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Chemical Genetic Identification of Peptidoglycan Inhibitors Potentiating Carbapenem Activity against Methicillin-Resistant *Staphylococcus aureus*

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SUMMARY

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial and community-acquired pathogen for which few existing antibiotics are efficacious. Here we describe two structurally related synthetic compounds that potentiate β-lactam activity against MRSA. Genetic studies indicate that these agents target SAV1754 based on the following observations: (i) it has a unique chemical hypersensitivity profile, (ii) overexpression or point mutations are sufficient to confer resistance, and (iii) genetic inactivation phenocopies the potentiating effect of these agents in combination with β-lactams. Further, we demonstrate these agents inhibit peptidoglycan synthesis. Because SAV1754 is essential for growth and structurally related to the recently reported peptidoglycan flippase of *Escherichia coli*, we speculate it performs an analogous function in *S. aureus*. These results suggest that SAV1754 inhibitors might possess therapeutic potential alone, or in combination with β-lactams to restore MRSA efficacy.

INTRODUCTION

Staphylococcus aureus is a major human pathogen causing both hospital and community-acquired infections. Successive acquisition of resistance to most classes of antibiotics has seriously limited treatment options. In particular, methicillin-resistant *S. aureus* (MRSA), which is highly cross-resistant to essentially all other β-lactam antibiotics in this class, constitutes over 50% of all clinical isolates identified in U.S. hospitals during a surveillance period spanning 1998–2005 (Styers et al., 2006). Moreover, the number of serious infections caused by MRSA alone in the United States throughout 2005 approached nearly 95,000 cases, of which almost 19,000 related fatalities were estimated, thus exceeding the U.S. death toll attributed to HIV/AIDS in the same year (Klevens et al., 2007; Bancroft, 2007).

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 with 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 assemble peptidoglycan precurors and adequately crosslink these polymers within the cell wall (Pinho et al., 2001a; Scheffers and Pinho, 2005).

PBP2A and PBP2 represent important antibiotic targets that might be exploited to develop new efficacious agents against MRSA (Pinho et al., 2001b; Bush et al., 2007; Projan and Bradford, 2007). However, a number of auxiliary genes have also been identified by transposon-based screens, which, if inactivated, also dramatically increase MRSA susceptibility to β-lactams (Berger-Bächi et al., 1992; de Lencastre and Tomasz, 1994). Interestingly, many of these genes that are required for the maximal expression of resistance by PBP2A are also involved in peptidoglycan precursor biosynthesis and turnover (Berger-Bächi and Rohrer, 2002; de Lencastre et al., 1999; Sobral et al., 2003; Gardete et al., 2004). These and other studies (Pinho and Errington, 2003; Miller et al., 2004; Memmi et al., 2008; Sieradzki et al., 2008) emphasize the diversity of achievable points of genetic potentiation with β-lactams that could be mimicked by cognate inhibitors. Therefore, an alternative approach to developing MRSA active agents would involve identifying inhibitors that target these factors, which in combination with a β-lactam, would restore the activity of the latter.

Conceptually, target-specific inhibitors of β-lactam potentiating determinants should phenocopy their genetic inactivation. Moreover, provided such inhibitors are target specific, they serve as chemical probes to identify essential factors that might be refractory to transposon insertional inactivation screens. To identify compounds that synergize in combination with a β-lactam, a high-throughput screen (HTS) against our chemical library was performed using the MRSA clinical isolate, COL, grown in the presence of a sub-minimal inhibitory concentration (MIC) of ertapenem. Here we describe a structurally related class of inhibitors that synergize with the queried β-lactam and

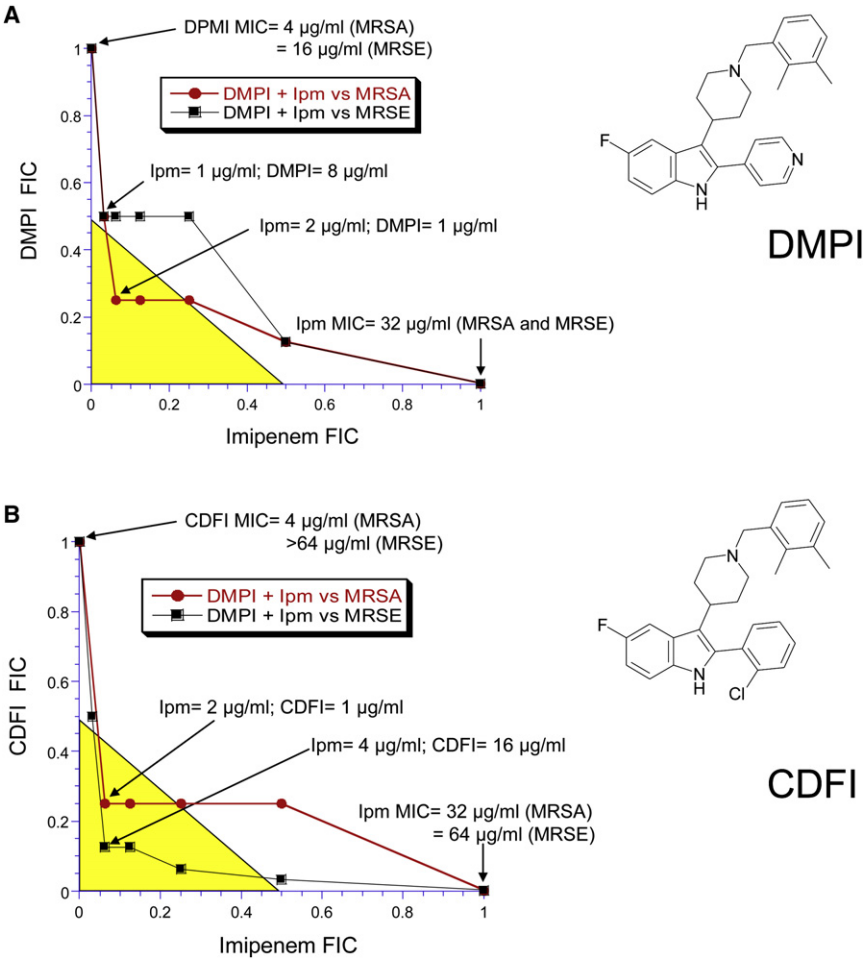


Figure 1. DMPI and CDFI Chemical Structures and Analysis of Synergy in Combination with Imipenem

Synergy of DMPI (A) and CDFI (B) in combination with imipenem were tested against MRSA and MRSE clinical isolates, COL and CLB26329, respectively. Drug concentrations and fraction inhibitory concentrations (FIC) used to evaluate synergism are indicated. Note synergism is achieved by the combination of the two agents fully inhibiting growth provided their individual drug concentrations sum to a FICI value $\leq$ 0.5 (indicated by the diagonal line).

potentiate its activity against MRSA COL. As predicted by mechanism-of-action (MOA) profiling (Donald et al., 2009 [this issue of *Chemistry & Biology*]) and confirmed by resistance mapping, these agents target the uncharacterized gene SAV1754. In this study, we demonstrate that SAV1754 is an essential gene and show that its inactivation by chemical or genetic means results in cell wall defects, including quantitative defects in peptidoglycan synthesis and aberrant cell wall peptidoglycan staining. In addition, genetic inactivation of SAV1754 by antisense interference dramatically restores COL susceptibility to β-lactams, similar to that achieved by antisense interference of *pbp2*. Based on SAV1754 phenotypes and its structural similarity to the recently described *E. coli* protein, MurJ (Ruiz, 2008; Inoue et al., 2008), we propose SAV1754 might function similarly as a peptidoglycan flippase required to translocate lipid-linked peptidoglycan precursors to nascent sites of cell wall synthesis.

RESULTS

Characterization of an Indole Series of Small Molecules that Potentiate the Effects of Carbapenem Antibiotics

To identify chemical entities that restore the activity of the carbapenem class of β-lactams against MRSA, we performed an HTS of our synthetic chemical library (8 μM final concentration) against the MRSA clinical isolate, COL, grown in the presence of a sub-MIC (2 μg/ml) of ertapenem. Here we characterize two structurally-related compounds identified that potentiate the effects of carbapenems; 3-{1-[(2,3-Dimethylphenyl)methyl]piperidin-4-yl}-1-methyl-2-pyridin-4-yl-1*H*-indole (DMPI) and 2-(2-Chlorophenyl)-3-[1-(2,3-dimethylbenzyl)piperidin-4-yl]-5-fluoro-1*H*-indole (CDFI) (Figure 1). To confirm DMPI and CDFI potentiate the activity of carbapenems, their synergistic effects in combination with imipenem were tested. DMPI and CDFI both displayed strong synergy in combination with imipenem against COL; whereas 32 μg/ml imipenem was required as a single agent to achieve a minimum inhibitory concentration (MIC), as little as 2 μg/ml imipenem in combination with 1 μg/ml of either DMPI or CDFI was sufficient to inhibit COL (Figure 1; see Table S1 available online). Indeed such synergistic effects were observed over multiple imipenem concentrations tested. CDFI also displayed synergy in combination with imipenem against the methicillin resistant *Staphylococcus epidermidis* (MRSE) clinical isolate CLB26329 across multiple imipenem concentrations, despite MRSE displaying reduced susceptibility to either agent alone. Further, CDFI displayed marked potentiation of imipenem against methicillin-resistant coagulase negative *Staphylococci* (MRCNS) as well as the community-acquired MRSA strain, USA300 (data not shown). Interestingly, no synergism between any of the above agents was observed against methicillin-sensitive *S. aureus* (MSSA) (Table S1), suggesting that their synergism is specific in the context of the mechanism of β-lactam resistance among these bacteria.

Antibacterial activity of DMPI and CDFI was assessed against a panel of Gram-positive and Gram-negative bacteria using standard CLSI protocols (Table S2). DMPI displays comparable activity against MSSA and MRSA (MIC = 16 μg/ml) but weak growth inhibitory activity versus other pathogenic bacteria tested. CDFI displays significantly improved activity against MSSA and MRSA (MIC = 2 μg/ml) as well as improved spectrum including *Bacillus subtilis* (MIC = 4 μg/ml), *Streptococcus pneumoniae* (MIC = 4 μg/ml), and *Enterococcus faecium* (MIC80 = 4 μg/ml). Although both compounds lack activity against all

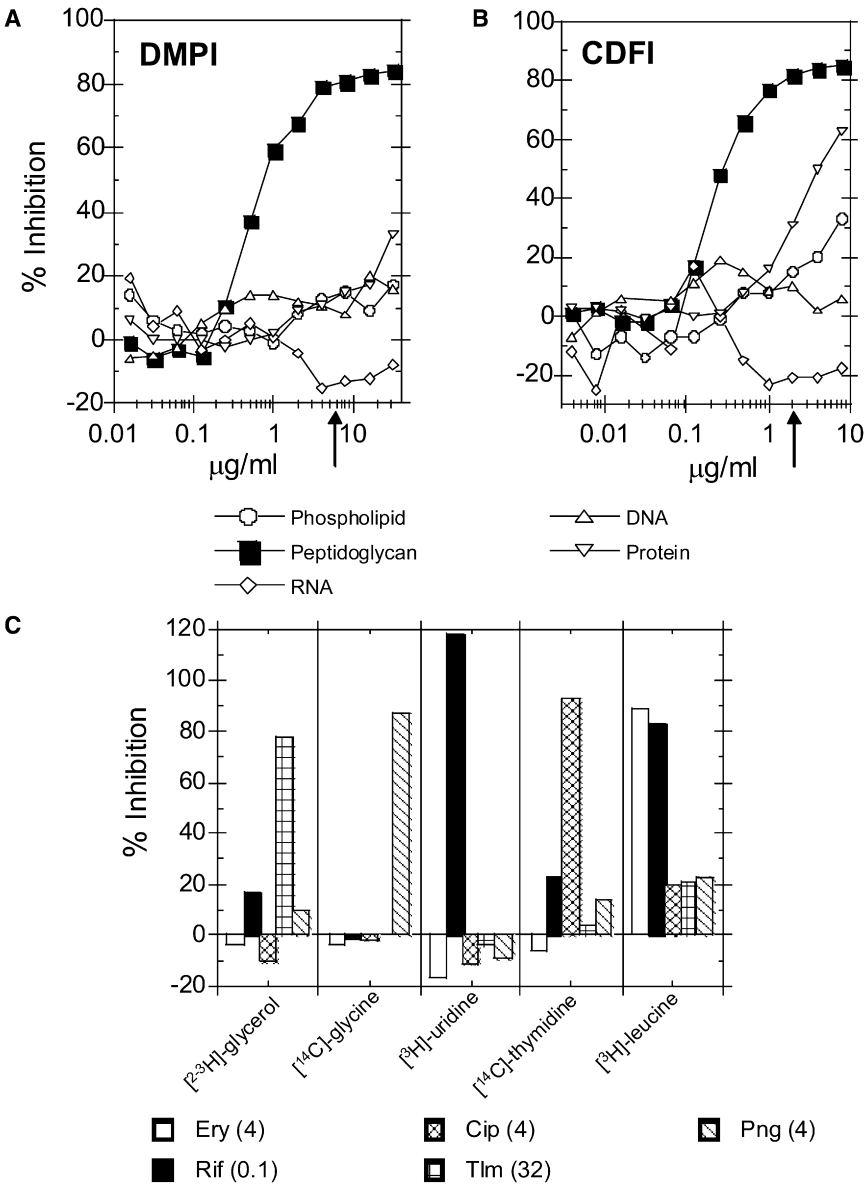


Figure 2. Inhibitory Effects of DMPI and CDFI on Macromolecular Synthesis

(A and B) DMPI (A) and CDFI (B) inhibitory effects across the indicated drug concentrations were determined by measuring incorporation of radiolabeled precursors of either DNA (^{14}C-thymidine), RNA (^{3}H-uridine), phospholipid ($^{2\text{-}3}$H-glycerol), protein (^{3}H-leucine), or cell wall (^{14}C-glycine) synthesis in *S. aureus* strain EP167. Arrows indicate MIC for each compound.

(C) Control compounds for inhibiting synthesis of DNA (Ciprofloxacin; Cip), RNA (Rifampicin; Rif), phospholipid (thiolactomycin; Tlm), protein (erythromycin; Ery), and cell wall (penicillin G; Png) are shown at the single sub MIC concentration (μg/ml) indicated in brackets. Note that protein synthesis is inhibited by both erythromycin and rifampicin control drug treatments, as expected.

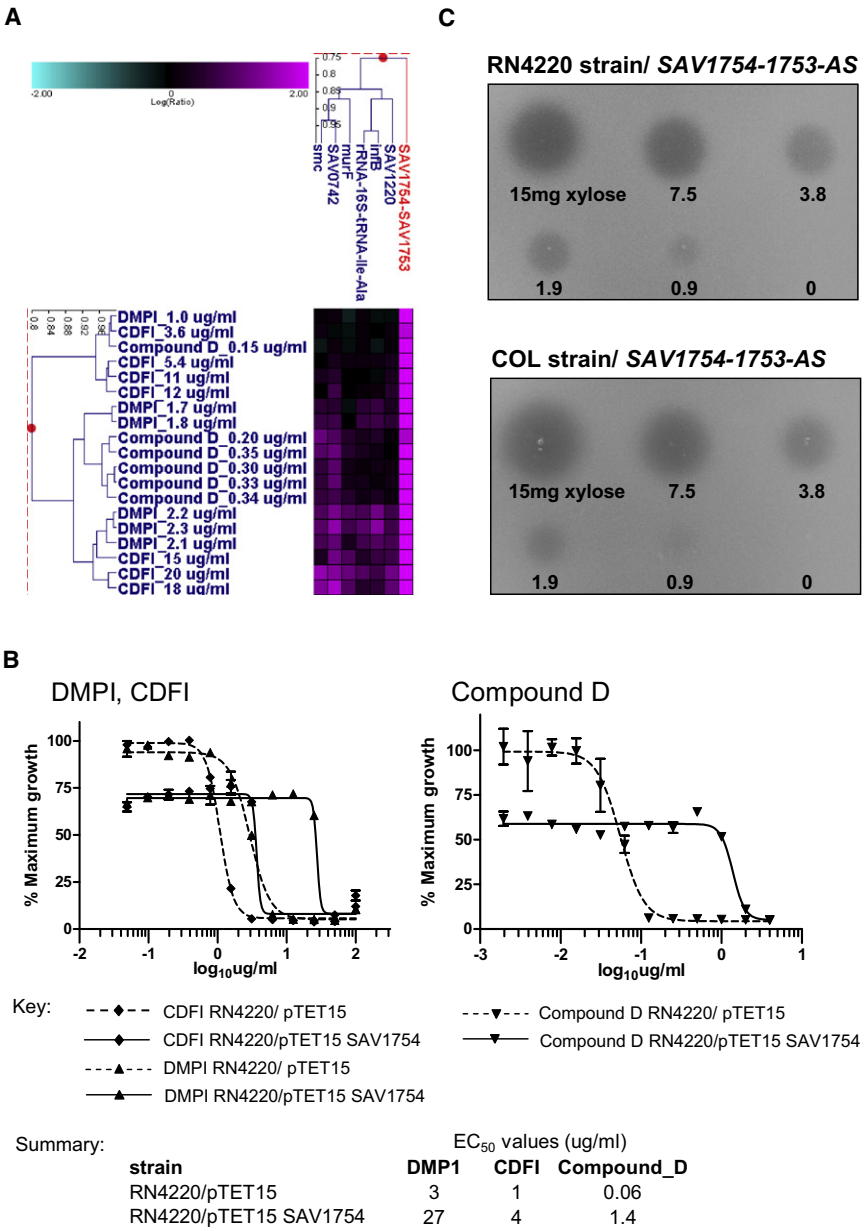
Gram-negative bacteria tested (MIC > 64 μg/ml for *H. influenza* and *Escherichia coli*), this might reflect their inability to penetrate the outer membrane and/or efficient efflux from the cell. Consistent with this possibility, measurable growth inhibitory activity of DMPI and CDFI (MICs were 32 μg/ml and 4 μg/ml, respectively) were obtained against a permeablized *E. coli* strain, MB5746 (Onishi et al., 1996), which is defective for TolC-mediated efflux and has the lpxC^{101} mutation which impairs lipopolysaccharide biosynthesis and increases permeability of the outer membrane.

Macromolecular Labeling Studies Demonstrate that Imipenem Potentiators Affect Cell Wall Synthesis

The effects of DMPI and CDFI on macromolecular synthesis pathways were determined in MSSA *S. aureus* strain EP167 by measuring the relative incorporation of radiolabeled precursors of DNA, RNA, protein, phospholipid, and cell wall synthesis across a range of concentrations. Treatment with either compound at multiple sub-MIC levels resulted in marked inhibition of peptidoglycan synthesis (Figure 2). Inhibition was selective for cell wall synthesis; all other macromolecular pathways assayed were either unaffected or only modestly inhibited (e.g., CDFI's effect on protein synthesis) at concentrations approaching their MIC's. Thus the primary growth inhibitory effect of DMPI and CDFI relate to perturbing cell wall biosynthesis.

Antisense Interference Profiling Predicts Imipenem Potentiators Target SAV1754

To corroborate that DMPI and CDFI act on cell wall biosynthesis, and to potentially identify the step in the peptidoglycan biosynthetic pathway at which these compounds might act, we applied an antisense-induced strain sensitivity (AISS) profiling assay described in the accompanying paper by Donald et al. published in this issue of *Chemistry & Biology* (Donald et al., 2009). Briefly, AISS profiling relies on gene-specific antisense interference transcripts under the control of a xylose-inducible promoter to reduce expression of the cognate target, thereby sensitizing the strain to compounds that act through the partially depleted gene product (Forsyth et al., 2002). By performing this analysis in parallel with a compendium of 245 essential genes across a range of partially inhibitory compound doses, target-specific chemical hypersensitivity phenotypes might be identified that predict the MOA of a compound of interest, including the drug target and/or affected cellular pathway. Indeed, in the accompanying report by Phillips and colleagues, AISS profiling has been successfully applied to reflect the known MOA among a spectrum of known antibiotics affecting DNA, RNA, fatty acid, protein synthesis, and cell wall biosynthesis (Donald et al., 2009).



Summary:

strain	EC_{50} values (ug/ml) DMP1	CDFI	Compound_D
RN4220/pTET15	3	1	0.06
RN4220/pTET15 SAV1754	27	4	1.4

Figure 3. DMPI and CDFI Mechanism of Action Studies and SAV1754 Target Validation

(A) Two-dimensional cluster of 19 experiments and seven antisense strains displaying significant hypersensitivity for DMPI, CDFI, and/or compound D. Strain depletions are depicted in magenta, strain resistances in cyan. The thresholds for including strains in the cluster analysis are > 5-fold depletion, p value $\leq$ 0.01 in three or more experiments. The compound name is followed by the treatment concentration. All three compounds share similar strain depletion profiles at all compound concentrations, with the most significantly depleted strain identified expressing an antisense interference fragment complementary to 131 nucleotides of the 3′ end of the SAV1754 transcript as well as 30 nucleotides of the adjacent 5′ end of SAV1753 (shown in red).

(B) Episomal expression of SAV1754 confers resistance to inhibitors. Dose response for inhibition of RN4220 by DMPI, CDFI, and compound D. Compared with the control strain (plasmid pTETl5, dotted lines), EC_{50} values with the SAV1754 expression vector strain (plasmid pTET15 SAV1754, solid lines) are increased approximately 10-fold for DMPI and 4-fold for CDFI and 20-fold for compound D. Cells were grown for 10 hr in LB media supplemented with chloramphenicol (34 μg/ml) and anhydrotetracycline inducer (20 ng/ml). Dose-response curves were fitted using a four parameter logistic equation (GraphPad Prism Software) with error bars representing the standard error of the mean (SEM) of duplicate samples.

(C) SAV1754 is essential for *S. aureus* growth. RN4220 and COL strains maintaining the SAV1754 antisense plasmid were seeded in LB agar and xylose induction of the SAV1754 antisense achieved by spotting xylose as indicated. Note prominent and xylose-dependent zones of growth inhibition specific to SAV1754 antisense induction. No growth inhibition was observed in parallel spottings with RN4220 or COL strains harboring the empty antisense vector pEPSA5 (not shown).

AISS profiles in response to DMPI and CDFI were unique and highly selective, with a single hypersensitive strain (SAV1754-AS) identified as prominently and reproducibly depleted from the population (Figure 3A). Based on *S. aureus* Mu50 genome sequence annotation, antisense RNA expressed by SAV1754-AS is complementary to 131 nucleotides of the 3′ end of the SAV1754 gene transcript as well as 30 nucleotides of the 5′ end of the adjacent SAV1753 gene transcript. The open reading frame (ORF) of these genes overlap with the terminal nucleotide of SAV1754 (an adenosine) comprising the first nucleotide of the start codon of the distal SAV1753 gene.

To explore the significance of the SAV1754-AS strain sensitivity to DMPI and CDFI, we tested whether episomal SAV1754 overexpression confers resistance to these agents. Growth inhibition was compared between RN4220 maintaining full-length SAV1754 placed under the control of a tetracycline-inducible promoter (pTET15-SAV1754) versus the vector alone (pTET15). Overexpression of SAV1754 under inducing conditions shifted the EC_{50} value of both DMPI and CDFI approximately 10-fold and 4-fold higher than observed for the vector control strain (Figure 3B). Further, genetic depletion of SAV1754 by xylose-induced antisense interference abolished growth in a xylose-dependent manner, confirming that SAV1754 is essential in *S. aureus* RN4220 (Figure 3C) as previously predicted (Mott et al., 2008). SAV1754 gene essentiality was similarly demonstrated in MRSA where, following transformation of the SAV1754-AS plasmid into COL, xylose-dependent growth inhibition was also observed (Figure 3C). These results suggest that the growth inhibitory effects of DMPI and CDFI might be targeting the product of the essential gene, SAV1754.

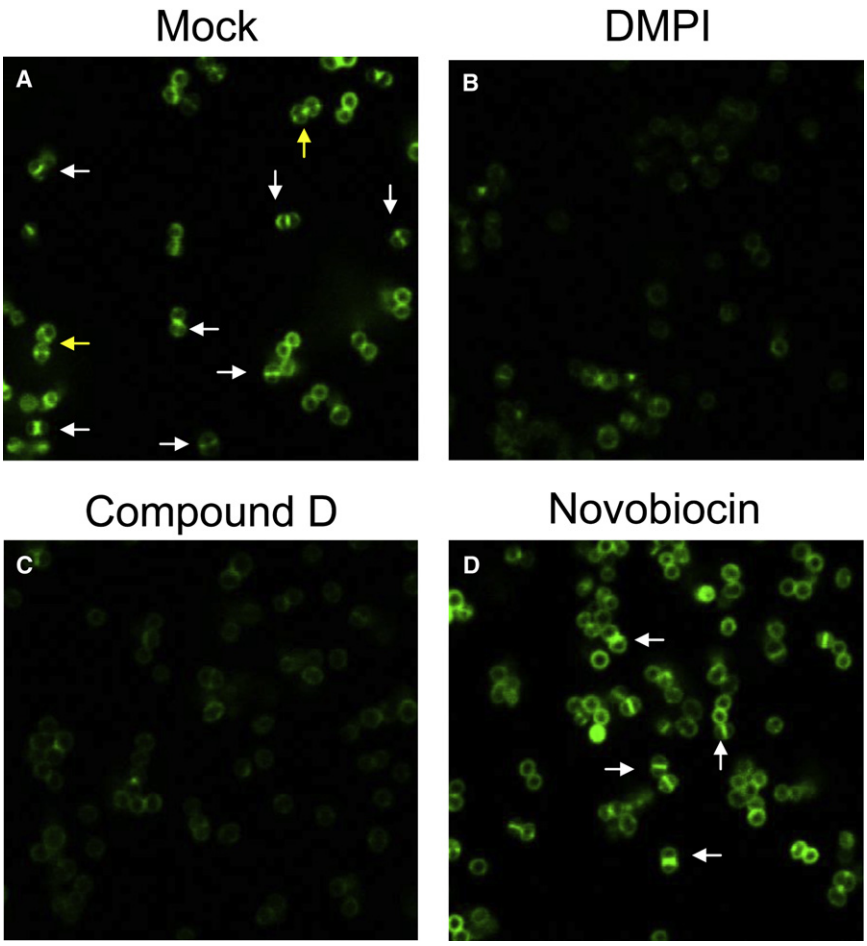


Figure 4. Cell Wall Staining of Nascent Peptidoglycan and Cell Division Septa following Drug Treatment

Fluorescence images of Van-FL labeled *S. aureus* RN4220 cells grown with a transient incubation of excess D-serine (Pinho and Errington, 2003) and subsequently grown 30 min in TSB medium at 30°C in the presence of 2% DMSO as a mock treatment (A) or treated at 2X MIC with DMPI (B), compound D (C), or the control compound, novobiocin (D). As highlighted in (A), arrows indicate different stages of septum formation; cells with two fluorescent spots (yellow arrows) correspond to early septum ring formation, and a solid fluorescent bar across the mid plane of the cell (white arrows) corresponds to a complete septum. Note that Van-FL staining of nascent peptidoglycan localized to septa is markedly reduced by DMPI and compound D, whereas strong Van-FL labeling of peptidoglycan and septa are detected among novobiocin-treated cells.

SAV1754 is predicted to encode a 553 amino acid protein containing 14 transmembrane helices. However, its functional role is unknown. Genomic BLAST searches against NCBI microbe proteome databases (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) reveal that SAV1754 orthologs are restricted to Gram-positive bacteria, including *S. epidermidis* (0.0), *Bacillus cereus* (7e-89), *B. anthracis* (4e-87), *B. subtilis* (1e-85), *E. faecalis* (1e-49), *S. pneumoniae* (3e-40), and *Clostridium difficile* (2e-35) (Figure S1A). No close homolog to SAV1754 is present in human genome databases. Interestingly, a recent report describes SAV1754 (also known as SA1575 based on *S. aureus* strain N315 genome annotation) as the target of a novel class of antibacterial anthranilic acids that also inhibit cell wall biosynthesis and their resistance maps to the SAV1754 locus (Mott et al., 2008). Evaluating the most potent member of this class (compound D) by AISS profiling corroborated its selective inhibition of SAV1754, because the SAV1754-AS strain was uniquely identified and markedly hypersensitive to compound D across all drug concentrations tested (Figure 3A, and Donald et al., 2009). As expected, overexpression of SAV1754 also conferred resistance to compound D (Figure 3B), as previously reported by Mott and colleagues. Further, macromolecular labeling experiments confirm its primary effect on cell wall synthesis (Figure S2; Mott et al., 2008), and like DMPI and CDFI, compound D prominently synergized the activity of imipenem against COL (Figure S3). Thus, DMPI, CDFI, and compound D likely share a common drug target whose cognate inhibitors effect cell wall biosynthesis and synergize with carbapenems.

SAV1754 Inhibitors Disrupt Peptidoglycan Deposition into the Cell Wall

To test for additional cell wall inhibitory effects caused by DMPI, CDFI, or compound D, we utilized a previously described fluorescein-conjugated vancomycin (Van-FL) staining procedure (Pinho and Errington, 2003) to examine peptidoglycan deposition following compound treatment of the MSSA strain RN4220. As expected, fluorescent staining of control treated cells (n = 88 cells evaluated) identified nascent peptidoglycan largely localized to the cell division site and concentrated along the division septum (n = 48 or 55% of cells scored), with only minor staining throughout the rest of the cell wall (Figure 4). However, cells treated with DMPI at 2XMIC for 50 min resulted in a striking reduction in overall fluorescence of new cell wall material and only 7% of cells displaying clear septum straining. CDFI-treated cells were similarly affected (data not shown). Further, compound-D-treated cells resulted in a significant reduction in overall Van-FL staining of new wall material, with weak staining of septa observed among only 4% of cells (n = 137) scored. Novobiocin treatment under identical conditions served as a negative control; strong Van-FL staining of nascent peptidoglycan was localized to the septum among 30% of 124 cells analyzed. Therefore, Van-FL staining of cells treated with these putative SAV1754 inhibitors supports their proposed primary effect on cell wall biosynthesis.

Characterization of DMPI-Resistant Mutants

To corroborate the MOA of these compounds as SAV1754 inhibitors and imipenem potentiators, DMPI was chosen to select for resistance mutants in *S. aureus* strain COL using 2 μg/ml DMPI in

Table 1. Susceptibility of SAV1754 Mutants to Compound/Imipenem Combinations Compared with the Parental COL Strain

Compound ID	Additive (μg/ml)	Value	COL	COL-1	COL-2	COL-3
Imipenem	None	MIC[a]	16	16	16	16
DPMI	None	MIC	4	128	128	128
DPMI	Imipenem (4)	MIC	0.13	32	32	64
DPMI	Imipenem (4)	FICI[b]	0.28	0.5	0.5	0.75
CDFI	None	MIC	2	128	128	128
CDFI	Imipenem (4)	MIC	0.25	64	64	64
CDFI	Imipenem (4)	FICI	0.38	0.75	0.75	0.75
Compound D	None	MIC	0.13	0.13	0.06	0.13
Compound D	Imipenem (4)	MIC	0.008	0.031	0.016	0.031
Compound D	Imipenem (4)	FICI	0.31	0.50	0.50	0.50

[a] MIC values are defined as the concentration of test compound that inhibits bacterial growth and were determined using standard CLSI protocols.
[b] FICI (fraction inhibitory concentration index) as a measure of synergy; FICI values $\leq$ 0.5 defining synergy between the two agents.

combination with 4 μg/ml imipenem. After 24 hr growth, spontaneous resistance occurred at a frequency of $\sim 1 \times 10^{-9}$, suggesting a single mutation is likely responsible for the resistance phenotype. Three independent resistant mutants, COL-1, COL-2, and COL-3, were evaluated for their susceptibility to DMPI alone or in combination with a sub-inhibitory concentration of imipenem at 4 μg/ml. Each of the isolated mutants was resistant to DMPI as a single agent, with MICs > 64 μg/ml and > 16-fold relative the parental COL strain (Table 1). Further, COL-1, COL-2, and COL-3 required 250–500-fold higher DMPI levels than the parental COL strain to achieve the desired MIC in combination with imipenem at 4 μg/ml. Because the resistant isolates lack an altered susceptibility to imipenem as a single agent, we conclude that the mutants are resistant to DMPI, not imipenem. In addition, the synergistic effects of DMPI and imipenem were not observed with the COL1-2, COL-2, and COL-3 mutants (Figure S4 and Table S3). Similarly, each of the three DMPI-resistant isolates displayed cross-resistance to the structural analog, CDFI, and suppressed the synergistic activity between CDFI and imipenem. No cross-resistance to compound D was detected; only 2–4-fold differences in compound D MIC values were observed among the DMPI-resistant strains (Table 1; Figure S3).

To assess whether these DMPI resistant mutants map to mutations within SAV1754 or the adjacent ORF, SAV1753, (which might also be potentially affected by the SAV1754 antisense transcript) as predicted by AISS profiling, both genes were fully sequenced from COL-1, COL-2, and COL-3 isolates. In fact, all three strains contain a distinct single missense mutation in the SAV1754 ORF compared to the COL parental strain (Figure S5) and no mutations were identified within SAV1753. COL-1 contains a G to T transition at nucleotide 131 of the SAV1754 ORF, causing a proline to glutamine amino acid substitution at position 44 of the protein. COL-2 possesses a C to T transition at nucleotide 766 of the SAV1754 ORF, resulting in a proline to serine substitution at amino acid 257 of the protein. A base change transition from A to G at nucleotide 54 of the SAV1754 ORF was also identified in COL-3, resulting in an isoleucine to methionine substitution at amino acid position 18 of the protein. Because each of these mutants conferred marked resistance to DMPI and CDFI without cross resistance to multiple cell wall inhibitors (e.g., vancomycin, moenomycin, D-cycloserine, fosfomycin, tunicamycin, or compound D) or other agents (ciprofloxacin, linezolid, platensimycin, or clindamycin), it is unlikely SAV1754 plays a general role in drug import or efflux (Table S4). Further, allelic introduction of each of the three SAV1574 mutations into RN4220 was sufficient to confer DMPI resistance (Figure 5). As expected, each of these mutations was sufficient to provide cross-resistance to CDFI but not compound D (Figure 5). Collectively, these results strongly implicate SAV1754 as the direct target of DMPI and CDFI and predict that its inactivation potentiates the activity of imipenem.

Genetic Inactivation of SAV1754 Potentiates Imipenem Activity against MRSA

Provided DMPI and CDFI potentiate imipenem activity against COL by inhibiting SAV1754, a prediction can be made that genetic inactivation of SAV1754 should similarly restore imipenem activity in this genetic background. To test this possibility, we examined antibiotic susceptibility of COL maintaining the SAV1754-AS plasmid versus a control strain with empty vector. Strains were seeded in agar media with or without the antisense inducer (50 mM xylose). Genetic depletion of SAV1754 resulted in a xylose-dependent hypersensitivity to imipenem as judged by markedly increased zones of growth inhibition over a broad range of imipenem levels tested (Figure 6; data not shown). Further, depletion of SAV1754 resulted in pronounced hypersensitivity across a wide range of additional β-lactams tested, including ertapenem, cephalosporins (cefepime, ceftazidime, and ceftriaxone), and piperacillin in combination with tazobactam (Figure 6). Remarkably, SAV1754-based genetic potentiation of β-lactams exceeded that achieved by PBP2-AS inactivation, which is a known target of these agents and thus serves as a positive control (Figure 6). The PBP2-AS antibiotic sensitivity profile was restricted to a subset of cell wall inhibitors including imipenem, ertapenem, and vancomycin. Notably, mutations in multiple cell wall genes, including *murE* (Gardete et al., 2004) and *fmhB* (Rohrer et al., 1999), which are also known to restore MRSA susceptibility to β-lactams, also conferred genetic potentiation to imipenem and other β -lactams by antisense interference (data not shown).

To further examine drug sensitivity phenotypes associated with partial inactivation of SAV1754, we tested the susceptibility of the COL SAV1754-AS strain to a broad panel of known

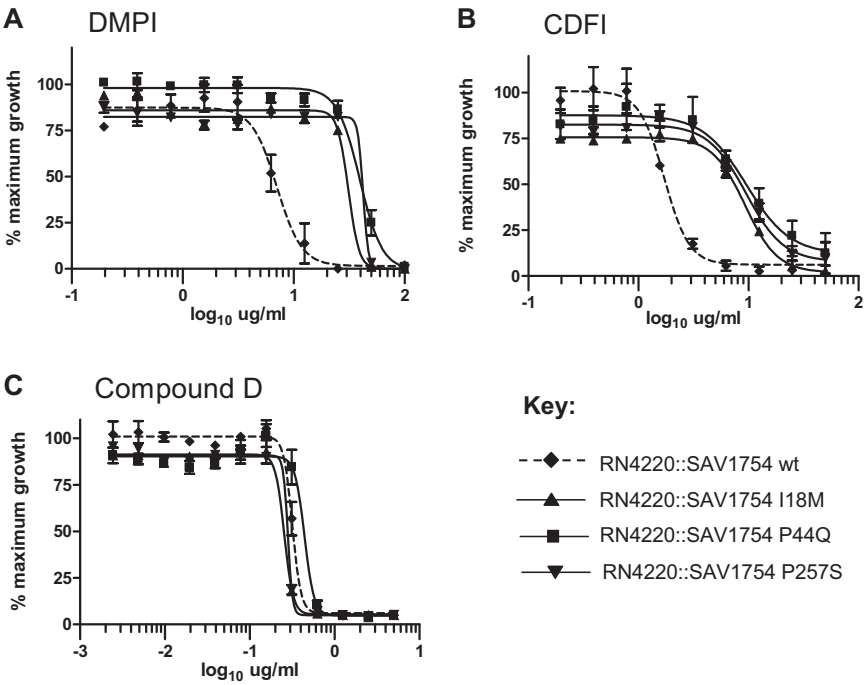


Figure 5. Mutations Associated with DMPI and CDFI Resistance in (MRSA) COL Are Sufficient to Confer Resistance in (MSSA) RN4220

Dose response for inhibition of representative *S. aureus* strains transformed with I18M, P44Q, or P257S mutations by DMPI (A), CDFI (B) and Compound D (C). Allelic copies of SAV1754 were introduced by homologous recombination yielding a tandem duplication at the SAV1754 locus (Figure S6). EC_{50} values for DMPI and CDFI with the mutant strains (solid lines) are increased approximately 5-fold compared with a control strain containing a tandem wild-type SAV1754 duplication (dotted lines). In contrast, cross-resistance of these alleles to compound D is not observed. EC_{50} value determinations of allelic pseudo-diploids to DMPI, CDFI, and compound D are summarized. Cells were grown for 10 hr in LB media supplemented with tetracycline (5 μg/ml). Dose-response curves were fitted using a four parameter logistic equation (GraphPad Prism Software) with error bars representing the standard error of the mean (SEM) of duplicate samples.

Summary:

	EC_{50} values (ug/ml)		
strain	**DMPI**	**CDFI**	**Compound_D**
RN4220::SAV1754 wt	7	2	0.4
RN4220::SAV1754 I18M	31	9	0.3
RN4220::SAV1754 P44Q	39	10	0.3
RN4220::SAV1754 P257S	42	10	0.3

antibiotics. As expected, SAV1754-AS displayed marked hypersensitivity to DMPI, CDFI, and compound D (Figure 6). However, SAV1754-AS chemical sensitivity phenotypes did not extend to other mechanistic classes of antibiotics including inhibitors of DNA gyrase (fluoroquinolones), DHFR (trimethoprim), RNA synthesis (rifampicin), protein synthesis (polyketide, macrolide, or oxazolidinones), or fatty acid synthesis (cerulein). In addition, knockdown of SAV1754 expression did not result in hypersensitivity to other non β-lactam cell wall inhibitors tested, including fosfomycin, moenomycin, teicoplanin, bacitracin, and tunicamycin (data not shown). Thus genetic depletion of SAV1754 in MRSA provides chemical hypersensitivity phenotypes specific to target-selective inhibitors as well as β-lactams rather than a pleiotropic antibiotic hypersensitization. Although genetic inactivation of SAV1754 potentiates the activity of β-lactams, no reciprocal relationship was observed; specifically, genetic inactivation of PBP2 did not enhance the effect of DMPI, CDFI, or compound D. Notwithstanding additional effects potentially elicited by the SAV1754 antisense interference (for example off-target SAV1753 effects), these results demonstrate that genetic inactivation of SAV1754 in the MRSA COL strain

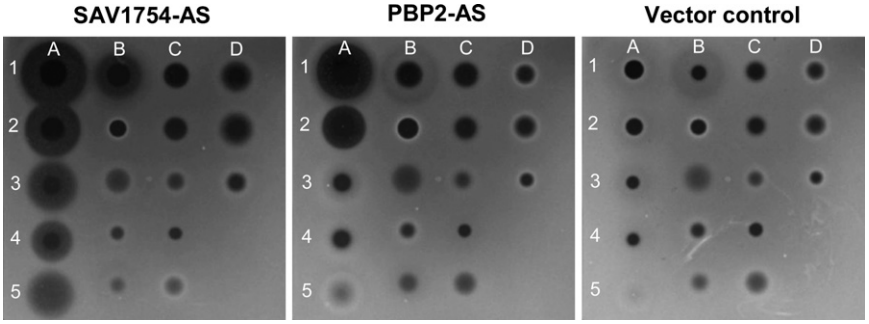


Figure 6. Genetic Depletion of SAV1754 by Antisense Interference Restores COL Susceptibility to β-Lactams

COL strains bearing either SAV1754-AS, the PBP2 positive control (PBP2-AS), or empty vector (pEPSA5) were seeded in LB agar with 50 mM xylose to induce antisense interference. Drugs spotted are as follows: imipenem (12.8 μg; A1); ertapenem (51.2 μg; A2); cefepime (102.4 μg; A3); ceftazidime (102.4 μg; A4); ceftriaxone (102.4 μg; A5); piperacillin/tazobactam (25.6 μg; B1); vancomycin (0.8 μg; B2); cerulein (12.8 μg; B3); rifampicin (0.003 μg; B4); azithromycin (0.4 μg; B5); ciprofloxacin (0.1 μg; C1); levofloxacin (0.1 μg; C2); trimethoprim (0.8 μg; C3); tetracycline (25.6 μg; C4); linezolid (1.6 μg; C5); compound D (0.0125 μg; D1); DMPI (0.8 μg; D2); CDFI (0.2 μg; D3); DMSO (10 μl 20% stock; D4); 0.1M PBS (pH 6) and 10 mM MOPS (pH 7) (10 μl of each buffer spotted adjacent to one another; D5). Susceptibility was scored according to zones of inhibition after 24 hr growth at 37°C. SAV1754 hypersensitivity is restricted to two classes of antibiotics: (i) β-lactams which target PBPs, and (ii) the proposed SAV1754 inhibitors (DMPI, CDFI, and compound D). Zone sizes for parallel spottings on inoculated media lacking xylose were identical to the vector control (not shown).

background phenocopys the imipenem potentiation effect produced by DMPI, CDFI, and compound D. Further, chemical hypersensitivity phenotypes of SAV1754 reflect those achieved by PBP2 and other cell wall mutants (manuscript in preparation), implicating SAV1754 as performing a novel function that directly or indirectly affects peptidoglycan synthesis or assembly.

DISCUSSION

Targeting SAV1754 Restores MRSA Susceptibility to β-Lactams

By phenotypic screening for synthetic compounds that restore MRSA susceptibility to carbapenems, we identified a new indole class of compounds, DMPI and CDFI, which potentiate imipenem activity across multiple methicillin-resistant *Staphylococci*. DMPI and CDFI are predicted to elicit their carbapenem potentiating effect by inhibiting SAV1754, a previously uncharacterized gene product. AISS profiling of DMPI and CDFI, as well as the previously reported inhibitor (compound D), for target-specific chemical sensitivity across 245 essential genes tested revealed SAV1754 as uniquely and prominently hypersensitive to each of these agents, suggesting they share a common MOA. Further, SAV1754 overexpression significantly reduced RN4220 susceptibility to DMPI and CDFI, analogous to that previously reported for compound D (Mott et al., 2008). Indeed, isolation of independent DMPI resistant mutants in MRSA COL mapped to three distinct mutations in SAV1754, each reducing COL susceptibility to DMPI > 16-fold (MIC > 64 μg/ml) and each suppressing DMPI's potentiation of imipenem. Because these mutations were not cross-resistant to multiple cell wall inhibitors tested, it is unlikely they reflect a compensatory or bypass mechanism of resistance. Interestingly, these mutations all map to the amino terminal half of the SAV1754 protein, with the DMPI resistance mutation P44Q mapping particularly close to the compound D resistance mutation A48T previously reported (Mott et al., 2008). These results, combined with allelic resistance studies, demonstrate their sufficiency in MSSA strain RN4220 to confer DMPI and CDFI resistance and strongly implicate SAV1754 as their likely target. Consistent with this possibility, genetic depletion of SAV1754 in MRSA dramatically increased COL sensitivity to imipenem, congruent with the synergistic effects of both the indole and anthranilic acid class inhibitors in combination with imipenem. Thus, opposing phenotypes of β-lactam resistance or restored susceptibility were achieved in MRSA by distinct genetic alterations in SAV1754.

Transposon-mediated insertional mutants generated in MRSA COL have identified an extensive list of genetic determinants, which, if inactivated, restore susceptibility to β-lactams (Berger-Bächi and Rohrer, 2002; de Lencastre et al., 1999). With few exceptions, such ''auxillary'' genes (e.g., *femA*, *femB*, *murE*, and *pbp2*) are directly involved in cell wall biosynthesis. The failure to identify SAV1754 by these means might reflect its essential functional role and/or intrinsic transposon biases precluding the likely occurrence of such insertion mutations. The application of an antisense interference strategy, as used here to demonstrate genetic inactivation of SAV1754 augments imipenem activity against COL, suggests the broader utility of this approach to identify additional genes sharing this phenotype. Indeed, preliminary results using targeted antisense interference in COL validates a number of cell wall genes whose inactivation is known to restore MRSA susceptibility to β-lactams, including *murE* (Gardete et al., 2004), *fmhB* (Rohrer et al., 1999), *murF* (Sobral et al., 2003), and *glmS* (Komatsuzawa et al., 2004) and identifies additional cell wall genes such as *murB*, *murC*, and *pbpA* that share this phenotype (data not shown).

Possible Role of SAV1754 as a Gram-Positive Peptidoglycan Flippase

Although essentially all the genes comprising the peptidoglycan biosynthetic pathway have been long since identified (Bugg and Walsh 1992) and extensively characterized across Gram-positive and Gram-negative bacteria (van Heijenoort 2001, Green, 2002, El Zoeiby et al., 2003), one central participant—a flippase required to translocate lipid II peptidoglycan precursors from the cytoplasmic side of the cell membrane to the periplasmic surface of the membrane—has remained largely elusive (Scheffers and Pinho, 2005). However, two recent reports describe the *E. coli* gene, *murJ* (*mviN*), as likely fulfilling this function (Ruiz, 2008; Inoue et al., 2008). *MurJ* is predicted to encode a 14-membrane-spanning inner membrane protein that is broadly conserved among Gram-negative bacteria and essential for growth. Moreover, *murJ* conditional mutants possess reduced peptidoglycan levels and a concomitant increase of cell wall precursors, resulting in an osmotic remedial lysis phenotype. Consistent with this claim, MurJ is absent from the *Mollicutes* class of enodosymbiotic bacteria (including *Mycoplasma* spp.), which lack a peptidoglycan cell wall. Therefore, an important question raised from this work relates to the nature of the flippase activity in Gram-positive bacteria.

We speculate that SAV1754, which is conserved among Gram-positive bacteria (Figure S1A), might perform an analogous peptidoglycan lipid II flippase function. Like *murJ*, SAV1754 is essential for cell viability and is predicted to encode a 14-transmembrane-spanning integral membrane protein, which, if inactivated, results in similarly striking cell wall phenotypes, including (i) decreased macromolecular synthesis of peptidoglycan, (ii) accumulation of peptidoglycan precursors (Mott et al., 2008), (iii) reduced peptidoglycan deposition by Van-FL staining, and (iv) restored susceptibility of MRSA to a broad diversity of β-lactams. In accordance with this suggestion, SAV1754 is also a member of the same multidrug/oligosaccharidyl-lipid/polysaccharide protein superfamily as MurJ. Moreover, SAV1754 shares three Pfam motifs characteristic of MurJ; an ~250 N-terminal domain polysaccharide biosynthesis motif to which DMPI, CDFI, and compound D resistance mutations map, a MVIN motif spanning essentially the entire length of SAV1754, and a C-terminal MatE motif (Figure S1B).

Like *murJ*, SAV1754 is also absent among *Mycoplasma* spp., which, as endosymbiont-living bacteria, lack a peptidoglycan cell wall. Because 16S rRNA sequence roots *Mycoplasma* as ancestrally related to Gram-positive bacteria but diverging from the latter as recently as 65 million years ago, its parallel genome reduction and cell wall loss further support the relationship between SAV1754 function and cell wall biosynthesis. We also note that by applying the bioinformatic deduction strategy used to identify MurJ (N. Ruiz, 2008), a SAV1754 homolog (2e-26) is identified in *Eubacterium dolichum*. Despite the report

that *E. dolichum* lacks a peptidoglycan cell wall as judged by wheat germ agglutinin fluorescent staining (Maymó-Gatell et al., 1997), clear homologs of MurA through MurG, as well as MraY and PBPs, were also identified. Thus, the assertion that *E. dolichum* lacks a peptidoglycan cell wall must be revisited (Maymó-Gatell et al., 1997).

Limited similarity between SAV1754 and MurJ—despite our assertion that they are functional orthologs—might reflect the substantial evolutionary distance between *E. coli* and *S. aureus* as well as their hydrophobic transmembrane domain structure (Figure S5). Sequence divergence between these proposed peptidoglycan flippases might also be attributed, in part, to structural differences between lipid II peptidoglycan substrates common to Gram-positive and Gram-negative bacteria. Notwithstanding this possibility, *B. subtilis* encodes a protein (BSU30050) sharing strong homology (1e-85) with SAV1754. Yet *B. subtilis* and *E. coli* peptidoglycan share a common stem peptide distinct from *S. aureus. B. subtilis* and Gram-negative derived stem peptides contain meso-diamoinopimelic acid (DAP) and no pentaglycine bridge, whereas *S. aureus* and most other Gram-positive bacteria substitute DAP with L-lysine, from which a pentaglycine bridge is conjugated (Scheffers and Pinho, 2005). Therefore, biochemical assays are required to measure the predicted flippase activity of SAV1754 and MurJ, as well as confirm in vitro the effects of SAV1754 inhibitors, and examine whether their possible inhibitory spectrum extends to MurJ, as suggested by their modest activity against the permeabilized *E. coli* strain MB5746.

Chemical Genetic Synergism as an Approach to Antibiotic Lead Discovery

An urgent need exists to develop new efficacious agents to treat MRSA infections. Whereas significant efforts have focused on single agents that fulfill this role (Silver, 2003, 2007; Payne et al., 2007), the use of combination agents that augment spectrum gaps of existing therapeutics with proven safety profiles has also gained general acceptance as a valid drug development strategy (Walsh, 2000; Keith et al., 2005; Yeh et al., 2006; Hugonnet et al., 2009). Indeed, whole-cell compound potentiation screens offer significant advantages over biochemical or target-based whole-cell screens. First, as in the case of an ertapenem synergism screen, subinhibitory levels of ertapenem broadly inhibit the penicillin-binding protein family, which is a significant technical challenge by genetic means. Second, provided the MOA of the queried antibiotic is specific and mechanistically conserved across bacteria, it serves as a chemical surrogate to genetic strategies as a means to identify synergistic agents targeting specific pathogens not amenable to mutagenesis. Finally, whereas the rationale of target-based screens is to identify cognate inhibitors (which may or may not exist among a finite screening library), synergism-based screens emphasize the entire genome as possible targets of potentiation, thereby increasing the likelihood that compounds of interest will be identified. However, critical to the success of this approach is the need to rapidly determine the MOA of such growth inhibitory compounds. This is typically achieved by classical drug-resistant mutant analysis (Mott et al., 2008). AISS profiling as demonstrated here and in the accompanying report (Donald et al., 2009), provides a rapid chemical-genetic strategy linking growth inhibitory compounds to their target according to gene-specific compound hypersensitivity phenotype(s) identified among a compendium of potential targets tested in parallel.

Although none of the compounds we report are sufficiently potent in their current form—and significant chemical modifications would be required to advance their development—their discovery and determined MOA strongly implicate SAV1754 as a druggable target, which, if chemically inhibited, elicits the desired potentiation of β-lactams. Because SAV1754 is broadly conserved across Gram-positive bacteria and its phenotypes implicate a central role in *S. aureus* cell wall biogenesis, it represents a new participant of this important pathway that a broad diversity of antibiotics target. Thus, target-based screens are warranted to identify new SAV1754 inhibitory classes with improved antibiotic lead properties. More broadly, empiric screening for synergistic agents to other existing antibiotics that similarly restore susceptibility to MRSA or expand spectrum to other bacterial pathogens, provides timely and pragmatic alternatives to antibacterial drug discovery.

SIGNIFICANCE

Staphylococcus aureus is a major nosocomial and community-acquired pathogen that has acquired an alarming resistance to practically all antibiotics. Here we identify antibacterial inhibitors (DMPI and CDFI) that restore susceptibility of methicillin-resistant _S. aureus_ (MRSA) to carbapenem class β-lactam antibiotics. Genetic studies confirm that these agents as well as the structurally distinct inhibitor, compound D (Mott et al., 2008), target the functionally unannotated protein, SAV1754. First, we demonstrate SAV1754 is specifically hypersensitive to each of these agents among an extensive collection of essential genes examined by AISS profiling, as described in the accompanying paper (Donald et al., 2009) in this issue of _Chemistry & Biology_. Conversely, SAV1754 overexpression confers resistance to these compounds. Further, DMPI-resistant mutations in MRSA map to SAV1754 and are sufficient to confer resistance to DMPI and CDFI as well as suppress the synergistic effects of these agents in combination with imipenem. Importantly, SAV1754 antisense-induced inactivation in MRSA phenocopies the potentiating effect of these compounds in restoring β-lactam activity, thereby providing a mechanistic basis for their synergistic effects. SAV1754 inhibitors also disrupt peptidoglycan synthesis as judged by whole cell macromolecular labeling and fluorescence staining of nascent peptidoglycan. Interestingly, SAV1754 is essential for _S. aureus_ growth and is structurally related to the recently reported MviN/MurJ peptidoglycan precursor flippase in _Escherichia coli_ (Ruiz, 2008). However, whereas MurJ homologs are restricted to Gram-negative bacteria, SAV1754 family members are conserved across Gram-positive bacteria. Based on these data, we propose SAV1754 provides a peptidoglycan flippase function in cell wall biosynthesis among Gram-positive bacteria and that inhibitors of this target might possess therapeutic potential alone or as combination agents with β-lactams. More broadly, our work highlights an antibacterial discovery strategy for new agents that synergize with existing antibiotics, thereby

providing a rational framework for expanding their current efficacy.

EXPERIMENTAL PROCEDURES

S. aureus Strains

MSSA strain EP167 is the RN450 lab strain transformed with antisense vector pEPSA5 (Forsyth et al., 2002) and was used in macromolecular labeling experiments and for compound susceptibility testing. MSSA strain RN4220 was used as host for antisense or overexpression plasmids, or for chromosomal gene targeting vectors. RN4220 is a lab strain, selected originally from a phage-cured wild-type isolate of NCTC 832 (RN450) for enhanced transformation competency (Novick, 1991). Methicillin-resistant *S. aureus* MB 5393 (COL) and *S. epidermidis* MB 6255 (CLB 26329) were used in synergy studies.

Synergy Studies

Standard protocols were used to determine the MIC values of compounds (Amsterdam, 2005 and available from the Clinical and Laboratories Standards Institute as protocol M07-A7; www.clsi.org). MIC values are defined as the lowest concentration of agents either singly or in combination that completely inhibited visible growth. The "checkerboard" technique was used to determine whether combinations of two antibacterial compounds demonstrate synergy (Amsterdam, 2005). The analysis was performed using 96-well polystyrene microtiter plates. All strains were diluted to a final concentration of 5×10^5 CFU/ml in fresh cation-adjusted Mueller Hinton broth with or without 2% NaCl. Data were interpreted by calculating the fractional inhibitory concentration (FIC) values and indexes (FICI). The FIC was determined for each compound by dividing the MIC of each compound when used in combination, by the MIC of that compound when used alone. The FICI is the sum of the two FIC values for each compound in an active combination. That is, $FIC_A = MIC_{A\text{-}comb} / MIC_{A\text{-}alone}$; $FICI = FIC_A + FIC_B$. An FICI value ≤ 0.5 was interpreted as synergistic.

Plasmid and Strain Construction

Polymerase chain reaction (PCR) primers (IDT) used in plasmid and strain construction are listed in Table S5. Plasmid pTET15 (GenBank accession FJ859896) was used for episomal expression of SAV1754. This shuttle vector contains a tetracycline-inducible promoter, the regulatory tetR repressor gene and chloramphenicol (for *S. aureus*) and ampicillin (for *E. coli*) selectable markers. Genomic *S. aureus* DNA was prepared with a Wizzard® kit (Promega) and SAV1754 fragments were PCR amplified with Platinum *pfx* polymerase (Invitrogen). The SAV1754 COL strain ORF was subcloned using vector and PCR primer BamHI and SmaI sites. The resulting expression plasmid was verified by DNA sequencing prior to introduction into the RN4220 strain by electroporation (Kraemer and Iandolo 1990). Transformants were selected on LB agar supplemented with 34 μg/ml chloramphenicol. Integrating plasmid pSA3182-UC was used to introduce additional copies of the SAVl754 gene at the endogenous chromosomal locus (RN4220 strain) via homologous recombination. This vector was derived from pSA3182 (Xia et al., 1999) by substituting a 1.1 kb Eco*R*I DNA fragment (from phage phi-11) for a 0.8 kb fragment containing the pUC plasmid replication origin (to permit propagation in *E. coli*) and by adding a *Sal*I site to facilitate subcloning. Genomic 2.l kb fragments spanning the SAVl754 gene and flanking sequence were PCR amplified from the COL parental strain and DMPI-resistant mutants and subcloned into vector *BamH*I and *Sal*I sites. After DNA sequence confirmation, the resulting plasmids were electroporated into RN4220 and stable transformants selected on LB agar supplemented with 5 μg/ml tetracycline. Primary colonies were purified by two rounds of streaking on this selective media. The structure of the SAV1754 locus in transformants harboring cointegrated vector was confirmed by genomic PCR using a combination of vector-specific primers and primers external to the introduced DNA fragment (primer pairs P3/P4, P3/P5, P4/P6, and P5/P6; Table S5 and Figure S6). The majority of transformants contained only two genomic copies of targeting vector. The presence of additional copies (in ~10%) was detected by PCR with vector-specific primers P5/P6. The tandem copies of SAV1754 were PCR amplified (primers P3/P5 and P4/P6) and DNA sequenced to confirm the presence of wild-type and targeted P44Q, I18M, and P257S alleles.

Macromolecular Labeling

Selective inhibition of peptidoglycan synthesis was measured in living cells of *S. aureus* using the macromolecule radiolabeling assay described by Onishi et al. with adaptations for *Staphylococcus* (Onishi, 1996). MSSA strain EP167 was grown in nutrient broth (Difco) with 1% NaCl and chloramphenicol (34 μg/ml) to select for antisense plasmid pEPSA5 (Forsyth et al., 2002). Aliquots were transferred to wells of microtitration plates containing compound dilutions and radioactive precursors of macromolecules. Radiolabeled substrates were: peptidoglycan, [^{14}C-(U)]-glycine (0.5 μCi/ml); DNA, [2-^{14}C]-thymidine (0.25 μCi/ml); phospholipid, [2-^{3}H]-glycerol (0.25 μCi/ml), RNA, [5, 6-^{3}H]-uridine (0.075 μCi/ml), and protein L-[4, 5-^{3}H]-leucine (2.5 μCi/ml). Erythromycin (10 μg/ml) was included in all assays except protein synthesis to counteract the effects of the stringent control. After 30 min incubation at 37°C, TCA 5% (final) was added to stop incorporation. Precipitates were collected on glass microfiber filtermats using a cell harvester (Micro 96, Molecular Devices).

AISS Profiling Assay

AISS profiles were generated for compounds as described in the accompanying paper by Phillips and colleagues published in this issue of *Chemistry & Biology* (Donald et al., 2009). Briefly, 245 strains each containing a different inducible antisense interference plasmid targeting an essential gene in *S. aureus* RN4220 (MSSA) were pooled into 24 different bins of 6–12 strains each. The bins were then grown each at a different concentration of xylose inducer (ranging from 1.8 to 55 mM) for ~20 population doublings either in the presence of compound being tested or as a 2% DMSO mock treatment control. Strain bin growth was performed in 384-well plates (Costar 3680) on a fully automated system using LB media containing chloramphenicol (34 μg/ml) over three cycles of growth ranging from 5 to 7 hr each for a total time of ~18 hr in a total volume of 50 μl. At the end of the growth period cells from all 24 bins were pooled for each compound treatment or DMSO mock control. Pooled cells were then lysed and subjected to multiplex PCR to amplify specific antisense markers for each strain. Strain specific markers were subsequently identified and peak areas quantitated by DNA fragment analysis on an ABI 3730 genetic analyzer. Peak areas were then normalized and strain depletion ratios were calculated. Statistical significance was determined as a p value utilizing an error model generated for each individual strain across a discrete set of known standards and unknown test samples selected to represent total coverage of all strains within the array set (see also Donald et al., 2009). Log depletion ratios and p values for all strains for each of the compound treatments shown in Figure 3A can be found in Tables S6 and S7. The antisense strains described in this work are available for noncommercial use following the standard Merck Material Transfer Agreement (MTA) and clearance procedures.

Fluorescence Staining

An equimolar ratio of vancomycin antibiotic (Sigma) and a BODIPY FL conjugate of vancomycin (Van-FL, Invitrogen), at a sub-inhibitory concentration of 0.5 μg ml^{-1}, was used to label newly synthesized peptidoglycan precursors as previously described (Pinho and Errington, 2003). In short, a culture of *S. aureus* RN4220 (MSSA) was grown for 16 hr in tryptic soy broth (TSB) at 37°C in the presence of 0.125 M D-Serine amino acid (Sigma), to allow for incorporation of D-serine amino acid in place of D-alanine amino acid into the propagating cell wall. The culture was diluted 1/50 into fresh TSB supplemented with 0.125 M D-Serine and continued to grow at 30°C until mid-log phase (OD_{600} = 0.5). The culture was then divided into four 1ml aliquots and appropriate antibiotics were added at 2X their respective MIC: DMPI 2 μg ml^{-1}, Compound D 0.04 μg ml^{-1}, Novobiocin 0.3 μg ml^{-1}, and no drug treatment control. The cultures were further incubated at 30°C for 50 min. The cultures were then washed once and resuspended in the same volume of fresh TSB in the absence of D-serine but with appropriate antibiotics (as stated above) and incubated at 30°C for 15 min, to allow for incorporation of D-alanine amino acid. The cells were spun and Van/Van-FL mixture was added to each culture and incubated at room temperature for 10 min. The samples were applied to poly-lysine coated chambered slides and washed once with PBS and resuspended with VECTASHIELD (Vector Laboratories). The samples were immediately observed by fluorescence microscopy.

Antisense Interference in MRSA COL

COL strains transformed with antisense interference plasmids or vector control were grown overnight in Luria Bertani (LB) Miller broth at 37°C containing 34 μg/ml chloramphenicol. 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.

SUPPLEMENTAL DATA

Supplemental Data include six figures and seven tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00241-5.

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REFERENCES

Bancroft, E.A. (2007). Antimicrobials resistance: It's not just for hospitals. JAMA *298*, 1803–1804.

Berger-Bächi, B., and Rohrer, S. (2002). Factors influencing methicillin resistance in *Staphylococci*. Arch. Microbiol. *178*, 165–171.

Berger-Bächi, B., Strässle, A., Gustafson, J.E., and Kayser, F.H. (1992). Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aure*us. Antimicrob. Agents Chemother. *36*, 1367–1373.

Bugg, T.D.H., and Walsh, C.T. (1992). Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. Nat. Prod. Rep. *9*, 199–215.

Bush, K., Heep, M., Macielag, M.J., and Noel, G.J. (2007). Anti-MRSA β-lactams in development, with a focus on ceftobiprole: the first anti-MRSA β-lactam to demonstrate clinical efficacy. Expert Opin. Investig. Drugs *16*, 419–429.

de Lencastre, H., and Tomasz, A. (1994). Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. *38*, 2590–2598.

de Lencastre, H., Wu, S.W., Pinho, M.G., Ludovice, A.M., Filipe, S., Gardete, S., Sobral, R., Gill, S., Chung, M., and Tomasz, A. (1999). Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. *5*, 163–175.

de Lencastre, H., Oliveira, D., and Tomasz, A. (2007). Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. Curr. Opin. Microbiol. *10*, 428–435.

Donald, R.G.K., Skwish, S., Forsyth, A.R., Anderson, J.W., Zhong, T., Burns, C., Lee, S., Meng, X., LoCastro, L., Wang, L., et al. (2009). A novel *Staphylococcus aureus* fitness test platform for mechanism-based profiling of antibacterial compounds. Chem. Biol. *16*, this issue, 826–836.

El Zoeiby, A., Sanschagrin, F., and Levesque, R.C. (2003). Structure and function of the Mur enzymes: development of novel inhibitors. Mol. Microbiol. *47*, 1–12.

Forsyth, R.A., Haselbeck, R.J., Ohlsen, K.L., Yamamoto, R.T., Xu, H., Trawick, J.D., Wall, D., Wang, L., Brown-Driver, V., Froelich, J.M., et al. (2002). A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. Mol. Microbiol. *43*, 1387–1400.

Gardete, S., Ludovice, A.M., Sobral, R.G., Filipe, S.R., de Lencastre, H., and Tomasz, A. (2004). Role of murE in the Expression of beta-lactam antibiotic resistance in *Staphylococcus aureus*. J. Bacteriol. *186*, 1705–1713.

Green, D.W. (2002). The bacterial cell wall as a source of antibacterial targets. Expert Opin. Ther. Targets *6*, 1–19.

Hugonnet, J.E., Tremblay, L.W., Boshoff, H.I., Barry, C.E., 3rd, and Blanchard, J.S. (2009). Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. Science *323*, 1215–1218.

Inoue, A., Murata, Y., Takahashi, H., Tsuji, N., Fujisaki, S., and Kato, J. (2008). Involvement of an essential gene, mviN, in murein synthesis in *Escherichia coli*. J. Bacteriol. *190*, 7298–7301.

Keith, C.T., Borisy, A.A., and Stockwell, B.R. (2005). Multicomponent therapeutics for networked systems. Nat. Rev. Drug Discov. *4*, 71–78.

Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., et al. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA *298*, 1763–1771.

Komatsuzawa, H., Fujiwara, T., Nishi, H., Yamada, S., Ohara, M., McCallum, N., Berger-Bächi, B., and Sugai, M. (2004). The gate controlling cell wall synthesis in *Staphylococcus aureus*. Mol. Microbiol. *53*, 1221–1231.

Kraemer, G.R., and Iandolo, J.J. (1990). High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. *21*, 373–376.

Matsuhashi, M., Song, M.D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N., and Konno, M. (1986). Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. J. Bacteriol. *167*, 975–980.

Maymó-Gatell, X., Chien, Y., Gossett, J.M., and Zinder, S.H. (1997). Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science *276*, 1568–1571.

Memmi, G., Filipe, S.R., Pinho, M.G., Fu, Z., and Cheung, A. (2008). *Staphylococcus aureus* PBP4 is essential for β-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob. Agents Chemother. *52*, 3955–3966.

Miller, C., Thomsen, L.E., Gaggero, C., Mosseri, R., Ingmer, H., and Cohen, S.N. (2004). SOS response induction by β-lactams and bacterial defense against antibiotic lethality. Science *305*, 1629–1631.

Mott, J.E., Shaw, B.A., Smith, J.F., Bonin, P.D., Romero, D.L., Marotti, K.R., and Miller, A.A. (2008). Resistance mapping and mode of action of a novel class of antibacterial anthranilic acids: evidence for disruption of cell wall biosynthesis. J. Antimicrob. Chemother. *62*, 720–729.

Novick, R.P. (1991). In Methods In Enzymology; Bacterial Genetic Systems, *Volume 204*, J.H. Miller, ed. (New York: Academic Press), pp. 587–636.

Onishi, H.R., Pelak, B.A., Gerckens, L.S., Silver, L.L., Kahan, F.M., Chen, M.H., Patchett, A.A., Galloway, S.M., Hyland, S.A., Anderson, M.S., and Raetz, C.R. (1996). Antibacterial agents that inhibit lipid A biosynthesis. Science *274*, 980–982.

Payne, D.J., Gwynn, M.N., Holmes, D.J., and Pompliano, D.L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat. Rev. Drug Discov. *6*, 29–40.

Pinho, M.G., and Errington, J. (2003). Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery. Mol. Microbiol. *50*, 871–881.

Pinho, M.G., de Lencastre, H., and Tomasz, A. (2001a). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant *Staphylococci*. Proc. Natl. Acad. Sci. USA *98*, 10886–10891.

Pinho, M.G., Filipe, S.R., de Lencastre, H., and Tomasz, A. (2001b). Complementation of the essential peptidoglycan transpeptidase function of

penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J. Bacteriol. *183*, 6525–6531.

Projan, S.J., and Bradford, P.A. (2007). Late stage antibacterial drugs in the clinical pipeline. Curr. Opin. Microbiol. *10*, 441–446.

Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., and Berger-Bächi, B. (1999). The essential *Staphylococcus aureus* gene fmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc. Natl. Acad. Sci. USA *96*, 9351–9356.

Ruiz, N. (2008). Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA *105*, 15553–15557.

Ruiz, N. (2009). *Streptococcus pyogenes* YtgP (Spy_390) complements *Escherichia coli* strains depleted of the putative peptidoglycan flippase MurJ. Antimicrob. Agents Chemother. *53*, 3604–3605.

Scheffers, D.J., and Pinho, M.G. (2005). Bacterial cell wall synthesis: new insights from localization studies. Microbiol. Mol. Biol. Rev. *69*, 585–607.

Sieradzki, K., Chung, M., and Tomasz, A. (2008). Role of a sodium-dependent symporter homologue in the thermosensitivity of β-lactam antibiotic resistance and cell wall composition in *Staphylococcus aureus*. Antimicrob. Agents Chemother. *52*, 505–512.

Silver, L.L. (2003). Novel inhibitors of bacterial cell wall synthesis. Curr. Opin. Microbiol. *6*, 431–438.

Silver, L.L. (2007). Multi-targeting by monotherapeutic antibacterials. Nat. Rev. Drug Discov. *6*, 41–55.

Sobral, R.G., Ludovice, A.M., Gardete, S., Tabei, K., De Lencastre, H., and Tomasz, A. (2003). Normally functioning murF is essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*. Microb. Drug Resist. *9*, 231–241.

Song, M.D., Wachi, M., Doi, M., Ishino, F., and Matsuhashi, M. (1987). Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett. *221*, 167–171.

Styers, D., Sheehan, D.J., Hogan, P., and Sahm, D.F. (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann. Clin. Microbiol. Antimicrob. *5*, 1–9.

van Heijenoort, J. (2001). Recent advances in the formation of the bacterial peptidoglycan monomer unit. Nat. Prod. Rep. *18*, 503–519.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. Nature *406*, 775–781.

Xia, M.M., Lunsford, R.D., McDevitt, D., and Iordanescu, S. (1999). Rapid method for the identification of essential genes in *Staphylococcus aureus*. Plasmid *42*, 144–149.

Yeh, P., Tschumi, A.I., and Kishony, R. (2006). Functional classification of drugs by properties of their pairwise interactions. Nat. Genet. *38*, 489–494.

Note Added in Proof

Natividad Ruiz has recently reported the SAV1754 homolog from *Streptococcus pyogenes*, YtgP (Spy_0390), complements *Escherichia coli* strains depleted of the putative peptidoglycan flippase, MurJ (Ruiz, 2009).