.

Bacteriolytic activity was measured by following changes in optical density at 600 nm of a suspension of live or heat-killed (100°C, 30 min) *S. aureus* or *B. subtilis* bacteria (at OD<sub>600</sub> = 0.1 to 0.4), incubated with bactericidal PGRPs or peptidoglycan-lytic enzymes (lysostaphin or mutanolysin, positive controls) or BSA (negative control) in 5 mM TRIS (pH 7.6), with 150 mM NaCl, 4 µM ZnSO<sub>4</sub>, and 4% glycerol at 37°C for 48 h.

### **Gene expression and qRT-PCR**

*B. subtilis* or *E. coli* were treated with PGRPs or control proteins or antibiotics for 15 to 120 min under the same conditions as used for bacterial killing assays. RNA was isolated from bacteria using RiboPure-Bacteria kit from Ambion according to the manufacturer's protocol. The amounts of mRNA were measured using quantitative reverse transcription real-time PCR (qRT-PCR) as previously described<sup>44</sup>. cDNA was synthesized from 100 ng of RNA using RT<sup>2</sup> PCR Array First Strand Kit (SA Biosciences). Gene expression was quantified by qRT-PCR using the ABI 7000 Sequence Detection System with 1 cycle 10-min at 95°C and 40 cycles 15 sec at 95°C and 1 min at 60°C using SA Biosciences SYBR Green Master Mix and the following gene-specific primers: *htrA* (CACCAGCGATTGTCGGTATTAC and

ACCCTGAACCGCTTTCTGTATC) or *cpxP* (TGCTGAAGTCGGTTCAGGCGATAA and TCTGCTGACGCTGATGTTCCGGTTA)<sup>23</sup>, and common primers for 16s rRNA from all Eubacteria (ACTCCTACGGGAGGCAGCAGT and ATTACCGCGGCTGCTGGC)<sup>52</sup> as a housekeeping gene. For each gene,  $\Delta Ct$  was calculated followed by normalization to the housekeeping gene, followed by calculation of  $\Delta\Delta Ct$  for each gene:  $\Delta\Delta Ct = \Delta Ct_1 - \Delta Ct_2$ , where  $\Delta Ct_1$  is the PGRP- or antibiotic-treated bacteria and  $\Delta Ct_2$  is BSA-treated bacteria. This calculation gives the fold increase in expression of each gene in PGRP- or antibiotic-treated bacteria versus BSA-treated bacteria. Preliminary kinetics experiments in *B. subtilis* demonstrated that gentamicin- or PGRP-induced *htrA* expression began to increase in 15 min, reached the maximum in 30 min, and did not significantly increase at 60 and 120 min, and thus 30-min (*B. subtilis*) or 60 min (*E. coli*) treatments were used in all subsequent experiments. The results are expressed as means  $\pm$  SEM from 3-4 experiments and compared using *t*-test. Similar results were obtained when recombinant mouse serum albumin, cloned, expressed, and purified by the same methods as PGRPs, was used instead of BSA as a negative control (data not shown).

### **Complementation of $\Delta cssS$ , $\Delta cssR$ , $\Delta htrA$ , $\Delta cpxA$ , and $\Delta cpxR$ mutants**

*cssS*, *cssR* and *htrA* were amplified from *B. subtilis* 168 DNA using the following forward and reverse primers: *cssS*, AATTGCCGTCTCCTCGTATCG and CGAAGCAGACCTTGTCAGAGA; *cssR*, GTTGAAAGGATGTGAAGAGCC and GCGGCTTGTTCATGATGACATC; and *htrA*, TCTGTTCCATCGACTCAGTCC and GGCCTGAGGCATTATGTCTTA. The PCR products were gel purified and subjected to a second round of PCR using the following forward and reverse primers tagged with restriction sites (for *cssR* and *htrA* the tagging sequence was also switched to obtain reverse orientation as a negative complementation control):

*cssS*-F: CGGgtcgacAATTGCCGTCTCCTCGTATCG (tagged with *Sall*)

*cssS*-R: GCCgtcgacCGAAGCAGACCTTGTCAGAGA (tagged with *Sall*)

*cssR*-F: CGGgtcgacGTTGAAAGGATGTGAAGAGCC (tagged with *Sall*)

*cssR*-R: GCTgaattcGCGGCTTGTTCATGATGACAT (tagged with *EcoRI*)

*htrA*-F: AGCgtcgacTCTGTTCCATCGACTCAGTCC (tagged with *Sall*)

*htrA*-R: TCGgaattcGGCCTGAGGCATTATGTCTTA (tagged with *EcoRI*)

After amplification with a proofreading polymerase and gel purification using QIAGEN gel extraction kit, TA overhangs were added to the post-amplification product using a low-proofreading polymerase (GoTaq Flexi), and the product was subcloned into a TOPO vector (PCR4, InVitrogen) according to the manufacturer's protocol. All the clones were verified by colony PCR and restriction enzyme digestion with *Sall* and *EcoRI*, sub-cloned into *B. subtilis* pGDL48, which is a derivative of a natural low-copy expression plasmid<sup>36</sup> as *Sall* (*cssS*) and *Sall* and *EcoRI* (*cssR* and *htrA*) inserts, amplified in *E. coli* MC1061, and verified by colony PCR using pGDL48 forward (GAGGTGTAATTCGTAAGTCC) and reverse (AGCAAACCCGTATTCCACGA) primers. The sequences and orientation of the inserts were verified by sequencing, and pGDL48 plasmids with the inserts were transfected into the respective *B. subtilis* mutant strains (BV2006, BFA2461 and BV2003), and the presence of the pGDL48 with the inserts was verified by colony PCR using pGDL48 forward and reverse primers. The bactericidal effects of PGRPs on *B. subtilis*  $\Delta cssS$ ,  $\Delta cssR$  and  $\Delta htrA$  mutants

complemented with forward orientation sequences were compared to the effects on mutants transfected with reverse orientation sequences or with empty vector (pGDL48) as negative controls.

The entire *cpxR-cpxA* operon (including 309 bp 5' of the *cpxR* start codon, which includes the ribosomal binding site and the promoter<sup>53</sup>) or promoter-*cpxR* sequence only were amplified from WT *E. coli* MG1655 DNA using the following forward and reverse primers: *cpxRA* (*cpxR-cpxA*), TCGAACATATGGCTCTGCGTACTG and GAAGTTTAACTCCGCTTATACAGC; and *cpxR*, TCGAACATATGGCTCTGCGTACTG and GCTGCCTATCATGAAGCAGAAACC. As a negative control, we amplified the periplasmic loop of *cpxA* (bp 88-489, which is devoid of transmembrane and kinase domains and cannot activate CpxR) using the following forward and reverse primers: CTCGATTCACGCCAGAT and GCGGTCAAACAGTAAGT. The PCR products were gel purified, cloned into linearized pCR2.1 using TOPO TA cloning kit (Invitrogen), and confirmed by sequencing. For complementation, chemically competent  $\Delta cpxA$  and  $\Delta cpxR$  *E. coli* mutants were prepared by the modified chemical procedure<sup>54</sup>: 250 ml of each bacteria was grown overnight to OD<sub>660</sub> = 0.45 at 18°C with shaking (200 rpm) in SOB medium (5 g NaCl, 5 g yeast extract, 20 g trypton, and 2.5 ml 1M KCl per liter), centrifuged at 2500xg at 4°C, suspended in 20 ml of ice-cold Inoue solution (10.9 g MnCl<sub>2</sub>, 2.2 g CaCl<sub>2</sub>, 18.7 g KCl, and 20 ml 0.5 M piperazine-1,2-bis[2-ethanesulfonic acid] per liter), centrifuged, and re-suspended in 5 ml of ice-cold Inoue solution. Then 1.5 ml of 37°C DMSO was added, followed by incubation at 22°C for 10 min, freezing of aliquots in liquid nitrogen, and storage at -80°C. Competent cells were transfected with *cpxRA*, *cpxR*, or control *cpxA*(88-489) plasmids, selected with ampicillin, and used in bactericidal assays. The bactericidal effects of PGRPs on *E. coli*  $\Delta cpxA$  and  $\Delta cpxR$  mutants complemented with *cpxRA* or *cpxR* were compared to the effects on mutants transfected with inactive *cpxA*(88-489) periplasmic fragment as a negative control. *cpxRA* was used for  $\Delta cpxA$  complementation because the expression of both *cpxR* or *cpxA* is coordinated and is controlled by the same promoter 5' of *cpxR*.

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**Supplementary Table 1. Bacterial strains and plasmids used in this study.**

Strain	Relevant genotype	Source or reference
<i>B. subtilis</i>		
ATCC 6633	Wild type	ATCC
168	<i>trpC2</i> (parental strain for mutants)	18, 22
ESFKD	<i>trpC2 lytF::spc lytE::kan</i>	18
EFKYOJLp	<i>trpC2 lytF::spc lytE::kan cwIS::pM4SDΔojL</i>	18
1A304	<i>trpC2 metB5 xin-1 SPβ(s)</i> (168 parental strain for $\Delta$ <i>lytC</i> and $\Delta$ <i>lytD</i> )	20
SH115	<i>trpC2 metB5 xin-1 SPβ(s) lytC::ble</i>	20
SH119	<i>trpC2 metB5 xin-1 SPβ(s) lytD::spc</i>	20
SH128	<i>trpC2 metB5 xin-1 SPβ(s) lytC::ble lytD::spc</i>	20
BV2006	<i>trpC2 cssS::pMutin2</i>	22
BFA2461	<i>cssR(yvqA)::pMutin4</i>	22
BV2003	<i>htrA::pMutin2</i>	22
BV2002	<i>cssS::Sp htrA(ykdA)::pMutin2</i>	22
<i>S. aureus</i>		
Rb	Wild type clinical isolate	47
<i>L. monocytogenes</i>		
ATCC 19115	Wild type	ATCC
<i>E. coli</i>		
MG1655	Wild type parental K-12 strain (ATCC 700926) for mutants	ATCC
MAK004	MG1655 <i>cpxA</i>	23
MAK005	MG1655 <i>cpxR</i>	23
MC1061	Wild type strain for amplification of pGDL48 plasmid	ATCC
<b>Plasmid</b>	<b>Characteristics</b>	<b>Source or reference</b>
<i>B. subtilis</i>		
pGDL48	Low-copy <i>B. subtilis</i> expression plasmid for complementation	36
cssS-pGDL48	<i>cssS</i> -expressing plasmid for complementation	This study
cssR-pGDL48	<i>cssR</i> -expressing plasmid for complementation	This study
htrA-pGDL48	<i>htrA</i> -expressing plasmid for complementation	This study
cssS-Rev-pGDL48	<i>cssS</i> in reverse orientation (negative control for complementation)	This study
cssR-Rev-pGDL48	<i>cssR</i> in reverse orientation (negative control for complementation)	This study
htrA-Rev-pGDL48	<i>htrA</i> in reverse orientation (negative control for complementation)	This study
<i>E. coli</i>		
pCR2.1	Cloning and expression vector for complementation	InVitrogen
cpxRA-pCR2.1	<i>cpxR:cpxA</i> -expressing plasmid for complementation	This study
cpxR-pCR2.1	<i>cpxR</i> -expressing plasmid for complementation	This study
cpxA(88-489)-pCR2.1	<i>cpxA</i> periplasmic loop bp 88-489 (negative control for complementation)	This study