

Mutations in the Primary Sigma Factor σ^A and Termination Factor Rho That Reduce Susceptibility to Cell Wall Antibiotics

Yong Heon Lee,^{a,b} John D. Helmann^a

Department of Microbiology, Cornell University, Ithaca, New York, USA^a; Department of Biomedical Laboratory Science, Dongseo University, Busan, South Korea^b

Combinations of glycopeptides and β -lactams exert synergistic antibacterial activity, but the evolutionary mechanisms driving resistance to both antibiotics remain largely unexplored. By repeated subculturing with increasing vancomycin (VAN) and cefuroxime (CEF) concentrations, we isolated an evolved strain of the model bacterium *Bacillus subtilis* with reduced susceptibility to both antibiotics. Whole-genome sequencing revealed point mutations in genes encoding the major σ factor of RNA polymerase (*sigA*), a cell shape-determining protein (*mreB*), and the ρ termination factor (*rho*). Genetic-reconstruction experiments demonstrated that the G-to-C substitution at position 336 encoded by *sigA* (*sigA*^{G336C}), in the domain that recognizes the -35 promoter region, is sufficient to reduce susceptibility to VAN and works cooperatively with the *rho*^{G56C} substitution to increase CEF resistance. Transcriptome analyses revealed that the *sigA*^{G336C} substitution has wide-ranging effects, including elevated expression of the general stress σ factor (σ^B) regulon, which is required for CEF resistance, and decreased expression of the *glpTQ* genes, which leads to fosfomicin (FOS) resistance. Our findings suggest that mutations in the core transcriptional machinery may facilitate the evolution of resistance to multiple cell wall antibiotics.

The evolution of multidrug resistance among pathogenic bacteria is an increasing threat to global public health because the rate of resistance evolution far outpaces that of development of new antibiotics (1–3). Combinations of antibiotics have emerged as a promising strategy to enhance antibiotic effectiveness (4, 5), and they are commonly classified as additive (no interaction), synergistic (greater than the additive effect), or antagonistic (less than the additive effect) (6, 7). The synergistic interactions between glycopeptides and β -lactams have been demonstrated, especially against staphylococci with reduced susceptibilities to vancomycin (VAN) (8, 9). Glycopeptide antibiotics, such as VAN, inhibit cell wall biosynthesis by binding tightly to the D-Ala-D-Ala terminus of the peptidoglycan pentapeptide, thereby blocking the transpeptidation and transglycosylation reactions in peptidoglycan assembly (10). The β -lactam antibiotics, including cephalosporins, such as cefuroxime (CEF), inhibit cell wall cross-linking by inactivating specific transpeptidases known as penicillin-binding proteins (PBPs) (11, 12). Although synergistic combinations of drugs have clinical benefits, in some cases, resistance to drug combinations may evolve at an even higher rate than resistance to individual drugs (13).

Whole-genome resequencing has enormous resolving power for identifying the genetic changes underlying adaptation. Thus, it has been widely used to explore the evolution of antibiotic resistance that results from the accumulation of random mutations (14–17). Thousands of bacterial genome sequences are available in the public domain and can be used as reference genomes for comparison (18).

Mutations affecting bacterial RNA polymerase (RNAP) arise during many selections (2, 19–23), suggesting that alterations to RNAP can facilitate adaptation. Bacterial RNAP is an essential, highly conserved, multisubunit enzyme that is responsible for transcription (24). It consists of the core ($\alpha_2\beta\beta'\omega$) enzyme, a dissociable σ factor (24), and, in Gram-positive bacteria, an additional δ subunit that enhances transcriptional specificity (25, 26). For transcription initiation, the core enzyme must bind one of various σ factors, each of which recognizes a specific promoter

sequence (27). Bacteria have one housekeeping σ factor (σ^{70} in *Escherichia coli*; σ^A in *Bacillus subtilis*) and several alternative σ factors that are activated in response to either environmental or developmental signals. Thus, the replacement of one σ factor by another redirects transcription of different subsets of genes (28–30).

The transcription termination factor Rho is required for one of two bacterial termination mechanisms, Rho dependent and intrinsic (31, 32). Rho is broadly distributed among the *Bacteria* and highly conserved, but some bacterial species do not have a homolog (33, 34). Rho is essential in enterobacteria, such as *E. coli* (35), but dispensable in the Gram-positive bacteria *B. subtilis* and *Staphylococcus aureus* (33, 36). In *B. subtilis*, the cellular level of Rho is less than 5% that of *E. coli*, and initially, *rho* itself and the *trp* operon were the only genes known to be regulated by Rho-dependent termination (37, 38). In addition, Gram-positive bacteria, except for *Micrococcus luteus*, are resistant to the Rho-inhibiting antibiotic bicyclomycin, suggesting that Rho is usually nonessential in these bacteria (33, 39).

Here, we describe mutations resulting from the selection of *B. subtilis* for reduced susceptibility to VAN and CEF. Whole-genome resequencing and genetic-reconstruction studies demonstrate that single-nucleotide substitutions within *sigA* and *rho* are responsible for the observed effects. These results highlight the ability of mutations affecting the core transcriptional machinery to mediate adaptation to cell envelope antibiotics.

Received 24 June 2014 Accepted 5 August 2014

Published ahead of print 11 August 2014

Address correspondence to John D. Helmann, jdh9@cornell.edu.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.02022-14

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Null mutant strains were constructed by using long-flanking homology PCR (LFH-PCR) as described previously (40). Cells were routinely cultured at 37°C in lysogeny broth (LB) medium or minimal medium supplemented with either 2% glucose or 2% glycerol-3-phosphate (glycerol-3-P). Minimal medium contained 40 mM potassium morpholinepropanesulfonate (MOPS) (adjusted to pH 7.4 with KOH), 2 mM potassium phosphate buffer (pH 7.0), glucose (2% [wt/vol]), (NH₄)₂SO₄ (2 g/liter), MgSO₄ · 7H₂O (0.2 g/liter), trisodium citrate · 2H₂O (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), 3 nM (NH₄)₆Mo₇O₂₄, 400 nM H₃BO₃, 100 μM FeCl₃, 30 nM CoCl₂, 10 nM CuSO₄, 10 nM ZnSO₄, and 80 nM MnCl₂. For *B. subtilis*, the following antibiotics were used for selection: spectinomycin (100 μg/ml), kanamycin (15 μg/ml), chloramphenicol (10 μg/ml), or macrolide-lincosamide-streptogramin B (containing 1 μg/ml erythromycin and 25 μg/ml lincomycin).

Selection of antibiotic resistance. The MICs for vancomycin and cefuroxime in combination were determined using a Bioscreen C microbial-growth analyzer. Overnight cultures of *B. subtilis* W168 were subcultured by 1:100 dilution in 5 ml of Mueller-Hinton (MH) medium (Difco) containing sub-MICs of the combination of the antibiotics (vancomycin, 0.1 μg/ml; cefuroxime, 2 μg/ml). Then, 24-h cultures were inoculated 1:100 into 5 ml fresh medium containing both antibiotics, with the concentrations incrementally increasing for 10 days. Bacterial glycerol stocks were frozen at -80°C every day. The day 6 cultures were streaked on an LB agar plate, and the susceptibilities of 10 colonies to vancomycin and cefuroxime were compared. Finally, a representative colony (HB13513) was selected for whole-genome resequencing.

Whole-genome resequencing. *B. subtilis* wild-type and HB13513 mutant strains were grown in LB medium to an optical density at 600 nm (OD₆₀₀) of 0.4, and their genomic DNAs were purified using the Qiagen DNeasy blood and tissue kit. The quantity and purity of DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technology, Inc., Wilmington, DE), and DNA was sequenced using an Illumina HiSeq 2000 at the Cornell University Life Sciences Core Laboratories Center. To identify single-nucleotide polymorphisms (SNPs), the resulting genomic sequence data were assembled with MOSAIC, using the reference sequence (41) under GenBank accession number ABQK00000000.

Genetic reconstruction. Each point mutation was moved into the wild-type chromosome using LFH PCR as described previously (2). The upstream and downstream fragments were amplified from either the wild type or HB13513 using four primers for each SNP (Table 1) and then joined to an antibiotic resistance cassette (*mls*, *kan*, or *spc*), which was introduced as a selectable marker after the stop codon or before the promoter region. The final PCR products were transformed into the wild-type *B. subtilis* strain, and the correct transfer of the point mutation was verified by DNA sequencing.

Plasmid construction. Molecular techniques were performed as described by Sambrook and Russell (42). Plasmid pPL82 was used for the expression of *glpTQ* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{spac}(hy) (43). To construct pYH006 (pPL82-*glpTQ*), the promoterless *glpTQ* operon was amplified from *B. subtilis* wild-type chromosomal DNA by PCR using the primers 6205 (*glpTQ* Pspac-F)/6206 (*glpTQ* Pspac-R). Construct integrity was verified by DNA sequencing. The resulting plasmid was then integrated by double-crossover homologous recombination into the *amyE* locus.

Disk diffusion assays. Disk diffusion assays were performed as described previously (2). Briefly, strains were grown in LB at 37°C with shaking to an OD₆₀₀ of 0.4. A 100-μl aliquot of these cultures was added to 4 ml of 0.7% MH soft agar (kept at 45°C) and directly poured onto MH plates (containing 15 ml of 1.5% MH agar). After 30 min incubation at room temperature, the plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing the antibiotics to be tested were placed on top of the MH agar, and the plates were incubated at 37°C for 16 to 18

h. The diameters of the inhibition zones were measured after subtraction of the diameter of the filter paper disk (6.5 mm). The following antibiotics and quantities were used in the disk diffusion assays: cefuroxime, 50 μg; ceftazidime, 30 μg; ceftriaxone, 30 μg; ampicillin, 50 μg; penicillin G, 50 μg; piperacillin, 100 μg; oxacillin, 1 μg; and fosfomycin (FOS), 100 μg or 500 μg.

MIC assays. The Etest assay was performed similarly to the disk diffusion assay. Etest strips (bioMérieux) impregnated with VAN or CEF (at concentrations ranging from 0.016 to 256 μg/ml) were applied to MH agar plates, and the plates were incubated at 37°C for 16 to 18 h. The MIC was determined from the scale at the intersection of bacterial growth with the Etest strip. For the growth inhibition assay, strains were grown to an OD₆₀₀ of 0.4 and then diluted 1:400 in MH broth. Aliquots (200 μl) of the diluted cultures were dispensed in a Bioscreen 100-well microtiter plate, and a range of at least 9 antibiotic concentrations close to the MIC were added to each well. Growth was measured spectrophotometrically (OD₆₀₀) every 30 min for 24 h using a Bioscreen C microbial-growth analyzer at 37°C with continuous shaking. The MIC was defined as the lowest concentration of antibiotic that completely inhibited growth (OD₆₀₀ < 0.2) at the 16-h or 24-h time point.

RNA preparation and microarray analyses. Total RNA was isolated from strains HB13679 (*sigA*^{WT}-*mls*) and HB13680 (*sigA*^{G336C} *mls* [where *sigA*^{G336C} represents the G-to-C substitution at position 336 of *sigA*]) grown in LB medium to an OD₆₀₀ of 0.4, using the RNeasy minikit (Qiagen) followed by DNase treatment with Turbo DNA free (Ambion). The quantity and purity of RNA were determined using a NanoDrop spectrophotometer. Two microarray analyses were performed in biological triplicate with a dye swap. cDNA labeling and microarray analysis were performed as described previously (44). The GenePix Pro software package (version 6.0) was used for image processing and analysis. The normalized microarray data sets were filtered to remove those genes that were not expressed at levels significantly above background under either condition (sum of mean fluorescence intensities, <20). In addition, the mean and standard deviation of the fluorescence intensities were computed for each gene, and those for which the standard deviation was greater than the mean value were ignored. The fold change was calculated by using the average signal intensities for HB13680 divided by those for HB13679.

RT-PCR assay. For reverse transcription (RT)-PCR, total RNA (1 μg) was reverse transcribed into cDNA by using random hexamers and a TaqMan reverse transcription kit (Roche). The cDNA was then amplified by PCR using gene-specific primer sets (Table 1). The reaction mixture was denatured (95°C for 3 min) and then subjected to 20 thermal cycles (95°C for 30 s, 54°C for 30 s, and 72°C for 1 min) and a final extension (72°C for 10 min). Primer pair 6094 (*glpT* RT-F)/6095 (*glpT* RT-R) was used to detect the *glpT* transcript. 16S rRNA was used as a normalization control. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized.

Microarray data accession number. The microarray data set is available in the NCBI GEO database under accession number GSE55202.

RESULTS AND DISCUSSION

Development of resistance to the combination of VAN and CEF in *B. subtilis*. We combined experimental evolution and whole-genome sequencing to define a pathway enabling *B. subtilis* to develop increased resistance to the combination of VAN and CEF. The MICs of VAN and CEF in MH medium for *B. subtilis* W168 are 0.25 μg/ml and 6 μg/ml, respectively. When either antibiotic was present at 0.5× MIC, the observed MIC for the other was reduced by ~2-fold, indicative of synergism. To select for increased resistance against this combination of cell wall antibiotics, wild-type cells were continuously subcultured for 10 days with increasing concentrations of both antibiotics. Cells were taken every day before subculturing for frozen bacterial stocks, and their

TABLE 1 Strains, plasmids, and primers

Strain, plasmid, or primer	Genotype, description, or sequence (5'→3') ^a	Source or reference ^b
Strains		
W168	<i>trpC2</i>	BGSC no. 1A1
HB13513	W168 <i>sigA</i> ^{G336C} <i>mreB</i> ^{L201S} <i>rho</i> ^{G56C}	Evolved isolate
HB13679	W168 <i>sigA</i> ^{WT} - <i>mls</i>	LFH PCR with W168
HB13680	W168 <i>sigA</i> ^{G336C} - <i>mls</i>	LFH PCR with W168
HB13681	W168 <i>kan-mreB</i> ^{WT}	LFH PCR with W168
HB13682	W168 <i>kan-mreB</i> ^{L201S}	LFH PCR with W168
HB13683	W168 <i>spc-rho</i> ^{WT}	LFH PCR with W168
HB13684	W168 <i>spc-rho</i> ^{G56C}	LFH PCR with W168
HB13690	W168 <i>sigA</i> ^{WT} - <i>mls spc-rho</i> ^{G56C}	LFH PCR with HB13684
HB13691	W168 <i>sigA</i> ^{G336C} - <i>mls spc-rho</i> ^{G56C}	LFH PCR with HB13684
HB13685	W168 <i>rho::spc</i>	LFH PCR with W168
HB13686	W168 <i>penP::kan</i>	LFH PCR with W168
HB13689	W168 <i>rho::spc penP::kan</i>	HB13686 chr DNA with HB13685
HB0008	CU1065 <i>fosB::cat</i>	63
HB13698	W168 <i>fosB::cat sigA</i> ^{WT} - <i>mls</i>	HB0008 chr DNA with HB13679
HB13699	W168 <i>fosB::cat sigA</i> ^{G336C} - <i>mls</i>	HB0008 chr DNA with HB13680
HB11302	CU1065 <i>bshC::cat</i>	Laboratory stock
HB13700	W168 <i>bshC::cat sigA</i> ^{WT} - <i>mls</i>	HB11302 chr DNA with HB13679
HB13701	W168 <i>bshC::cat sigA</i> ^{G336C} - <i>mls</i>	HB11302 chr DNA with HB13680
HB13551	W168 <i>sigB::cat</i>	72
HB13709	W168 <i>sigB::cat sigA</i> ^{WT} - <i>mls</i>	HB13551 chr DNA with HB13679
HB13710	W168 <i>sigB::cat sigA</i> ^{G336C} - <i>mls</i>	HB13551 chr DNA with HB13680
HB13713	W168 <i>sigA</i> ^{WT} - <i>mls amyE::P_{spac}(hy)-glpTQ (cat)</i>	pYH006 with HB13679
HB13714	W168 <i>sigA</i> ^{G336C} - <i>mls amyE::P_{spac}(hy)-glpTQ (cat)</i>	pYH006 with HB13680
Plasmids		
pPL82	IPTG-inducible expression vector (<i>amyE</i> integration)	43
pYH006	<i>P_{spac}(hy)-glpTQ</i> in pPL82	This study
Primers (no. [name])		
6074 (<i>sigA</i> up-F)	CGCCTACGCTCAAAAGATTG	
6075 [<i>sigA</i> up-R (mls)]	GAGGGTTGCCAGAGTTAAAGGATCTTCAGTAAAATAAAGGCATA TTATCCA	
6076 [<i>sigA</i> do-F (mls)]	CGATTATGCTTTTTGCGCAGTCGGCTGCAAATGAACATTGTGGTG	
6077 (<i>sigA</i> do-R)	CACTTGTCATCACAACTTTTCTCAA	
6078 (<i>mreB</i> up-F)	ACAATGAGAGCTCTTCGCCA	
6079 [<i>mreB</i> up-R (kan)]	CCTATCACCTCAAATGGTTCGCTGGCAAAAATACCCTAAAGGGAAAA	
6080 [<i>mreB</i> do-F (kan)]	CGAGCGCCTACGAGGAATTTGTATCGAAAGGAAAAAGGATATTTG TAACACTT	
6081 (<i>mreB</i> do-R)	TGGATGTGCTCCAGTGCTTT	
6082 (<i>rho</i> up-F)	GTCGCAGTTGACCCCTTTGA	
6083 [<i>rho</i> up-R (<i>spc</i>)]	CGTTACGTTATTAGCGAGCCAGTCAAGGTTTTGACACGGAATTGA	
6084 [<i>rho</i> do-F (<i>spc</i>)]	CAATAAAACCCTTGCCCTCGCTACGTC CAATTCCGTGTCAAACCTT	
6085 (<i>rho</i> do-R)	AGCTCTGCTTCAGGCTGGTT	
6086 (<i>rho</i> up-F) for KO	GCGTTGATATCCTGTTCGGA	
6087 [<i>rho</i> up-R (<i>spc</i>)] for KO	CGTTACGTTATTAGCGAGCCAGTCCATAAAAACACCACGCTTTTCA	
6088 [<i>rho</i> do-F (<i>spc</i>)] for KO	CAATAAAACCCTTGCCCTCGCTACG ACCATCCTTGCTACGGCTCT	
6089 (<i>rho</i> do-R) for KO	GTACATAGGCTTCGCTCCCC	
6090 (<i>penP</i> up-F)	GATACTGCAGGCCCTTTTC	
6091 [<i>penP</i> up-R (kan)]	CCTATCACCTCAAATGGTTCGCTGCTATGGATTTTGCTTCGGCA	
6092 [<i>penP</i> do-F (kan)]	CGAGCGCCTACGAGGAATTTGTATCGGTGGCAATTCTGTCAAACCG	
6093 (<i>penP</i> do-R)	CAAAAGCTCCAGGAAAAGA	
6094 (<i>glpT</i> RT-F)	TCAACGGATGGTTTCAAGGA	
6095 (<i>glpT</i> RT-R)	GCCGTATCGCACGAAATAA	
5397 (16S rRNA-8F)	AGAGTTTATCCTGGCTCAG	
5398 (16S rRNA-519R)	GTATTACCGCGGCTGCTGG	
6205 (<i>glpTQ</i> P _{spac} -F)	GCGCCCCGGGCACAAACAGCAAAGGGGGA	
6206 (<i>glpTQ</i> P _{spac} -R)	GCGCTCTAGATTTTGTCTTTAATAACCCTTTT	

^a Restriction sites are underlined, and the sequences complementary to antibiotic resistance cassettes for LFH PCR are in boldface.

^b chr, chromosomal.

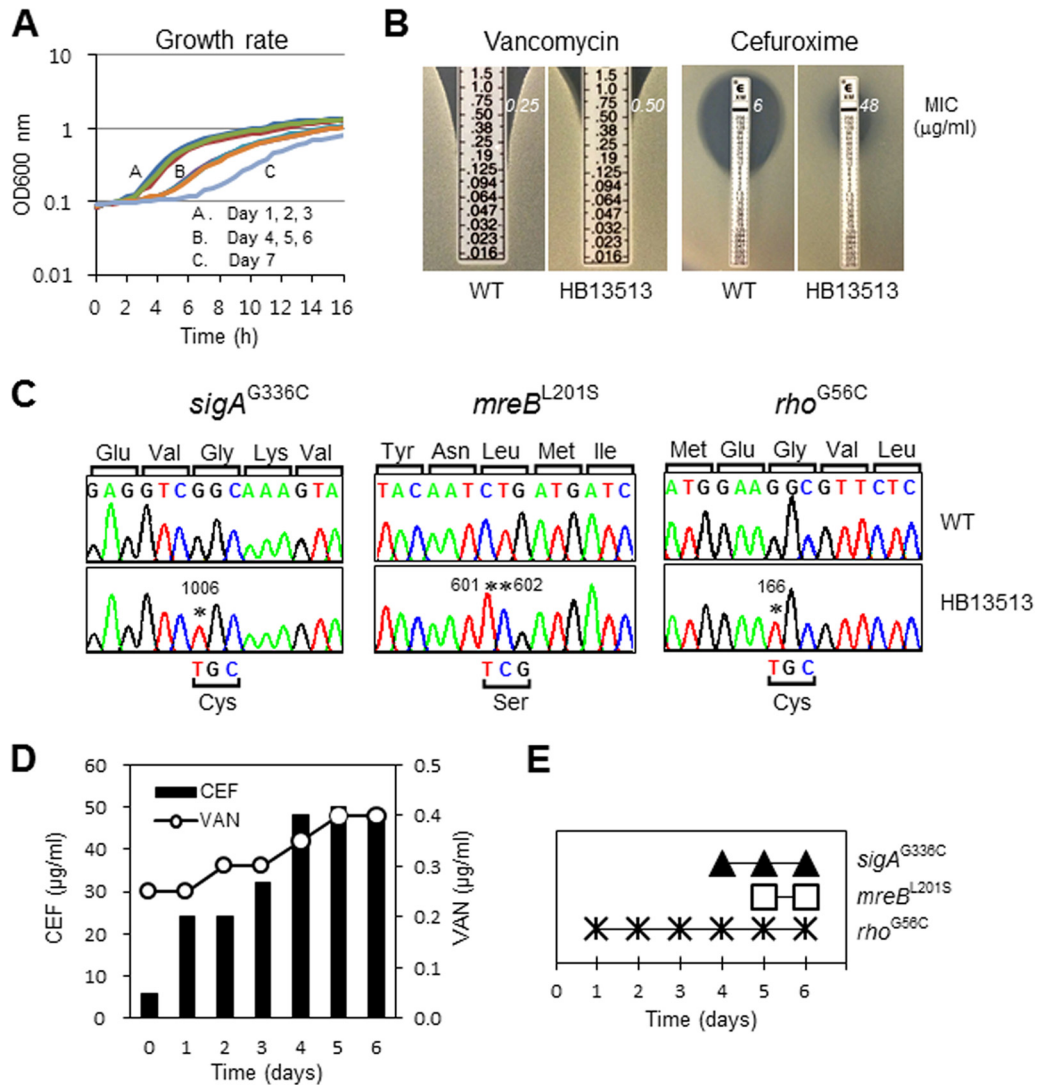


FIG 1 Experimental evolution of increased resistance to the combination of VAN and CEF in *B. subtilis*. (A) Growth rates of the evolved strains. Wild-type *B. subtilis* W168 (BGSC 1A1) cells were grown by repeated subculturing with selection for increasing resistance to both VAN and CEF. (B) Determination of MICs of VAN and CEF for the evolved isolate after 6 days of culturing. Etest strips (bioMérieux) with VAN or CEF (concentrations, 0.016 to 256 $\mu\text{g/ml}$) were applied to MH agar plates, and then the MIC values were read as the point of intersection or by interpolation after 18 h of incubation at 37°C. (C) Sanger sequencing for the confirmation of SNPs (as originally identified by Illumina-based whole-genome resequencing). Strain HB13513 has four point mutations in three genes. The mutated residues are indicated by asterisks, and the altered amino acids are listed at the bottom of each panel. (D) Determinations of the VAN and CEF MICs for evolved strains. The MICs of VAN and CEF were determined by the growth inhibition assay and Etest assay, respectively. (E) Sequential fixation of mutations during the evolution of resistance to the combination of VAN and CEF.

growth rates were measured spectrophotometrically using a Bioscreen C growth analyzer at 37°C. As the cells grew very slowly from day 7 (Fig. 1A), we focused on the day 6 strain. We first isolated a single colony of the evolved strain (HB13513) by streaking it onto an LB agar plate and determined the MICs of VAN and CEF by using an Etest assay. Strain HB13513 displayed an ~8-fold-increased MIC of CEF, together with a modest (≤ 2 -fold) increase in the MIC of VAN (Fig. 1B). These data suggest that *B. subtilis* HB13513 has undergone significant evolution of CEF resistance. Despite extended exposure to VAN, only a modest decrease in VAN susceptibility was observed in this *B. subtilis* isolate.

Identification of mutations that drive the evolution of reduced antibiotic susceptibility. To identify the genomic basis of

these changes in susceptibility to VAN and CEF, we used whole-genome resequencing of HB13513 and its parental wild type to reveal four SNPs that distinguish the two strains. These four SNPs affect three genes (*sigA*, *mreB*, and *rho*) and lead to single amino acid changes in each encoded protein. These mutations were confirmed by Sanger DNA sequencing (Fig. 1C).

When antibiotic susceptibility was evaluated by the growth inhibition assay or Etest assay, the MICs of VAN and CEF gradually increased over time, as expected (Fig. 1D). To determine the order of fixation of these mutations, we sequenced these loci in each sequential daily culture. As shown in Fig. 1E, evolution proceeded through the sequential fixation of mutations in the *rho*, *sigA*, and *mreB* genes. The first mutation arising (*rho*^{G56C}) correlates with an ~4-fold increase in CEF resistance, with another significant in-

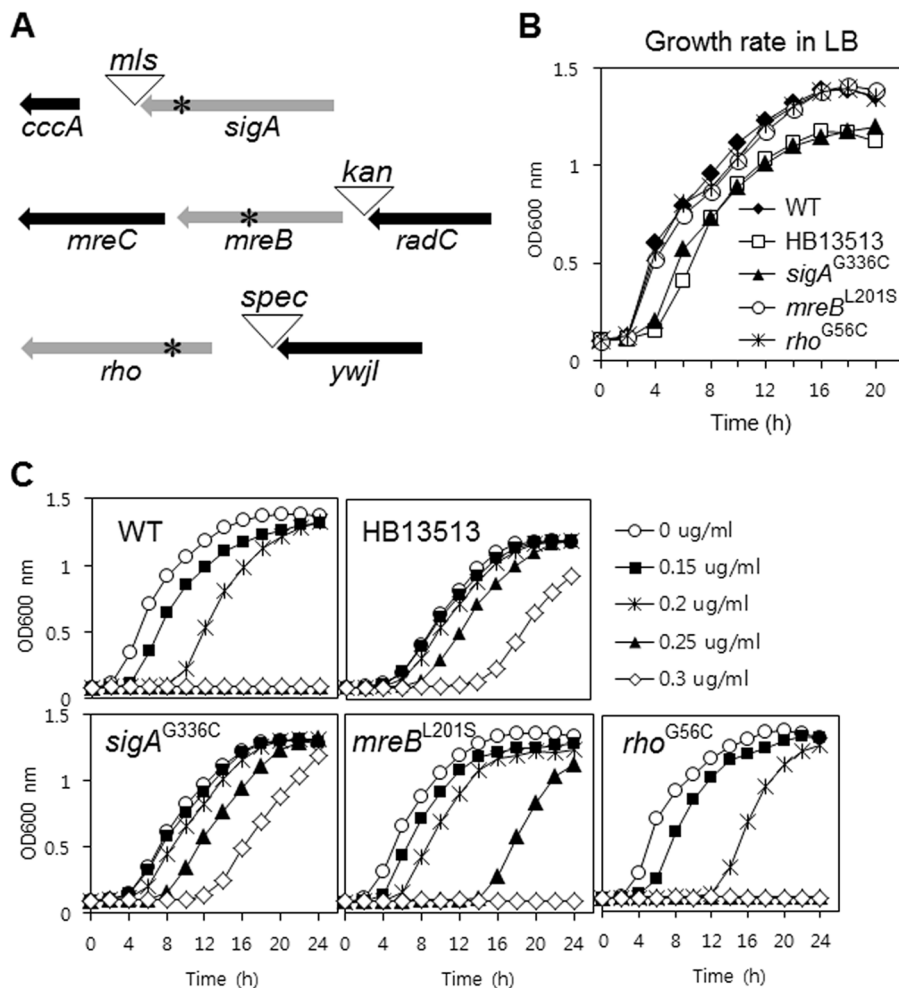


FIG 2 Identification of the genetic determinants for slow growth and reduced VAN susceptibility. (A) Genetic reconstruction of the substitutions in the wild-type chromosomal DNA. The antibiotic resistance cassette (*mls*, *kan*, or *spc*) was placed as a selectable marker after the stop codon or before the promoter region. The SNPs, indicated by asterisks, were moved into the parental wild-type strain by transformation of the LFH-PCR product. (B) Determinants of the slow-growth phenotype in the HB13513 mutant. Growth assays were performed in LB medium in the absence of antibiotic. (C) Determinants of reduced VAN susceptibility in the HB13513 mutant. Growth assays were performed in MH medium in the absence or presence of VAN. The data are representative of at least three independent experiments.

crease associated with the next two mutations at days 4 and 5 (which were also correlated with an increase in the MIC of VAN).

The *sigA*^{G336C} substitution is responsible for both altered growth and reduced VAN susceptibility. To determine which of these mutations confer the reduced susceptibility to VAN and/or CEF, we moved each point mutation into the parental wild-type strain, as described previously (2). An antibiotic resistance cassette was introduced as a selectable marker either after the stop codon or before the promoter region of each gene (Fig. 2A). These genetic-reconstruction experiments demonstrated that the altered growth phenotype (longer lag phase) results from the *sigA*^{G336C} substitution (Fig. 2B). This is consistent with the appearance of this mutation in the day 4 culture (Fig. 1E), which also had a longer lag phase (Fig. 1A). Our results provide an example of the general trend that antibiotic resistance mutations affecting essential genes often increase fitness in the presence of antibiotic but result in decreased fitness (including reductions in growth and virulence) in the absence of antibiotic (45).

To identify genetic determinants that affect susceptibility to

VAN, we compared the growth rates of the reconstructed strains in the presence of VAN using a Bioscreen C growth analyzer. As shown in Fig. 2C, the *sigA*^{G336C} substitution is largely responsible for the reduced VAN susceptibility, although the *mreB*^{L201S} substitution also has a slight effect. These results suggest that the *sigA*^{G336C} substitution is sufficient for reduced VAN susceptibility (comparable to that noted in strain HB13513) (Fig. 2C), but this change also results in overall reduced fitness (Fig. 2B).

Although the *sigA*^{G336C} substitution occurred at day 4 and the *mreB*^{L201S} substitution occurred at day 5 (Fig. 1E), the cultures from days 2 and 3 were also slightly less susceptible to VAN (Fig. 1D). This may be due to induction of an adaptive-tolerance response. For example, VAN is known to be an inducer of several cell envelope stress responses, including the σ^M regulon (30, 46) and the LiaRS two-component system (47).

The *sigA*^{G336C} and *rho*^{G56C} alleles together reduce susceptibility to CEF. A similar genetic-reconstruction strategy was used to identify the determinants of reduced CEF susceptibility in HB13513. These studies demonstrate that both *sigA*^{G336C} and

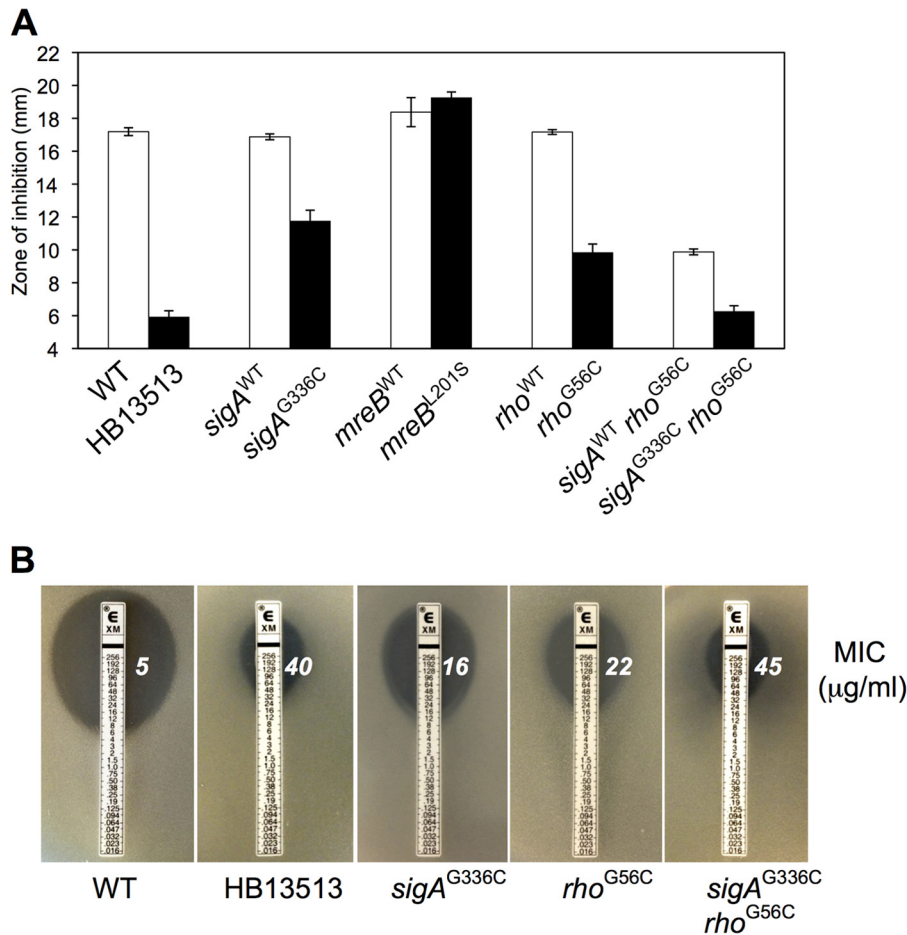


FIG 3 Identification of the genetic determinants for decreased CEF susceptibility. (A) Determination of the CEF susceptibilities of the reconstructed strains. Note that in the course of introducing each of these mutations into the parent strain (by selection for the linked antibiotic resistance marker [Fig. 2A]), recombination can occur either proximal to the SNP (leaving a wild-type allele in the chromosome) or distal to the SNP (leading to a strain with the SNP in the chromosome). The former integrants, which provide a control to rule out effects from the integrated antibiotic cassette, are designated by a superscript WT, and the latter are identified by the corresponding amino acid change. Disk diffusion assays were performed on MH agar plates with a filter paper disk containing 50 μg CEF. Each bar represents the average zone of inhibition, expressed as the total diameter minus the diameter of the filter paper disk (6.5 mm). Three independent experiments were performed for each strain, and the standard deviations are indicated by error bars. (B) Determination of MICs for CEF using an Etest assay. The Etest strips (bioMérieux) with CEF concentrations of 0.016 to 256 μg/ml were applied to MH agar plates, and the MIC was read by interpolation after 18 h of incubation at 37°C.

rho^{G56C} reduce CEF susceptibility, and the combination is sufficient to confer a 9-fold increase in the MIC (Fig. 3). In contrast, the *mreB*^{L201S} substitution was not directly involved in CEF resistance (Fig. 3A). Notably, the *rho*^{G56C} substitution led to an over 4-fold increase in the CEF MIC in the Etest assays (Fig. 3B). These results suggest that the reduced susceptibility to CEF in the HB13513 strain is attributable to the effects of both *sigA*^{G336C} and *rho*^{G56C}.

A *rho*-null mutant displays reduced susceptibility to β-lactam antibiotics. We next sought to determine how the *rho*^{G56C} mutation might affect Rho function. The active form of Rho is a ring-shaped homohexamer with RNA-binding, ATP hydrolysis, translocase, and RNA/DNA helicase activities (32, 48). Each monomer has two distinct functional domains: an N-terminal RNA-binding domain (RNA-BD) and a C-terminal ATP-binding domain (ATP-BD) (49), but the secondary RNA-binding site (SBS) also exists in the ATP-BD (50).

Analysis of conserved motifs in *B. subtilis* Rho using the NCBI

Conserved Domain database (51) revealed that the *rho*^{G56C} substitution lies close to the N-terminal RNA-binding domain (Fig. 4A). Since the 6 RNA-binding domains of the Rho hexamer together compose the primary RNA-binding site (52, 53), we hypothesized that this substitution might impair RNA binding to Rho and therefore would be a loss-of-function mutation. Indeed, the MIC of CEF for a *rho*-null mutant was exactly the same as that for the reconstructed *rho*^{G56C} strain (Fig. 4B). Bicyclomycin is an antibiotic that inhibits Rho function by interacting with its secondary RNA-binding site (54). Although *B. subtilis* is not sensitive to bicyclomycin (since Rho is not essential), bicyclomycin dramatically reduced CEF susceptibility (Fig. 4C), indicating that bicyclomycin works antagonistically with respect to β-lactams. Moreover, the *rho*-null mutant displayed reduced sensitivity to other broad-spectrum β-lactams, especially cephalosporins (Fig. 4D).

In *B. subtilis*, global transcriptomics approaches have recently revealed that a *rho*-null mutant results in numerous extended

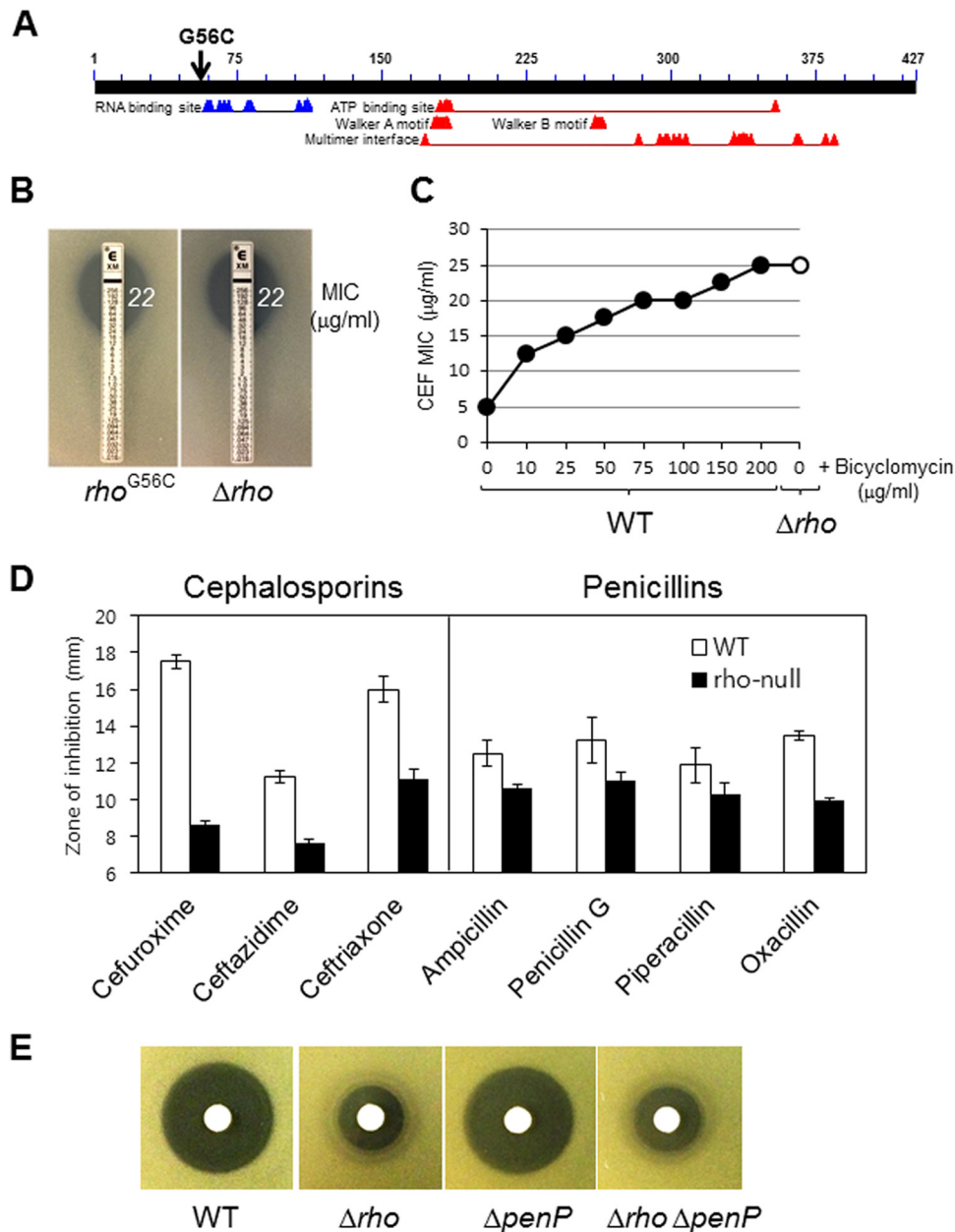


FIG 4 A ρ -null mutation or bicyclomycin reduces β -lactam susceptibility. (A) Schematic representation of the location of the ρ^{G56C} substitution. The conserved motifs were identified using the NCBI Conserved Domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). (B) Effect of the ρ gene deletion on the sensitivity of *B. subtilis* to CEF. MICs were determined using an Etest assay. (C) Effect of bicyclomycin inhibition on the sensitivity of *B. subtilis* to CEF. Treatment with the Rho inhibitor bicyclomycin increases the CEF MIC in a concentration-dependent manner. MICs were determined by the growth inhibition assay in MH broth. The data are reported as median values from three independent experiments. The MIC endpoint was established after 16 h of incubation. (D) Effect of the ρ gene deletion on the sensitivity of *B. subtilis* to broad-spectrum β -lactams. Disk diffusion assays were performed on MH agar plates. Three independent experiments were performed for each strain, and the standard deviations are indicated by error bars. (E) Effect of the $penP$ gene deletion on CEF resistance in the ρ -null mutant. A representative data set from the disk diffusion assay is shown.

mRNAs (up to 12 kb), thereby affecting the expression of dozens of downstream genes that collectively comprise $\sim 2\%$ of the genome (55). Interestingly, $penP$, which encodes a putative β -lactamase, is among the genes with 3'-extended mRNAs in a ρ -null mutant. We therefore hypothesized that a ρ -null mutation might affect the levels or translation of $penP$ mRNA, possibly accounting for the observed CEF resistance. However, $penP$ is not required for β -lactam resistance in the ρ -null mutant (Fig. 4E).

As β -lactams target the PBPs, we also examined the impact of pbp gene deletions on the reduced CEF susceptibility in the ρ -null mutant. Previously, we used a Bocillin-FL competition assay to demonstrate that CEF targets PBP1 ($ponA$), PBP2b ($pbpB$), PBP2c ($pbpF$), and PBP4 ($pbpD$) (56). To determine if increased expression of one or more of these PBPs might contribute to elevated CEF resistance, we measured the resistance of ρ -null strains additionally lacking one or more PBPs in a zone-of-inhi-

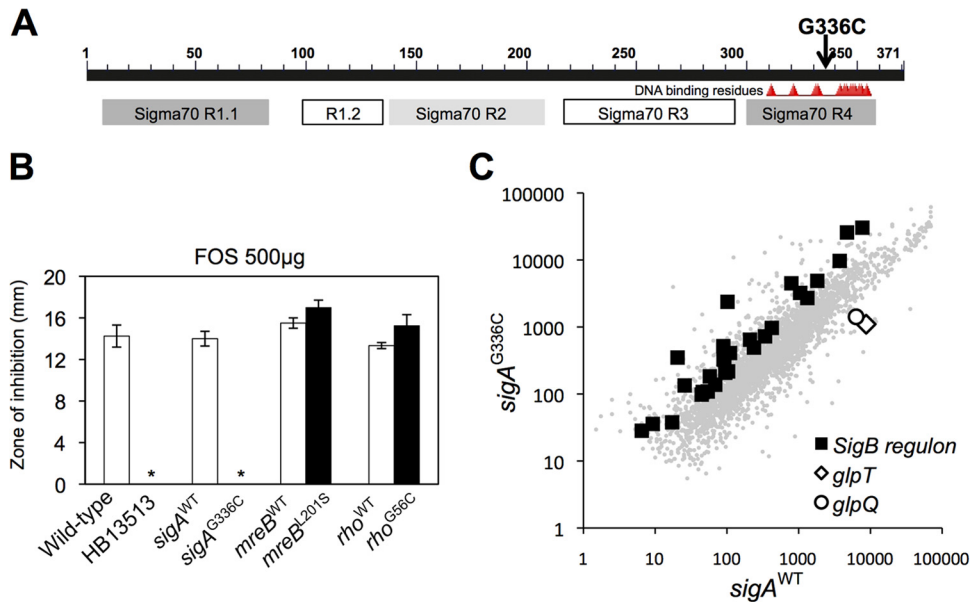


FIG 5 The *sigA*^{G336C} substitution drives the acquisition of FOS resistance. (A) Schematic representation of the location of the *sigA*^{G336C} substitution. The conserved motifs were identified by using the NCBI Conserved Domain database. (B) Determination of the FOS susceptibilities of the reconstructed strains. Disk diffusion assays were performed on MH agar plates with a filter paper disk containing FOS. The asterisks indicate no inhibition. The error bars indicate standard deviations. (C) Microarray transcriptional analysis of strains HB13679 (*sigA*^{WT}-*mls*) and HB13680 (*sigA*^{G336C}-*mls*) under nonstress conditions. RNA was extracted from cells grown in LB medium to an OD₆₀₀ of 0.4. The fluorescence intensity data are representative of two independent experiments, each of which was performed with dye swaps on each of three biological replicates. Each point represents the gene expression value for a single gene, with a subset of genes indicated by larger symbols as noted (see the text).

bition assay. However, none of the deletions tested led to a significant change in CEF resistance in the *rho*-null mutant. For example, *rho*-null mutant strains carrying an additional mutation(s) in *ponA*, *pbpD*, *pbpF*, *pbpG*, or *dacA* were at least as resistant to CEF as the *rho*-null mutant, and even a quadruple mutant (*rho*, *pbpD*, *pbpF*, and *pbpG*) was as resistant as the *rho* single mutant (data not shown). Thus, we conclude that inactivation of *rho* is a new genetic mechanism affecting β -lactam susceptibility in *B. subtilis*, but our results do not explain why this mutation confers resistance.

The *sigA*^{G336C} substitution affects sensitivity to multiple antibiotics. We next sought to determine how the *sigA*^{G336C} mutation alters antibiotic susceptibility. The *sigA*^{G336C} substitution is located in the helix-turn-helix (HTH) motif in region 4 that recognizes the -35 promoter element (Fig. 5A). Since *sigA* is essential, we hypothesized that this is an altered-function mutation likely affecting either core binding, promoter recognition, or possibly interaction with one or more regulatory proteins (57). We used disk diffusion assays to determine whether the *sigA*^{G336C} substitution affected susceptibility to other cell wall antibiotics, including fosfomycin, bacitracin, and D-cycloserine. Indeed, the *sigA*^{G336C} substitution made *B. subtilis* highly resistant to FOS, whereas the substitutions in *mreB* and *rho* did not (Fig. 5B). Our results demonstrate that the *sigA*^{G336C} substitution alone leads to reductions in susceptibility to CEF, VAN, and FOS.

The *sigA*^{G336C} substitution alters the transcriptome. We used microarray-based gene expression profiling to better understand the genetic basis for resistance to antibiotics in the *sigA*^{G336C} mutant. This mutation clearly leads to genome-wide changes in gene expression between the mutant (HB13680) and wild-type (HB13679) strains even under nonstress conditions (Fig. 5C). Sig-

nificantly, in the *sigA*^{G336C} mutant (HB13680), 28 genes of the σ^B general stress response regulon (58) were upregulated >2 -fold, and other operons (including *glpTQ*) were strongly (>4 -fold) downregulated (Fig. 5C). Since the mRNA level for *sigB* was not increased in the *sigA*^{G336C} mutant, the increased expression of the σ^B regulon is unlikely to be due to an increase in the σ^B protein level. One possible explanation is that the mutant σ^A has reduced stability or binding affinity to core, thereby enabling σ^B to compete more readily for RNA polymerase core enzyme. Alternatively, the mutant σ^A may alter the expression of proteins involved in the posttranslational control of σ^B activity.

The *sigA*^{G336C} substitution mediates CEF resistance by increased expression of the σ^B regulon. To determine whether σ^B is involved in the enhanced resistance to CEF, we introduced a null allele of *sigB* into the *sigA*^{G336C} mutant and performed disk diffusion assays. In contrast to the wild type, introduction of the *sigA*^{G336C} mutation did not lead to an increase in CEF resistance in the *sigB*-null mutant background. Indeed, the opposite was observed: the *sigB* *sigA*^{G336C} double mutant was more sensitive to CEF than the *sigB* single mutant, as judged by the diameter of growth inhibition (Fig. 6). However, after overnight growth, there was weak growth in the *sigA*^{G336C} mutant strain relatively close to the disk, consistent with some protective effect (Fig. 6, upper right). Nevertheless, these results suggest that *sigB* is critical for the ability of the *sigA*^{G336C} mutation to increase CEF resistance, consistent with the elevated expression of the σ^B regulon observed in the mutant strain (Fig. 5C). There are ~ 200 genes in the σ^B regulon (59–62), and the identity of the gene(s) that mediates reduced susceptibility to CEF is still unknown.

The *sigA*^{G336C} substitution mediates FOS resistance by decreased expression of *glpTQ*. We next sought to define the mech-

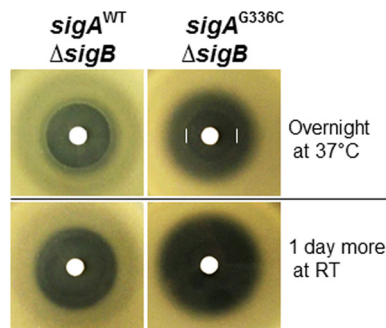


FIG 6 Reduced CEF susceptibility in the *sigA*^{G336C} mutant is dependent on σ^B . The *sigA*^{G336C} allele no longer decreases CEF susceptibility in strains carrying a *sigB* deletion. Disk diffusion assays were performed on MH agar plates with a filter paper disk containing 50 μ g CEF. Three independent experiments were performed for each strain, and one representative experiment is shown. Note that the *sigA*^{G336C} allele allows weak growth relatively close to the CEF disk, as visualized after overnight growth (upper right, white bars), but the overall diameters of the zones of growth inhibition are larger in the strains carrying the *sigA*^{G336C} and *sigB* alleles (right) than in the strains carrying the *sigB*-null mutant (left). RT, room temperature.

anism by which the *sigA*^{G336C} substitution confers resistance to FOS, which, unlike CEF and VAN, acts on the very early cytosolic steps of peptidoglycan biogenesis. We have shown previously that the σ^W -regulated *fosB* gene is a major FOS resistance determinant

(63), and a *bshC*-null mutant lacking bacillithiol (BSH), a cofactor for the FosB bacillithiol transferase, is also highly FOS sensitive (64). Although cells lacking FosB or BshC are very sensitive to FOS, they still displayed an increase in FOS resistance upon introduction of the *sigA*^{G336C} allele (Fig. 7A). We conclude that the *sigA*^{G336C} allele can act independently of FosB. Motivated by our characterization of the transcriptional changes resulting from the *sigA*^{G336C} substitution (Fig. 5C), we next asked whether σ^B might be involved in FOS resistance. In contrast with the results with CEF, disruption of *sigB* had no significant effect on the ability of the *sigA*^{G336C} substitution to confer FOS resistance (data not shown).

Mutations in *glpT* are known to be one mechanism for FOS resistance (65, 66), because GlpT is a glycerol-3-phosphate permease via which FOS enters the cell (67). The observed decrease in the transcription level of *glpT* seen in the microarray (Fig. 5C) was confirmed by RT-PCR analysis (Fig. 7B). This decreased expression is functionally significant, since the *sigA*^{G336C} mutant displayed a growth defect in minimal medium containing 2% glycerol-3-P, suggesting that it has lower levels of GlpT (Fig. 7C). To test whether this decreased expression of *glpT* was responsible for FOS resistance, the *glpT* operon was expressed under the control of the IPTG-inducible $P_{\text{spac(hy)}}$ promoter after integration into the *amyE* locus of the *sigA*^{G336C} mutant. Indeed, IPTG induction of *glpT* restored FOS sensitivity (Fig. 7D). These re-

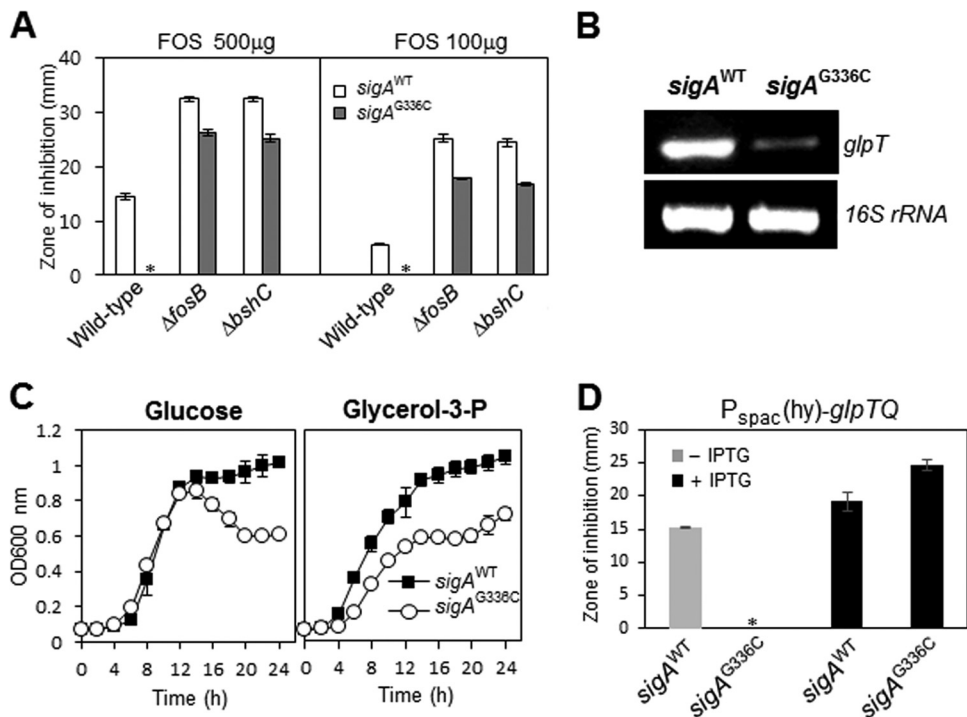


FIG 7 FOS resistance in the *sigA*^{G336C} mutant is largely due to decreased *glpT* transcription. (A) The *sigA*^{G336C} substitution can still confer FOS resistance in strains lacking *fosB* or *bshC*. Disk diffusion assays were performed on MH agar plates with a filter paper disk containing FOS. The asterisks indicate no inhibition. Each bar represents the average zone of inhibition, expressed as the total diameter minus the diameter of the filter paper disk (6.5 mm). Three independent experiments were performed for each strain, and the standard deviations are indicated by error bars. (B) RT-PCR analysis of *glpT* transcription. RNA was extracted from cells grown under the same conditions as for panel A. 16S rRNA was used as a loading control. (C) Growth comparison of strains HB13679 (*sigA*^{WT}-*mIs*) and HB13680 (*sigA*^{G336C}-*mIs*). Liquid growth assays were performed at 37°C in minimal medium supplemented with 2% glucose or 2% glycerol-3-P. The data are representative of at least three independent experiments. (D) IPTG induction of *glpT* restores FOS sensitivity in the *sigA*^{G336C} mutant. Disk diffusion assays were performed on MH agar plates with or without 0.1 mM IPTG. The asterisk indicates no inhibition. Three independent experiments were performed for each strain. The error bars indicate standard deviations.

sults suggest that the sigA^{G336C} substitution decreases the expression of *glpTQ* and that this decrease is sufficient to confer FOS resistance.

The molecular basis for the decreased expression of the *glpTQ* operon in the sigA^{G336C} mutant is unclear. We noticed that the residue affected by this mutation (G336) is close to residues previously implicated in contacting activator proteins. Specifically, amino acids near and within the first helix of the HTH motif in region 4 of the *E. coli* σ^{70} mediate contact with the response regulator PhoB (68). Therefore, we speculated that SigA^{G336C} may be defective for interaction with PhoP (the equivalent of the *E. coli* PhoB) or other, similar regulators. However, almost all PhoP-regulated genes are essentially unchanged in the sigA^{G336C} mutant (Fig. 5C and data not shown), suggesting that PhoPR activity is not affected by SigA^{G336C}. It is also known that only the *glpQ* gene of the *glpTQ* operon is under the control of PhoPR (69), and *glpT* has a SigA-type promoter (70). Thus, it is likely that transcription of the *glpTQ* operon is reduced directly due to a change in the promoter activation function of SigA^{G336C}.

Conclusions. Our data highlight the ability of mutational changes in the core transcriptional machinery to drive the evolution of decreased sensitivity to antibiotics in *B. subtilis*. To date, mutations in *rpoB* (β subunit) and *rpoC* (β' subunit) have been correlated with the emergence of changes in antibiotic resistance for *B. subtilis* and several human pathogens (2, 19, 20, 71). While the mechanistic bases for these changes are often unclear, we have shown previously that an *rpoC* mutation can decrease antibiotic susceptibility by increasing the activities of three extracytoplasmic function (ECF) σ factors (σ^M , σ^W , and σ^X) (72).

Here, we show that sigA^{G336C} is sufficient to reduce susceptibility to VAN and FOS and that it contributes (with *rho*^{G56C}) to decreased CEF susceptibility. In contrast with *rpoB* and *rpoC*, mutations in *sigA* and *rho* have not, to our knowledge, been previously linked to the emergence of antibiotic resistance in bacteria. Indeed, there are relatively few examples where altered-function mutations in a primary (essential) σ factor have emerged in random genetic selections (as opposed to experiments explicitly designed to recover such mutations). We are aware of three examples that include mutations leading to (i) an A-signaling defect in *Myxococcus xanthus* (73), (ii) suppression of defects associated with an inability to synthesize ppGpp in *E. coli* (74), and (iii) increased conversion of aminoimidazole ribotide to hydroxymethyl pyrimidine in *Salmonella enterica* (75). Here, we extend this list to include changes in antibiotic susceptibility. We also demonstrate that a *rho*-null mutation displays broad effects on susceptibility to β -lactam antibiotics in *B. subtilis*, but the mechanistic basis of this effect is presently unclear. Changes in Rho in *E. coli* have been previously shown to lead to widespread changes in the fitness landscape (76). Our results suggest that the Rho inhibitor bicyclomycin (which is used in agriculture as a feed additive) may increase the phenotypic resistance of Gram-positive bacteria to β -lactams. In sum, our data suggest that changes in the activity of the primary σ factor σ^A and the termination factor Rho may contribute to the evolution of antibiotic resistance.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (GM047446). Y.H.L. was partly supported by a research grant from Dongseo University (20140078).

REFERENCES

1. Taubes G. 2008. The bacteria fight back. *Science* 321:356–361. <http://dx.doi.org/10.1126/science.321.5887.356>.
2. Chopra I. 2013. The 2012 Garrod lecture: discovery of antibacterial drugs in the 21st century. *J. Antimicrob. Chemother.* 68:496–505. <http://dx.doi.org/10.1093/jac/dks436>.
3. Norrby SR, Nord CE, Finch R. 2005. Lack of development of new antimicrobial drugs: a potential serious threat to public health. *Lancet Infect. Dis.* 5:115–119. [http://dx.doi.org/10.1016/S1473-3099\(05\)10283-1](http://dx.doi.org/10.1016/S1473-3099(05)10283-1).
4. Berenbaum MC. 1989. What is synergy? *Pharmacol. Rev.* 41:93–141.
5. Eggimann P, Revelly JP. 2006. Should antibiotic combinations be used to treat ventilator-associated pneumonia? *Semin. Respir. Crit. Care Med.* 27:68–81. <http://dx.doi.org/10.1055/s-2006-933675>.
6. Yeh PJ, Hegreness MJ, Aiden AP, Kishony R. 2009. Drug interactions and the evolution of antibiotic resistance. *Nat. Rev. Microbiol.* 7:460–466. <http://dx.doi.org/10.1038/nrmicro2133>.
7. Yeh P, Tschumi AI, Kishony R. 2006. Functional classification of drugs by properties of their pairwise interactions. *Nat. Genet.* 38:489–494. <http://dx.doi.org/10.1038/ng1755>.
8. Climo MW, Patron RL, Archer GL. 1999. Combinations of vancomycin and beta-lactams are synergistic against staphylococci with reduced susceptibilities to vancomycin. *Antimicrob. Agents Chemother.* 43:1747–1753.
9. Perichon B, Courvalin P. 2006. Synergism between beta-lactams and glycopeptides against VanA-type methicillin-resistant *Staphylococcus aureus* and heterologous expression of the *vanA* operon. *Antimicrob. Agents Chemother.* 50:3622–3630. <http://dx.doi.org/10.1128/AAC.00410-06>.
10. Reynolds PE. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis.* 8:943–950. <http://dx.doi.org/10.1007/BF01967563>.
11. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* 32:234–258. <http://dx.doi.org/10.1111/j.1574-6976.2008.00105.x>.
12. Waxman DJ, Strominger JL. 1983. Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Annu. Rev. Biochem.* 52:825–869. <http://dx.doi.org/10.1146/annurev.bi.52.070183.004141>.
13. Hegreness M, Shores N, Damian D, Hartl D, Kishony R. 2008. Accelerated evolution of resistance in multidrug environments. *Proc. Natl. Acad. Sci. U. S. A.* 105:13977–13981. <http://dx.doi.org/10.1073/pnas.0805965105>.
14. Hachmann AB, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD. 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. *Antimicrob. Agents Chemother.* 55:4326–4337. <http://dx.doi.org/10.1128/AAC.01819-10>.
15. Tomasz A. 2013. The use of whole genome sequencing to solve an epidemiological puzzle. *EMBO Mol. Med.* 5:486–487. <http://dx.doi.org/10.1002/emmm.201302622>.
16. Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A. 2007. Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proc. Natl. Acad. Sci. U. S. A.* 104:9451–9456. <http://dx.doi.org/10.1073/pnas.0609839104>.
17. Hegreness M, Kishony R. 2007. Analysis of genetic systems using experimental evolution and whole-genome sequencing. *Genome Biol.* 8:201. <http://dx.doi.org/10.1186/gb-2007-8-1-201>.
18. Edwards DJ, Holt KE. 2013. Beginner's guide to comparative bacterial genome analysis using next-generation sequence data. *Microb. Inform. Exp.* 3:2. <http://dx.doi.org/10.1186/2042-5783-3-2>.
19. Cui L, Isii T, Fukuda M, Ochiai T, Neoh HM, Camargo IL, Watanabe Y, Shoji M, Hishinuma T, Hiramatsu K. 2010. An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 54:5222–5233. <http://dx.doi.org/10.1128/AAC.00437-10>.
20. Friedman L, Alder JD, Silverman JA. 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 50:2137–2145. <http://dx.doi.org/10.1128/AAC.00039-06>.
21. Watanabe Y, Cui L, Katayama Y, Kozue K, Hiramatsu K. 2011. Impact of *rpoB* mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. *J. Clin. Microbiol.* 49:2680–2684. <http://dx.doi.org/10.1128/JCM.02144-10>.

22. Conrad TM, Frazier M, Joyce AR, Cho BK, Knight EM, Lewis NE, Landick R, Pálsson BO. 2010. RNA polymerase mutants found through adaptive evolution reprogram *Escherichia coli* for optimal growth in minimal media. *Proc. Natl. Acad. Sci. U. S. A.* 107:20500–20505. <http://dx.doi.org/10.1073/pnas.0911253107>.
23. Brinkman CL, Bumgarner R, Kittichotirat W, Dunman PM, Kuechenmeister LJ, Weaver KE. 2013. Characterization of the effects of an *rpoC* mutation that confers resistance to the Fst peptide toxin-antitoxin system toxin. *J. Bacteriol.* 195:156–166. <http://dx.doi.org/10.1128/JB.01597-12>.
24. Murakami KS, Darst SA. 2003. Bacterial RNA polymerases: the whole story. *Curr. Opin. Struct. Biol.* 13:31–39. [http://dx.doi.org/10.1016/S0959-440X\(02\)00005-2](http://dx.doi.org/10.1016/S0959-440X(02)00005-2).
25. Juang YL, Helmann JD. 1994. The delta subunit of *Bacillus subtilis* RNA polymerase. An allosteric effector of the initiation and core-recycling phases of transcription. *J. Mol. Biol.* 239:1–14.
26. Lopez de Saro FJ, Woody AY, Helmann JD. 1995. Structural analysis of the *Bacillus subtilis* delta factor: a protein polyanion which displaces RNA from RNA polymerase. *J. Mol. Biol.* 252:189–202. <http://dx.doi.org/10.1006/jmbi.1995.0487>.
27. Borukhov S, Severinov K. 2002. Role of the RNA polymerase sigma subunit in transcription initiation. *Res. Microbiol.* 153:557–562. [http://dx.doi.org/10.1016/S0923-2508\(02\)01368-2](http://dx.doi.org/10.1016/S0923-2508(02)01368-2).
28. Gruber TM, Gross CA. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* 57:441–466. <http://dx.doi.org/10.1146/annurev.micro.57.030502.090913>.
29. Paget MS, Helmann JD. 2003. The sigma70 family of sigma factors. *Genome Biol.* 4:203. <http://dx.doi.org/10.1186/gb-2003-4-1-203>.
30. Cao M, Wang T, Ye R, Helmann JD. 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma(W) and sigma(M) regulons. *Mol. Microbiol.* 45:1267–1276. <http://dx.doi.org/10.1046/j.1365-2958.2002.03050.x>.
31. Peters JM, Vangeloff AD, Landick R. 2011. Bacterial transcription terminators: the RNA 3'-end chronicles. *J. Mol. Biol.* 412:793–813. <http://dx.doi.org/10.1016/j.jmb.2011.03.036>.
32. Richardson JP. 2002. Rho-dependent termination and ATPases in transcript termination. *Biochim. Biophys. Acta* 1577:251–260. [http://dx.doi.org/10.1016/S0167-4781\(02\)00456-6](http://dx.doi.org/10.1016/S0167-4781(02)00456-6).
33. Washburn RS, Marra A, Bryant AP, Rosenberg M, Gentry DR. 2001. *rho* is not essential for viability or virulence in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 45:1099–1103. <http://dx.doi.org/10.1128/AAC.45.4.1099-1103.2001>.
34. Opperman T, Richardson JP. 1994. Phylogenetic analysis of sequences from diverse bacteria with homology to the *Escherichia coli rho* gene. *J. Bacteriol.* 176:5033–5043.
35. Das A, Court D, Adhya S. 1976. Isolation and characterization of conditional lethal mutants of *Escherichia coli* defective in transcription termination factor *rho*. *Proc. Natl. Acad. Sci. U. S. A.* 73:1959–1963. <http://dx.doi.org/10.1073/pnas.73.6.1959>.
36. Quirk PG, Dunkley EA, Jr, Lee P, Krulwich TA. 1993. Identification of a putative *Bacillus subtilis rho* gene. *J. Bacteriol.* 175:647–654.
37. Yakhnin H, Babiarez JE, Yakhnin AV, Babbitzke P. 2001. Expression of the *Bacillus subtilis trpEDCFBA* operon is influenced by translational coupling and Rho termination factor. *J. Bacteriol.* 183:5918–5926. <http://dx.doi.org/10.1128/JB.183.20.5918-5926.2001>.
38. Ingham CJ, Dennis J, Furneaux PA. 1999. Autogenous regulation of transcription termination factor Rho and the requirement for Nus factors in *Bacillus subtilis*. *Mol. Microbiol.* 31:651–663. <http://dx.doi.org/10.1046/j.1365-2958.1999.01205.x>.
39. Nowatzke WL, Keller E, Koch G, Richardson JP. 1997. Transcription termination factor Rho is essential for *Micrococcus luteus*. *J. Bacteriol.* 179:5238–5240.
40. Mascher T, Margulis NG, Wang T, Ye RW, Helmann JD. 2003. Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol. Microbiol.* 50:1591–1604. <http://dx.doi.org/10.1046/j.1365-2958.2003.03786.x>.
41. Srivatsan A, Han Y, Peng J, Tehranchi AK, Gibbs R, Wang JD, Chen R. 2008. High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genet.* 4:e1000139. <http://dx.doi.org/10.1371/journal.pgen.1000139>.
42. Sambrook J, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. Quisel JD, Burkholder WF, Grossman AD. 2001. In vivo effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. *J. Bacteriol.* 183:6573–6578. <http://dx.doi.org/10.1128/JB.183.22.6573-6578.2001>.
44. Hachmann AB, Angert ER, Helmann JD. 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. *Antimicrob. Agents Chemother.* 53:1598–1609. <http://dx.doi.org/10.1128/AAC.01329-08>.
45. Andersson DI, Levin BR. 1999. The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* 2:489–493. [http://dx.doi.org/10.1016/S1369-5274\(99\)00005-3](http://dx.doi.org/10.1016/S1369-5274(99)00005-3).
46. Eiamphungporn W, Helmann JD. 2008. The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. *Mol. Microbiol.* 67:830–848. <http://dx.doi.org/10.1111/j.1365-2958.2007.06090.x>.
47. Mascher T, Zimmer SL, Smith TA, Helmann JD. 2004. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob. Agents Chemother.* 48:2888–2896. <http://dx.doi.org/10.1128/AAC.48.8.2888-2896.2004>.
48. Skordalakes E, Berger JM. 2006. Structural insights into RNA-dependent ring closure and ATPase activation by the Rho termination factor. *Cell* 127:553–564. <http://dx.doi.org/10.1016/j.cell.2006.08.051>.
49. Bear DG, Andrews CL, Singer JD, Morgan WD, Grant RA, von Hippel PH, Platt T. 1985. *Escherichia coli* transcription termination factor Rho has a two-domain structure in its activated form. *Proc. Natl. Acad. Sci. U. S. A.* 82:1911–1915. <http://dx.doi.org/10.1073/pnas.82.7.1911>.
50. Wei RR, Richardson JP. 2001. Identification of an RNA-binding site in the ATP binding domain of *Escherichia coli* Rho by H₂O₂/Fe-EDTA cleavage protection studies. *J. Biol. Chem.* 276:28380–28387. <http://dx.doi.org/10.1074/jbc.M102444200>.
51. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwartz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullochandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH. 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* 39:D225–D229. <http://dx.doi.org/10.1093/nar/gkq1189>.
52. Modrak D, Richardson JP. 1994. The RNA-binding domain of transcription termination factor rho: isolation, characterization, and determination of sequence limits. *Biochemistry* 33:8292–8299. <http://dx.doi.org/10.1021/bi00193a016>.
53. Skordalakes E, Berger JM. 2003. Structure of the Rho transcription terminator: mechanism of mRNA recognition and helicase loading. *Cell* 114:135–146. [http://dx.doi.org/10.1016/S0092-8674\(03\)00512-9](http://dx.doi.org/10.1016/S0092-8674(03)00512-9).
54. Magyar A, Zhang X, Kohn H, Widger WR. 1996. The antibiotic bicyclomycin affects the secondary RNA binding site of *Escherichia coli* transcription termination factor Rho. *J. Biol. Chem.* 271:25369–25374. <http://dx.doi.org/10.1074/jbc.271.41.25369>.
55. Nicolas P, Mader U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, Bidnenko E, Marchadier E, Hoebeke M, Aymerich S, Becher D, Bisicchia P, Botella E, Delumeau O, Doherty G, Denham EL, Fogg MJ, Fromion V, Goelzer A, Hansen A, Hartig E, Harwood CR, Homuth G, Jarmer H, Jules M, Klipp E, Le Chat L, Lecointe F, Lewis P, Liebermeister W, March A, Mars RA, Nannapaneni P, Noone D, Pohl S, Rinn B, Rugheimer F, Sappa PK, Samson F, Schaffer M, Schwikowski B, Steil L, Stulke J, Wiegert T, Devine KM, Wilkinson AJ, van Dijl JM, Hecker M, Volker U, Bessieres P, Noirot P. 2012. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* 335:1103–1106. <http://dx.doi.org/10.1126/science.1206848>.
56. Luo Y, Helmann JD. 2012. Analysis of the role of *Bacillus subtilis* sigma(M) in beta-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol. Microbiol.* 83:623–639. <http://dx.doi.org/10.1111/j.1365-2958.2011.07953.x>.
57. Gruber TM, Markov D, Sharp MM, Young BA, Lu CZ, Zhong HJ, Artsimovitch I, Geszvain KM, Arthur TM, Burgess RR, Landick R, Severinov K, Gross CA. 2001. Binding of the initiation factor sigma(70) to core RNA polymerase is a multistep process. *Mol. Cell* 8:21–31. [http://dx.doi.org/10.1016/S1097-2765\(01\)00292-1](http://dx.doi.org/10.1016/S1097-2765(01)00292-1).
58. Hecker M, Pane-Farre J, Volker U. 2007. SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu. Rev. Microbiol.* 61:215–236. <http://dx.doi.org/10.1146/annurev.micro.61.080706.093445>.
59. Helmann JD, Wu MF, Kobel PA, Gamo FJ, Wilson M, Morshedi MM, Navre M, Paddon C. 2001. Global transcriptional response of *Bacillus*

- subtilis* to heat shock. J. Bacteriol. 183:7318–7328. <http://dx.doi.org/10.1128/JB.183.24.7318-7328.2001>.
60. Nannapaneni P, Hertwig F, Depke M, Hecker M, Mader U, Volker U, Steil L, van Hijum SA. 2012. Defining the structure of the general stress regulon of *Bacillus subtilis* using targeted microarray analysis and random forest classification. Microbiology 158:696–707. <http://dx.doi.org/10.1099/mic.0.055434-0>.
 61. Petersohn A, Brigulla M, Haas S, Hoheisel JD, Volker U, Hecker M. 2001. Global analysis of the general stress response of *Bacillus subtilis*. J. Bacteriol. 183:5617–5631. <http://dx.doi.org/10.1128/JB.183.19.5617-5631.2001>.
 62. Price CW, Fawcett P, Ceremonie H, Su N, Murphy CK, Youngman P. 2001. Genome-wide analysis of the general stress response in *Bacillus subtilis*. Mol. Microbiol. 41:757–774. <http://dx.doi.org/10.1046/j.1365-2958.2001.02534.x>.
 63. Cao M, Bernat BA, Wang Z, Armstrong RN, Helmann JD. 2001. FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*. J. Bacteriol. 183:2380–2383. <http://dx.doi.org/10.1128/JB.183.7.2380-2383.2001>.
 64. Gaballa A, Newton GL, Antelmann H, Parsonage D, Upton H, Rawat M, Claiborne A, Fahey RC, Helmann JD. 2010. Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli. Proc. Natl. Acad. Sci. U. S. A. 107:6482–6486. <http://dx.doi.org/10.1073/pnas.1000928107>.
 65. Bordi C, Butcher BG, Shi Q, Hachmann AB, Peters JE, Helmann JD. 2008. In vitro mutagenesis of *Bacillus subtilis* by using a modified Tn7 transposon with an outward-facing inducible promoter. Appl. Environ. Microbiol. 74:3419–3425. <http://dx.doi.org/10.1128/AEM.00476-08>.
 66. Lindgren V. 1978. Mapping of a genetic locus that affects glycerol 3-phosphate transport in *Bacillus subtilis*. J. Bacteriol. 133:667–670.
 67. Santoro A, Cappello AR, Madeo M, Martello E, Iacopetta D, Dolce V. 2011. Interaction of fosfomycin with the glycerol 3-phosphate transporter of *Escherichia coli*. Biochim. Biophys. Acta 1810:1323–1329. <http://dx.doi.org/10.1016/j.bbagen.2011.07.006>.
 68. Makino K, Amemura M, Kim SK, Nakata A, Shinagawa H. 1993. Role of the sigma 70 subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. Genes Dev. 7:149–160. <http://dx.doi.org/10.1101/gad.7.1.149>.
 69. Antelmann H, Scharf C, Hecker M. 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. J. Bacteriol. 182:4478–4490. <http://dx.doi.org/10.1128/JB.182.16.4478-4490.2000>.
 70. Nilsson RP, Beijer L, Rutberg B. 1994. The *glpT* and *glpQ* genes of the glycerol regulon in *Bacillus subtilis*. Microbiology 140:723–730. <http://dx.doi.org/10.1099/00221287-140-4-723>.
 71. Howden BP, Peleg AY, Stinear TP. 2014. The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. Infect. Genet. Evol. 21:575–582. <http://dx.doi.org/10.1016/j.meegid.2013.03.047>.
 72. Lee YH, Nam KH, Helmann JD. 2013. A mutation of the RNA polymerase beta' subunit (*rpoC*) confers cephalosporin resistance in *Bacillus subtilis*. Antimicrob. Agents Chemother. 57:56–65. <http://dx.doi.org/10.1128/AAC.01449-12>.
 73. Davis JM, Mayor J, Plamann L. 1995. A missense mutation in *rpoD* results in an A-signalling defect in *Myxococcus xanthus*. Mol. Microbiol. 18:943–952. <http://dx.doi.org/10.1111/j.1365-2958.1995.18050943.x>.
 74. Hernandez VJ, Cashel M. 1995. Changes in conserved region 3 of *Escherichia coli* sigma 70 mediate ppGpp-dependent functions in vivo. J. Mol. Biol. 252:536–549. <http://dx.doi.org/10.1006/jmbi.1995.0518>.
 75. Dougherty MJ, Downs DM. 2004. A mutant allele of *rpoD* results in increased conversion of aminoimidazole ribotide to hydroxymethyl pyrimidine in *Salmonella enterica*. J. Bacteriol. 186:4034–4037. <http://dx.doi.org/10.1128/JB.186.12.4034-4037.2004>.
 76. Freddolino PL, Goodarzi H, Tvazoie S. 2012. Fitness landscape transformation through a single amino acid change in the Rho terminator. PLoS genetics 8:e1002744. <http://dx.doi.org/10.1371/journal.pgen.1002744>.