Chemistry & Biology
Article

Antagonism of Chemical Genetic Interaction Networks Resensitize MRSA to β-Lactam Antibiotics

Sang Ho Lee,[1] Lisa Wang Jarantow,[1] Hao Wang,[1] Susan Sillaots,[1] Henry Cheng,[1] Timothy C. Meredith,[1,3] John Thompson,[2] and Terry Roemer[1,*]
[1]Department of Infectious Diseases, Merck Research Laboratories, Merck and Company, Kenilworth, NJ 07033, USA
[2]Department of Molecular Modeling, Merck Research Laboratories, Merck and Company, Rahway, NJ 07065, USA
[3]Present address: Infectious Diseases, Novartis Institute for Biomedical Research, 500 Technology Square, Cambridge, MA 02139, USA
*Correspondence: terry_roemer@merck.com
DOI 10.1016/j.chembiol.2011.08.015

SUMMARY

Antibiotic drug resistance among hospital and community acquired methicillin resistant *Staphylococcus aureus* (MRSA) has dramatically eroded the efficacy of current therapeutics. We describe a chemical genetic strategy using antisense interference to broadly identify new drug targets that potentiate the effects of existing antibiotics against both etiological classes of MRSA infection. Further, we describe the resulting chemical genetic interaction networks and highlight the prominent and overlapping target sets that restore MRSA susceptibility to penicillin, cephalosporins, and carbapenems. Pharmacological validation of this approach is the potent synergy between a known inhibitor to a member of this genetic potentiation network (GlmS) and a broad set of β-lactam antibiotics against methicillin resistant *Staphylococci*. Developing drug-like leads to these targets may serve as rational and effective combination agents when paired with existing β-lactam antibiotics to restore their efficacy against MRSA.

INTRODUCTION

Staphylococcus aureus is the leading Gram-positive bacterial pathogen causing both hospital and community-acquired infections and for which antibiotic treatment options are compromised by emerging drug resistance. Methicillin resistant *S. aureus* (MRSA), which is highly cross resistant to essentially all other β-lactam antibiotics, now represents over half of all *S. aureus* clinical isolates identified in the USA (Styers et al., 2006). In fact, the number of serious infections caused by MRSA alone in the United States as monitored in 2005 approached nearly 95,000 cases, of which almost 19,000 related fatalities were estimated. Consequently, mortality associated with MRSA infection now exceeds those attributed to HIV/AIDS in the United States (Klevens et al., 2007; Bancroft, 2007).

Despite the growing resistance to β-lactam antibiotics across both Gram-positive and Gram-negative bacterial pathogens, they remain the single most clinically relevant antibiotic drug class (Walsh, 2003). Mechanistically, β-lactams acetylate an essential transpeptidation activity common to a family of penicillin binding proteins (PBPs), thereby inhibiting peptidoglycan crosslinking and integrity of the cell wall (van Heijenoort, 2001). The key resistance determinant to these agents is an acquired gene, *mecA*, which encodes a penicillin binding protein (PBP2A) with markedly reduced affinity to β-lactams compared to native *S. aureus* PBPs (Matsuhashi et al., 1986; Song et al., 1987; de Lencastre et al., 2007). MRSA resistance to β-lactams is achieved by the cooperative function of PBP2A and PBP2, which provide transpeptidation and transglycosylation activities, respectively, necessary to crosslink peptidoglycan precursors and construct the cell wall under conditions when other susceptible PBPs are otherwise inactivated by these agents (Pinho et al., 2001a; Scheffers and Pinho, 2005).

PBP2A and PBP2 represent important antibiotic targets that may be exploited to develop new efficacious agents against MRSA (Pinho et al., 2001b; Moyá et al., 2010). However, a growing number of auxiliary genes have also been identified by transposon-based screens that are typically involved in peptidoglycan precursor biosynthesis and turnover and are required for PBP2A-mediated β-lactam resistance. (de Lencastre and Tomasz, 1994; Berger-Bächi and Rohrer, 2002; de Lencastre et al., 1999; Sobral et al., 2003; Gardete et al., 2004). Additional studies also emphasize an emerging network of genetic perturbations capable of potentiating the effects of β-lactams against MRSA (Pinho and Errington, 2003; Memmi et al., 2008; Kuroda et al., 2003; Gardete et al., 2006; Beltramini et al., 2009). Recently, we identified SAV1754 as a new member of this auxiliary gene set that is required for peptidoglycan biosynthesis and whose cognate inhibitors display potent synergy in combination with β-lactams (Huber et al., 2009). Therefore, further genetic characterization of the β-lactam resistance of MRSA, combined with the identification of inhibitory compounds to members of this β-lactam genetic potentiation network provide a rational framework for discovering combination agents potentially effective in restoring β-lactam activity against MRSA.

Here we describe a *S. aureus* antisense interference-based screen of 245 essential genes in the hospital and community-acquired clinical isolates MRSA COL and USA300 to genetically

identify additional auxiliary factors, which if partially depleted, enhance MRSA susceptibility to β-lactams or other clinically used antibiotics. Demonstrating the reliability of this approach, several previously described auxiliary genes including *murE*, *femX* (*fmhB*), *femD* (*glmM*), *pbp2*, SAV1220, and SAV1754 were faithfully identified to restore β-lactam susceptibility of both MRSA strain backgrounds (Memmi et al., 2008; Berger-Bächi and Rohrer, 2002; Gardete et al., 2004; Beltramini et al., 2009; Huber et al., 2009). Additional essential genes involved in peptidoglycan precursor synthesis that share this phenotype by antisense modulation include the majority of the *mur* and *pbp* genes, albeit to differing extents among different β-lactam antibiotic classes. Genes not previously known to affect MRSA β-lactam susceptibility were also identified, including those involved in cell division (*ftsZ*, *ftsW*, and *ftsA*), wall teichoic acid biosynthesis (*tarL*), secretion (*spsB*), transcription (hu), and SAV1892; the latter of which is largely uncharacterized but predicted to participate in cell wall biogenesis or remodeling. Here, the resulting chemical genetic interaction networks are described for antibiotics targeting *S. aureus* cell wall, DNA synthesis and replication, RNA biogenesis, fatty acid biosynthesis, and protein synthesis. The utility of these genetic potentiation maps is that target-specific inhibitors to antibiotic susceptibility determinants should phenocopy their genetic inactivation. Consistent with this, we demonstrate potent synergy between a previously described GlmS inhibitor, Nva-FMDP (Chmara et al., 1998), and a broad set of β-lactam antibiotics against MRSA and methicillin resistant *Staphylococcus epidermidis*, MRSE.

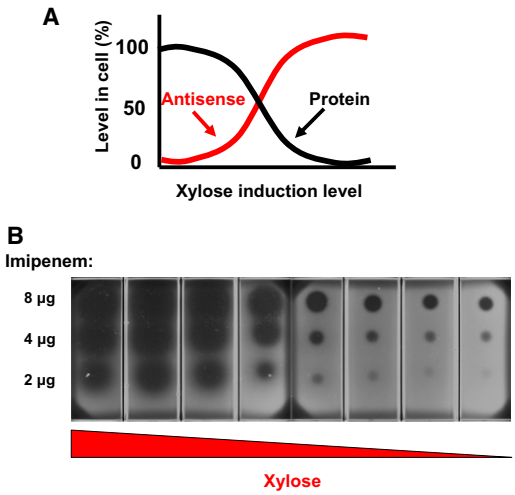


Figure 1. Antisense Induction of *mecA* (Pbp2A) and Restored MRSA COL Susceptibility to Imipenem

(A) Schematic of antisense interference and relationship between antisense induction and protein depletion as a function of xylose induction level.

(B) Example of restored susceptibility of MRSA COL to imipenem by xylose inducible antisense depletion of *mecA*. MRSA COL are seeded in agar chambers with varying concentrations of xylose (0 μM, right, to 100 μM, left), and imipenem are spotted horizontally at three concentrations. The increase in xylose concentration results in increasing susceptibility of MRSA COL to imipenem due to depletion of Pbp2A protein by antisense interference.

See also Table S1.

RESULTS

Antisense-Mediated Genetic Depletion and Antibiotic Hypersusceptibility Screening

Clinically relevant antibiotics used in the study were selected according to their microbiological activity against an extensive panel of current *S. aureus* clinical isolates. (Fritsche et al., 2008). These include the β-lactam antibiotics whose activity has been most extensively eroded by commonly occurring MRSA isolates, such as the penicillin/β-lactamase combination agent, piperacillin-tazobactam, cephalosporins (cefepime, ceftazidime, and ceftriaxone), and carbapenems (imipenem and ertapenem). Other clinically used antibiotics selected for this study include fluoroquinolones that target DNA replication (ciprofloxacin and levofloxacin), protein synthesis inhibitors (tetracycline, linezolid, and azithromycin), as well as the mechanistically distinct agents, trimethoprim, (DNA synthesis), rifampicin (RNA biogenesis), and cerulenin (fatty acid biosynthesis). Three early stage antibacterial leads, all targeting SAV1754, were also selected for study (Huber et al., 2009).

To genetically screen MRSA for altered sensitivity to the above antibiotics, we transformed COL strain with individual members of a previously described plasmid library (Donald et al., 2009) containing xylose inducible antisense interference fragments specifically targeting one of 245 *S. aureus* essential genes. Briefly, the library was constructed by subcloning short antisense fragments (ranging in size from 200 to 800 bp in length) behind the pT5X xylose inducible promoter (Forsyth et al., 2002). Upon xylose addition into the growth medium, antisense expression targets the complementary mRNA, leading to transcription and translation interference of the target protein (Forsyth et al., 2002; Donald et al., 2009; Xu et al., 2010). Although full xylose induction leads to growth arrest, partial antisense induction affords titratable knockdown of gene expression to sensitize cells to antibiotics (Donald et al., 2009) (Figure 1). As an attempt to normalize genetic knockdown across all targets, a subminimal inhibitory concentration (MIC) of the inducer was determined so that growth was routinely impaired 10%–20% (IC_{10}-IC_{20}) for each antisense strain. Antibiotic hypersensitivity screens were performed by seeding individual COL-AS (antisense) strains in LB medium containing suitable xylose levels to partially knockdown target expression (10–50 mM depending on strain) and spotting 10 μl of each antibiotic on the plate. Target-specific hypersensitivities were identified by comparing the antibiotic zone of inhibition of antisense knockdown strains in the presence versus absence of xylose. Qualitative antibiotic hypersensitivity scores were assigned to AS strains as being strong (3+), medium (2+), mild (1+), or lacking a phenotype (0+) (Figure 2; see Table S1 available online).

Consistent with the reliability of the screen to detect target-based antibiotic hypersensitivity, a number of known drug-target interactions were identified (Figures 2A and 2B). These include the *dfrA*-AS strain (3+), depleted of dihydrofolate reductase, and which specifically displayed a pronounced hypersensitivity to its known inhibitor, trimethoprim (Walsh, 2003). The AS strain corresponding to *thyA* (thymidylate synthase) also displayed detectable (1+) and specific hypersensitivity to trimethoprim, consistent with the observed synergy between trimethoprim and sulfonamides, the latter of which targets dihydropteroate

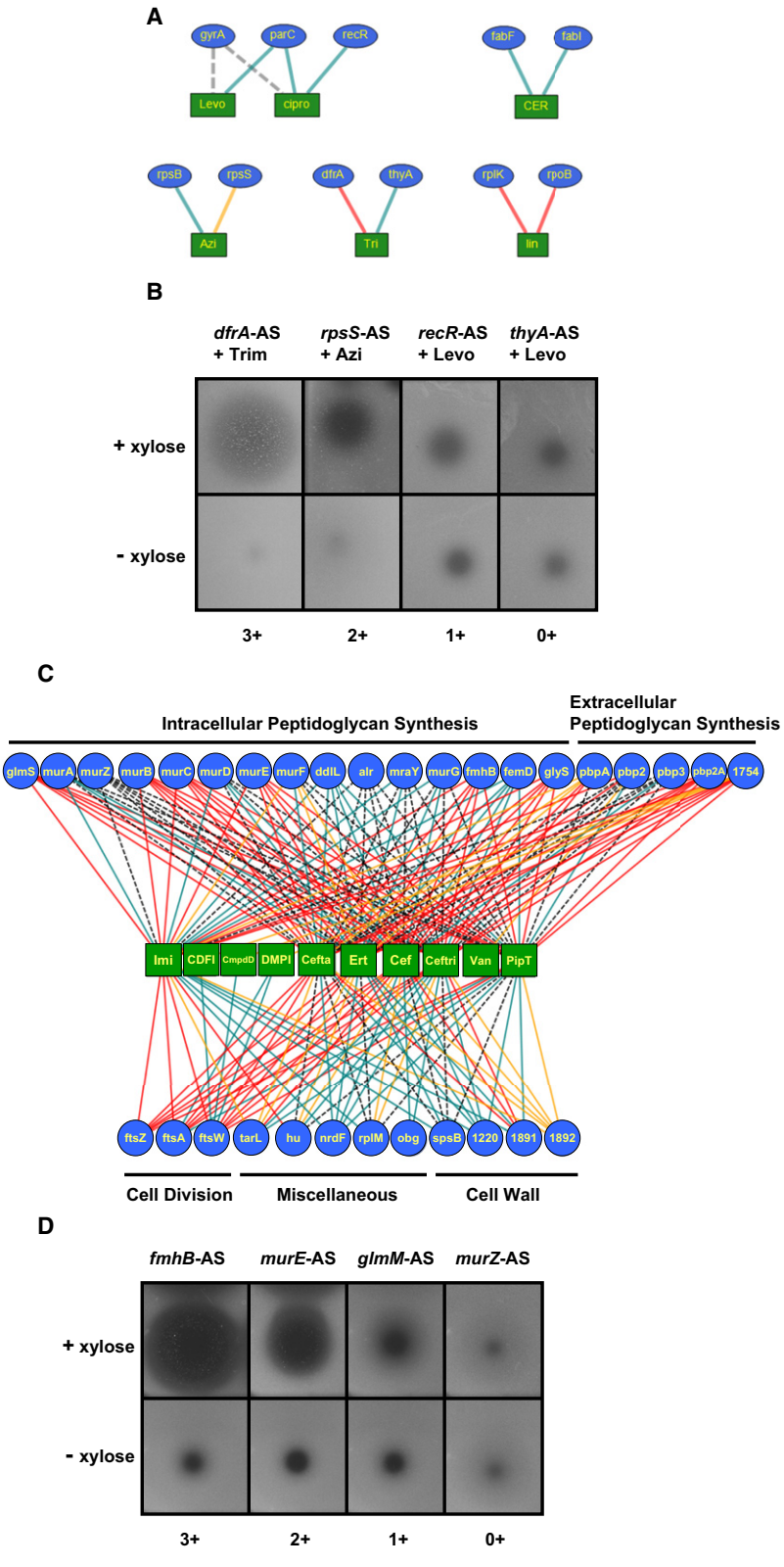


Figure 2. MRSA COL Chemical Genetic Interaction Map by Antisense Interference

(A) MRSA COL chemical genetic interaction map of noncell wall antibiotics. Chemical genetic interactions are depicted as connecting lines between genes (blue circle) and antibiotics (green squares). Lines are colored according to qualitative scores assigned for the level of hypersensitivity between compound and target gene as red (3+, strong), orange (2+, medium), blue (1+, mild), or dashed black (0+, no hypersensitivity despite expectation a phenotype would be observed). Antibiotics tested include levofloxacin (Levo), ciprofloxacin (Cipro), cerulenin (Cer), azithromycin (Azi), trimethoprim (Tri), and linezolid (Lin).

(B) Representative hypersensitization phenotypes of members of the noncell wall antibiotic set. COL antisense bearing strains were seeded in LB agar plates supplemented with 50 mM xylose to induce modest antisense expression (top row) or without xylose (bottom row) that serves as negative control for gene-specific hypersusceptibility phenotypes. Representative chemical genetic qualitative scores are shown.

(C) COL genetic potentiation map of cell wall antibiotics. Chemical genetic interactions are depicted as described in (A). Antibiotics tested include imipenem (Imi), ertapenem (Ert), cefepime (Cef), ceftazidime (Ceftra), ceftriaxone (Ceftri), piperacillin/tazobactam (PipT), vancomycin (Van), and the SAV1754 inhibitors, DMPI, CDFI, and compound D (CmpdD).

(D) Representative qualitative scores for cefepime hypersusceptibility was performed as described in (B), using antisense strains corresponding to the known auxiliary genes reported to contribute to β-lactam susceptibility; *fmhB* (3+), *murE* (2+), and *glmM* (1+). Note, *murZ* (0+) is used as a negative control based on the presence of a functional paralog, *murA*, which buffers the effect of a mild depletion of *murZ*.

See also Figures S2, S3, and S4 and Table S1.

synthase and leads to the incorporation into dead end tetrahydrofolate analogs (Walsh, 2003). Striking and specific hypersensitivity was also detected between the *pbp2A*-AS strain (3+) and all six β-lactams tested, as expected based on the essential role PBP2A performs in β-lactam resistance (Figure 2C). Similarly, the SAV1754-AS strain displayed marked hypersensitivity (2+) to cognate inhibitors, DMPI, CDFI, and compound D (Huber et al., 2009). Further, *rpoB* and *rplK* AS strains (both 3+) as well as *rpsS*-AS (2+) and *rpsB*-AS (1+) revealed a range of antibiotic hypersensitivities, specifically against the protein synthesis inhibitors linezolid and azithromycin, respectively. Target or mechanism-based hypersensitivity, albeit mild, was also detected in screens using ciprofloxacin (*recR*-AS (1+), *parC*-AS (1+)) and levofloxicin (*parC* AS (1+)), as well as cerulenin (*fabI*-AS (1+), *fabF*-AS (1+)). Screening β-lactam antibiotics revealed several *fem* or auxiliary (*aux*) genes previously reported to participate in MRSA resistance to these agents, including *femX* (*fmhB*), *femD* (*glmM*), *femF* (*murE*), and *pbp2* (Figures 2C and 2D). In all but one case, (*glmM*-AS; 1+), the corresponding AS strains displayed strong hypersusceptibility (3+) to both imipenem and ertapenem, as well as multiple penicillins and cephalosporins. Reciprocally,

inactivation of *pbp3* is reported to not significantly alter MRSA β-lactam susceptibility (Pinho et al., 2000), consistent with our findings (Pbp3-AS; 0+). Collectively these results demonstrate that relevant xylose concentrations were used to appropriately sensitize AS strains broadly across antibiotic screening conditions to identify known target-pathway-antibiotic interactions.

Antisense-Based Genetic Potentiation of β-Lactams

Mild AS depletion in COL identifies multiple well characterized *S. aureus* genes required for β-lactam resistance (Figure 2C; Table S1). Functionally, these genes can be grouped to five general cellular processes. Group 1 members include GlmS (3+ across all β-lactams tested), a glucosamine-6-phosphate synthase involved in the first committed step in peptidoglycan precursor synthesis, as well as MurA-G and MraY, which participate in eight sequential steps starting with UDP-GlcNAc to build the peptidoglycan monomer also known as murein pentapeptide or lipid II (van Heijenoort, 2001) (Figure 3). Interestingly, *murB*, *murC*, and *murE* AS strains displayed reproducibly strong hypersensitization (3+) across all six β-lactams whereas *murA*, *murD*, *murF*, *mraY*, and *murG*-AS strains all displayed only mild to moderate sensitization specifically to imipenem, ertapenem, and cefepime. Group 2 members include SAV1754 and the *pbp* gene family. Remarkably, SAV1754-AS strain hypersusceptibility to all β-lactams is restored to levels comparable to that of the *pbp2A*-AS strain (3+), whereas the *pbp3*-AS strain was unaltered in its hypersensitivity to these agents. Interestingly, *pbp1* and *pbp2*-AS strains displayed a reciprocal relationship in their β-lactam hypersensitivity to these agents, with the *pbp2*-AS strain preferentially strongly susceptible to imipenem and ertapenem, whereas *pbp1*-AS produced only moderate susceptibility to carbapenems but strong resensitization to all cephalosporins tested (Figures 2B and 4A). Group 3 genes include *ftsA*, *ftsW*, and *ftsZ*, and encode core components of a macromolecular complex (termed the Z ring) that is required for coordinating cell division and the position of the resulting division plane, processes that directly affect peptidoglycan synthesis and septum formation (Adams and Errington, 2009). Indeed, AS strains corresponding to each of these Z-ring components displayed strong and largely identical hypersusceptibility (3+) to all β-lactams except piperacillin-tazobactam (Figures 2C and 4B).

Group 4 genes participate in diverse cellular processes. For example, *glyS* encodes a glycyl tRNA synthetase; the *glyS*-AS strain is uniquely and strongly hypersensitive (3+) to the full set of β-lactams tested. Although perhaps counterintuitive, these results corroborate biochemical studies that demonstrate a charged glycyl tRNA synthetase serves as an essential substrate donor of Fem proteins to reconstitute in vitro pentaglycine bridge synthesis (Schneider et al., 2004). Modest depletion of TarL (an essential enzyme involved in wall teichoic acid synthesis) also resensitizes MRSA to all β-lactams, although to varying extents (1–3+) (Figure 4C). These results are corroborated by a recent report that deletion of the first committed step in this pathway (*tarO*) also dramatically resensitizes MRSA to this family of antibiotics (Campbell et al., 2011). We speculate that the mild phenotypes associated with *spsB*-AS may relate to the established function of *spsB*, a signal peptidase required for signal peptide cleavage of cell wall localized proteins (Cregg et al., 1996) and which is transcriptionally induced by β-lactam mediated cell wall damage (Gardete et al., 2006). Likewise, a homolog to the histone-like protein, hu, has been reported to localize in the cell wall and control cell wall assembly in *Mycobacterium smegmatis* (Katsube et al., 2007). Mild depletion of hu by antisense induction resulted in considerable hypersusceptibility (2–3+); particularly to carbapenems (Figure 4C).

Group 5 genes are predicted to participate in cell wall biogenesis and are represented by SAV1891, SAV1892, and SAV1220. Whereas the AS strain corresponding to SAV1892 produced robust hypersensitivity (3+) to all β-lactams, SAV1891-AS displayed intermediate and varied β-lactam sensitivities, whereas the SAV1220-AS strain sensitivity to each of these agents was mild (Figure 4C). SAV1220 has recently been demonstrated to function as a serine/threonine kinase of a phospho relay signal transduction pathway critical for maintaining proper cell wall architecture (Beltramini et al., 2009). Interestingly, SAV1892 is structurally related to UDP-N-acetylmuramyl tripeptide synthetase-like proteins, including MurF and Mpl whereas SAV1891 is predicted to encode a cobyric acid synthase. SAV1891 and SAV1892 are predicted to be coexpressed from a common operon. Based on SAV1892 similarity to MurF, which we demonstrate shares this β-lactam hypersusceptibility phenotype, as well as the reported hypersensitivity of the *Acinetobacter baylyi mpl* mutant to β-lactams (Gomez and Neyfakh, 2006), we favor the hypothesis that SAV1892 functions directly in cell wall biogenesis, whereas antisense interference of SAV1891 likely mediates these phenotypes by polar effects on upstream expression of SAV1892.

Extending the Identification of Genetic Potentiators to β-Lactams in the Community-Acquired MRSA Strain USA300

Essential genes that when partially depleted display a conserved β-lactam hypersusceptibility phenotype in both hospital and community acquired MRSA clinical isolates represent a privileged set of β-lactam potentiation targets. Accordingly, antisense interference plasmids corresponding to genes with demonstrated β-lactam hypersensitivity in MRSA COL were shuttled into the community acquired MRSA strain, USA300, a highly virulent and intrinsically antibiotic resistant and epidemic strain common in the United States (David and Daum, 2010). USA300 antisense containing strains were evaluated for altered susceptibility to the following subset of antibiotics; imipenem, penicillin, cefepime, cerulenin, vancomycin, ceftriaxone, trimethoprim, linezolid, and ciprofloxacin. Generally, a strong correlation was observed between the specific antibiotic hypersusceptibility phenotypes by genetic depletion performed in COL versus USA300 (Figure 5; Table S1). In particular, *murB*, *murC*, *glyS*, SAV1754, *pbpA*, *pbp2A*, and *ftsZ*-AS strains all displayed dramatically restored USA300 susceptibility (3+) specific to the representative penicillin, cephalosporins, and cerbapenem tested. We also observe that *murA*, *murD*, *murG*, *mraY*, and *spsB* appear to preferentially affect USA300 susceptibility (all 2–3+ with respect to these antibiotics) versus that detected in COL. These results demonstrate that reproducible β-lactam hypersusceptibility phenotypes are achieved by antisense

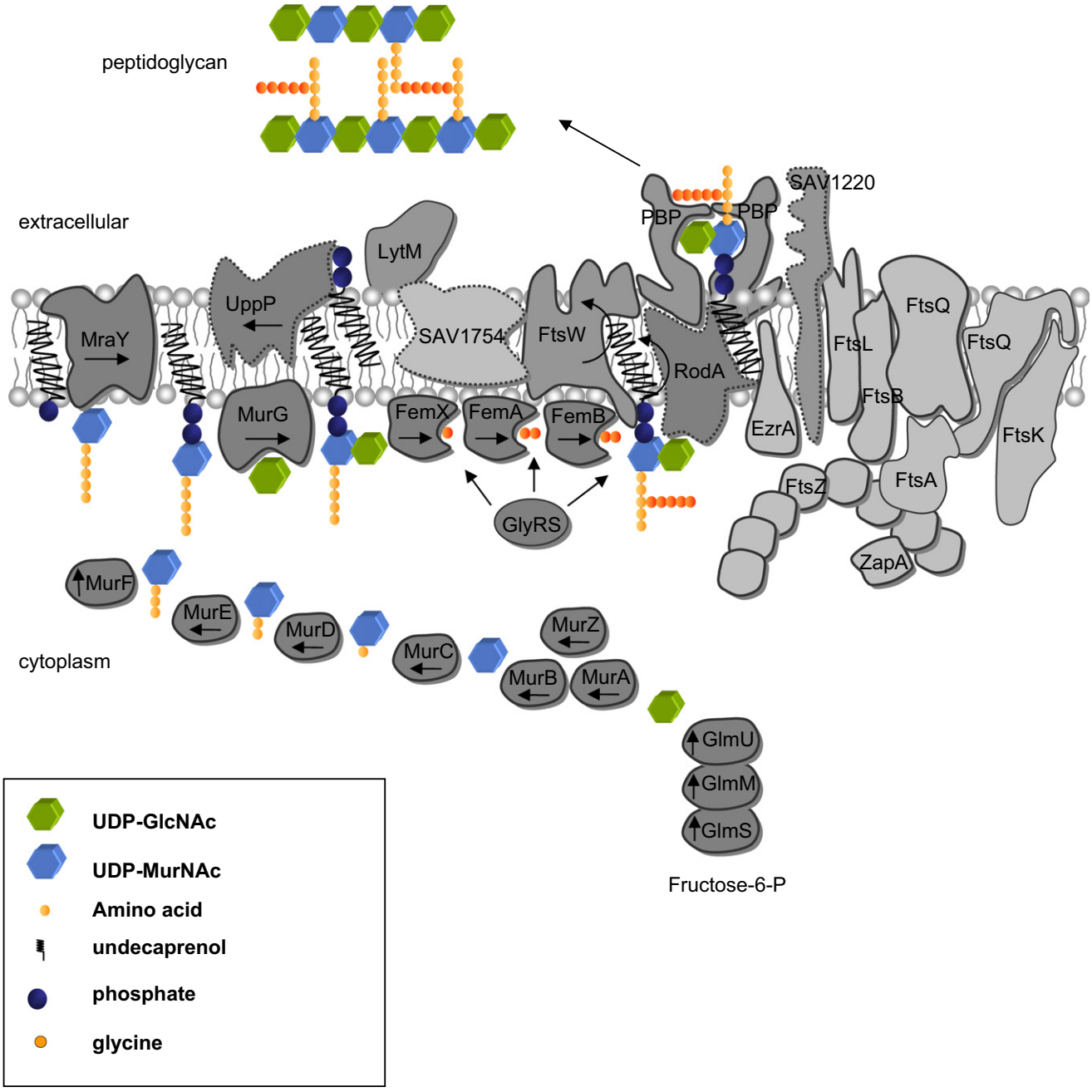


Figure 3. Schematic Representation of Coordinated Cell Wall Biosynthesis and Cell Division in *S. Aureus*

Peptidoglycan synthesis starts in the cytoplasm with the conversion of fructose-6-P to UDP-N-acetyl-glucosamine (UDP-GlcNAc) by GlmS, GlmM, and GlmU enzymes. The final soluble precursor, UDP-MurNAc-pentapeptide (UDP-MurNAc) is produced by the sequential action of the MurA to MurF enzymes. Lipid I (undecaprenylphosphate-MurNAc-pentapeptide) is formed at the membrane by MraY, which transfers the soluble UDP- MurNAc-pentapeptide to undecaprenylphosphate (C55-P). MurG links UDP-GlcNAc to lipid I, producing lipid II (undecaprenylphosphate-GlcNAc-MurNAc-pentapeptide). Lipid II is further modified in *S. aureus*, by the addition of 5 glycine residues, catalyzed by FemXAB enzymes; such interpeptide bridges occur in most Gram-positive bacteria, but may vary in composition and length. Finally, lipid II is translocated across the membrane by an unknown mechanism, of which both SAV1754 and FtsW have been proposed. Extracellular peptidoglycan units are incorporated into the nascent peptidoglycan network through the activity of PBPs by transglycosylation (TG) and transpeptidation (TP) reactions.

knockdown of Group 1–5 genes in USA300. Further, the resulting gene set defines a genetic potentiation map of targets for which compound-specific inhibitors are predicted to synergize with β-lactams against hospital and community-acquired MRSA.

Synergistic Chemical-Chemical Interactions between β-Lactam Antibiotics and GlmS Inhibitor, Nva-FMDP

To investigate whether our genetic findings could be confirmed pharmacologically, we examined the growth inhibitory effects

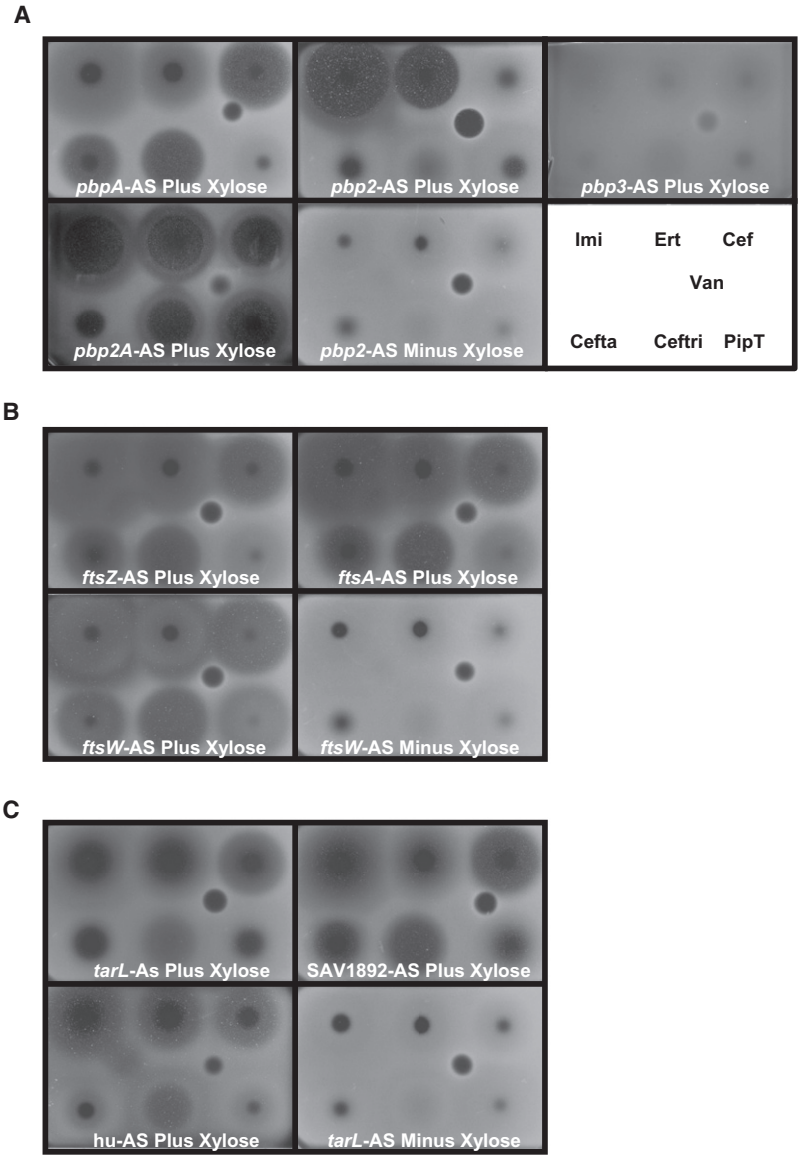


Figure 4. Representative Hypersusceptibility Phenotypes of Members of the Cell Wall Genetic Potentiation Map

(A) PBP hypersusceptibility of antisense bearing strains to *pbpA* (top left), *pbp2* (top right), *pbp3* (middle left), and *pbp2A* (middle right) under partial antisense induction (plus 50 mM xylose) versus the negative control, *pbp2*-AS (bottom left) assayed identically but without antisense induction (minus xylose). Antibiotics are spotted according to grid coordinates (bottom right).

(B) Cell division (Fts) hypersusceptibility of antisense bearing strains to *ftsZ* (top left), *ftsA* (top right), and *ftsW* (bottom left) under partial antisense induction (plus 50 mM xylose) versus the negative control, *ftsW*-AS assayed identically but without antisense induction (bottom right).

(C) Antisense bearing strains corresponding to *tarL* (top left), SAV1892 (top right), and hu (bottom left) under partial antisense induction (plus 50 mM xylose) versus the negative control, *tarL*-AS assayed identically but without antisense induction (bottom right).

and potential synergistic activity of Nva-FMDP (Chmara et al., 1998) in combination with distinct β-lactam antibiotic classes and clinically relevant *Staphylococci*. Nva-FMDP (whose bioactive pharmacophore is an analog of glutamine) is a competitive inhibitor of GlmS, the first committed step in peptidoglycan synthesis in *S. aureus* (Komatsuzawa et al., 2004). GlmS uses glutamine as an ammonia source to convert D-fructose-6-phosphate into D-glucosamine 6-phosphate, which is subsequently converted by GlmM and GlmU to UDP-N-acetylglucosamine, the fundamental amino sugar building block of peptidoglycan (van Heijenoort, 2001). Although Nva-FMDP displays potent whole cell activity against *Bacillus subtilis* (Chmara et al., 1998) and *S. epidermidis* (Figure S1), *S. aureus* growth is largely unaffected by the compound (Figure 6A; Figure S1). This likely reflects species-specific differences between permease and protease-specific import and activation mechanisms required for Nva-FMDP bioactivity (Chmara et al., 1998). Nva-FMDP does however strongly potentiate imipenem activity against MRSA COL (Figure 6A; Figure S1). Moreover, potentiation between Nva-FMDP and imipenem is fully suppressed by supplementing 20 mM *N*-acetylglucosamine (GlcNAc) into the medium, demonstrating the target-specific inhibitory effect of Nva-FMDP (Figure 6B). Further, modest depletion of GlmS by antisense induction markedly increases MRSA COL sensitivity to Nva-FMDP, recapitulates the chemical genetic hypersensitivity to imipenem, and enhances the potentiating effect of these agents in a GlcNAc-suppressible manner (Figures 6C and 6D). Analogous conclusions are drawn by targeting folate biosynthesis using *dfrA*-AS, trimethoprim, sulfamethoxazole, and thymidine supplementation to suppress inactivation of the pathway (Figure S1B and Supplemental Information).

Antibiotic synergy is routinely determined by standard checkerboard methodology using a 64-point dose matrix and is formally defined as a fractional inhibitory concentration index (FICI) ≤ 0.5, where the FICI is the sum of the fractional inhibitory concentrations (FIC) of each compound (Amsterdam, 2005). Accordingly, we quantified the observed potentiation between these agents against MRSA COL, USA300, and methicillin resistant *Staphylococcus epidermidis* (MRSE). Nva-FMDP and imipenem produce FICI values of 0.08 and 0.02 against MRSA COL and MRSE respectively, with up to 256-fold reduced levels of imipenem being required to inhibit growth when combined with Nva-FMDP (Figure 6E; Table S2). Although USA300 is intrinsically resistant to β-lactam antibiotics, its imipenem susceptibility is cell density dependent (this is referred to as an inoculum effect; see Sabath et al., 1975). Under such high inoculum conditions, significant synergy in combination with Nva-FMDP (FIC1 0.19) is also observed (Figure 6E; Table S2). Interestingly, Nva-FMDP mediated potentiation is not restricted to imipenem but extensively demonstrated among a broad set of β-lactam

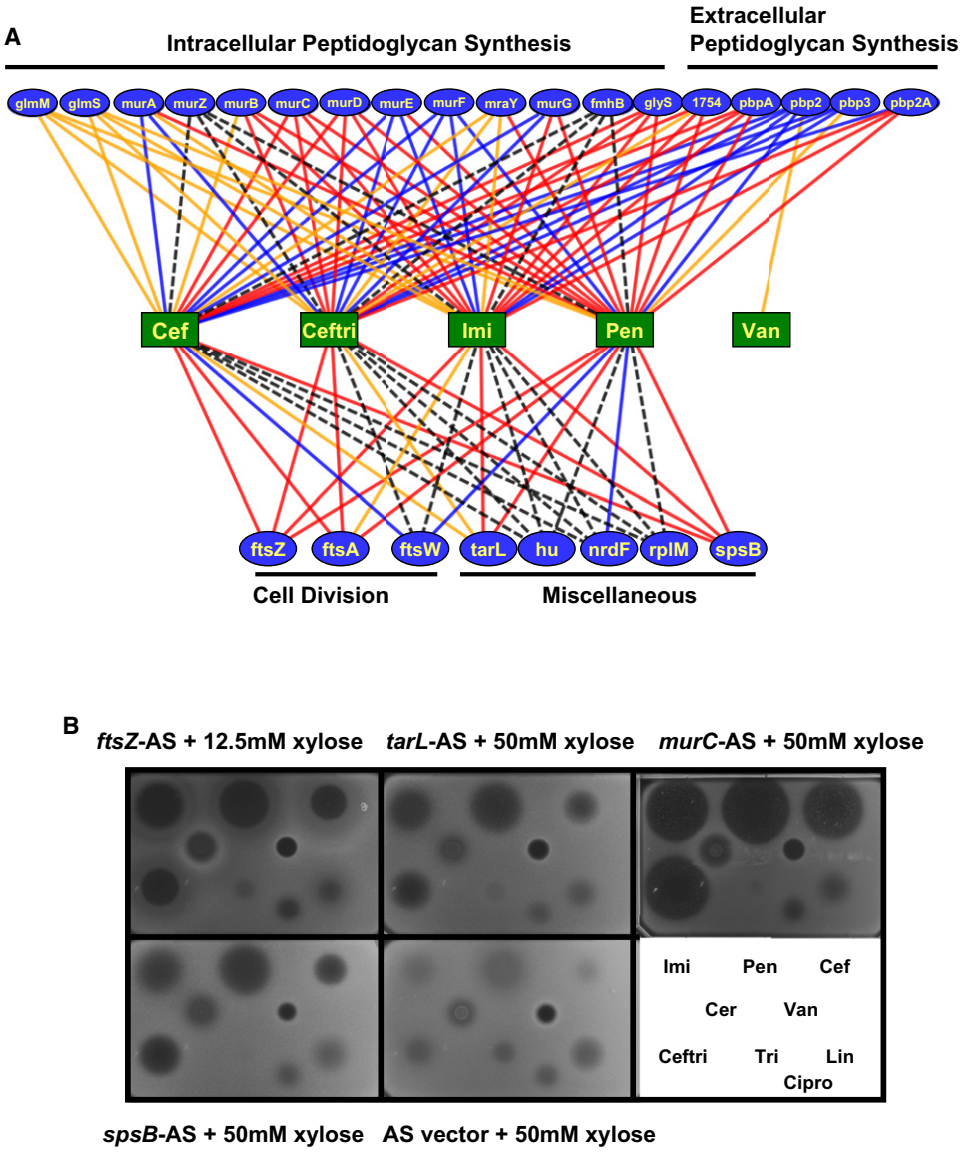


Figure 5. USA300 Genetic Potentiation Map of Cell Wall Antibiotics

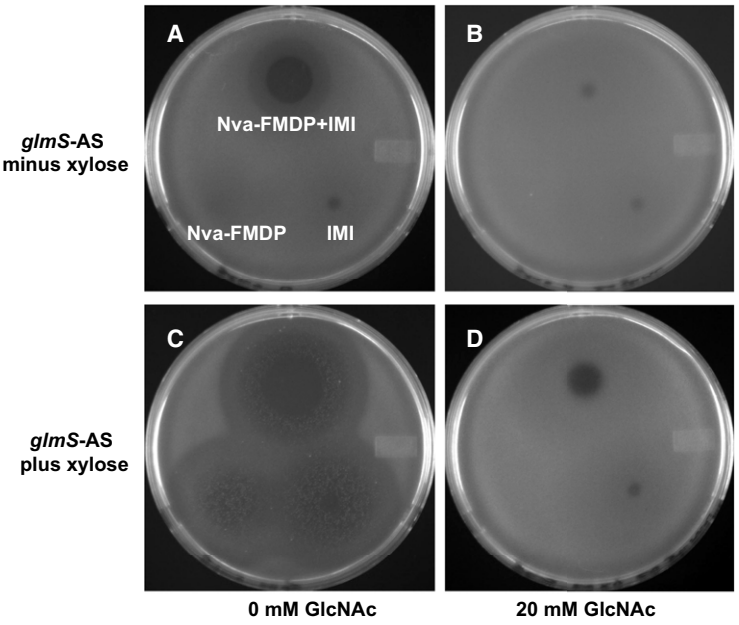
(A) Chemical genetic interactions are depicted as described in Figure 2.

(B) Antisense bearing strains corresponding to *ftsZ* (top left), *tarL* (top right), and *spsB* (bottom left) under partial antisense induction (plus 50 mM xylose) versus the negative control (USA300 maintaining the antisense vector without antisense interference fragment) assayed identically in the presence of optimized xylose concentrations (12.5–50 mM).

antibiotics tested, including additional cabapenems (ertapenem), cephalosporins (cefepime, ceftriaxone, and ceftazidime), and penicillin G (Figure S1). Therefore, these chemical-chemical interactions underscore a mechanism-based potentiation strategy to restore β-lactam antibiotic activity broadly against methicillin resistant *Staphylococci*, as originally predicted by our chemical genetic interaction map.

DISCUSSION

Chemical genetic interaction networks, albeit historically restricted to nonessential genes, provide important mechanistic insights into how model microbes tolerate antibiotic stress (Gomez and Neyfakh, 2006; Girgis et al., 2009; Barker et al., 2010; Costanzo et al., 2010). Here we describe an antisense interference-based genetic knock down strategy to identify essential genes that are required for the maintenance of β-lactam antibiotic resistance among clinical isolates of *S. aureus*. Beyond previously described auxiliary genes, multiple well characterized genes not previously demonstrated to participate in MRSA β-lactam susceptibility, including multiple genes involved in peptidoglycan and wall teichoic acid glycopolymer synthesis, protein synthesis, cell division, transcription, and signal transduction were identified to similarly and strongly reset susceptibility of MRSA to β-lactams in hospital and community-acquired MRSA clinical isolates. The extent of the



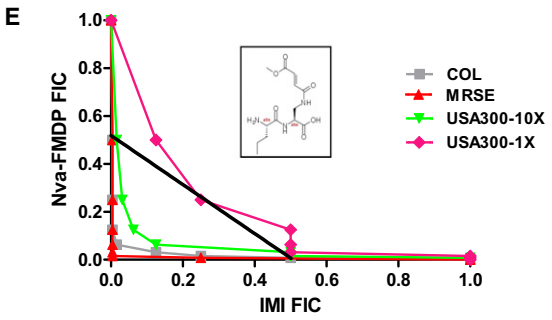


Figure 6. Synergistic Activity between Nva-FMDP and β-Lactam Antibiotics

(A) Nva-FMDP (256 μg) and imipenem (3 μg) spotted separately or co-spotted on COL seeded plate reveals potentiation between the two agents.

(B) Same as (A) but with 20 mM GlcNAc supplemented to the plate.

(C) COL *glmS*-AS strain under partial antisense induction (20 mM xylose) confers prominent hypersensitivity to Nva-FMDP and imipenem; enhanced potentiation is observed by co-spotting these agents.

(D) Same as (C) but with 20 mM GlcNAc supplemented to the plate.

(E) Nva-FMDP and imipenem checkerboard assays against COL, USA300, and MRSE. FIC of each antibiotic pair are plotted. Synergism is achieved with combinations of the two agents fully inhibiting growth with individual FIC values yielding a sum FICI of $\leq$0.5 (indicated by the black diagonal line). USA300 β-lactam susceptibility is inoculum dependent; accordingly checkerboard assays were performed under standard (1×) inoculum cell density and 10× normal cell density.

See also Figure S1 and Table S2.

β-lactam potentiating gene network we describe likely reflects our focus on screening essential genes through conditional expression, thereby overcoming the intrinsic difficulty of identifying such phenotypes among essential genes by previous transposon-based strategies (de Lencastre and Tomasz, 1994; de Lencastre et al., 1999; Berger-Bächi and Rohrer, 2002). Importantly, these results predict that small molecules that interdict gene products comprising the β-lactam genetic potentiation maps should synergize the effects of β-lactams against drug resistant *Staphylococci*. Accordingly, we provide pharmacological validation that the GlmS inhibitor Nva-FMDP is a remarkably effective synergist in combination with a broad set of β-lactams, restoring the efficacy of this antibiotic class well below their clinically defined drug resistance breakpoint concentrations against MRSA and MRSE clinical isolates. In addition, the interdicting compound does not need to fully abrogate the activity of its cognate target to be an effective synergist to β-lactams, as demonstrated by the modest knock down levels (IC_{10}-IC_{20}) using antisense interference against essential genes to hypersensitize MRSA to such antibiotics. Further, MRSA strains are viable in the presence of Nva-FMDP at concentrations that achieve strong β-lactam synergy (Figure 6A). This phenomenon may be extended to future studies to identify low potency compounds that only partially inactivate the target gene or possibly even nonessential genes but are still effective in restoring β-lactam susceptibility against MRSA or other bacterial pathogens (Tamae et al., 2008).

We postulate that as a consequence of the adapted mechanism of β-lactam resistance that MRSA has acquired, it is highly vulnerable to genetic potentiation of this drug class over non-β-lactam antibiotic drug classes. This vulnerability reflects a network of cellular processes that are indirectly required to maintain the residual activity of PBP2 transglycosidase and PBP2A transpeptidase function under β-lactam stress, and that if breached, dramatically exacerbate the effects of these antibiotics (Figure 3). For example, FtsZ is required for proper localization of PBP2 to the division site to mediate localized peptidoglycan synthesis, peptidoglycan cross linking, and septum formation (Pinho and Errington, 2003). Therefore, under conditions where PBP2 may be partially mislocalized from its normal site of action, it is reasonable to expect that the resulting MRSA strain would be hypersensitized to β-lactam antibiotics, as we demonstrate. Extending this view, as the Z ring is a multisubunit complex, depletion of other components (e.g., FtsA and FtsW) of the complex may phenocopy FtsZ β-lactam hypersensitivity. Pinho and colleagues have also demonstrated that normal PBP2 localization to the septum is achieved by binding to its peptidoglycan monomer substrate, which itself is synthesized at sites of nascent cell wall synthesis (Pinho and Errington, 2005). Consequently, depletion of peptidoglycan precursor synthesis by genetic knockdown of elements of this pathway as early as *glmS* could adversely affect PBP2 localization and confer β-lactam hypersensitivity of these MRSA strains. Localization of other PBPs is also affected in the context of specific genetic mutations. Recently, normal septum localization of PBP4 has been demonstrated to be dependent on TarO, the first committed enzymatic step required for the production of the cell wall glycopolymer, wall teichoic acid (Atilano et al., 2010). These results, combined with the recent demonstration that *tarO* deletion

mutants are hypersusceptible to β-lactams (Campbell et al., 2011), likely explain the observed hypersusceptibility phenotype of *tarL* to these agents. However, in addition to PBP mislocalization as a mechanism for elevated β-lactam susceptibility, alternative possibilities exist. These include the possible transcriptional repression of PBPs, as demonstrated by *murE* depletion resulting in reduced transcription of both *pbp2A* and *pbp2* (Gardete et al., 2004). Importantly, these PBP defects need not be mutually exclusive and in fact may be exacerbated by depletion of peptidoglycan polymer levels below those which are required for viability resulting from the combined effects of disrupting peptidoglycan synthesis by chemical and genetic means at multiple points within the pathway.

Interestingly, in MRSA COL, PBPs display unique hypersensitivities among distinct β-lactam antibiotic classes. For example, modest antisense induction targeting *pbp2*-AS strain results in marked hypersensitivity to carbapenems, whereas *pbpA*-AS strain is reciprocally hypersensitive to cephalosporins. Further, *pbp3*-AS strain lacks hypersensitivity to all β-lactams, as previously reported (Pinho et al., 2000) whereas *pbp2A*-AS strain is hypersensitive to all β-lactams tested. As *pbp2A* provides the molecular basis for MRSA resistance to β-lactams (Matsuhashi et al., 1986; Song et al., 1987; de Lencastre et al., 2007), its genetic depletion is expected to confer pan-hypersensitivity to these agents. However, the basis for the hypersensitivity profiles of the remaining PBPs is unclear. One possibility is that carbapenems preferentially inhibit PBP2 whereas cephalosporins possess greater affinity to the *pbpA* encoded PBP1, recapitulating that demonstrated for their *Pseudomonas aeruginosa* PBP orthologs (Moyá et al., 2010 and references therein). Alternatively, these phenotypes may manifest as a direct result of their distinct functional roles in *S. aureus* peptidoglycan biosynthesis, in combination with genetic interactions between individual PBP members as well as their distinct binding affinities to different β-lactams.

Genetic potentiation maps also highlight an important limitation of target based screening approaches that rely on genetic knockdown of a target to hypersensitize cells to cognate inhibitors. Indeed, this approach can be successful, as demonstrated by the prominent target-specific hypersensitivity of glmS-AS to Nva-FMDP (Figures 6C and 6D) as well as the discovery of platensimycin (Wang et al., 2006). However, our results highlight an alternative outcome; one in which chemical hypersensitivity need not directly reflect the chemical inhibition of the depleted target, but instead hypersensitivity resulting from genetic depletion of a member of the genetic interaction network of the target that is chemically inhibited. The broad β-lactam hypersensitivity as demonstrated among peptidoglycan, cell division, and other pathways indirectly affecting PBP function underscores this potential complexity. Further, the hypersensitivity phenotype of a target to its cognate inhibitor (e.g., SAV1754-DMPI, CDFI, or Compound D) may also be markedly less pronounced than the target's hypersensitivity to compounds that do not directly target it, such as β-lactams (Figures S2 and S3). Similarly, PBP2A displays pan-hypersusceptibility to all β-lactams tested, yet the protein has poor affinity and is not inhibited by these agents. Therefore, chemical hypersensitivity of a strain genetically depleted for a particular target is, on its own, insufficient to surmise compound mode of action and requires independent genetic and/or biochemical confirmation. Despite this potential complexity, antisense knockdown strains with particular antibiotic susceptibilities serve as potential whole cell screening assays for inhibitors of particular pathways comprising the genetic interaction network of a given target (see Supplemental Information for details).

Recently, an elegant genetic proof-of-concept of the achievable efficacy that combination agents could provide in treating β-lactam resistant *S. aureus* infections has been demonstrated (Jo et al., 2011). Deletion of the nonessential *vraSR* two component signal transduction pathway restores β-lactam susceptibility to a broad collection of MRSA isolates (Kuroda et al., 2003; Gardete et al., 2006; Jo et al., 2011). Jo et al. (2011) extend these results and demonstrate that deletion of the *vraSR* operon also restored the efficacy of oxacillin in murine models of MRSA USA300 pneumonia and skin infection (Kalan and Wright, 2011). β-lactam genetic potentiation maps therefore provide an important first step to discovering target-specific inhibitors that, if coadministered, coformulated, or chemically linked to existing β-lactams, provide a rational strategy to restoring the efficacy of this important class of antibiotic drugs against drug resistant *Staphylococci*.

SIGNIFICANCE

Antibiotic drug resistance, as has emerged among hospital and community acquired methicillin resistant *Staphylococcus aureus* (MRSA), has dramatically eroded the efficacy of current therapeutic agents and created an urgent need for new treatment options. We describe an antisense interference strategy to modulate essential gene function in this important bacterial pathogen and identify genes required for MRSA resistance to β-lactams. In total, 245 essential genes are screened under partially repressing conditions in the hospital-acquired MRSA isolate COL for altered susceptibility to 17 clinically relevant antibiotics. We describe an extensive chemical genetic interaction network of β-lactam hypersensitivity determinants that contribute to β-lactam resistance of MRSA. Members of this network include genes known or predicted to be principally involved in various aspects of cell wall and cell division processes, including peptidoglycan synthesis (*glm*, *mur*, *pbp*), cell wall signaling (SAV1220), cell division (*fts*), and genes of unknown function (SAV1892). Further, we demonstrate the requirement of this genetic potentiation network for β-lactam resistance of the community-acquired MRSA epidemic strain, USA300. Recapitulating these genetic findings, a small molecule inhibitor of the earliest step in peptidoglycan biosynthesis (achieved using the GlmS inhibitor, Nva-FMDP), dramatically potentiates the activity of several β-lactam antibiotics against these MRSA strains as well as methicillin resistant *Staphylococcus epidermidis*. In so doing, we provide a rational framework for developing inhibitors of targets within this chemical genetic interaction network, which if paired with existing β-lactam antibiotics would display synergistic antibacterial activity against methicillin resistant *Staphylococci* and offer a combination agent strategy to restore the efficacy of this important antibiotic drug class. More broadly, this approach is generally

applicable to other microbial pathogens and antibiotics as well as cancer cell lines and anticancer agents in which synergistic agents for combination therapy are sought.

EXPERIMENTAL PROCEDURES

S. Aureus Strains, Antisense Interference Plasmids, and Synergy Studies

All studies utilize MRSA COL (MB 5393), USA300 (MB 6256) or MRSE strain MB6255. A detailed description of all antisense interference fragments, including DNA sequence, BLAST scores of best *S. aureus* match, and gene nomenclature in MSSA strain RN4220, MRSA COL, and USA300 is provided (Table S3). COL and USA300 were transformed with antisense interference plasmids or vector control as previously described (Huber et al., 2009). Assay plates were prepared by seeding 10^7 cells/ml of each culture into 48°C cooled LB Miller agar containing 34 μg/ml chloramphenicol, and 0, 25, 50, 100, or 200 mM xylose. Agar plates were allowed to set and then spotted with 10 μl of each drug and incubated at 37°C with humidity for 18 hr. Qualitative scores of all chemical genetic interactions summarized in Figures 2, 3, 4, and 5 are provided (Table S1). The qualitative hypersensitivity scores are based on visual inspection of zone of inhibition of xylosed induced plate versus uninduced plate. A score of (0) is given when no differential zone sizes are observed between the plates, and a score of 1 or 2 is given when differential zone sizes are at least 2-fold greater when compared to the uninduced plate with a score of 3 given zone differential >4-fold. Plate photos of antibiotic zone of inhibition for all confirmed chemical genetic interactions in MRSA COL and USA300, including relevant controls, is provided as searchable pdfs (Figures S3 and S4). The checkerboard technique was used to quantify synergy between antibiotic agents (Amsterdam, 2005). FICI values were determined by adding the FIC value of each compound required to achieve a MIC when paired with the second agent. As Nva-FMDP is inactive against COL, Nva-FMDP FIC values are based on the assumption that its MIC would be two times the highest concentration (32.8 mg/ml) used in the checkerboard assay.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at doi:10.1016/j.chembiol.2011.08.015.

ACKNOWLEDGMENTS

We thank Howard Bussey, Juliana Malinverni, Huseyin Mehmet, and Malcolm Whiteway for critical reading of the manuscript. We also thank Tanja Schneider for constructing Figure 3. All authors are present or past employees of Merck as stated in the affiliations and potentially own stock and/or hold stock options in the company.

Received: June 17, 2011
Revised: August 19, 2011
Accepted: August 22, 2011
Published: November 22, 2011

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