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Article

Probing Teichoic Acid Genetics with Bioactive Molecules Reveals New Interactions among Diverse Processes in Bacterial Cell Wall Biogenesis

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SUMMARY

The bacterial cell wall has been a celebrated target for antibiotics and holds real promise for the discovery of new antibacterial chemical matter. In addition to peptidoglycan, the walls of Gram-positive bacteria contain large amounts of the polymer teichoic acid, covalently attached to peptidoglycan. Recently, wall teichoic acid was shown to be essential to the proper morphology of *Bacillus subtilis* and an important virulence factor for *Staphylococcus aureus*. Additionally, recent studies have shown that the dispensability of genes encoding teichoic acid biosynthetic enzymes is paradoxical and complex. Here, we report on the discovery of a promoter (P_{ywaC}), which is sensitive to lesions in teichoic acid synthesis. Exploiting this promoter through a chemical-genetic approach, we revealed surprising interactions among undecaprenol, peptidoglycan, and teichoic acid biosynthesis that help explain the complexity of teichoic acid gene dispensability. Furthermore, the new reporter assay represents an exciting avenue for the discovery of antibacterial molecules.

INTRODUCTION

In *B. subtilis* 168 the major wall teichoic acid is a poly(glycerol phosphate) polymer. The identification of temperature-sensitive mutants that were impaired for teichoic acid biosynthesis led to the isolation of the *tag* (*t*eichoic *a*cid *g*lycerol) gene cluster for poly(glycerol phosphate) biogenesis in *B. subtilis* (Briehl et al., 1989). Together, sequence-based homology analysis, biochemical studies of biosynthetic proteins and the chemical structure of wall teichoic acid have led to a plausible biosynthetic model (Figure 1) (Bhavsar and Brown, 2006). The model predicts that synthesis is initiated on the cytoplasmic face of the membrane on an undecaprenol-phosphate molecule. The teichoic acid polymer is thought to be assembled step-wise, through addition at the nonreducing end, of *N*-acetylglucosamine-1-phosphate (TagO), *N*-acetylmannosamine (TagA), and several residues of glycerol phosphate (TagB and F) to the membrane embedded prenol-phosphate substrate. Glycerol phosphate is derived from the activated precursor cytidine 5′-diphosphoglycerol (CDP-glycerol) synthesized by the glycerol phosphate cytidylyltransferase, TagD.

The genetics of the dispensability of *tag* genes has proven to be remarkably complex. Work from our laboratory using precise gene deletions, coupled with ectopic conditional complementation, showed that the late-acting gene products (TagB, TagD, and TagF) were indispensable for cell viability in culture (Bhavsar et al., 2001, 2004). Astonishingly, although these genes were essential for viability, the first step (TagO) of teichoic acid biosynthesis proved to be dispensable in both *B. subtilis* and *S. aureus* (D'Elia et al., 2006a, 2006b; Weidenmaier et al., 2004). This apparent contradiction was addressed when we demonstrated that the late-acting genes *tagB, tagD*, and *tagF* could be deleted in both *B. subtilis* and *S. aureus* only in the presence of an accompanying deletion in *tagO* (D'Elia et al., 2006a, 2006b). Thus the essential phenotypes of the late genes in wall teichoic acid synthesis are contextual and subject to unusual genetic interactions with the initial step of the pathway. Here we report on investigations using a library of small molecules of known biological activity to probe this unusual genetic complexity.

Biologically active small molecules are finding increasing use in a research paradigm that emphasizes the value of these as probes. Recently, Silhavy, Kahne and co-workers reported on the use of antibiotics in a hyperpermeable strain of *Escherichia coli* to identify suppressors with a key role in outer membrane assembly (Ruiz et al., 2005; Wu et al., 2005). This approach highlights the use of bioactive small molecules in illuminating a process that has been refractory to conventional genetics and about which remarkably little is known. Indeed, the concept of systematic "phenotyping" with a large library of compounds annotated for biological activity was first reported by Root and co-workers (Root et al., 2003) but has not been broadly used to characterize biological complexity. In work reported here, we have developed a cell-based, promoter-reporter, luminescence screen for lesions in the late steps in teichoic acid synthesis and find that it is perturbed most acutely

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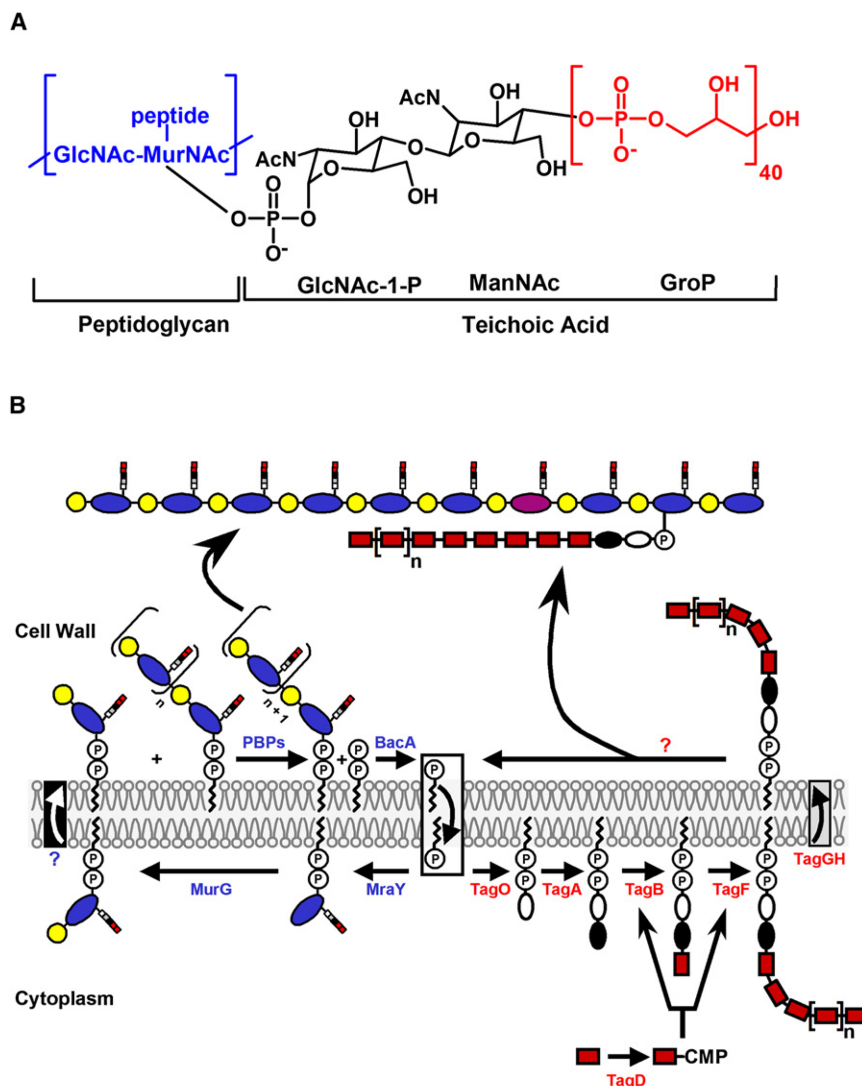

Figure 1. Teichoic Acid Biogenesis

(A) Shown is the chemical structure of the wall teichoic acid polymer from *B. subtilis* 168. Its attachment to peptidoglycan is through the 6-hydroxyl of *N*-acetylmuramic acid.

(B) Depicted is the biosynthesis of peptidoglycan and teichoic acid precursors on the left and right side of the figure, respectively. Solid arrows denote catalysis by the indicated enzyme. The convergence of both pathways on the common substrate undecaprenol-phosphate is highlighted in the center rectangle. As shown, peptidoglycan synthesis proceeds with the addition of an *N*-acetylmuramic pentapeptide (blue oval) added by MraY, generating lipid I. Subsequent addition of *N*-acetyl glucosamine (yellow circle) by MurG generates lipid II before export by an unknown flipase (denoted "?"). Penicillin-binding proteins (PBPs) add a murein unit to the existing nascent peptidoglycan strand on the outside of the cell. Similarly, wall teichoic acid synthesis proceeds with the addition of *N*-acetylglucosamine-1-phosphate (open oval) and *N*-acetylmannosamine (shaded oval) by transferases TagO and TagA, respectively. TagD catalyzes the synthesis of CDP-glycerol, a source of activated glycerol phosphate (red rectangle) for poly(glycerol phosphate) synthesis by the primase TagB and polymerase TagF. The polymer is exported to the cell surface by TagGH and transferred by an unknown enzyme (denoted "?") to the 6-hydroxyl of *N*-acetylmuramic acid of peptidoglycan.

by cell-wall-active antibiotics that target undecaprenol-linked steps in cell wall assembly. These findings have provided substantial evidence for a hypothesis explaining the complex genetic interactions seen in our gene dispensability experiments, namely that cell death associated with lesions in the late steps of teichoic acid synthesis is due to the sequestration of undecaprenol-linked teichoic acid intermediates from the essential process of peptidoglycan synthesis. These investigations have likewise revealed surprising genetic connectivity among undecaprenol, peptidoglycan, and teichoic acid biogenesis.

RESULTS

Transcriptional Profiling on Depletion of a Late Enzyme in Teichoic Acid Synthesis

Our work began with a search for genes transcriptionally activated by TagD depletion. The TagD protein is a cytidylyltransferase that provides activated glycerol phosphate for the late steps of poly(glycerol phosphate) synthesis and therefore its depletion leads to lethality (D'Elia et al., 2006a). TagD depletion was accomplished with a *B. subtilis* mutant (EB240; all strain and primer information are in Tables S1 and S2, respectively, available online) where the wild-type copy of *tagD* was deleted and a complementing ectopic copy was under strict control of a xylose inducible promoter (Bhavsar et al., 2001). Transcriptional profiling using microarray analysis was done on the conditional mutants comparing genes expressed during replete (2% xylose) and deplete (0% xylose) inducer concentrations (Figure 2 and supplementary information online [http://www.ncbi.nlm.nih.gov/projects/geo/, GEO accession: GSE7373]). One hundred and seventy six genes were identified as significantly activated in response to TagD depletion (Table S3). The transcriptional profiles of these genes were compared to data sets of *B. subtilis* genes previously identified to be transcriptionally activated by known antibiotics (for details see http://www.gpc-biotech.com/supplementary_material.htm) (Hutter et al., 2004). We noted that genes activated by TagD depletion in our study were often also upregulated by cell-wall active antibiotics. For further analysis, we short-listed 10 genes that were conserved among Gram-positive bacteria and were highly upregulated by TagD depletion. These fell into two general classifications: 5 genes that were upregulated solely by TagD depletion and 5 genes that were also upregulated in response to cell wall-active antibiotics (highlighted in Figure 2 and Table S3).

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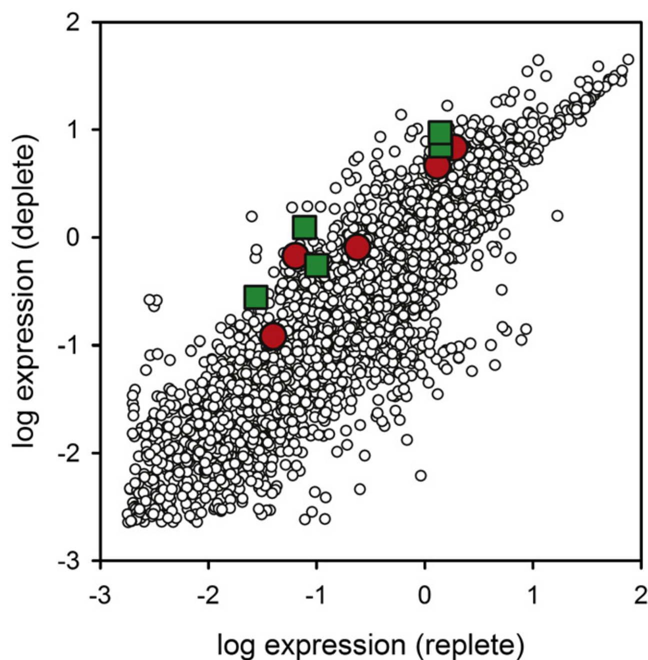

Figure 2. Global Transcriptional Consequences of Depletion of the TagD Protein in *B.* Subtilis 168

The transcriptional response of all open reading frames was tested using a strain (EB240) where the expression of TagD is controlled by the inducer xylose. Gene expression coordinates are for replete (2% xylose) and deplete (0% xylose) inducer conditions. Ten upregulated genes were selected for further study and are highlighted according to their response in a previous microarray study of transcriptional responses to some 37 antibiotics (Hutter et al., 2004): genes that were activated almost exclusively by TagD depletion (red circle) and genes activated by both TagD depletion and cell-wall-active antibiotics (green square).

P_{ywaC} Is a Faithful Reporter of Lesions in Late Steps of Teichoic Acid Biogenesis

For the 10 short-listed genes, promoter-reporter strains were generated using an autonomous, real-time, luminescence (*lux*) reporter system (Qazi et al., 2001). The promoter regions of the short-listed genes were transcriptionally fused to the *lux* genes and introduced into *B. subtilis* using Campbell-type integration that conserves the native context of the surrounding genomic sequence. These reporter systems were introduced into *tagB, tagD*, and *tagF* conditional mutants and luminescence was monitored continuously as each gene product was depleted. One promoter, P_{ywaC}, showed the most promise as a reporter of lesions in teichoic acid biosynthesis showing 4-, 12-, and 20-fold increases in luminescence, normalized for cell density, on depletion of TagB, TagD, and TagF, respectively (Table S4). Figure 3 depicts a typical response seen from the P_{ywaC} reporter strain to late step Tag protein depletion, where an increase in luminescence was observed on TagD depletion and was marked by a corresponding decrease in growth. Additionally, the P_{ywaC} *lux* system was placed into TagO and YacN (YgbB) depletion strains. Similar to the late-step Tag protein depletion, a depletion of the isoprenoid synthesis enzyme, YacN, showed a marked 3-fold increase in luminescence upon depletion, whereas depletion of TagO, an early teichoic acid enzyme, did not show any

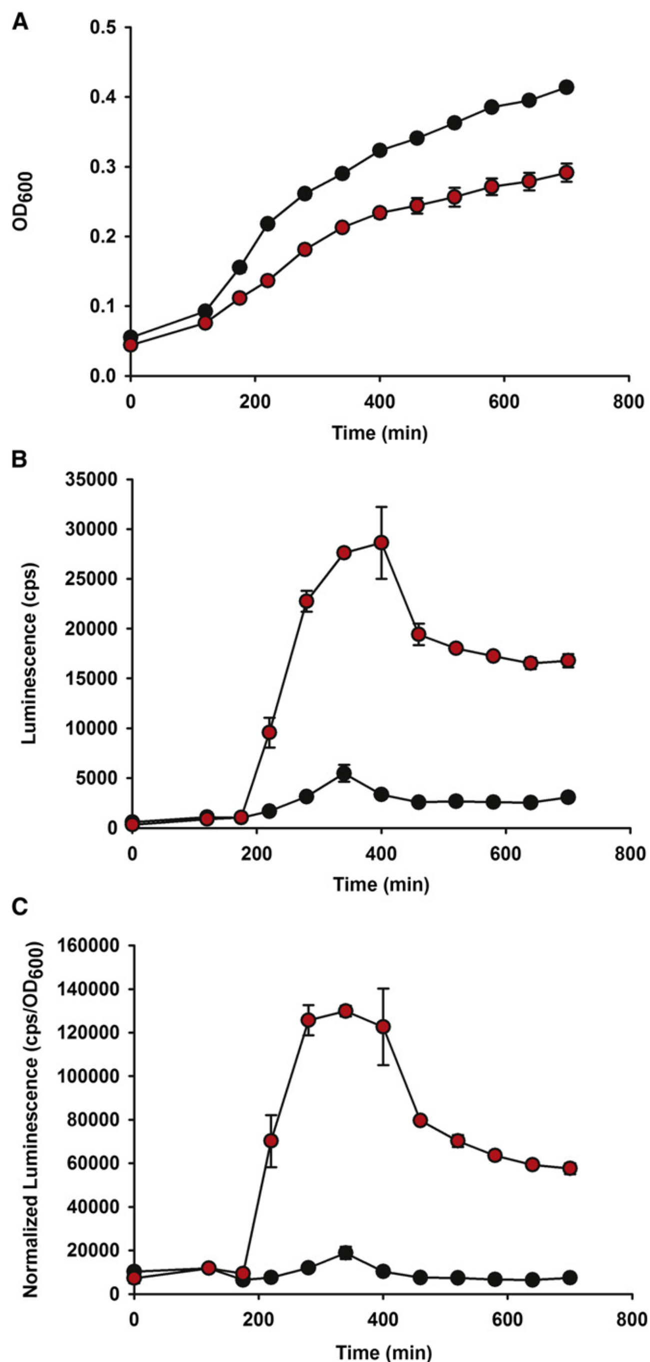

Figure 3. Effects of TagD Depletion on the P_{ywaC} Reporter Strain

Cell density (OD_{600}) and reporter gene expression (luminescence) from the P_{ywaC} promoter were measured over time in a strain (EB1402) of *B. subtilis* where TagD expression is under xylose control. The strain was subcultured in LB media containing erythromycin and spectinomycin in the absence (black circle) and presence (red circle) of the inducer (2% xylose). Growth, optical density at 600 nm (A), and luminescence, counts per second (cps) (B), were monitored. Normalized luminescence (cps/OD_{600}) was also calculated (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

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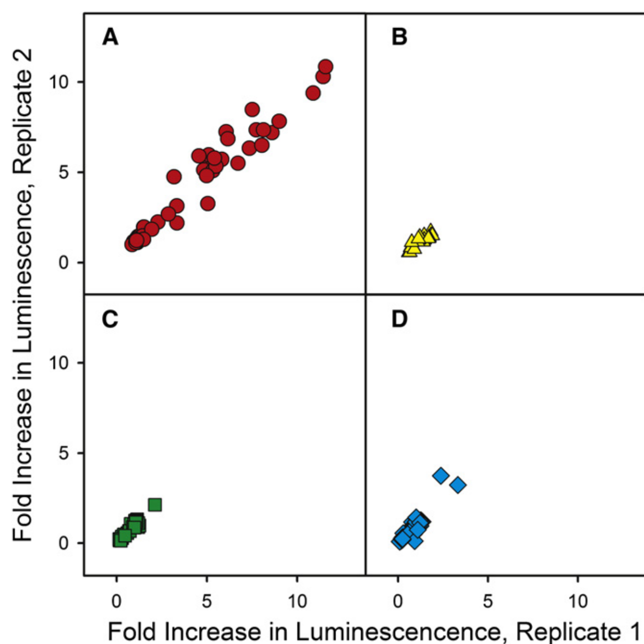

Figure 4. Response of the Promoter-Reporter System to 167 Known Antibiotics in the Primary Screen

Replicate plots show the fold increase in luminescence of the P_{ywaC} reporter strain (EB1385) in response to 167 compounds (10 μM) annotated for antimicrobial activity, a subset of the Prestwick collection of 1120 bioactive compounds. Fold increase in luminescence was calculated from the normalized luminescence (cps/OD_{600}) value of the maximal response of the reporter strain to a particular compound, divided by the normalized luminescence value of the low control (DMSO). Panels (A–D) plot the responses to compounds with well-characterized activity against bacterial cell wall synthesis, protein synthesis, DNA synthesis, and other targets, respectively.

significant P_{ywaC} response (Table S4). Thus, the P_{ywaC} *lux* system was a faithful reporter of lesions in the late, but not early, steps of teichoic acid biogenesis and also responded to an interruption in polyisoprenoid (undecaprenol) synthesis.

Response of the P_{ywaC} Reporter Strain to Small Molecule Probes of Cell Physiology

In order to systematically probe the P_{ywaC} reporter, we used a commercially available library (Prestwick) of 1120 small molecules annotated for biological activity. The collection contained a large number of previously approved drugs and some 167 antibiotics. The response of a small subset of the library, the 167 antibiotics in duplicate, is shown in Figure 4. The plots are categorized according to the known activity of these molecules against cell wall synthesis, protein synthesis, DNA synthesis, and "other" targets. The data reveal that the reporter responded almost exclusively to cell-wall-active antibiotics. In the entire collection of 1120 compounds, some 27 chemicals led to at least a 3-fold increase in normalized luminescence (Table S5). Twenty-four of these are well-known antibiotics that impact on wall peptidoglycan synthesis. The remaining compounds (methyl-benzethonium chloride, trimethylcolchinic acid, and homosalate) are molecules that have been reported to have antibacterial activity but have unknown mechanisms. Perhaps these too impact on bacterial cell wall synthesis.

Having identified almost exclusively cell-wall-active antibiotics as activators of the reporter P_{ywaC}, we further characterized the response with a more careful secondary screen using a panel of well-characterized antibiotics. Figure 5 shows the response of our reporter to a panel of 18 antibiotics. These probes were used at concentrations that bracketed the minimum inhibitory concentration (MIC), two and four times above and below the MIC for the reporter strain. Again, the P_{ywaC} reporter responded almost exclusively to cell-wall-active antibiotics, particularly bacitracin, ramoplanin, vancomycin, and fosfomycin, and it was also activated by fosmidomycin. These molecules led to at least an 8-fold increase in normalized luminescence. Interestingly, a subset of cell-wall-active antibiotics, specifically the beta lactams (i.e., ampicillin, cefotaxime) and cycloserine, showed a much smaller response (2- to 4-fold).

These two groups of P_{ywaC}-activating compounds had mechanisms of action that focused on dissectible steps in peptidoglycan biosynthesis—those involving undecaprenol phosphate-linked steps and those involving peptide crosslinking. As detailed below, we believe that the results are consistent with the hypothesis that the C_{55} polyisoprenoid lipid carrier undecaprenol-phosphate is at the center of interactions among teichoic acid, peptidoglycan, and undecaprenol synthesis, and that these interactions explain the complex gene dispensability patterns in teichoic acid biosynthesis. Furthermore, these results situate YwaC as an important new signaling protein with a broad role in cell stress that is likewise impacted principally by lesions in undecaprenol-linked steps.

Genomic Context Examination of Teichoic Acid, Peptidoglycan, and Undecaprenol Biosynthetic Enzymes

To further investigate the possibility for interactions among the enzymes involved in the biosynthesis of teichoic acid, peptidoglycan, and undecaprenol, we examined functional interactions inferred by genomic context. Computational analyses of genomic organization have been used to provide novel insights into the functional associations among gene products (Bowers et al., 2004; Huynen et al., 2000; Janga et al., 2005; von Mering et al., 2007). A very promising analysis has been the examination of rearranged operons to find new members of particular pathways (Janga et al., 2005). Using inferred associations derived from gene fusions (Moreno-Hagelsieb and Collado-Vides, 2002), conservation of gene order (Janga and Moreno-Hagelsieb, 2004), operon predictions by intergenic distances (Moreno-Hagelsieb and Collado-Vides, 2002), and rearranged operons (Janga et al., 2005), we examined the connectivity among known genes coding for teichoic acid, peptidoglycan, and undecaprenol biosynthetic enzymes (Figure 6). This analysis identified a large number of interactions among these three processes. Although there seemed to be interactions connecting teichoic acid genes directly to peptidoglycan genes, there appeared to be very strong connectivity of these pathways through undecaprenol biosynthesis. This bioinformatics approach provided additional evidence for strong interactions between teichoic acid and peptidoglycan biosynthesis through the polyisoprenoid lipid carrier, supporting our explanation for the genetic complexity surrounding teichoic acid gene dispensability.

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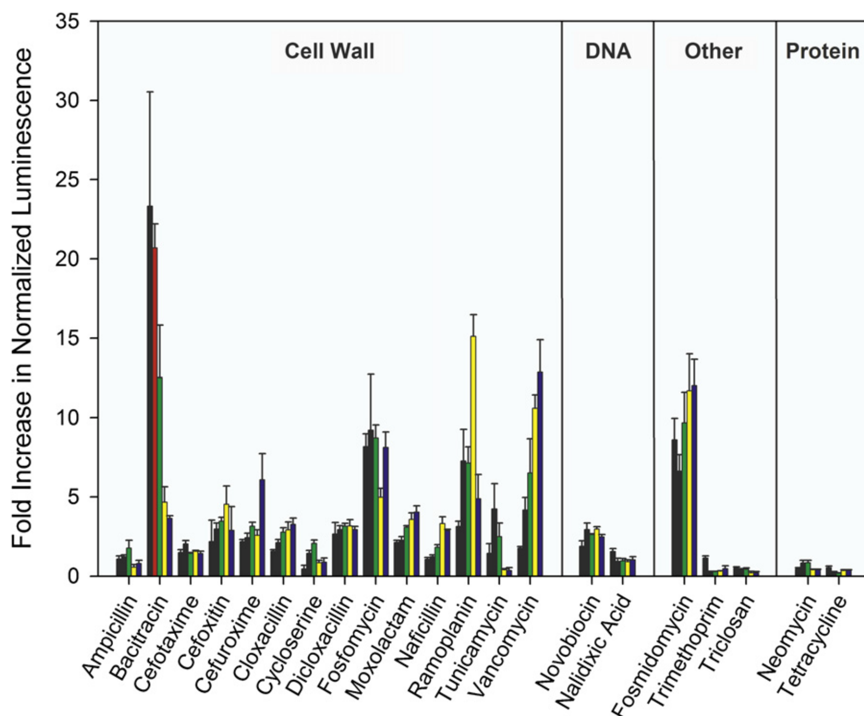

Figure 5. Analysis of P_{ywaC} Reporter Response to a Panel of 18 Antibiotics

The P_{ywaC} reporter strain (EB1385), grown in LB supplemented with erythromycin, was exposed to the a panel of 18 antibiotics of varying chemical class and mechanism of action at concentrations that bracketed the MIC for each antibiotic: 4-fold lower (black bar), 2-fold lower (red bar), MIC (green bar), 2-fold higher (yellow bar), and 4-fold higher (blue bar). Fold increase in luminescence was calculated from the normalized luminescence (cps/OD_{600}) value of the reporter strain divided by the normalized luminescence value of no antibiotic at the same time point. The experiments were performed in triplicate and the mean and standard deviation are indicated on the plot.

Late Step Teichoic Acid Enzyme Depletion Impacts Peptidoglycan Synthesis

The profound impact of peptidoglycan-directed probes on our *ywaC* reporter was striking given that the reporter system showed a similarly intense response to blocks in the late, but not early, steps of teichoic acid biogenesis. Furthermore, the reporter responds significantly to impacts on isoprenoid synthesis, specifically YacN. Additionally, the computational analysis supported a strong connectivity between teichoic acid and peptidoglycan, with many interactions mediated through undecaprenol synthesis. This prompted us to investigate the impact of depleting teichoic acid biosynthetic enzymes on peptidoglycan synthesis. Using pulse-chase methodology and cultured cells, we showed that depletion of the essential late acting enzyme, TagF, led to a significant reduction in the incorporation of D-[^{14}C]glutamic acid into cell wall peptidoglycan (Figure 7). In contrast, depletion of the early dispensable enzyme, TagO, showed no such reduction, but rather displayed a small but significant increase in D-[^{14}C]glutamic acid incorporation.

DISCUSSION

Small molecule probes are increasing being used to investigate the biological consequences of processes that are difficult to study by conventional genetic or biochemical techniques. Recently, genetic dispensability studies in *B. subtlis* have uncovered unusual genetic complexity among the *tag* genes (D'Elia et al., 2006a). The chemical genetic experiments described herein provide new evidence for genetic connectivity among teichoic acid, peptidoglycan, and polyisoprenoid synthesis.

We discovered *ywaC* in a genome-wide search for *loci* transcriptionally sensitive to lesions in late-acting teichoic acid synthesis genes. The gene product of *ywaC* is a largely uncharacterized protein that only recently has been demonstrated to possess GTP pyrophosphokinase activity (Nanamiya et al., 2008). Such enzymes synthesize guanosine 5′-diphosphate 3′-diphosphate and guanosine 5′-triphosphate 3′-diphosphate, better known as (p)ppGpp, a signaling molecule that is well recognized for its role in the stringent response to amino acid starvation (Justesen et al., 1986). Previous studies have revealed that *ywaC* is a member of the σ^W regulon (Cao et al., 2002a, 2002b; Hyyrylainen et al., 2005) that includes genes that protect the cell from antibiotics that block peptidoglycan synthesis (Cao et al., 2002a). Although it might have been anticipated that the P_{ywaC} reporter system would respond to inhibitors of peptidoglycan synthesis, it was not anticipated to respond to lesions in teichoic acid synthesis. Indeed, the P_{ywaC} reporter construct responds almost exclusively to cell-wall-active compounds. However, these compounds appear to fall into two distinct groups with mechanisms of action that focus on dissectible steps in peptidoglycan biosynthesis: those involving undecaprenol-phosphate-linked steps (bacitracin, ramoplanin, vancomycin and fosfomycin, and fosmidomycin) and those involving peptide crosslinking (cycloserine and the beta lactams).

The perturbation of our promoter-reporter strain by antibiotics that target undecaprenol-phosphate-linked steps is especially compelling. These probes elicited significantly higher responses and target steps that would alter the concentration of free undecaprenol-phosphate levels. Bacitracin inhibits the dephosphorylation of undecaprenol-pyrophosphate (the byproduct of peptidoglycan polymerization) to yield undecaprenol-phosphate the membrane-linked carrier for teichoic acid and peptidoglycan biosynthesis (Stone and Strominger, 1971). Ramoplanin affects transglycosylation by blocking the polymerization of Lipid II (Lo et al., 2000), the undecaprenol-phosphate-linked disaccharide pentapeptide that is the basic building block of peptidoglycan. Upon transglycosylation, the disaccharide pentapeptide is added to the growing peptidoglycan layer (Walsh, 2003), ultimately yielding free undecaprenol-phosphate. Although vancomycin is well known for its role in binding to D-alanine-D-alanine residues in peptidoglycan and preventing

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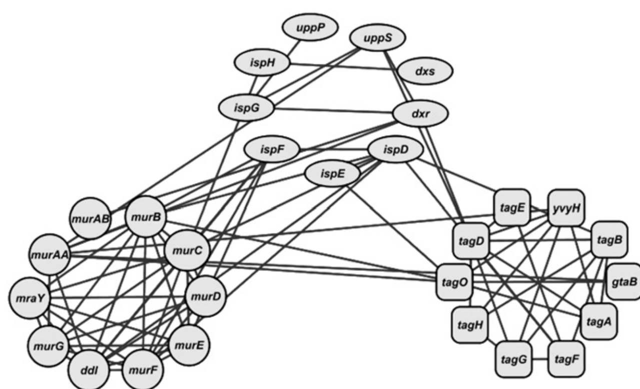

Figure 6. High-Confidence Genomic Context Inferred Interactions among Gene Products Related to Teichoic Acid, Peptidoglycan, and Undecaprenol (Isoprenoid) Synthesis
Genetic interaction links were established among all genes by integrating high-confidence (higher or equal to 0.90) predictions based on gene fusions, predicted operons by both intergenic distances and conservation of gene order, and by examining operon rearrangements across more than 760 prokaryotic genomes. We extracted the connections made among gene products involved in peptidoglycan (circles), teichoic acid (squares), and undecaprenol (isoprenoid) biosynthesis (ellipse) from the complete set of genomic context predictions in *B. subtilis*.

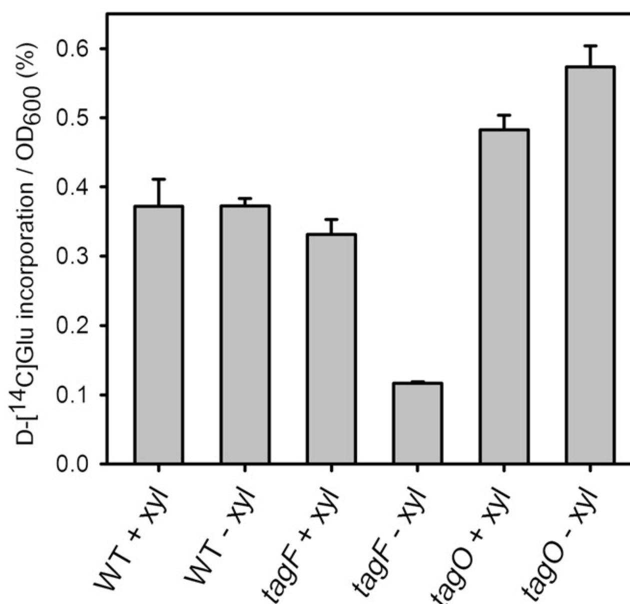

Figure 7. D-Glutamic Acid Incorporation into Peptidoglycan
The rate of peptidoglycan synthesis was measured for mid log cultures of the wild-type strain (WT), a complemented *tagF* deletion strain (*tagF*), and a complemented *tagO* deletion strain (*tagO*) in the presence (+) and absence (−) of inducer (2% xylose). The percent D-[^{14}C]Glu incorporated into the cell wall after a 1 min pulse, followed by a 5 min chase, was calculated as a measure of the OD_{600} for each condition. Each sample was done in triplicate with the standard deviation indicated on the plot.

peptide crosslinking, it likewise causes a steric blockade that indirectly affects transglycosylation by interfering with the penicillin-binding proteins (Walsh, 2003). Thus, vancomycin also inhibits an undecaprenol-phosphate-linked step. Fosfomycin targets MurA, catalyzing the first committed step in the peptidoglycan biosynthetic pathway (Marquardt et al., 1994) and would ultimately impact on the levels of undecaprenol-phosphate-linked intermediates available to peptidoglycan biosynthesis.

Another key probe was fosmidomycin, a molecule that targets IspC, the enzyme catalyzing the first committed step of isoprenoid biosynthesis in *B. subtilis* (Jomaa et al., 1999), which showed an approximately 12-fold response in the reporter assay. Indeed, previous work from our group has shown that this pathway is essential to the viability of *B. subtilis* and that genetic perturbation of the pathway impacts primarily on cell wall biosynthesis (Campbell and Brown, 2002), presumably by limiting the formation of undecaprenol-phosphate. Similar to the response to the chemical probe, fosmidomycin, depletion of YacN, a key enzyme in polyisoprenoid synthesis, stimulated the P_{ywaC} reporter.

The lower responses of the promoter-reporter strain to cycloserine and the beta-lactams (ampicillin, cefoxitin, cloxacillin, and moxolactam) are likewise consistent with the emergent conclusion that lipid-linked steps were particularly sensitive to perturbation. Cycloserine targets D-ala-D-ala ligase (Neuhaus and Lynch, 1964). The inhibition of D-ala-D-ala ligase might not affect undecaprenol-phosphate-linked steps because the Lipid II tripeptide is known to be a substrate for transglycosylation (van Heijenoort et al., 1992). The impact of inhibition by cycloserine would instead be on peptidoglycan peptide crosslinking. Indeed, beta-lactams inhibit the transpeptidation step of peptidoglycan synthesis, preventing peptidoglycan crosslinking.

We believe our results are consistent with the hypothesis that the C_{55} polyisoprenoid lipid carrier undecaprenol-phosphate is at the center of interactions among teichoic acid, peptidoglycan, and undecaprenol synthesis, and that these interactions explain the complex gene dispensability patterns in wall teichoic acid biosynthesis. Given that lipid-linked steps in peptidoglycan synthesis were the focus of chemical-genetic interactions with the *ywaC* reporter, undecaprenol-phosphate levels emerge as a likely origin for interactions between peptidoglycan and teichoic acid biosynthesis. This link was supported by our computational analyses of genomic context and by biochemical experiments described herein. Our analyses of genomic context identified strong genetic interactions between peptidoglycan and teichoic acid acting largely through the undecaprenol (isoprenoid) network. These findings prompted us to investigate the activity of peptidoglycan synthesis on perturbation of wall teichoic acid synthesis. We found that depletion of a late-acting teichoic acid enzyme, TagF, causes a significant reduction in D-glutamic acid incorporation into the cell wall. We posit that lesions in late steps of teichoic acid synthesis lead to the sequestration of undecaprenol-phosphate in lipid-linked teichoic acid intermediates. As a shared lipid intermediate in both peptidoglycan and teichoic acid biogenesis, the reduction in free undecaprenol-phosphate pools would be manifest in a decrease in peptidoglycan synthesis as we have demonstrated here. This scenario would be consistent with previous studies that have suggested that undecaprenol-phosphate pools are limiting to peptidoglycan synthesis (Kohlrausch et al., 1989; Mengin-Lecreulx et al., 1991). Indeed, our results with the *tagO* mutant further support the thesis that undecaprenol-phosphate is limiting. We showed a small but significant increase in

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peptidoglycan synthesis on depletion of the nonessential protein TagO, catalyzing the first step of teichoic acid synthesis. This suggests that depletion of TagO blocks the use of undecaprenol-phosphate by the teichoic acid pathway and allows for an increased pool of lipid precursor for peptidoglycan synthesis.

Our characterization of the promoter P_{ywaC} with a library of highly characterized, biologically active small molecules provides new evidence for a hypothesis to explain the genetic complexity of wall teichoic acid biogenesis. We suggest that cell death associated with lesions in the late steps of teichoic acid synthesis is due to the accumulation of lipid-linked teichoic acid intermediates resulting in the sequestration of undecaprenol-phosphate from peptidoglycan assembly. We have shown previously, in both *B. subtilis* and *S. aureus*, that the lethal phenotype associated with depletion of late acting enzymes (TagD, TagB, and TagF) can be suppressed by preventing the initiation of teichoic acid production through a deletion of the first step encoded in *tagO* (D'Elia et al., 2006a, 2006b). This suppression is consistent with the nonessential phenotype of gene *tagO* and presumably works by freeing up undecaprenol-phosphate for the process of peptidoglycan synthesis. This thesis is supported by physiological experiments presented here that reveal decreased peptidoglycan synthesis upon depletion of a late-acting but not early-acting step. Interestingly, this argument is further supported by phenotypic examination of conditional mutants in these metabolic pathways. Disruptions of teichoic acid production (Bhavsar et al., 2001), peptidoglycan biogenesis (Wei et al., 2003), and isoprenoid synthesis (Campbell and Brown, 2002) in *B. subtilis* result in remarkably similar cell ultrastructure when examined by electron microscopy. Indeed, the observed loss of rod shape, aberrations in septation, and altered wall architecture are common features among these and classically associated with collapse of the Gram-positive cell wall.

SIGNIFICANCE

Our findings have revealed surprising genetic connectivity among isoprenoid, peptidoglycan, and teichoic acid biogenesis that is evident in the regulation of expression of the gene *ywaC.* Never before has such a profound link been drawn between these biosynthetic pathways supported by genetic, computational, and biochemical evidence. Our results suggest that the dispensability of teichoic acid biosynthetic enzymes centers on the impact of their depletion on peptidoglycan synthesis. We propose that, as a shared lipid intermediate in peptidoglycan and teichoic acid pathways, undecaprenol-phosphate is susceptible to sequestration by blocks in the late steps of teichoic acid synthesis. That this genetic and mechanistic connectivity was revealed through an analysis of phenotypic response to a library of highly annotated compounds points to the power of such an approach in describing cellular complexity. These findings open the door to work aimed at better understanding and potentially exploiting these complex genetic and biochemical interactions with strategies to target cell wall biogenesis with new chemical matter that is insensitive to existing antibiotic resistance mechanisms. Indeed, the promoter reporter system described here provides a fresh and now validated approach to search for small molecules that impinge broadly on cell wall biogenesis in Gram-positive bacteria.

EXPERIMENTAL PROCEDURES

General Methods

Materials, general methods, the details of the growth and analysis of the *tagD* mutant for the microarray experimentation, and the construction of the complemented *tagO* deletion are described in Supplemental Experimental Procedures. Tables S1–S5 can be found online.

Construction of Luciferase Promoter-Reporter Plasmids

An erythromycin resistance cassette amplified from pMUTIN4 using primers Erm-F and Erm-R (Table S2), digested with PstI and *Xho*I restriction enzymes was inserted into pSB2025 digested with the same sites to generate pLuxErm. To clone candidate promoters into pLuxErm, 750 base pairs upstream of the putative translational start site of the gene of interest was amplified from *B. subtilis* 168 genomic DNA using the primers listed in Table S2 (P_{gene} − F / P_{gene} − R primer pair). This polymerase chain reaction (PCR) product was cloned into pBluescript SKII+ digested with EcoRV and subsequently moved into pLuxErm by ligation into the multiple cloning site upstream of *luxA* using a combination of the following restriction enzymes: EcoRI, NcoI, and SalI, yielding pLuxErm containing the promoter of interest (POI).

Generation of Luciferase Promoter-Reporter Strains

The reporter plasmids pLuxErmPOI were transformed into the following *B. subtilis* strains after passage through *E. coli* MC1061 to concatamerize the DNA: EB6, EB240, EB323, EB633, EB669, and EB1599 and selected on LB supplemented with spectinomycin, lincomycin, erythromycin, and xylose for EB240, EB323, EB633, EB669, and EB1599 and only erythromycin for selection in EB6. To verify that a single integration event had occurred, genomic DNA was prepared from resistant transformants and analyzed by PCR using a reverse primer that annealed to *luxA* and a forward primer that annealed upstream of the amplified promoter region of the gene of interest (P_{gene}ver) (Table S2).

Luminescent Reporter Assay: TagB, TagD, TagF, TagO, and YacN Depletion Studies

One milliliter of an overnight culture of EB240, EB323, EB633, EB669, and EB1599 transformants, grown in LB supplemented with spectinomycin, erythromycin, and xylose, was pelleted and resuspended into 1 ml fresh LB containing spectinomycin and erythromycin with or without xylose. An initial 100-fold dilution was made into LB supplemented with erythromycin and spectinomycin or LB supplemented with erythromycin, spectinomycin, and xylose in white, clear-bottom 96-well plates (Perkin Elmer, Woodbridge, ON). A second 100-fold dilution was made as required if growth in the absence of xylose approached an OD_{600} ~0.2 after 4 hr of growth. Growth and luminescence were monitored in triplicate for a period of 12 hr using the Envision (Luminescence plate reader) (Perkin Elmer).

Luminescent Reporter Assay: Chemical Library Screen

A high throughput screen was done using the Prestwick Chemical Library (Prestwick Chemical Inc., Washington, DC), which contains 1120 compounds. Dimethyl sulfoxide (DMSO) was used as the low control. The screen was conducted essentially the same way as described above for the antibiotic studies. The P_{ywaC} reporter in a wild-type background (EB1385; Table S1) was grown to an OD_{600} ~0.2, at which time compounds were added using the Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, CA) to a final concentration of 10 μM. Growth and luminescence were monitored for 12 hr using the Envision (Luminescence plate reader).

Luminescent Reporter Assay: Secondary Studies of 18 Antibiotics

The minimum inhibitory concentration (MIC) of each antibiotic was determined in white, clear-bottom 96-well plates (Perkin Elmer). Antibiotic concentrations at MIC and 2-fold and 4-fold above and below were tested in triplicate. A 100-fold dilution of EB1385 was made into 200 μl LB supplemented with

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erythromycin from an overnight culture grown in the same medium. Cells were grown in white, clear-bottom 96-well plates (Perkin Elmer). As cells reached an OD_{600} ~0.2, antibiotics were added to the cells at the following concentrations (in μg/ml): 0.5, 1, 2, 4, 8 for ampicillin, fosmidomycin, cefotaxime, and trimethoprim; 1, 2, 4, 8, 16 for nalidixic acid and tetracycline; 128, 256, 512, 1024, 2048 for cycloserine and fosfomycin; 64, 128, 256, 512, 1024 for bacitracin; 0.25, 0.5, 1, 2, 4 for novobiocin and triclosan; 0.125, 0.25, 0.5, 1, 2 for ramoplanin and vancomycin; and 2, 4, 8, 16, 32 for tunicamycin and neomycin. Growth and luminescence were monitored for 12 hr using the Envision.

Computational Genomic Context Analyses of Teichoic Acid, Peptidoglycan, and Undecaprenol (Isoprenoid) Biosynthesis

Predictions of functional associations were performed for the published genome of *B. subtilis* (Kunst et al., 1997). Gene fusions were predicted as published previously (Moreno-Hagelsieb and Collado-Vides, 2002). Operons were predicted in all available prokaryotic genomes downloaded from the National Center for Biotechnology Information's RefSeq database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) (Pruitt et al., 2005), which contained around 760 genomes as of October of 2008. These predictions were made by both intergenic distances (Moreno-Hagelsieb and Collado-Vides, 2002) and conservation of gene order across evolutionarily distant genomes (Janga and Moreno-Hagelsieb, 2004). Predicted operons were used to find functional interactions by operon rearrangements as described in the Nebulon system (Janga et al., 2005). Briefly, if the orthologs of two genes in *B. subtilis* were found to be in an operon in any of the genomes available, the two genes were predicted to functionally interact. Orthologs were determined as reciprocal best hits as described previously (Moreno-Hagelsieb and Latimer, 2008). The scores from all sources of predictions were integrated using a naive Bayes approach that has been used for integration of other genomic context databases such as PROLINKS (Bowers et al., 2004) and STRING (von Mering et al., 2007). We extracted known genes directly involved in teichoic acid, peptidoglycan, and isoprenoid/undecaprenol biosynthesis from the resulting large network of high-confidence (more than or equal to 0.90) functional interactions.

Pulse-Chase Labeling with D-[^{14}C]Glutamic Acid and Isolation of Peptidoglycan

Strains EB6 (WT), EB669 (*tagF* deletion) and EB1437 (*tagO* deletion) were grown O/N in a Spizizen minimal medial (SMM) (Spizizen, 1958) supplemented with yeast extract (0.1%), the appropriate amino acids (His, Arg, Met at 0.1 mg/ml) and xylose. Fresh cultures of the modified SMM (as above), but in the presence and absence of xylose, were inoculated to a starting OD_{600} of 0.01 for EB6 and EB669, and 0.05 in the case of EB1437 and shaken at 37°C at 275 rpm. At mid log, 1 ml cultures were removed from the shaker and pulsed at room temperature with 1 μCi D-[^{14}C]Glu (American Radiolabeled Chemicals, St. Louis, MO) for 1 min, followed by a chase with cold D-Glu (500 μg/ml). After 5 min, the cultures were chilled on ice for 10 min and the OD_{600} was determined before pelleting the cells for 10 min. The cells were washed once with saline before harvesting the cell walls. Cell walls were isolated by boiling in 25 mM citrate (pH 6.0) in 4% SDS for 6 hr. Solutions were stored at −20°C O/N before being boiled again for 1 hr. After cooling, the solution was applied to a 30,000 MWCO filter (Pall, Mississauga, ON) and spun at 13,000 rpm for 30 min. In the filter unit, the precipitate was resuspended in 150 μl citrate buffer (25 mM [pH 6.0]) containing DNase (0.04 mg/ml) and RNase (0.06 mg/ml) and incubated at room temperature (RT) for 30 min before trypsin (final concentration 0.6 mg/ml) was added and incubated at RT for an additional 30 min. The filtrate was spun through before the precipitate was washed in 25 mM citrate (pH 6.0) and spun a final time to collect the cell wall on the membrane. All filtrates were kept and analyzed. The membrane, along with all the washes and filtrates were analyzed by liquid scintillation counting. For each sample, the radioactive counts collected on the membrane were compared with the total radioactive counts, determined, and divided against the OD_{600} of the harvested cultures, giving a measure of the percentage D-[^{14}C]Glu incorporation/OD_{600}.

SUPPLEMENTAL DATA

Supplemental Data include five tables and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00142-2.

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