

A Mutation of the RNA Polymerase β' Subunit (*rpoC*) Confers Cephalosporin Resistance in *Bacillus subtilis*

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In bacteria, mutations affecting the major catalytic subunits of RNA polymerase (encoded by *rpoB* and *rpoC*) emerge in response to a variety of selective pressures. Here we isolated a *Bacillus subtilis* strain with high-level resistance to cefuroxime (CEF). Whole-genome resequencing revealed only one missense mutation affecting an invariant residue in close proximity to the C-terminal DNA-binding domain of RpoC (G1122D). Genetic reconstruction experiments demonstrate that this substitution is sufficient to confer CEF resistance. The G1122D mutation leads to elevated expression of stress-responsive regulons, including those of extracytoplasmic function (ECF) σ factors (σ^M , σ^W , and σ^X) and the general stress σ factor (σ^B). The increased CEF resistance of the *rpoC*^{G1122D} strain is lost in the *sigM rpoC*^{G1122D} double mutant, consistent with a major role for σ^M in CEF resistance. However, a *sigM* mutant is very sensitive to CEF, and this sensitivity is still reduced by the G1122D mutation, suggesting that other regulatory effects are also important. Indeed, the ability of the G1122D mutation to increase CEF resistance is further reduced in a triple mutant strain lacking three ECF σ factors (σ^M , σ^W , and σ^X), which are known from prior studies to control overlapping sets of genes. Collectively, our findings highlight the ability of mutations in RNA polymerase to confer antibiotic resistance by affecting the activity of alternative σ factors that control cell envelope stress-responsive regulons.

Transcription in all cellular organisms is driven by a multisubunit DNA-dependent RNA polymerase (RNAP) with a conserved crab claw-like shape that embraces the template DNA (1). The bacterial RNAP holoenzyme consists minimally of the core ($\alpha_2\beta\beta'\omega$) enzyme and one of a family of σ factors (2–4). Gram-positive bacteria have an additional δ subunit that enhances transcriptional specificity by blocking RNAP binding at weak promoter sites (5, 6). For transcription initiation, the core enzyme must associate with one of various σ factors that recognize different promoter sequences and thus enable specific binding of RNAP to gene promoters (7). The resulting holoenzyme forms an open complex by melting the DNA near the transcription start site and then begins to synthesize RNA (8, 9). Once the initial 10 or so nucleotides of RNA have been synthesized, the σ factor is released from the core enzyme, which elongates the RNA until it encounters a termination signal (10).

Bacteria have one housekeeping σ factor, σ^{70} , and a variable number of alternative σ factors that are activated in response to different environmental conditions (2, 7, 11). The extracytoplasmic function (ECF) σ factors, which often respond to cell envelope stress, are the largest group of alternative σ factors (11, 12). The core enzyme is highly conserved in sequence and structure from bacteria to humans, particularly among the large-subunit (the bacterial β and β' subunits) homologs (1, 13). Four structurally conserved modules, known as the β' jaw, β' clamp, β lobes, and β flap, play a crucial role in proper accommodation of the σ factor and DNA within RNAP (10, 14).

RNAP is one of the most central transcriptional regulatory hubs, and mutations affecting RNAP can arise during many selections (15–19). Mutant versions of the α subunit (RpoA) are able to substantially alter the cellular phenotype (20), and mutations in β (RpoB) that lead to resistance to rifampin in *Bacillus subtilis* also lead to altered expression of regulons involved in growth, competence, sporulation, and germination (21). In addition, mutations of the β' subunit (RpoC) allow regulatory adaptation for optimal growth in minimal medium (15). The substitution of one σ factor

for another reprograms the transcriptional profile, and it is therefore plausible that mutations to the core enzyme that affect σ activity could effect broad and complex changes to the transcriptome.

Cephalosporins are β -lactam antibiotics that interfere with bacterial cell wall cross-linking catalyzed by transpeptidases known as penicillin-binding proteins (PBPs) (22). Three primary mechanisms lead to β -lactam resistance (23–25). The most common is the production of β -lactamases, which serve to hydrolyze the β -lactam ring. Alternatively, changes in the active site of PBPs, which reduce the affinity for β -lactams, may arise. Finally, efflux pumps may eject β -lactams from the periplasm before they reach the PBP targets. Interestingly, none of these mechanisms appears to account for the development of β -lactam resistance in *B. subtilis*. Previously, we have reported that the intrinsic level of resistance to cefuroxime (CEF), an expanded-spectrum β -lactam cephalosporin, is substantially reduced in cells lacking multiple ECF σ factors (26), in particular σ^M (27). Whereas loss of ECF σ factors leads to a significant increase in CEF sensitivity, the mechanisms that might lead to high-level CEF resistance remain unknown.

Here we employed whole-genome resequencing to identify a single point mutation (G1122D) in *B. subtilis rpoC* that imparts CEF resistance. Homology modeling suggests that this mutation affects an absolutely conserved G1122 residue in close proximity to the DNA-binding site of RpoC that may thereby affect processes of promoter engagement. Consistent with our prior studies,

Received 13 July 2012 Returned for modification 14 September 2012

Accepted 6 October 2012

Published ahead of print 15 October 2012

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doi:10.1128/AAC.01449-12

TABLE 1 Bacterial strains and plasmid used in this study

Strain or plasmid	Genotype or description	Source or reference ^a
Strains		
W168	<i>trpC2</i>	BGSC accession no. 1A1
HB13506	W168 <i>rpoC</i> ^{G1122D} <i>citM</i> ::Tn7(CEF-resistant isolate)	Tn7 library with W168
HB13576	W168 <i>rpoC</i> ^{WT} - <i>kan</i>	LFH PCR with W168
HB13577	W168 <i>rpoC</i> ^{G1122D} - <i>kan</i>	LFH PCR with W168
HB10016	168 <i>sigM</i> :: <i>tet</i>	56
HB13591	W168 <i>sigM</i> :: <i>tet</i>	HB10016 chr DNA with W168
HB7007	CU1065 <i>sigX</i> :: <i>spec</i>	29
HB13592	W168 <i>sigX</i> :: <i>spec</i>	HB7007 chr DNA with W168
HB13549	W168 <i>sigW</i> :: <i>mls</i>	30
HB13634	W168 <i>sigM</i> :: <i>tet sigX</i> :: <i>spec</i>	HB7007 chr DNA with HB13591
HB13635	W168 <i>sigM</i> :: <i>tet sigW</i> :: <i>mls sigX</i> :: <i>spec</i>	HB13549 chr DNA with HB13634
HB553	CU1065 <i>sigB</i> :: <i>cat</i>	Laboratory stock
HB13551	W168 <i>sigB</i> :: <i>cat</i>	HB553 chr DNA with W168
HB13552	W168 <i>sigB</i> :: <i>cat sigW</i> :: <i>mls</i>	HB13551 chr DNA with HB13549
HB13528	W168 <i>rocG</i> :: <i>spec</i>	30
HB10348	168 <i>spx</i> :: <i>mls</i>	27
HB13599	W168 <i>spx</i> :: <i>mls</i>	HB10348 chr DNA with W168
HB10381	168 <i>disA</i> :: <i>cat</i>	27
HB13601	W168 <i>disA</i> :: <i>cat</i>	HB10381 chr DNA with W168
HB13547	W168 <i>yuaFG</i> :: <i>mls</i>	30
HB13618	W168 <i>yuaFG</i> :: <i>spec</i>	ECE79 with HB13547
HB13605	W168 <i>spx</i> :: <i>mls disA</i> :: <i>cat</i>	HB10381 chr DNA with HB13599
HB13619	W168 <i>spx</i> :: <i>mls yuaFG</i> :: <i>spec</i>	HB13618 chr DNA with HB13599
HB13579	W168 <i>sigB</i> :: <i>cat rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13551
HB13580	W168 <i>sigB</i> :: <i>cat rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13551
HB13581	W168 <i>sigW</i> :: <i>mls rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13549
HB13582	W168 <i>sigW</i> :: <i>mls rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13549
HB13583	W168 <i>sigB</i> :: <i>cat sigW</i> :: <i>mls rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13552
HB13584	W168 <i>sigB</i> :: <i>cat sigW</i> :: <i>mls rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13552
HB13593	W168 <i>sigM</i> :: <i>tet rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13591
HB13594	W168 <i>sigM</i> :: <i>tet rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13591
HB13595	W168 <i>sigX</i> :: <i>spec rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13592
HB13596	W168 <i>sigX</i> :: <i>spec rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13592
HB13636	W168 <i>sigM</i> :: <i>tet sigW</i> :: <i>mls sigX</i> :: <i>spec rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13635
HB13637	W168 <i>sigM</i> :: <i>tet sigW</i> :: <i>mls sigX</i> :: <i>spec rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13635
HB13597	W168 <i>rocG</i> :: <i>spec rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13528
HB13598	W168 <i>rocG</i> :: <i>spec rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13528
HB13602	W168 <i>spx</i> :: <i>mls rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13599
HB13603	W168 <i>spx</i> :: <i>mls rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13599
HB13614	W168 <i>disA</i> :: <i>cat rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13601
HB13615	W168 <i>disA</i> :: <i>cat rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13601
HB13621	W168 <i>yuaFG</i> :: <i>spec rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13618
HB13622	W168 <i>yuaFG</i> :: <i>spec rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13618
HB13608	W168 <i>spx</i> :: <i>mls disA</i> :: <i>cat rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13605
HB13609	W168 <i>spx</i> :: <i>mls disA</i> :: <i>cat rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13605
HB13623	W168 <i>spx</i> :: <i>mls yuaFG</i> :: <i>spec rpoC</i> ^{WT} - <i>kan</i>	HB13576 chr DNA with HB13619
HB13624	W168 <i>spx</i> :: <i>mls yuaFG</i> :: <i>spec rpoC</i> ^{G1122D} - <i>kan</i>	HB13577 chr DNA with HB13619
Plasmid ECE79	pErm::Spec (cassette switching vector)	31

^a chr, chromosomal.

this mutation leads to elevated activity of ECF σ factors (σ^M , σ^W , and σ^X), which can thereby lead to CEF resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study, unless otherwise noted, are derivatives of wild-type *B. subtilis* W168 (*Bacillus* Genetic Stock Center [BGSC] accession number 1A1), and all are listed in Table 1. Cells were routinely cultured in Luria-Bertani (LB) broth at 37°C with vigorous shaking or on LB agar plates containing 1.5% (wt/

vol) Bacto agar (Difco). The following antibiotics were used for selection: spectinomycin (Spec; 100 μ g/ml), tetracycline (Tet; 5 μ g/ml), kanamycin (Kan; 15 μ g/ml), chloramphenicol (Cat; 10 μ g/ml), or macrolide-lincosamide-streptogramin B (MLS) (contains 1 μ g/ml erythromycin and 25 μ g/ml lincomycin). Mutations were introduced into *B. subtilis* by transformation as described previously (32).

Tn7 transposon mutagenesis. The modified Tn7 transposon mutagenesis system mTn7SX was used to select mutants with increased resistance to CEF (33). The amplified Tn7 library DNA was introduced by

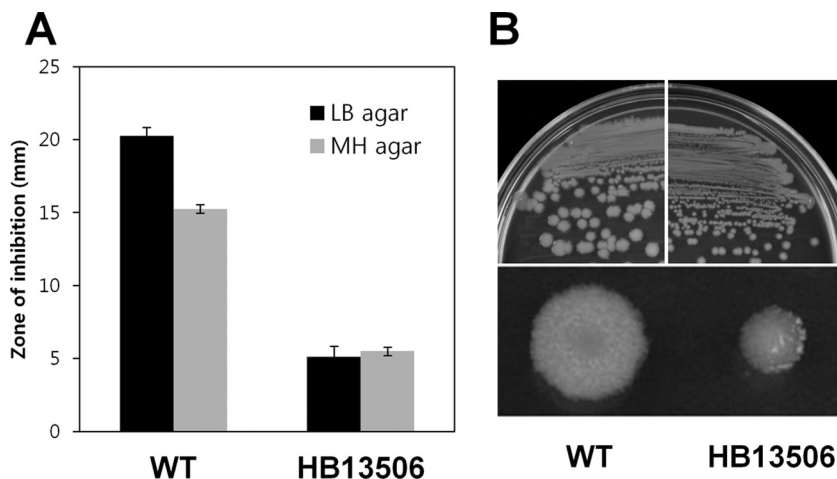


FIG 1 Isolation of a slow-growing *B. subtilis* strain with high-level resistance to CEF. (A) Susceptibility to CEF was determined by disk diffusion assays, which were performed on LB or MH agar plates with a filter paper disk containing 30 μg CEF. Each bar represents the average zone of inhibition, 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 deviation is indicated by error bars. (B) Sizes of wild-type and HB13506 mutant colonies grown on LB agar plates. Cells were streaked or spread onto plates and were incubated for 1 day at 37°C.

transformation into the wild-type *B. subtilis* strain, and cells were spread onto LB agar plates containing 100 $\mu\text{g}/\text{ml}$ Spec and 15 $\mu\text{g}/\text{ml}$ CEF. To confirm that the increased CEF resistance of the Tn7 mutants is due to the transposon insertion, linkage tests were performed by transforming the chromosomal DNA of the Tn7 mutants into the wild-type strain and selecting with 100 $\mu\text{g}/\text{ml}$ Spec. The 10 transformants were restreaked onto LB agar plates containing 100 $\mu\text{g}/\text{ml}$ Spec and 15 $\mu\text{g}/\text{ml}$ CEF in order to determine whether the CEF resistance is linked to a transposon insertion. The Tn7 insertion site was determined by PCR amplification and sequencing of the Tn7-chromosomal DNA junction (34).

Whole-genome resequencing. Chromosomal DNA was isolated from strains W168 and HB13506 grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.4 by using the Qiagen DNeasy blood and tissue kit. The quantity and purity of DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and DNA was sequenced and analyzed by the Cornell University Life Sciences Core Laboratories Center using Illumina DNA sequencing. The sequence data were assembled with MOSAIC by using the reference sequence (35) under GenBank accession number [ABQK000000000](https://www.ncbi.nlm.nih.gov/nuccore/ABQK000000000).

Genetic reconstruction. The mutated *rpoC* allele was introduced into the wild-type chromosome by using long-flanking homology (LFH) PCR followed by DNA transformation as described previously (36, 37). The upstream fragment, ending 34 nucleotides after the stop codon of the *rpoC*^{G1122D} allele, was amplified from the HB13506 mutant using primers *rpoC* up-fwd (5'-TATCACACAGGGTCTTCCGC-3') and *rpoC* up-rev (5'-CCTATCACCTCAAATGGTTTCGCTGCTGTTTTTGCAGTCTTTCAGCA-3'). A control wild-type *rpoC* allele was also amplified using the same primer set. The downstream fragment starting 24 nucleotides after the stop codon of *rpoC* was amplified using primers *rpoC* do-fwd (5'-CGAGCGCCTACGAGGAATTTGTATCGTGCAAAAACAGTCTTTCAGCAG-3') and *rpoC* do-rev (5'-AAGCAGTAACCTCGATTCCGT-3'). A kanamycin antibiotic resistance cassette, followed by a downstream fragment, was introduced as a selectable marker after the stop codon of the wild-type and mutant *rpoC* alleles by using LFH PCR. The resulting constructs were introduced by transformation into the wild-type *B. subtilis* strain, and the presence of the mutation was confirmed by DNA sequencing.

Antibiotic susceptibility tests. Susceptibility testing was performed by using Etest and disk diffusion assays. Cells were grown in LB medium to an OD_{600} of 0.4. Aliquots (100 μl) of these cultures were mixed with 4 ml of 0.7% LB or Mueller-Hinton (MH) soft agar (kept at 50°C) and were

directly poured onto LB or MH agar plates (containing 15 ml of 1.5% LB or MH agar). After 30 min at room temperature, the plates were dried for 20 min in a laminar airflow hood and were then used for the assays. The Etest assays were performed on MH agar to determine the MIC. Etest strips (bioMérieux, Durham, NC) impregnated with CEF (at concentrations ranging from 0.016 to 256 $\mu\text{g}/\text{ml}$) were applied to agar plates. The plates were incubated at 37°C and were read after 18 h. The MIC (in micrograms per milliliter) was determined directly from the scale where the ellipse edge intersects the Etest strip. Disk diffusion assays were performed as described previously (38). Whatman filter paper disks containing the antibiotics to be tested were placed on top of the LB or MH agar, and the plates were incubated at 37°C overnight. The diameters of the inhibition zones were measured after subtraction of the diameter of the filter paper disk (6.5 mm). The following chemicals and quantities were used in the disk diffusion assays: Triton X-100, 20 μl of a 25% solution; fosfomycin, 500 μg ; and CEF, 30 μg or 50 μg .

RNA preparation and microarray analyses. Total RNA was isolated from three biological replicates of HB13576 (*rpoC*^{WT}-*kan*) and HB13577 (*rpoC*^{G1122D}-*kan*) grown in LB medium to mid-log phase (OD_{600} , 0.4) by using the RNeasy minikit (Qiagen), followed by DNase treatment with a Turbo DNA-free kit (Ambion). The quantity and purity of RNA were determined using a NanoDrop spectrophotometer. cDNA labeling and microarray analysis were performed as described previously (39). Two microarrays were performed in biological triplicates with a dye swap, and the GenePix Pro software package (version 6.0) was used for image processing and analysis. Each expression value is representative of four separate measurements (duplicate spots on each of two arrays). Mean values and standard deviations for the normalized microarray data sets were calculated with MS Excel. 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. For the regulon-based assignment of transcriptional changes, we further analyzed genes that had fluorescence signal intensities higher than 150 for at least one RNA sample. The fold change was calculated by using the average signal intensities for HB13577 divided by those for HB13576.

RNAP homology modeling. Sequence alignments of RNAP subunits from *B. subtilis*, *Thermus thermophilus*, and *Escherichia coli* were created using ClustalW (40). The homology models of the *B. subtilis* RNAP sub-

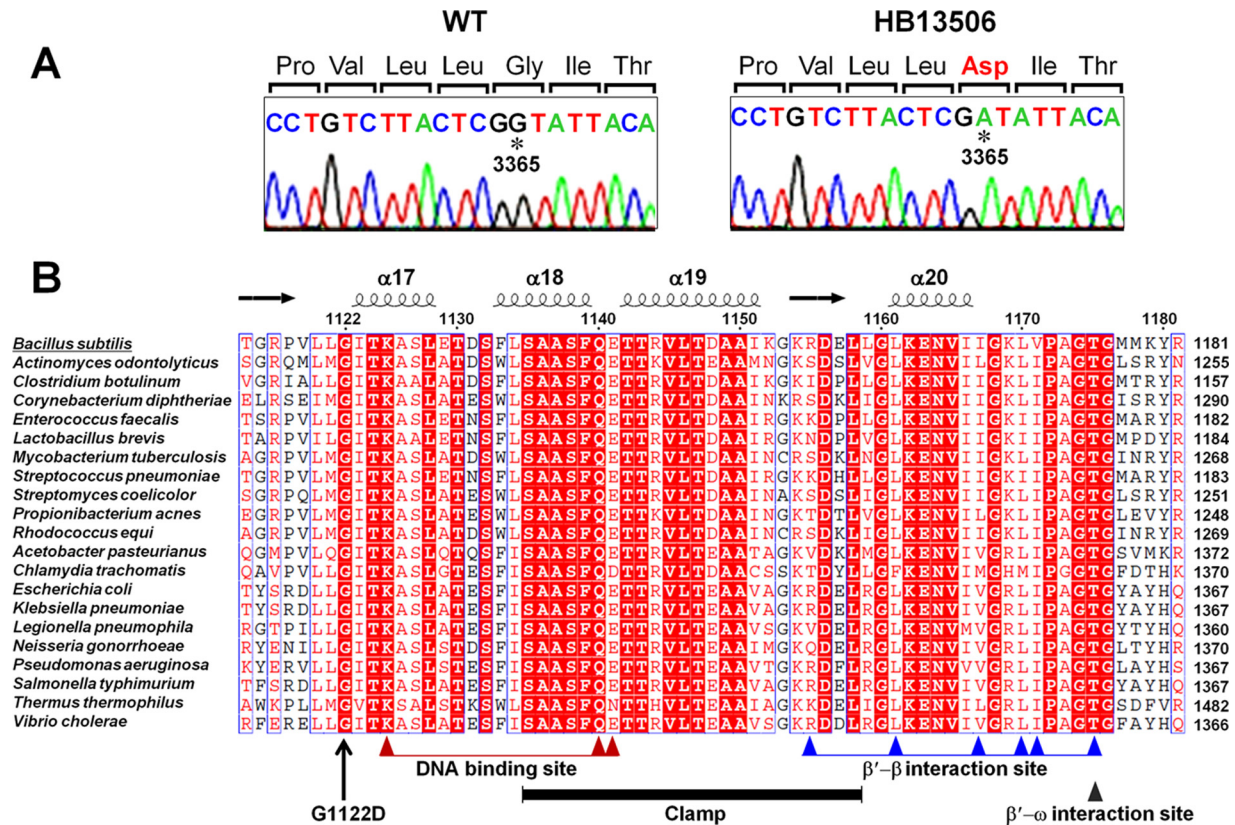


FIG 2 Whole-genome resequencing and single-nucleotide polymorphism identification. (A) Sanger sequencing of genomic DNA confirms a G-to-A alteration at nucleotide 3365 of *rpoC* in strain HB13506 (as initially identified by Illumina whole-genome resequencing). The mutated residue is indicated by an asterisk, and the altered amino acid at position 1122 is shown in red. (B) Structure-based multiple-sequence alignment of the C-terminal domain of RpoC from *B. subtilis* (Swiss-Prot accession no. P37871), *Actinomyces odontolyticus* (A7B9Q3), *Clostridium botulinum* (A7FZ76), *Corynebacterium diphtheriae* (Q6N1F6), *Enterococcus faecalis* (Q8Z241), *Lactobacillus brevis* (Q03PV0), *Mycobacterium tuberculosis* (A5U053), *Streptococcus pneumoniae* (Q97N08), *Streptomyces coelicolor* (Q8CJT1), *Propionibacterium acnes* (G7U6X7), *Rhodococcus equi* (E9T446), *Acetobacter pasteurianus* (C71FQ4), *Chlamydia trachomatis* (O84316), *E. coli* (P0A8T7), *Klebsiella pneumoniae* (B5XYF4), *Legionella pneumophila* (Q5X865), *Neisseria gonorrhoeae* (Q5F5R6), *Pseudomonas aeruginosa* (Q9HW09), *Salmonella enterica* serovar Typhimurium (P0A2R4), *T. thermophilus* (Q8RQE8), and *Vibrio cholerae* (Q9KV29). The absolutely conserved residues are boxed in red, and the highly conserved residues are represented by red letters. The secondary structure was adopted from the crystal structure of *T. thermophilus* RNAP (PDB ID 2O5J) (43). The conserved motifs were identified by using the NCBI Conserved Domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and by structural analysis of *T. thermophilus* RNAP (13).

units were constructed from the crystal structure of *T. thermophilus* RNAP (Protein Data Bank [PDB] identification code [ID] 2O5J) and the electron microscopy (EM) structure of *E. coli* RNAP (PDB ID 3IYD) by using the Swiss-Model Alignment mode (41–43). The individual models were superimposed onto the *E. coli* RNAP initiation complex structure (PDB ID 3IYD) using the Coot program (44), and all-atom contact and geometry were checked with MolProbity (45). Structures were visualized using PyMOL (<http://www.pymol.org>).

Microarray data accession number. All microarray data are available in the NCBI GEO database under accession number GSE37742.

RESULTS AND DISCUSSION

Isolation of a spontaneous mutant of *B. subtilis* highly resistant to CEF. To investigate the development of cephalosporin resistance in the model bacterium *B. subtilis*, we initially used transposon-based genome-wide random mutagenesis (33). We introduced an amplified mTn7SX library into the wild-type *B. subtilis* strain by transformation and isolated a mutant (HB13506) that was able to grow on LB agar containing 15 μg/ml of CEF, an expanded-spectrum cephalosporin antibiotic. To directly compare the levels of CEF resistance of wild-type and HB13506 mutant strains, we performed disk diffusion assays. Indeed, the iso-

lated strain exhibited remarkably reduced susceptibility to CEF (Fig. 1A). Strain HB13506 also displays a small-colony phenotype: colonies are about 4-fold smaller than wild-type colonies when grown on LB agar plates (Fig. 1B). By sequencing of chromosomal DNA flanking the transposon (33), we found that mTn7SX was inserted into *citM*, encoding a Mg²⁺ citrate transporter, but the CEF resistance and small-colony phenotypes were not linked to the transposon insertion. We therefore suspected that other spontaneous mutations were responsible for the CEF resistance of HB13506.

Identification of a point mutation in an absolutely conserved glycine residue of RpoC. The resistance phenotype of strain HB13506 was stably maintained after many transfers in the absence of CEF. We therefore performed whole-genome resequencing of the HB13506 mutant and its parental wild-type strain in order to identify the mutations conferring CEF resistance. Indeed, sequencing of whole genomes has been used successfully to directly determine novel mutations that result in increased resistance to certain antibiotics (46–49). Whole-genome sequence comparison identified a single-base substitution (G to A) at nucleotide 3365 of the *rpoC* gene, which encodes the RNA polymer-

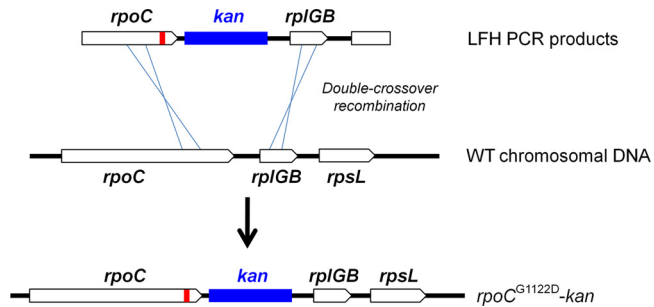


FIG 3 Genetic reconstruction of the mutant *rpoC* allele in the wild-type background. The schematic diagram shows the strategy for genetic reconstruction. The G1122D mutation is indicated by a thick red vertical line. A mutant allele of *rpoC* was amplified from the HB13506 mutant, and then a kanamycin antibiotic resistance cassette was introduced after the stop codon, followed by a 752-bp downstream fragment, using LFH PCR. The mutant allele was moved into the parental wild-type strain by transformation and was confirmed by DNA sequencing.

ase β' subunit, resulting in the G1122D (codon GGT to GAT) alteration. The presence of this mutation (designated *rpoC*^{G1122D}) was further confirmed by PCR and DNA sequencing (Fig. 2A).

To determine whether the mutated residue is related to any known structural or functional domains, we performed a multiple-sequence alignment of RpoC proteins from 11 Gram-positive (including *B. subtilis*) and 10 Gram-negative bacterial genera by using ClustalW and ESPrict (40, 50). The results show that the G1122D mutation is within a highly conserved C-terminal region of RpoC (Fig. 2B). Analysis of conserved motifs in the C terminus of *B. subtilis* RpoC, using the NCBI Conserved Domain database and structural analysis of *T. thermophilus* RNAP (13, 28), demonstrates that G1122 lies adjacent to the DNA-binding sites and β' clamp. The RNAP clamp, with mobile domains on the β and β' subunits, is part of the crab claw structure that opens and closes the active-site channel (51). Strikingly, we found that the G1122 residue of *B. subtilis* RpoC is absolutely conserved in all 21 RpoC proteins. We note that a recent study demonstrated that a high proportion

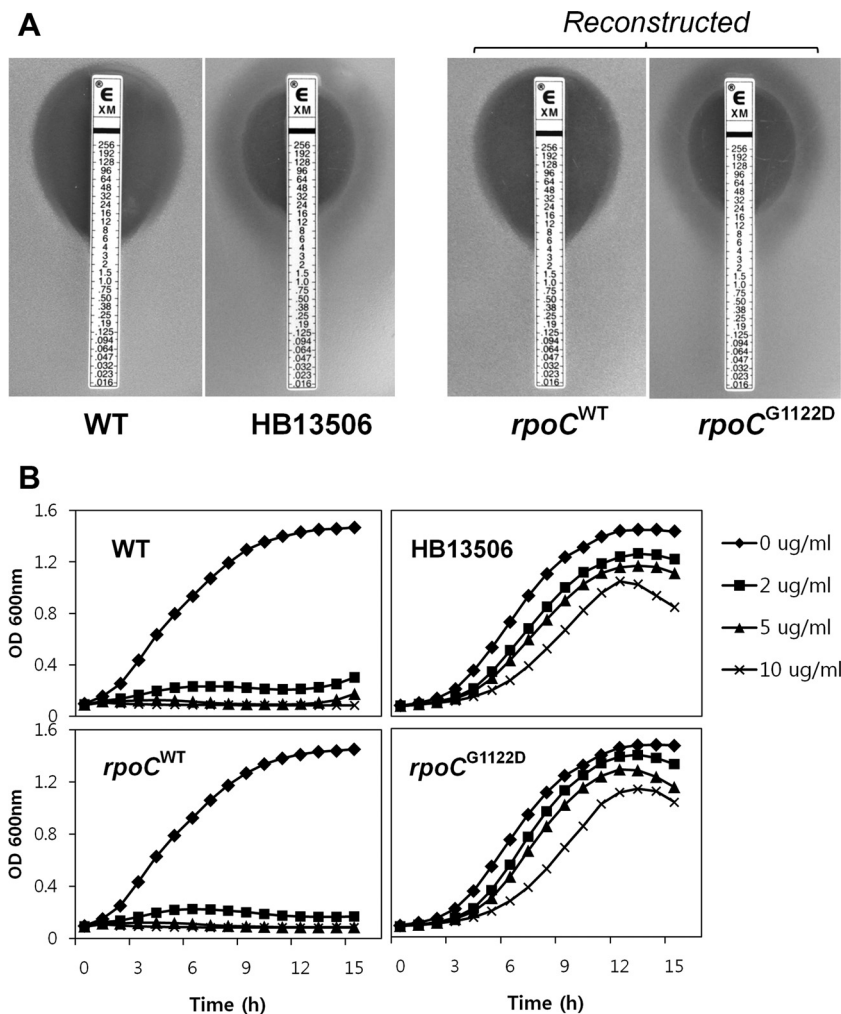


FIG 4 The G1122D substitution alone is responsible for both the CEF resistance and slow-growth phenotypes. (A) Determination of MICs of CEF by use of an Etest assay. Etest strips (bioMérieux) with CEF concentrations of 0.016 to 256 $\mu\text{g/ml}$ were applied to MH agar plates, and then the plates were incubated at 37°C for 18 h. The MIC (in micrograms per milliliter) was determined by identifying where bacterial growth intersects the Etest strip. (B) Growth assays, in the absence or presence of CEF, were performed in MH medium by using a Bioscreen C growth analyzer. The data are representative of at least three independent experiments.

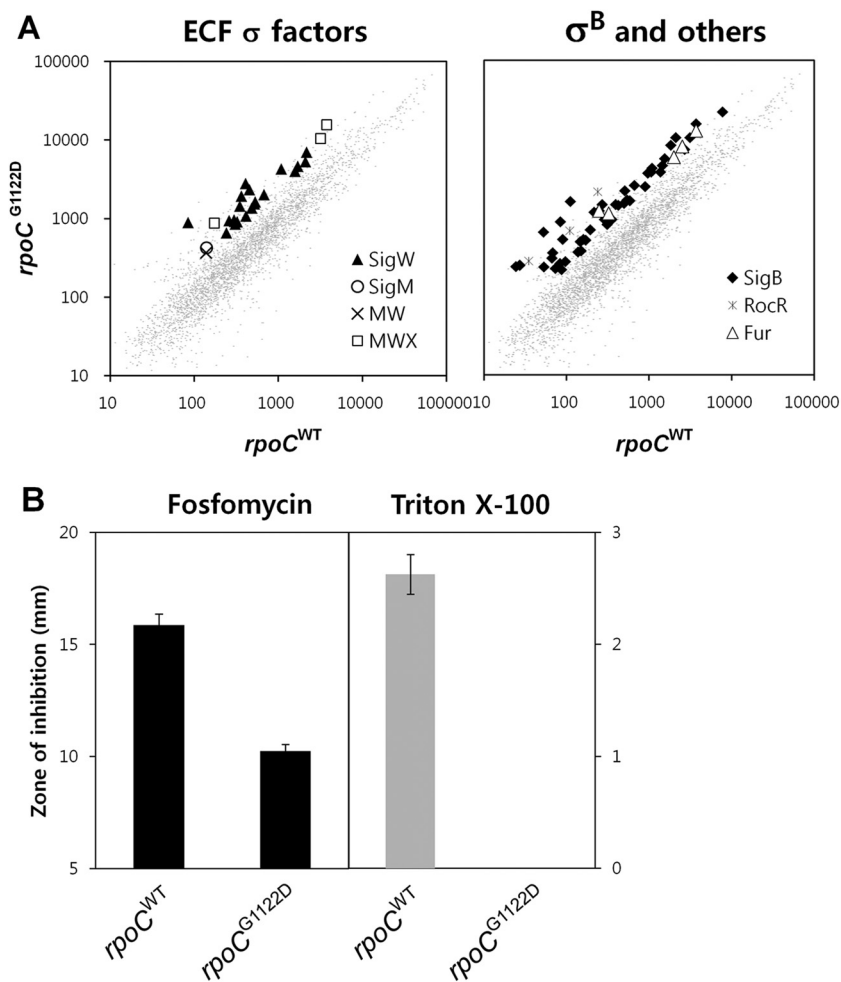


FIG 5 Influence of the G1122D substitution on the transcription of the σ factor regulons. (A) Microarray transcriptional analysis of strains HB13576 (*rpoC*^{WT}-*kan*) and HB13577 (*rpoC*^{G1122D}-*kan*). RNA was extracted from cells grown in LB medium to an OD₆₀₀ of 0.4. Analysis was focused on genes whose expression changed at least 2.5-fold. Data shown are mean fluorescence intensities from two independent experiments. Each comparison was performed with dye-swaps on each of three biological replicates. (B) Disk diffusion assays were performed on LB agar plates with fosfomycin and Triton X-100. Three independent experiments were performed for each strain. Error bars indicate standard deviations.

(~30%) of the absolutely conserved residues in both the β and the β' subunits are Gly. Some of these Gly residues are known to have distinct functional roles (13), but to our knowledge, no mutations in residue G1122 (or its equivalent) have been reported previously for any bacterial RNAP.

The *rpoC*^{G1122D} allele is sufficient to confer high-level CEF resistance. To determine whether the G1122D substitution in *rpoC* is sufficient for CEF resistance, we moved the mutant allele into the parental wild-type strain as outlined in Fig. 3. A kanamycin antibiotic resistance cassette was introduced as a selectable marker after the stop codon of the control wild-type and mutated *rpoC* alleles, respectively. The correct allelic replacements were confirmed by DNA sequencing. Determination of the MICs of the reconstructed HB13576 (*rpoC*^{WT}-*kan*) and HB13577 (*rpoC*^{G1122D}-*kan*) strains, by use of the Etest assay, revealed an increase from 5 to 20 $\mu\text{g}/\text{ml}$ on MH agar by the introduction of the *rpoC* G1122D point mutation (Fig. 4A). The reconstructed mutant also exhibited a lower growth rate than the wild type in the absence of CEF and a remarkable reduction in susceptibility to CEF, as measured using a Bioscreen C growth analyzer (Fig. 4B).

These results indicate that both the CEF resistance and slow-growth phenotypes of HB13506 are replicated in the reconstructed mutant strain HB13577 and are therefore due to the G1122D substitution in RpoC.

RpoC^{G1122D} facilitates transcriptional reprogramming by σ factors. To better understand the molecular basis of the CEF resistance conferred by the G1122D substitution, the transcriptome of the *rpoC*^{G1122D} mutant was assessed using DNA microarrays. There were genome-wide differences in expression patterns between the mutant (HB13577) and wild-type (HB13576) strains. Of the 175 genes upregulated >2.5-fold in the *rpoC*^{G1122D} mutant, we found that 68 (approximately 40%) belong to ECF σ factor (σ^M , σ^W , and σ^X) and σ^B regulons (Fig. 5A). These results are in good agreement with the recently observed role of ECF σ factors in CEF resistance (26, 27, 30). The remaining 60% of genes with altered expression are not associated with known cell envelope stress regulons, although this group includes both RocR-regulated (*rocA*, *rocB*, *rocE*, *rocF*, and *rocG*) and Fur-regulated (*yfkM* and *feuABC-ybbA* operon) genes.

Previously, σ^W was shown to be a major determinant for resis-

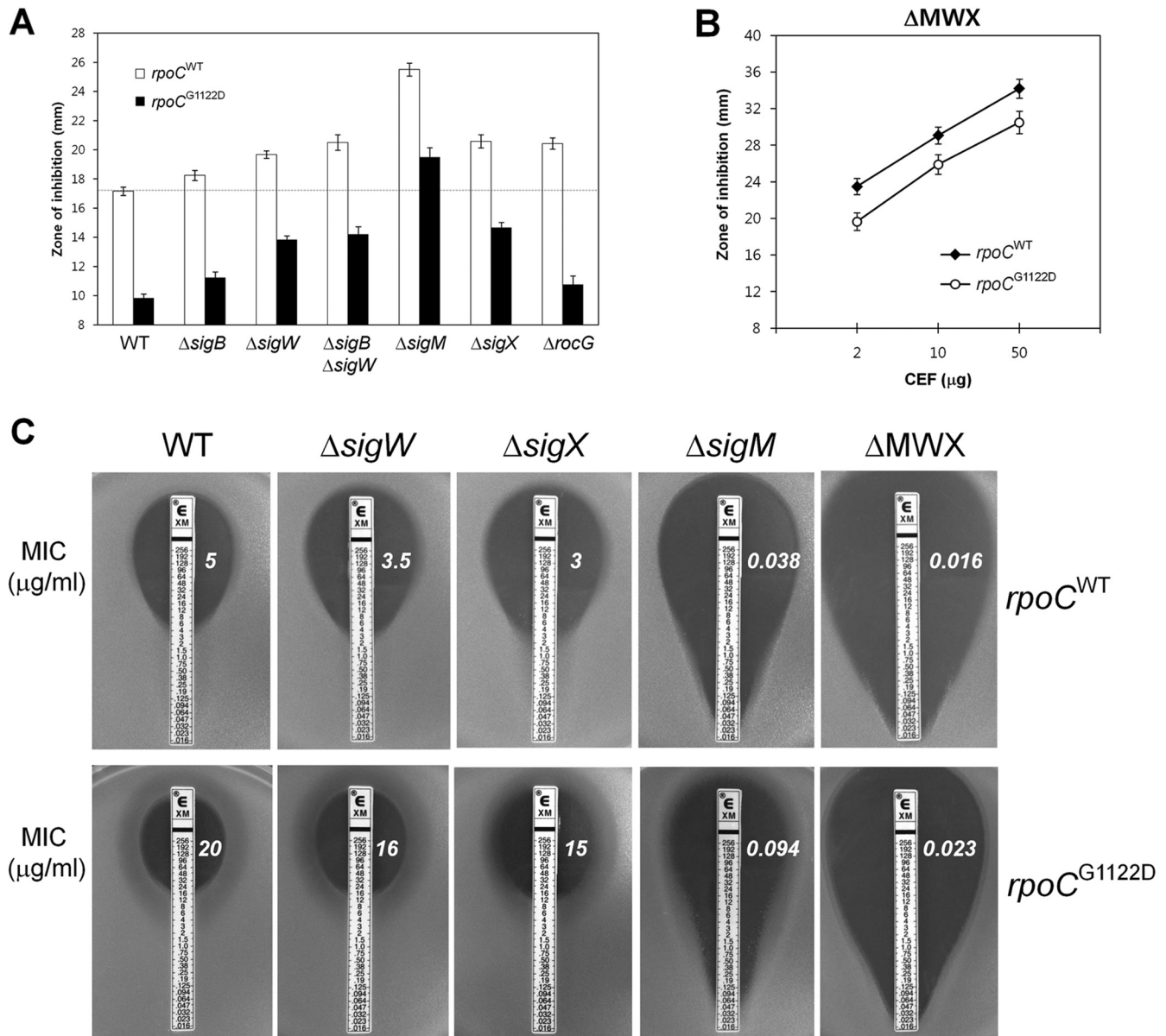


FIG 6 The ECF σ factors (σ^M , σ^W , and σ^X) are involved in CEF resistance conferred by the *rpoC* point mutation. (A) Determination of the CEF susceptibilities of several *B. subtilis* strains. The dotted horizontal line indicates the level of CEF susceptibility of strain HB13576 (*rpoC*^{WT}-*kan*). (B) Effect of the *rpoC*^{G1122D} allele on the CEF susceptibility of the ΔMWX triple mutant strain. Disk diffusion assays were performed on MH agar plates with filter paper disks containing three different amounts of CEF (2, 10, and 50 μ g). Three independent experiments were performed for each strain. Error bars indicate standard deviations. (C) Determination of MICs for CEF using an Etest assay. Etest strips (bioMérieux) with CEF concentrations of 0.016 to 256 μ g/ml were applied to MH agar plates, and the plates were then incubated at 37°C for 18 h. The MICs (in micrograms per milliliter), shown to the right of each Etest strip, were determined by identifying where bacterial growth intersects the Etest strip.

tance to antibiotics that impair cell wall synthesis, such as fosfomycin, and detergents that disrupt membrane function, such as Triton X-100 (38, 52). Since the microarray data suggest a general upregulation of the σ^W regulon, we performed disk diffusion assays under the same conditions as those used for the microarray analysis. As predicted, an increase in resistance to fosfomycin and Triton X-100 was noted for the *rpoC*^{G1122D} mutant compared to controls (Fig. 5B). These results support the hypothesis that the RpoC^{G1122D} substitution affects the global transcription profile, and thereby antibiotic resistance, by facilitating the transcriptional reprogramming of RNAP.

RpoC^{G1122D}-induced CEF resistance is due primarily to increased σ^M activity. We used disk diffusion assays to examine the individual contributions of alternative σ factors to the enhanced CEF resistance. Consistent with the known roles of σ^M , σ^W , and σ^X in CEF resistance (26, 27, 30), the disruption of *sigM* led to the greatest increase in CEF sensitivity, with more-modest effects attributed to disruption of *sigW* and *sigX* (Fig. 6A). Significantly, inactivation of σ^M in the *rpoC*^{G1122D} strain decreased resistance to a level lower than that normally seen in the wild-type parental strain. Although this *sigM* *rpoC*^{G1122D} mutant strain is somewhat more sensitive than the wild type to CEF (Fig. 6A), it retains the

slow-growth phenotype. Thus, growth rate reduction is not, by itself, sufficient to confer CEF resistance and is likely only one result of the broad changes in the transcriptome profile of the *rpoC*^{G1122D} mutant.

In contrast to the results obtained with the ECF σ factors, the CEF resistance of strains lacking σ^B or RocG was relatively unaffected in either the wild-type or the *rpoC*^{G1122D} mutant background (Fig. 6A). The RocG glutamate dehydrogenase has recently been linked to intrinsic CEF resistance in *B. subtilis* (30) but does not appear to be responsible for the increased resistance of the *rpoC*^{G1122D} strain (Fig. 6A). These results suggest that the G1122D substitution directs the cell toward CEF resistance by altering the transcription of the σ^M , σ^W , and σ^X (ECF σ factor) regulons.

It has been reported previously that these ECF σ factors (σ^M , σ^W , and σ^X) have overlapping promoter selectivities (53–57). As a result, increased sensitivity to some antibiotics is revealed only when two or more ECF σ factors are inactivated (26, 38). Indeed, deletion of all three ECF σ factor genes (*sigM*, *sigW*, and *sigX*) (Δ MWX) renders *B. subtilis* more sensitive to CEF than the single deletions (26). We therefore hypothesized that much (or even all) of the CEF resistance noted in the *rpoC*^{G1122D} mutant was due to increased activity of σ^M , σ^W , and σ^X . In support of this hypothesis, there was comparatively little change in CEF susceptibility when the *rpoC* point mutation was introduced into the Δ MWX triple mutant strain, as measured either by the zone of inhibition (Fig. 6B) or by Etest assays (Fig. 6C). In the Etest assays, the *rpoC*^{G1122D} mutation led to a 4-fold increase in the MIC for the wild type and increased the MICs for the *sigW* and *sigX* strains between 3- and 5-fold. In contrast, a <3-fold effect was noted for the *sigM* single mutant and a <2-fold effect for the Δ MWX triple mutant strain (Fig. 6C). We conclude that the increased level of CEF resistance in the *rpoC*^{G1122D} mutant is mediated mainly by σ^M , with additional roles played by both σ^W and σ^X .

Inactivation of the *spx*, *disA*, and *yuaFG* genes reduces CEF resistance. The ECF σ factors σ^M and σ^X contribute to CEF resistance by three distinct pathways involving the *B. subtilis* genes *spx*, *disA*, and *abh* (27). It has also been reported that overexpression of *yuaFG* rescues the CEF-sensitive phenotype of the *sigW* mutant (30). We used disk diffusion assays to determine if these genes are involved in CEF resistance in the G1122D mutant strain. As predicted, inactivation of either *spx*, *disA*, or *yuaFG* reduced CEF resistance levels, although the effects of *disA* and *yuaFG* were only slight compared to that of *spx*. In contrast, the *abh* null mutation had no effect in this genetic background (data not shown). The *spx disA* and *spx yuaFG* multiple mutant strains displayed further increases in CEF sensitivity, consistent with the hypothesis that these genes act in parallel pathways (Fig. 7). These results suggest that *spx*, *disA*, and *yuaFG* are among the genes controlled by ECF σ factors that contribute to CEF resistance in the *rpoC*^{G1122D} mutant, but these are unlikely to be the only relevant resistance determinants.

Structural homology models of *B. subtilis* RNAP. To understand the possible structural effects of the G1122D substitution in the β' subunit, and to explore whether this change might directly affect σ factor-RNAP complex formation, we performed homology modeling of the *B. subtilis* RNAP subunits (41, 58). The models generated were superimposed onto the EM structure of the *E. coli* RNAP transcription initiation complex (PDB ID 3IYD) (42), because no high-resolution crystal structure of a bacterial RNAP-

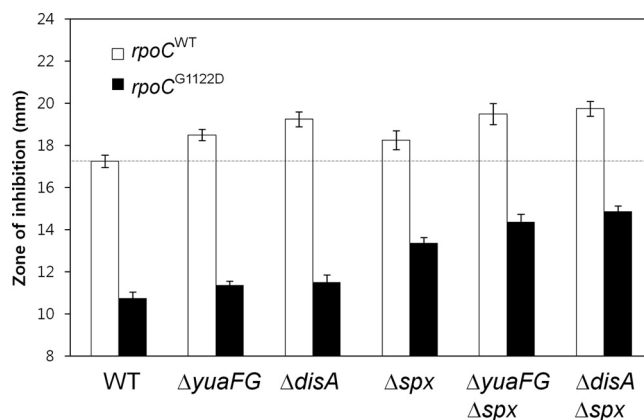


FIG 7 Involvement of *spx*, *disA*, and *yuaFG* genes in CEF resistance conferred by the *rpoC* point mutation. CEF susceptibility was determined by disk diffusion assays, which were performed on MH agar plates with a filter paper disk containing 50 μ g CEF. The dotted horizontal line indicates the level of CEF susceptibility in the HB13576 (*rpoC*^{WT}-*kan*) strain. Three independent experiments were performed for each strain. Error bars indicate standard deviations.

promoter complex is available. The absolutely conserved β' -G1122 residue (Fig. 2B) is positioned at the DNA entrance channel in the α 17-helix (numbered as for the β' subunit of *T. thermophilus* RNAP) containing the DNA-binding K1125 residue (Fig. 8A).

Since σ factors are known to interact with many regions of the core enzyme (59), we asked whether the β' -G1122 residue has any interaction with the σ factor. However, G1122 is located \sim 50 Å from the σ factor. Moreover, the distance of closest approach between G1122 and the β' clamp is \sim 10 Å; thus, they also are not likely to interact directly. We therefore undertook a closer inspection of the surroundings of the β' -G1122 residue. In the modeling of wild-type RNAP, β' -G1122 does not directly interact with the DNA recognition helix or neighbor molecules, whereas the ζ N atom of β' -K1125 interacts with the phosphate backbone of promoter DNA within 3.5 Å (Fig. 8B). In the modeling of mutant RNAP (β' -G1122D), however, the δ O atom of D1122 makes a salt bridge with the ζ N atom in the DNA-binding residue β' -K1125 within 3.5 Å (Fig. 8C). Thus, the G1122D substitution may affect interactions between K1125 and the DNA backbone, and these changes may differentially affect the activities of the various holoenzyme forms.

Conclusions. Our data demonstrate that a single point mutation in the absolutely conserved G1122 residue of the RNAP β' subunit is sufficient to impart CEF resistance. The slow-growth phenotype is also directly linked to the G1122D substitution but is by itself insufficient to confer CEF resistance. A recent study reported that mutations in the β subunit (*rpoB*) of RNAP enhance cephalosporin resistance in enterococci (60). However, so far no mutations affecting cephalosporin resistance have been identified in the bacterial *rpoC* gene. Moreover, we are unaware of any mutations reported at the residue equivalent to G1122 of *B. subtilis* RpoC in any bacteria.

The present study indicates that the *rpoC*^{G1122D} substitution increases the activity of ECF σ factors (σ^M , σ^W , and σ^X) and thereby contributes directly to CEF resistance in *B. subtilis*. As reported elsewhere (26, 27, 30) and confirmed here, these three

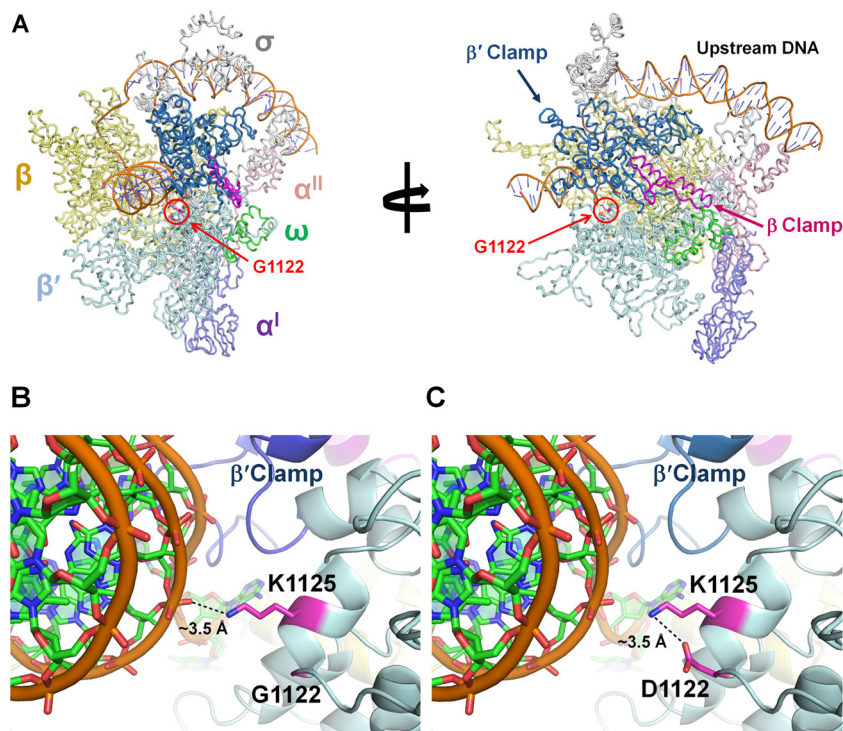


FIG 8 Modeling of *B. subtilis* RNAP. (A) Model structure of the *B. subtilis* RNAP transcription initiation complex. RNAP subunits are colored as follows: α' , slate; α'' , pink; β , yellow; β' , cyan; ω , green; σ^{70} , gray. The β clamp and β' clamp are shown in magenta and blue, respectively. (B) Wild-type RNAP. The β' -G1122 residue is located at the DNA entrance channel and in close proximity to the DNA-binding residue β' -K1125, which interacts with the phosphate backbone of downstream DNA within 3.5 Å. (C) Mutant RNAP (β' -G1122D). The side chain of the substituted D1122 interacts with β' -K1125 through a salt bridge within 3.5 Å. Modeling suggests that the G1122D substitution could affect DNA binding by K1125.

ECF σ factors are known to control genes that contribute to CEF resistance, including *spx* (σ^{MWX} regulon), *disA* (σ^{M} regulon), and *yuaFG* (σ^{W} regulon). In summary, we have identified novel phenotypes associated with the alteration of an invariant β' Gly residue (G1122) and provide a mechanistic explanation of the contribution of RNAP mutations to global transcriptional changes and thereby to the emergence of antibiotic resistance.

ACKNOWLEDGMENTS

We thank Pete Chandransu for helpful discussions and suggestions regarding homology modeling.

This work was supported by a grant from the National Institutes of Health (GM047446).

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