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Glutamate Dehydrogenase Affects Resistance to Cell Wall Antibiotics in *Bacillus subtilis*

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The glutamate dehydrogenase RocG of *Bacillus subtilis* is a bifunctional protein with both enzymatic and regulatory functions. Here we show that the *rocG* null mutant is sensitive to β-lactams, including cefuroxime (CEF), and to fosfomycin but that resistant mutants arise due to gain-of-function mutations in *gudB*, which encodes an otherwise inactive glutamate dehydrogenase. In the presence of CEF, Δ*rocG* Δ*gudB* mutant cells exhibit growth arrest when they reach mid-exponential phase. Using microarray-based transcriptional profiling, we found that the σ^W regulon was downregulated in the Δ*rocG* Δ*gudB* null mutant. A survey of σ^W-controlled genes for effects on CEF resistance identified both the NfeD protein YuaF and the flotillin homologue YuaG (FloT). Notably, overexpression of *yuaFG* in the *rocG* null mutant prevents the growth arrest induced by CEF. The YuaG flotillin has been shown previously to localize to defined lipid microdomains, and we show here that the *yuaFGI* operon contributes to a σ^W-dependent decrease in membrane fluidity. We conclude that glutamate dehydrogenase activity affects the expression of the σ^W regulon, by pathways that are yet unclear, and thereby influences resistance to CEF and other antibiotics.

In *Bacillus subtilis*, a model system for the Gram-positive bacteria (36), the synthesis of glutamate is catalyzed uniquely by the heterodimeric product of the *gltAB* operon. Glutamate acts as a central metabolite providing the link between carbon and nitrogen metabolism (11, 40). The degradation of glutamate is catalyzed by the strictly catabolic glutamate dehydrogenase RocG (2). In addition to *rocG*, *B. subtilis* has a second glutamate dehydrogenase gene, *gudB*, whose product is cryptic due to an insertion of three amino acids close to the active site of this enzyme. However, null mutants of *rocG* rapidly accumulate spontaneous gain-of-function suppressor mutations in *gudB* that remove the repeat sequence encoding the three-amino-acid insertion, thereby resulting in the synthesis of active GudB (3, 12).

Recent studies have shown that RocG has a second activity as a regulatory protein. RocG, if glutamate is available, directly interacts with GltC, the transcription activator of the *gltAB* operon, thus inhibiting its activity (10, 15). However, whether it has additional functions remains largely unknown. In addition to RocG, several other bacterial enzymes are now known to regulate gene expression. Some act as transcription factors by direct binding to either DNA or RNA, and others modulate the activity of transcription factors either by covalent modification or by protein-protein interactions (9).

Cefuroxime (CEF) belongs to the group of broad-spectrum β-lactam cephalosporin antibiotics, with antimicrobial activity against both Gram-positive and Gram-negative bacteria (31). The mode of action of CEF is conventional: by binding to specific penicillin-binding proteins (PBPs), it inhibits the third and final stage of bacterial cell wall synthesis. In Gram-negative bacteria such as *Escherichia coli*, CEF shows high affinity for PBP3 (35). β-Lactams such as CEF are also known to exert their toxicity, at least in part, by generating reactive oxygen species (14, 22).

Three major mechanisms have been proposed for bacterial resistance to β-lactam antibiotics. The most common mechanism is the production of β-lactam-degrading enzymes (β-lactamases), which are widely disseminated among bacteria. The second mechanism, well studied in Gram-positive staphylococcal and streptococcal species, is alterations in PBPs, resulting in low affinities for β-lactams. The third mechanism is mediated by efflux pumps which prevent access of the β-lactams to the PBP targets (32, 43). *B. subtilis* also exhibits intrinsic resistance to a wide variety of β-lactams, including CEF. Currently, however, none of these mechanisms have been found to be applicable to *B. subtilis*. The extracytoplasmic function (ECF) sigma (σ) factors of *B. subtilis* regulate genes activated by cell wall antibiotics and are known, in several cases, to confer antibiotic resistance (18). The mechanism of activation by antibiotics is not well understood, but in the case of σ^W, stress activates a proteolytic cascade resulting in release of free σ factor from a transmembrane anti-σ, RsiW (17). A multiply mutant *B. subtilis* strain lacking all seven ECF sigma factors (σ^M, σ^X, σ^W, σ^V, σ^Y, σ^Z, and σ^{YlaC}) has an increased sensitivity to β-lactams, including CEF. A similar sensitivity was noted for a triple mutant strain lacking σ^M, σ^W, and σ^X (28).

Here we address the influence of glutamate dehydrogenase activity on CEF resistance in *B. subtilis*. We were motivated by the serendipitous observation that *rocG* null mutant strains displayed an enhanced sensitivity to CEF. Our results demonstrate that glutamate dehydrogenase affects the activity of the ECF σ factor σ^W. Of the ~60 genes in the σ^W regulon, we identify the *yuaFGI* operon as playing a pivotal role in CEF resistance. Our results reveal an unexpected link between central metabolism and antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Deletion mutants were constructed by replacing genes with antibiotic resistance cassettes using long-flanking-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference[a]
Strains		
W168	*trpC2*	BGSC 1A1
HB13528	W168 *rocG*::*spc*	LFH PCR with W168
HB13541	W168 *rocG*::*spc gudB*::*kan*	HB13543 chromosomal DNA with HB13528
HB13542	W168 *rocG*::*spc gudB*$^+$	Gain-of-function mutation in *gudB*
HB13545	W168 *rocG*::*spc gudB*::*kan amyE*::P_{spac}(hy)-*rocG* (*cat*)	pYH001 with HB13541
HB13543	W168 *gudB*::*kan*	LFH PCR with W168
HB6156	CU1065 *yuaFGI*::*kan*	6
HB13159	W168 *yuaFGI*::*kan*	HB6156 chromosomal DNA with W168
HB13547	W168 *yuaFG*::*mls*	LFH PCR with W168
HB13548	W168 *yuaGI*::*mls*	LFH PCR with W168
HB13568	W168 *yuaI*::*mls*	LFH PCR with W168
HB5331	CU1065 *yqeZ-yqfAB*::*kan*	6
HB13566	W168 *yqeZ-yqfAB*::*kan*	HB5331 chromosomal DNA with W168
HB13567	W168 *yqeZ-yqfAB*::*kan yuaFG*::*mls*	HB5331 chromosomal DNA with HB13547
HB10102	168 *sigW*::*mls*	27
HB13549	W168 *sigW*::*mls*	HB10102 chromosomal DNA with W168
HB6208	W168 *sigW*::*spc*	6
HB13558	W168 *sigW*::*spc yuaFG*::*mls*	HB6208 chromosomal DNA with HB13547
HB13557	W168 *sigW*::*mls rocG*::*spc gudB*::*kan*	HB13549 chromosomal DNA with HB13541
HB13571	W168 *yuaFG*::*mls amyE*::P_{spac}(hy)-*yuaFG* (*cat*)	pYH002 with HB13547
HB13572	W168 *rocG*::*spc gudB*::*kan amyE*::P_{spac}(hy)-*yuaFG*	pYH002 with HB13541
HB13574	W168 *sigW*::*mls amyE*::P_{spac}(hy)-*yuaFG* (*cat*)	pYH002 with HB13549
HB13042	W168 *amyE*::P_{xyl}-*sigW* (*cat*)	21
HB13122	W168 P_{fabHAF} *fabHA*(P5*)-*fabF amyE*::P_{xyl}-*sigW* (*cat*)	21
HB13160	W168 *yuaFGI*::*kan amyE*::P_{xyl}-*sigW* (*cat*)	HB6156 chromosomal DNA with HB13042
HB13226	W168 P_{fabHAF} *fabHA*(P5*)*fabF yuaFGI*::*kan amyE*::P_{xyl}-*sigW* (*cat*)	HB6156 chromosomal DNA with HB13122
HB13236	W168 *yqeZ-yqfAB*::*kan amyE*::P_{xyl}-*sigW* (*cat*)	HB13566 chromosomal DNA with HB13042
Plasmids		
pPL82	IPTG-inducible expression vector (*amyE* integration)	33
pYH001	P_{spac}(hy)-*rocG* in pPL82	This study
pYH002	P_{spac}(hy)-*yuaFG* in pPL82	This study

[a] LFH PCR (29, 38) was used to construct deletions using the primers listed in Table S1 in the supplemental material.

homology (LFH) PCR as described previously (30, 39) in *B. subtilis* W168 (BGSC 1A1). Cells were routinely cultured in Luria-Bertani (LB) broth at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco). 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), $(NH_4)_2SO_4$ (2 g/liter), $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), trisodium citrate $\cdot 2H_2O$ (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), 3 nM $(NH_4)_6Mo_7O_{24}$, 400 nM H_3BO_3, 100 μM $FeCl_3$, 30 nM $CoCl_2$, 10 nM $CuSO_4$, 10 nM $ZnSO_4$, and 80 nM $MnCl_2$. Difco sporulation medium (DSM) agar was used for spore formation and maintenance of *B. subtilis* strains. The following antibiotics were used when appropriate: spectinomycin (Spec) (100 μg/ml), kanamycin (Kan) (15 μg/ml), chloramphenicol (Cat) (10 μg/ml), or macrolide-lincosamide-streptogramin B (MLS) (contains 1 μg/ml erythromycin and 25 μg/ml lincomycin) for *B. subtilis* strains and ampicillin (100 μg/ml) for *E. coli* DH5α.

Plasmid construction. PCR and cloning for plasmid construction were performed by using standard techniques (34). The primers used in the present study are listed in Table S1 in the supplemental material. Ectopic expression of *rocG* and *yuaFG* at *amyE* was placed under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{spac}(hy) using plasmid backbone pPL82 (33). For the construction of pYH001 (pPL82-*rocG*), the promoterless *rocG* gene was amplified from *B. subtilis* chromosomal DNA by PCR using the primers 5395 (*rocG* Pspac-F) and 5396 (*rocG* Pspac-R). pYH002 (pPL82-*yuaFG*) was constructed in a similar manner using the pair of primers 5561 (*yuaFG* Pspac-F) and 5563 (*yuaFG* Pspac-R). Construct integrity was verified by DNA sequencing. Plasmids were amplified in *E. coli* DH5α before transformation of *B. subtilis* strains.

Disk diffusion assays. Disk diffusion assays were performed as described previously (29). Briefly, strains were grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.4. A 100-μl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB agar plates (containing 15 ml of 1.5% LB agar). After 30 min at room temperature (to allow the soft agar to solidify), the plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing the antibiotics to be tested were placed on the top of the 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 antibiotics and quantities were used in the disk diffusion assays: penicillin G, 100 μg; ampicillin, 100 μg; fosfomycin, 500 μg; vancomycin, 100 μg; and CEF, 1 μg or 3 μg.

RNA preparation and microarray analyses. Total RNA was isolated from three biological replicates of W168 and HB13541 (*rocG gudB* double null mutant) grown in LB to mid-log phase (OD_{600} 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 (NanoDrop Technology Inc., Wilmington, DE). cDNA labeling and microarray analysis were performed as described previously (16). Two microarrays were used in biological triplicates. The GenePix Pro 6.0 software package 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 (SDs) for the normalized microarray data

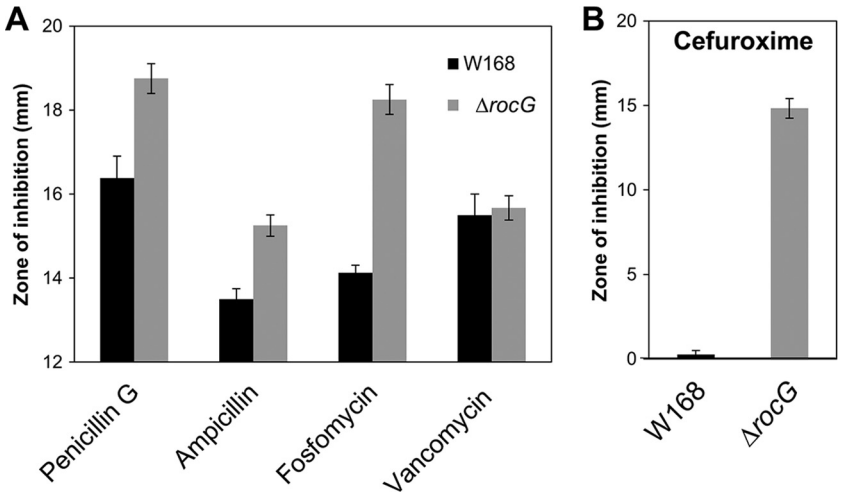

FIG 1 Disk diffusion susceptibility testing of the *rocG* mutant with several antibiotics affecting cell wall biosynthesis. (A) The *rocG* mutant is sensitive to fosfomycin and slightly sensitive to penicillin G and ampicillin. (B) Disruption of *rocG* markedly increases sensitivity to CEF (1 μg). Each bar represents the average zone of inhibition, expressed as total diameter minus diameter of the filter paper disk (6.5 mm). At least three assays were performed with three independent clones of each strain. Error bars indicate the standard deviations.

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 in either condition (sum of mean fluorescence intensity, <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 HB13541 divided by those for W168.

Quantitative real-time RT-PCR. Measurement of transcript abundance was performed by quantitative real-time RT-PCR (qRT-PCR). cDNA was synthesized by using random hexamer primers and a TaqMan reverse transcription kit (Roche). qRT-PCR was carried out by using SYBR green (Bio-Rad) and gene-specific primer pairs 5403 (*yqeZ* qRT-F)/5404 (*yqeZ* qRT-R) and 5411 (*yuaF* qRT-F)/5412 (*yuaF* qRT-R) according to the manufacturer's instructions. Expression of *yuaF* and *yqeZ* was calculated as the fold change based on the threshold cycle (C_T) values for each gene, as described previously (38). The level of 23S rRNA was used as a normalization control.

Fluorescence anisotropy. Fluorescence anisotropy analysis of *B. subtilis* strains treated with 1,6-diphenyl-1,3,5-hexatriene (DPH) was performed as described previously (37) with slight modifications. Strains were grown to mid-log phase (OD_{600} of 0.4 ± 0.01) in LB supplemented with 2% xylose. A 0.5-ml sample of each culture was then washed once and suspended in 2 ml of phosphate buffer (100 mM, pH 7.0) containing 5 μM DPH. After a 30-min incubation at room temperature, fluorescence anisotropy measurements (λ_{ex} = 358 nm, slit width = 10 nm; λ_{em} = 428 nm, slit width = 15 nm) were taken with a Perkin-Elmer LS55 luminescence spectrometer. The correction for the fluorescence intensity of nonlabeled cells was calculated as described by Kuhry et al. (23).

Microarray data accession number. The microarray data set is available in the NCBI GEO database under accession number GSE34383.

RESULTS AND DISCUSSION

A *rocG* null mutant shows increased susceptibility to CEF. We grew *B. subtilis* cells by repeated subculturing with selection for

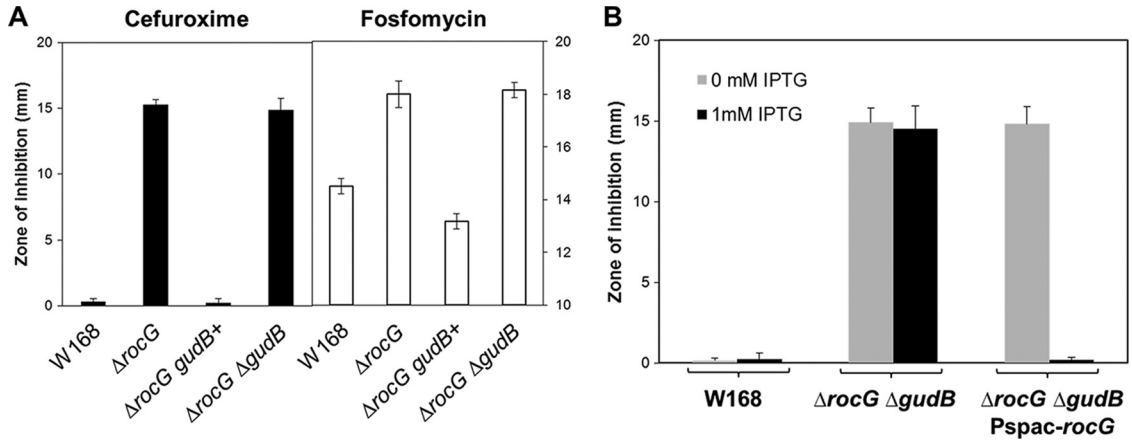

FIG 2 CEF resistance is distinctly influenced by the glutamate dehydrogenase activity. (A) Sensitivity to CEF and fosfomycin was determined by using disk diffusion assays. The spontaneous gain-of-function mutations in *gudB* restore normal resistance to these two antibiotics. (B) Induction of the *rocG* gene by IPTG (1 mM) complements the CEF-sensitive phenotype. Three independent experiments were performed for each strain, and the standard deviation is indicated by error bars.

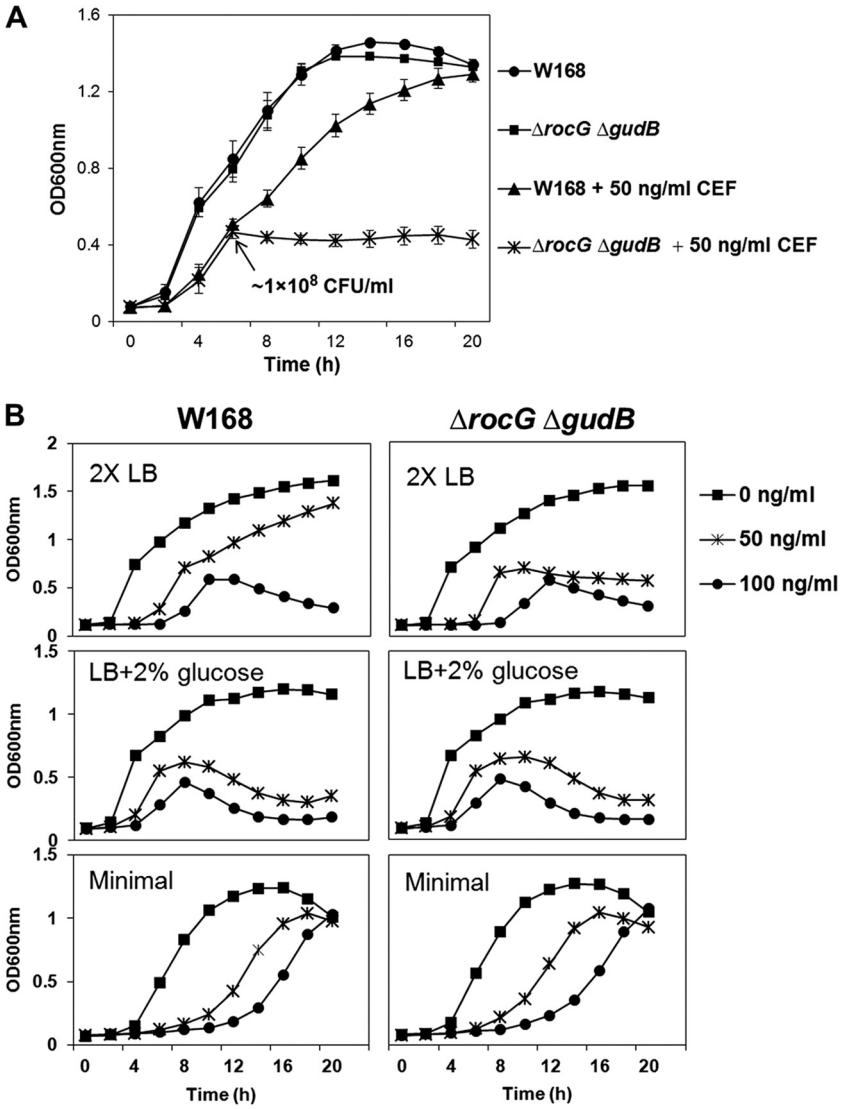

FIG 3 Mid-exponential-phase *rocG* mutant cells exhibit growth arrest in the presence of CEF. (A) Effect of CEF on the growth of the *rocG gudB* mutant. CEF (50 ng/ml) was added at the beginning of the culture. Liquid growth assays were performed in LB medium using a Bioscreen C growth analyzer. To determine CFU/ml, viable cell counts were estimated by plating diluted cultures on LB agar plates. Results from three independent experiments were averaged, and the standard deviation is indicated by error bars. (B) Effects of different culture media on the CEF-induced growth arrest of the *rocG gudB* mutant. Strains were grown in 2× LB, LB supplemented with 2% glucose, and glucose (2%) minimal medium in the presence or absence of CEF. Data are representative of at least three independent experiments.

increasing resistance to both vancomycin and cephalosporin. In studies to be presented in detail elsewhere, we found that the evolved strains were significantly altered in gene expression as judged by global transcriptome analyses using cDNA microarrays. Of relevance for the present study, the genes upregulated in the evolved strains included *rocG*, encoding the sole catabolic glutamate dehydrogenase in *B. subtilis*. However, mutational inactivation of *rocG* did not affect antibiotic resistance in these resistant strains, and the relevant genetic determinants were ultimately determined using whole-genome resequencing (data not shown). Although it is not an important determinant of cephalosporin resistance in these strains, we made the serendipitous observation that *rocG* mutant strains are more sensitive to some cell wall antibiotics in an otherwise wild-type background (Fig. 1A). Here, we define this unexpected link between glutamate dehydrogenase and cephalosporin sensitivity and identify the relevant genetic determinants.

To assess the potential role of *rocG* in conferring antibiotic resistance, we constructed an isogenic deletion mutant by homologous recombination in *B. subtilis* W168 (BGSC 1A1). Disk diffusion assays showed that disruption of *rocG* leads to susceptibility to β-lactams and fosfomycin but not to vancomycin (Fig. 1A). Although the *rocG* mutant is only slightly sensitive to penicillin G and ampicillin, it is notably sensitive to cefuroxime (CEF), a broad-spectrum cephalosporin (Fig. 1B). The *rocG* mutant showed a clear inhibition zone, but the wild type was only slightly affected by CEF, suggesting that RocG plays a crucial role in CEF resistance in *B. subtilis*. Since sensitivity to fosfomycin is less distinct than that to CEF, here we focused on identification of genetic factors that confer CEF resistance.

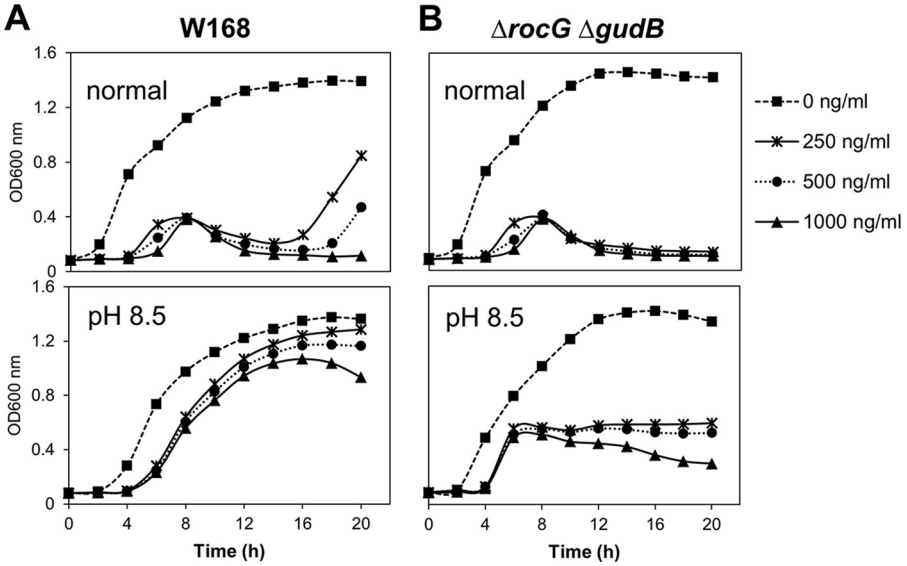

FIG 4 Effect of external alkaline pH on the susceptibility of wild-type (A) and *rocG gudB* mutant (B) strains of *B. subtilis* to CEF. Liquid growth assays were performed in LB medium (normal) or LB medium buffered with MOPS (pH 8.5) using a Bioscreen C growth analyzer. CEF was added at the beginning of the culture. A representative data set is shown.

The lack of glutamate dehydrogenase activity influences CEF resistance. The *rocG* mutant colonies grown on LB agar plates lyse more easily than wild-type colonies at room temperature. After 2 weeks, however, many new colonies arise and exhibit no lysis phenotype, eventually sporulating on LB agar plates (see Fig. S1 [left] in the supplemental material). Since it is known that *rocG* mutant strains rapidly accrue spontaneous gain-of-function mutations (previously designated *gudB1*) in *gudB*, which encodes an inactive glutamate dehydrogenase in *B. subtilis* 168 strains (3), we expected that this unusual phenotype would be due to mutations in *gudB*. Using DNA sequencing of *gudB*, we confirmed that these colonies are *gudB* gain-of-function mutants, as previously reported (3) (Fig. S1 [right] in the supplemental material). All of the sequenced colonies had a deletion of one copy of the 9-nucleotide direct repeat in the 5′ coding region of *gudB*.

To further examine whether the spontaneous gain-of-function mutations in *gudB*, here denoted *gudB*$^+$, suppresses the CEF-sensitive phenotype of a *rocG* mutant, we performed disk diffusion assays. These mutations restore normal resistance to CEF, and similar results were also obtained for fosfomycin resistance (Fig. 2A). Indeed, the *rocG gudB*$^+$ strain is slightly more resistant to these antibiotics than the wild type, possibly due to the constitutive expression of *gudB* (3). To avoid complications due to these suppressor mutations, we constructed a *rocG gudB* double mutant (HB13541) and used this as a glutamate dehydrogenase-negative strain. Moreover, when *rocG* expression is placed under the control of the P_{spac}(hy) promoter, it complements the CEF-sensitive phenotype of the double mutant (Fig. 2B). Control experiments show that IPTG itself does not reduce CEF sensitivity.

The glutamate dehydrogenase RocG functions not only as a central metabolic enzyme but also as a regulatory protein by interaction with GltC (10, 15). GudB shares with RocG both a common enzymatic activity and an ability to regulate GltC (3). Thus, the effects of glutamate dehydrogenase on antibiotic resistance could, in principle, be due to the enzymatic activity of the protein or the regulatory function.

CEF arrests the growth of the *rocG* mutant cells at mid-exponential phase. We next compared the growth behaviors of *B. subtilis* wild-type and *rocG gudB* double null mutant (Δ*rocG* Δ*gudB*) strains in the presence of CEF. CEF was added at the beginning of the culture, and growth was measured spectrophotometrically (by optical density at 600 nm) using a Bioscreen C microbial growth analyzer (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. After reaching mid-exponential phase (OD_{600} of ~0.5, which corresponds to about 1×10^8 CFU/ml), the *rocG gudB* mutant cells exhibit growth arrest in the presence of very low levels of CEF (50 ng/ml) (Fig. 3A).

Glutamate dehydrogenase is required for the catabolism of glutamate, arginine, ornithine, and proline, and transcription of *rocG* is strongly repressed by glucose and other easily metabolized carbon sources (3, 4, 5). Previously, in wild-type cells grown in nutrient broth, the total cellular activity of glutamate dehydrogenase was observed to be low in early exponential phase, with higher levels in the later stages of exponential growth (3). Thus, the CEF-induced growth arrest of the *rocG gudB* mutant appears to occur during the same growth phase when *rocG* would normally be upregulated. We therefore hypothesized that the CEF-induced growth arrest might be correlated with an inability of the *rocG gudB* mutant to metabolize alternative carbon sources and, as a corollary, that CEF somehow affects carbon source preferences.

We reasoned that if the growth arrest observed in the presence of CEF is due to a block in catabolism, then cells provided with a more abundant carbon supply should be delayed in growth arrest. Indeed, in 2× LB medium, growth impairment (for both the wild type and the *rocG gudB* double mutant) occurred at a somewhat higher OD_{600} value (approximately 0.7) than in LB (Fig. 3B versus A). However, wild-type cells actually had slightly increased sensitivity to CEF. We next tested the effects of providing cells with an abundant and easily metabolized carbon source (2% glucose). In LB supplemented with 2% glucose, both the wild-type and *rocG gudB* mutant strains exhibited growth arrest, and eventually lysis,

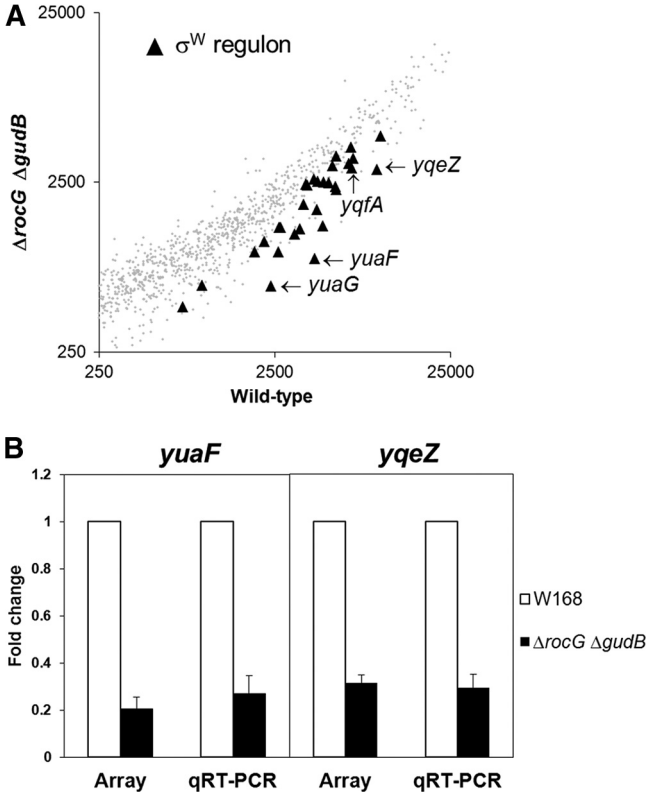

FIG 5 A *rocG* null mutant displays decreased expression of the σ^W regulon. (A) Gene expression variation as measured by cDNA microarray analysis under nonstress conditions. RNA was extracted from cells grown in LB medium to an OD_{600} of 0.4. Black triangles indicate the σ^W regulon genes. (B) Quantitative real-time RT-PCR analysis of *yuaF* and *yqeZ* expression. Data were expressed as fold change relative to wild-type cells. The level of 23S rRNA was used as a normalization control. Three independent experiments were performed for each gene, and the standard deviation is indicated by error bars.

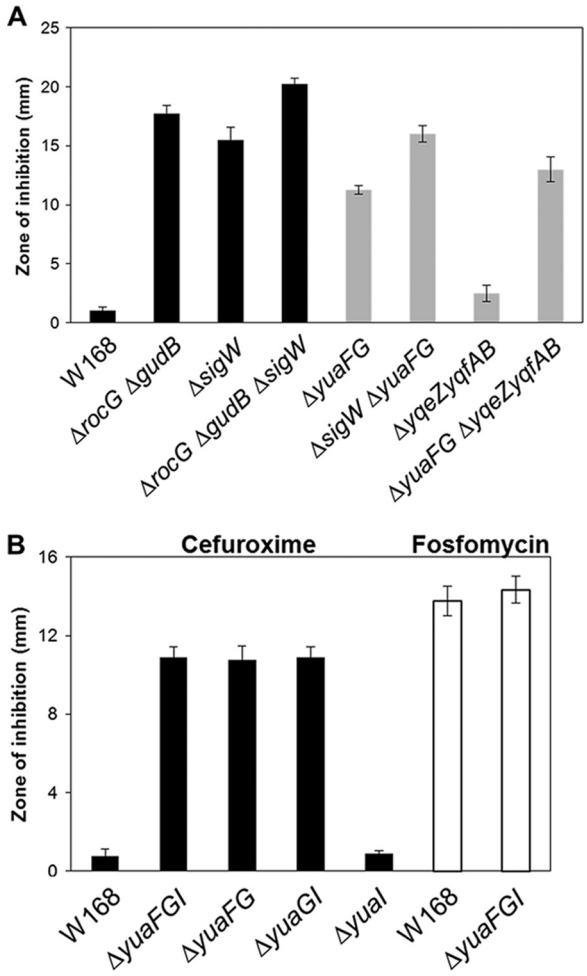

FIG 6 A survey of the σ^W regulon identifies *yuaFGI* as a major determinant of CEF resistance. The CEF sensitivity was determined by disk diffusion assay, which was performed on LB agar plates with a filter paper disk containing 3 μg CEF. (A) Detailed identification of genes conferring CEF resistance in the *yuaFGI* operon and determination of fosfomycin sensitivity. (B) Involvement of the σ^W regulon in RocG-mediated CEF resistance. Three independent experiments were performed for each strain, and the standard deviation is indicated by error bars.

in mid-logarithmic phase in the presence of low levels of CEF. Finally, in minimal medium containing 2% glucose, the wild-type and mutant strains showed similar responses to CEF: both strains showed a CEF-dependent growth lag but no longer displayed growth arrest in mid-logarithmic phase.

These results suggest that glucose-dependent repression of *rocG* leads even wild-type cells to behave phenotypically as glutamate dehydrogenase mutants. Moreover, simply providing cells with an easily metabolized carbon source is insufficient to bypass the growth arrest. Although the reasons for these medium-dependent differences are not entirely clear, these data suggest that CEF-induced growth arrest of the *rocG gudB* mutant in LB medium is correlated with a need for *rocG* activation.

Effect of alkaline growth pH on the susceptibility of *B. subtilis* to CEF. In addition to its role in carbon catabolism, RocG may play a role in pH homeostasis (43). The arginine catabolism (*roc*) operon has been shown to be upregulated at high pH, presumably because arginine breakdown can lead to acidic products that counteract base stress (43). However, in some species arginine catabolism is upregulated at acidic pH. Ammonia (NH_3), generated by glutamate dehydrogenase, can bind a proton (H^+), leading to an increase in intracellular pH (pH_i). It has been shown that during fermentation of amino acids by *B. subtilis* natto there is substantial ammonia production, much of which is due to glutamate dehydrogenase (20). We therefore reasoned that production of NH_3 by RocG might affect cellular pH and, since cell membranes are permeable to NH_3, also affect extracellular pH (pH_e). However, no significant differences in pH_e were observed between the *B. subtilis* wild-type and *rocG gudB* double mutant strains when measured at several different growth points in LB medium (data not shown).

Next, we examined the effect of alkaline growth pH (conditions known to induce *rocG* expression and the σ^W regulon [42, 44]) on the susceptibility of *B. subtilis* to CEF. The wild-type strain showed a remarkable reduction in susceptibility to CEF under alkaline growth conditions (Fig. 4A). However, as shown in Fig. 4B, the *rocG gudB* double mutant still shows high susceptibility to CEF even in MOPS-buffered LB medium (pH 8.5). Thus, simply raising the pH_e, in this case with buffer, is not sufficient to prevent growth arrest. We conclude that the role of RocG as being important for growth in the presence of low concentrations of CEF is not obviously linked to carbon catabolism or to a major role in pH

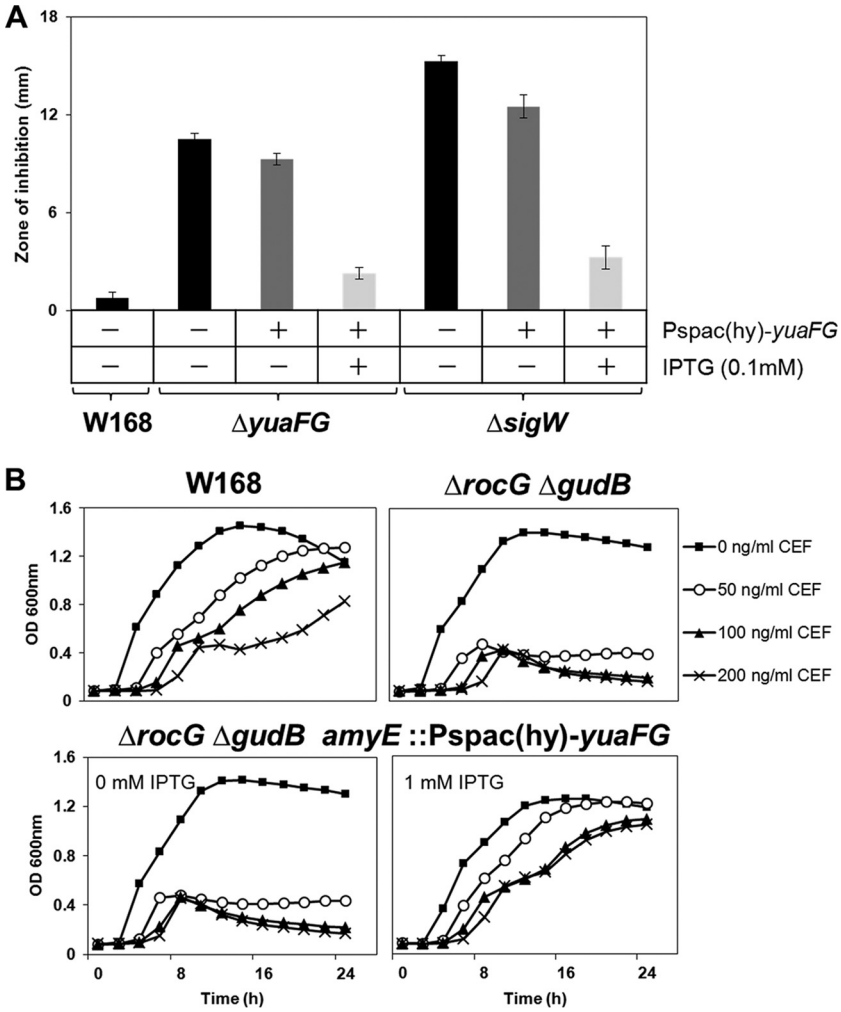

FIG 7 *yuaFG* overexpression by IPTG induction rescues the CEF-sensitive phenotype. (A) Induction of *yuaFG* expression by IPTG (0.1 mM) restores CEF resistance in the *yuaFG* mutant and the *sigW* mutant. Three independent experiments were performed for each strain, and the standard deviation is indicated by error bars. (B) IPTG-dependent induction of *yuaFG* prevents the growth arrest of *rocG* mutant cells. The maximal effect was observed at a final concentration of 1 mM IPTG. Liquid growth assays were performed in LB medium using a Bioscreen C growth analyzer. A representative data set is shown.

homeostasis. It remains possible that RocG affects intracellular pH or by other means alters cell physiology to help cells resist the deleterious effects of CEF, but determining the mechanism of this connection requires further study.

RocG is positively involved in controlling the expression of the σ^W regulon. In order to better understand the precise molecular mechanism(s) by which RocG exerts its effects on CEF resistance, the gene expression profile (transcriptome) of the *rocG gudB* mutant was assessed using DNA microarrays. The *rocG gudB* double null mutant showed significant changes in gene expression relative to a wild-type strain. Significantly, a regulon-based expression analysis revealed that most of the σ^W regulon (8, 19), which is known to be related to resistance to cell wall antibiotics (18), is downregulated in the *rocG gudB* mutant (Fig. 5A). This is consistent with the observed susceptibility of the *rocG* mutant to fosfomycin (Fig. 1A), since σ^W is required for expression of FosB, the major fosfomycin resistance determinant in *B. subtilis* (7).

Within the σ^W regulon, expression of genes in the *yuaFGI* and *yqeZ-yqfAB* operons was strongly downregulated. In *B. subtilis*, YuaF is a member of the NfeD family with a potential role in maintaining membrane integrity (1), and YuaG (recently renamed FloT) is a putative flotillin-like protein (24, 45). The *yqeZ* gene encodes a second NfeD family protein, while *yqfA* encodes another flotillin homologue that has functions partially redundant with those of YuaG (26). The observed changes in transcript abundance for *yuaF* (fold change ± standard deviation [SD] = 0.21 ± 0.049) and *yqeZ* (0.32 ± 0.035) were further confirmed by qRT-PCR analysis (0.27 ± 0.078 and 0.29 ± 0.058, respectively), as shown in Fig. 5B. The qRT-PCR results were in direct agreement with cDNA microarray data.

The *yuaFGI* operon is a major contributor to σ^W-dependent CEF resistance. To determine the contribution of the σ^W regulon to CEF resistance, disk diffusion assays were performed on LB agar plates (Fig. 6). As predicted, the *sigW* mutant exhibited increased sensitivity to CEF (Fig. 6A). A survey of σ^W-controlled genes for effects on CEF resistance revealed that *yuaFG* plays a major role in CEF resistance, with *yqeZ-yqfAB* playing an accessory role (Fig. 6A). We also found that the first two genes in the *yuaFGI* operon were enough for it to exert its full effect on CEF resistance (Fig. 6B). These results are also consistent with studies suggesting a

functional interaction between the NfeD protein YuaF and the flotillin YuaG (41). However, the *yuaFGI* operon does not confer fosfomycin resistance (Fig. 6B), consistent with the known involvement of another σ^W target gene, *fosB* (7). Together, these findings suggest that RocG affects CEF resistance by enhancing transcription of the σ^W regulon.

Overexpression of *yuaFG* in the *rocG* mutant prevents the growth arrest induced by CEF. To confirm the involvement of the *yuaFG* genes in CEF resistance, these two genes were placed under the control of an IPTG-inducible promoter and the fusion was integrated ectopically at the *amyE* locus [*amyE*::P_{spac}(hy)-*yuaFG*] of the *yuaFG* mutant, the *sigW* mutant, and the *rocG gudB* double mutant. We performed disk diffusion assays in the *yuaFG* and the *sigW* mutant backgrounds bearing the P_{spac}(hy)-*yuaFG* fusion (Fig. 7A). In both strains, induction of *yuaFG* expression by IPTG (0.1 mM) restored CEF resistance (Fig. 7). These strains also show slightly lower sensitivity to CEF under noninducing conditions, possibly because the P_{spac}(hy) promoter has low expression in the absence of IPTG.

We next determined whether the *yuaFG* genes can rescue the growth arrest induced by CEF in the *rocG gudB* mutant. Indeed, the *rocG gudB* mutant carrying the P_{spac}(hy)-*yuaFG* fusion showed growth arrest without IPTG induction but was rescued by induction of YuaFG synthesis (Fig. 7B). These effects occurred in an IPTG concentration-dependent manner, with a maximal effect at 1 mM (data not shown). These results suggest a pivotal role of the *yuaFG* genes in CEF resistance mediated by σ^W (and influenced by RocG) in *B. subtilis*.

The *yuaFGI* operon reduces membrane fluidity under σ^W-inducing conditions. To better understand how *yuaFGI* and *yqeZ-yqfAB* contribute to intrinsic CEF resistance, we investigated the influence of these genes on membrane fluidity. Both *yuaG* and *yqfA* encode putative flotillin-like proteins that are believed to organize the cell membrane into functional microdomains (1, 26). In addition, σ^W overexpression has previously been shown to reduce membrane fluidity by altering expression of fatty acid biosynthesis genes (21). The σ^W-dependent activation of a promoter (P_5) within the *fabHAF* operon leads to an increase in the proportion of straight-chain fatty acids and an increase in overall chain length. Since activation of P_5 accounts for some, but not all, of the σ^W-dependent decrease in membrane fluidity (21), we reasoned that upregulation of *yuaFGI* and/or *yqeZ-yqfAB* might alter membrane fluidity.

Membrane fluidity was assessed by measuring the fluorescence anisotropy of *B. subtilis* cells labeled with DPH (Fig. 8). Under normal growth conditions, both wild-type and *yuaFGI* knockout cells exhibited similar anisotropy levels. However, when *sigW* was overexpressed with a xylose-inducible promoter (P_{xyl}-*sigW*), the resulting increase in anisotropy was significantly lower in the *yuaFGI* knockout strain than in control cells. Since a higher anisotropy is indicative of a less-fluid membrane, these results indicate that expression of the *yuaFGI* operon reduces membrane fluidity when activated by σ^W. In contrast, deleting *yqeZ-yqfAB* had no effect on anisotropy levels, even under *sigW* overexpression conditions. The effect of *yuaFGI* on membrane fluidity is comparable to that of the σ^W-dependent promoter (P_5) within the *fabHAF* operon (21). In a σ^W overexpression strain both lacking *yuaFGI* and containing a mutation (P_5*) that abolishes P5 activity (P_{xyl}-*sigW yuaFGI* P_5*), anisotropy levels were the same as in wild-type cells. This demonstrates that both P_5 and *yuaFGI* function to

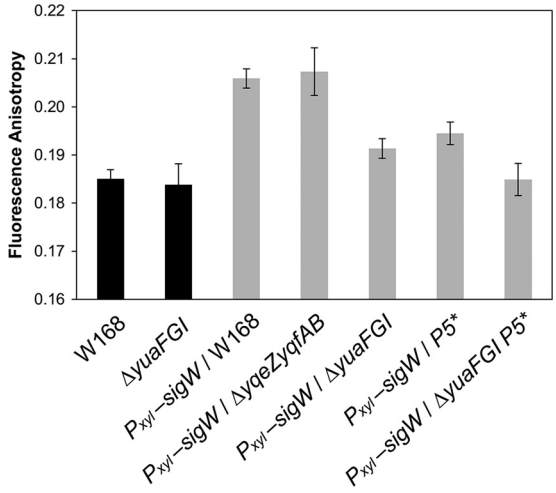

FIG 8 Inactivation of *yuaFGI* prevents the decrease in membrane fluidity induced by overexpression of σ^W. Cells were grown in LB medium with xylose (2%) to an OD_{600} of 0.4 and then incubated in phosphate buffer (100 mM, pH 7.0) with DPH (5 μM) at 25°C for 30 min. In strains containing the P_{xyl}-*sigW* construct, σ^W was expressed under the control of a xylose-inducible promoter. The membrane fluidity of each strain was determined via fluorescence anisotropy measurements. Data are presented as the average of at least three trials, and the standard error is indicated by error bars.

reduce membrane fluidity and that they are the primary components of the σ^W regulon to do so.

The effect of *yuaFGI* on membrane fluidity might explain how this operon contributes to CEF resistance. Adjustments in membrane fluidity can influence numerous properties of the lipid bilayer, such as permeability, protein mobility, and protein-protein interactions (25). However, not all changes in membrane fluidity result in CEF resistance, since the P_5-inactive strain was not any more susceptible to CEF than the wild-type strain (data not shown). YuaG (FloT) has also been linked to the formation of lipid domains, which have been shown to regulate sporulation, biofilm formation, and other signal transduction pathways (13, 26).

Concluding remarks. Our data demonstrate a previously unidentified regulatory effect of RocG on antibiotic resistance. Although glutamate dehydrogenase is relatively well studied in *B. subtilis*, the effects of glutamate dehydrogenase activity on the cell envelope stress response have thus far remained unknown. The present study indicates that the σ^W-dependent stress response is the link between RocG activity and CEF resistance. Glutamate dehydrogenase affects expression of the σ^W regulon, by mechanisms not yet resolved, and thereby contributes to CEF resistance. We specifically demonstrate that overexpression of the σ^W-regulated *yuaFG* operon prevents growth arrest of the *rocG* mutant in the presence of CEF. We also show that expression of *yuaFGI* operon reduces membrane fluidity under σ^W-inducing conditions and that this protein-based mechanism is additive with a previously described lipid-based pathway (21). These findings suggest that YuaFG influences CEF resistance by altering the physical properties of the membrane, but the origins of this effect are presently unclear. YuaFG are thought to help organize membrane microdomains (13, 26), and this could affect the assembly or activity of cell wall biosynthetic complexes known to be targeted by CEF. A future challenge will be to identify how glutamate dehy-

drogenase affects activity of the σ^W regulon (and thereby CEF and fosfomycin resistance) and how the activity of flotillin-like proteins affects cell wall biosynthesis pathways.

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REFERENCES

1. **Bateman A, et al.** 2004. The Pfam protein families database. Nucleic Acids Res. **32**:D138–D141.
2. **Belitsky BR.** 2002. Biosynthesis of amino acids of the glutamate and aspartate families, alanine and polyamines, p 203–231. *In* Sonenshein AL, Hoch JA, Losick R (ed), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
3. **Belitsky BR, Sonenshein AL.** 1998. Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. J. Bacteriol. **180**:6298–6305.
4. **Belitsky BR, Sonenshein AL.** 1999. An enhancer element located downstream of the major glutamate dehydrogenase gene of *Bacillus subtilis.* Proc. Natl. Acad. Sci. U. S. A. **96**:10290–10295.
5. **Belitsky BR, Kim H-J, Sonenshein AL.** 2004. CcpA-dependent regulation of *Bacillus subtilis* glutamate dehydrogenase gene expression. J. Bacteriol. **186**:3392–3398.
6. **Butcher BG, Helmann JD.** 2006. Identification of *Bacillus subtilis* σ^W-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by bacilli. Mol. Microbiol. **60**:765–782.
7. **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.
8. **Cao M, et al.** 2002. Defining the *Bacillus subtilis* sigma (W) regulon: a comparative analysis of promoter consensus search, runoff transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. J. Mol. Biol. **316**:443–457.
9. **Commichau FM, Stülke J.** 2008. Trigger enzymes: bifunctional proteins active in metabolism and in controlling gene expression. Mol. Microbiol. **67**:692–702.
10. **Commichau FM, Herzberg C, Tripal P, Valerius O, Stülke J.** 2007. A regulatory protein-protein interaction governs glutamate biosynthesis in *Bacillus subtilis*: the glutamate dehydrogenase RocG moonlights in controlling the transcription factor GltC. Mol. Microbiol. **65**:642–654.
11. **Commichau FM, Forchhammer K, Stülke J.** 2006. Regulatory links between carbon and nitrogen metabolism. Curr. Opin. Microbiol. **9**:167–172.
12. **Commichau FM, Gunka K, Landmann JJ, Stülke J.** 2008. Glutamate metabolism in *Bacillus subtilis*: gene expression and enzyme activities evolved to avoid futile cycles and to allow rapid responses to perturbations in the system. J. Bacteriol. **190**:3557–3564.
13. **Donovan C, Bramkamp M.** 2009. Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology **155**:1786–1799.
14. **Dwyer DJ, Kohanski MA, Collins JJ.** 2009. Role of reactive oxygen species in antibiotic action and resistance. Curr. Opin. Microbiol. **12**:482–489.
15. **Gunka K, et al.** 2010. Functional dissection of a trigger enzyme: mutations of the *Bacillus subtilis* glutamate dehydrogenase RocG that affect differentially its catalytic activity and regulatory properties. J. Mol. Biol. **400**:815–827.
16. **Hachmann AB, Angert ER, Helmann JD.** 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. Antimicrob. Agents Chemother. **53**:1598–1609.
17. **Heinrich J, Hein K, Wiegert TT.** 2009. Two proteolytic modules are involved in regulated intramembrane proteolysis of *Bacillus subtilis* RsiW. Mol. Microbiol. **74**:1412–1426.
18. **Helmann JD.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46**:47–110.
19. **Huang XJ, Gaballa A, Cao M, Helmann JD.** 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, σ^W. Mol. Microbiol. **31**:361–371.
20. **Kada S, Yabusaki M, Kaga T, Ashida H, Yoshida K.** 2008. Identification of two major ammonia-releasing reactions involved in secondary natto fermentation. Biosci. Biotechnol. Biochem. **72**:1869–1876.
21. **Kingston AW, Subramanian C, Rock CO, Helmann JD.** 2011. A σ^W-dependent stress response in *Bacillus subtilis* that reduces membrane fluidity. Mol. Microbiol. **81**:69–79.
22. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130**:797–810.
23. **Kuhry J-G, Duportail G, Bronner C, Laustriat G.** 1985. Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammoniumdiphenylhexatriene. Biochim. Biophys. Acta **845**:60–67.
24. **Langhorst MF, Reuter A, Stuermer CA.** 2005. Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cell. Mol. Life Sci. **62**:2228–2240.
25. **Los DA, Murata N.** 2004. Membrane fluidity and its roles in the perception of environmental signals. Biochim. Biophys. Acta **1666**:142–157.
26. **López D, Kolter R.** 2010. Functional microdomains in bacterial membranes. Genes Dev. **24**:1893–1902.
27. **Luo Y, Helmann JD.** 2009. Extracytoplasmic function sigma factors with overlapping promoter specificity regulate sublancin production in *Bacillus subtilis.* J. Bacteriol. **191**:4951–4958.
28. **Luo Y, Asai K, Sadaie Y, Helmann JD.** 2010. Transcriptomic and phenotypic characterization of a *Bacillus subtilis* strain without extracytoplasmic function σ factors. J. Bacteriol. **192**:5736–5745.
29. **Mascher T, Hachmann AB, Helmann JD.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J. Bacteriol. **189**:6919–6927.
30. **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.
31. **Neu HC, Fu KP.** 1978. Cefuroxime, a beta-lactamase-resistant cephalosporin with a broad spectrum of gram-positive and -negative activity. Antimicrob. Agents Chemother. **13**:657–664.
32. **Poole K.** 2004. Resistance to β-lactam antibiotics. Cell. Mol. Life Sci. **61**:2200–2223.
33. **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.
34. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed.Cold Spring Harbor Laboratory Press, New York, NY.
35. **Scott LJ, Ormrod D, Goa KL.** 2001. Cefuroxime axetil: an updated review of its use in the management of bacterial infections. Drugs **61**:1455–1500.
36. **Sonenshein AL, Hoch JA, Losick R (ed).** 2002. *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
37. **Svobodová J, Svoboda P.** 1988. Cytoplasmic membrane fluidity measurements on intact living cells of *Bacillus subtilis* by fluorescence anisotropy of 1,6-diphenyl 1,3,5-hexatriene. Folia Microbiol. **33**:1–9.
38. **Talaat AM, et al.** 2002. Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis.* Nucleic Acids Res. **30**:e104.
39. **Wach A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae.* Yeast **12**:259–265.
40. **Wacker I, et al.** 2003. The regulatory link between carbon and nitrogen metabolism in *Bacillus subtilis*: regulation of the *gltAB* operon by the catabolite control protein CcpA. Microbiology **149**:3001–3009.
41. **Walker CA, Hinderhofer M, Witte DJ, Boos W, Moller HM.** 2008. Solution structure of the soluble domain of the NfeD protein YuaF from *Bacillus subtilis.* J. Biomol. NMR **42**:69–76.
42. **Wiegert T, Homuth G, Versteeg S, Schumann W.** 2001. Alkaline shock induces the *Bacillus subtilis* sigma(W) regulon. Mol. Microbiol. **41**:59–71.
43. **Wilke MS, Lovering AL, Strynadka NC.** 2005. β-Lactam antibiotic resistance: a current structural perspective. Curr. Opin. Microbiol. **8**:525–533.
44. **Wilks JC, et al.** 2009. Acid and base stress and transcriptomic responses in *Bacillus subtilis.* Appl. Environ. Microbiol. **75**:981–990.
45. **Zhang HM, et al.** 2005. An alkali-inducible flotillin-like protein from *Bacillus halodurans* C-125. Protein J. **24**:125–131.