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Requirement of the CroRS Two-Component System for Resistance to Cell Wall-Targeting Antimicrobials in *Enterococcus faecium*

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ABSTRACT Enterococci are serious opportunistic pathogens that are resistant to many cell wall-targeting antibiotics. The CroRS two-component signaling system responds to antibiotic-mediated cell wall stress and is critical for resistance to cell wall-targeting antibiotics in *Enterococcus faecalis*. Here, we identify and characterize an orthologous two-component system found in *Enterococcus faecium* that is functionally equivalent to the CroRS system of *E. faecalis*. Deletion of *croRS* in *E. faecium* resulted in marked susceptibility to cell wall-targeting agents including cephalosporins and bacitracin, as well as moderate susceptibility to ampicillin and vancomycin. As in *E. faecalis*, exposure to bacitracin and vancomycin stimulates signaling through the CroRS system in *E. faecium*. Moreover, the CroRS system is critical in *E. faecium* for enhanced beta-lactam resistance mediated by overexpression of Pbp5. Expression of a Pbp5 variant that confers enhanced beta-lactam resistance cannot overcome the requirement for CroRS function. Thus, the CroRS system is a conserved signaling system that responds to cell wall stress to promote intrinsic resistance to important cell wall-targeting antibiotics in clinically relevant enterococci.

KEYWORDS enterococcus, antibiotic resistance, two-component regulatory systems

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Enterococcus faecalis and *Enterococcus faecium* represent serious opportunistic pathogens that are responsible for many nosocomial infections. Treatment of enterococcal infections is particularly challenging due to intrinsic and acquired resistance toward many clinically relevant antibiotics, including beta-lactams, aminoglycosides, glycopeptides, and trimethoprim (1). Because all clinical isolates of *E. faecalis* and *E. faecium* are intrinsically resistant to cephalosporins (a subset of beta-lactam antibiotics), disabling cephalosporin resistance with small molecule therapeutics may be a viable strategy to overcome antibiotic-resistant enterococcal infections. Both species use transpeptidase activity of a low-affinity penicillin-binding protein (Pbp5) in cooperation with the glycosyltransferase activity of the penicillin-binding proteins (PBPs) PonA or PbpF to continue transpeptidation and transglycosylation reactions required for cell wall assembly during cephalosporin exposure (2–5). However, additional determinants contributing to cephalosporin resistance have also been explored in *E. faecalis* and *E. faecium*.

In *E. faecalis*, two enzymes involved in cell wall synthesis (the UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase MurAA [6] and the alanine transferase BppA2 [7]) are known to be required for normal cephalosporin resistance. In addition, two signal transduction pathways mediate intrinsic resistance to cephalosporins and other cell wall-targeting antibiotics. One pathway includes a eukaryotic-like Ser/Thr kinase, IreK, and its cognate phosphatase, IreP, which act antagonistically to regulate a pathway leading to cephalosporin resistance (8, 9). An ortholog of IreK in *E. faecium* has

also been implicated in cephalosporin resistance of that species (10). In *E. faecalis*, a substrate for phosphorylation by IreK has been described, designated IreB, which acts as a negative regulator of the pathway (11). However, the specific output of the pathway that drives cephalosporin resistance remains unknown. In addition to the IreK/IreP signaling pathway, the two-component signal transduction system (TCS) consisting of the CroS sensor kinase and its cognate response regulator CroR has a role in resistance to cell wall-targeting antibiotics. Disruption of the CroRS TCS in *E. faecalis* renders strains more sensitive to diverse cell wall-targeting agents such as cephalosporins, ampicillin, bacitracin, and vancomycin (12, 13). Consistent with a role for the CroRS TCS in responding to antibiotic-mediated cell wall stress, these agents can also stimulate CroR-dependent transcription (12). However, only three genes regulated by CroR have been identified (12, 14, 15), with *croR* itself the only of those that possesses a clear role in antimicrobial resistance. Thus, the downstream effectors in the CroR regulon that drive resistance remain to be identified.

Although *E. faecium* is resistant to cephalosporins, most studies have analyzed ampicillin resistance in clinical isolates. High levels of ampicillin resistance have been associated with mutations in Pbp5. However, specific variants do not always correlate with MIC values in different *E. faecium* lineages (16–18), implying that additional factors modulate ampicillin resistance. A genome-wide study identified several determinants required for ampicillin resistance in *E. faecium*, including the L,D-transpeptidase Ldt_{fm}, the D-alanyl–D-alanine carboxypeptidase DdcP, and the glycosyltransferase Pgt (19). The Ldt_{fm} pathway was also identified as providing high-level ampicillin resistance after successive *in vitro* selections for ampicillin resistance (20–23). Collectively, these studies indicate that factors involved in enterococcal cell wall remodeling, distinct from the traditional biosynthetic PBPs, modulate resistance to ampicillin in *E. faecium*. However, the extent to which they also influence resistance to other beta-lactams such as cephalosporins remains largely unknown. Moreover, these factors are poorly conserved in *E. faecalis*, which tends to be considerably less prone to development of enhanced ampicillin resistance compared to *E. faecium*.

Ideally, any target for new therapeutics designed to disable enterococcal resistance to cephalosporins will be conserved in both *E. faecalis* and *E. faecium*. To explore whether mechanisms mediating cephalosporin resistance in *E. faecalis* are conserved in *E. faecium*, we identified and functionally characterized a TCS encoded in the *E. faecium* genome that is homologous to the CroRS TCS of *E. faecalis*. We report that deletion of the *E. faecium croRS* orthologs render *E. faecium* more susceptible to cell wall-targeting agents, some of which were observed to stimulate CroRS signaling in a wild-type strain. Moreover, the CroRS system is critical in *E. faecium* for enhanced cephalosporin and ampicillin resistance mediated by overexpression of Pbp5 and by expression of a Pbp5 variant that confers enhanced beta-lactam resistance. Thus, the CroRS system represents a conserved signaling system that responds to cell wall stress to promote intrinsic resistance to important cell wall-targeting antibiotics in clinically relevant enterococci.

RESULTS

Bioinformatic identification of CroRS orthologs in *E. faecium*. A BLASTn search of the *E. faecium* DO genome (24) with the *E. faecalis croR* and *croS* genes revealed candidates for *croR* and *croS* (HMPREF0351_12687 and HMPREF0351_12688). These genes are reciprocal best BLASTn hits with *croR* and *croS* of *E. faecalis*, suggesting they encode an orthologous TCS. Because functional studies (below) revealed that the *E. faecium* genes indeed encode an orthologous TCS, we will henceforth refer to the *croRS* genes and proteins using subscripts ("Efs" for *E. faecalis* and "Efm" for *E. faecium*). Figure 1A depicts the genomic architecture of the $croRS_{Efs}$ locus from the sequenced genome of *E. faecalis* OG1RF (25). The CroRS TCS is considered an "isolated" TCS, in that $croR_{Efs}$ and $croS_{Efs}$ are encoded on a bicistronic message lacking the downstream gene, *serS* (12). The genomic architecture surrounding the $croRS_{Efm}$ locus is identical to that of *E. faecalis*. The proteins encoded by the *croR* and *croS* orthologs exhibit high similarity over their entire length: $CroR_{Efs}$ and $CroR_{Efm}$ are 90% identical (96% similar), and $CroS_{Efs}$

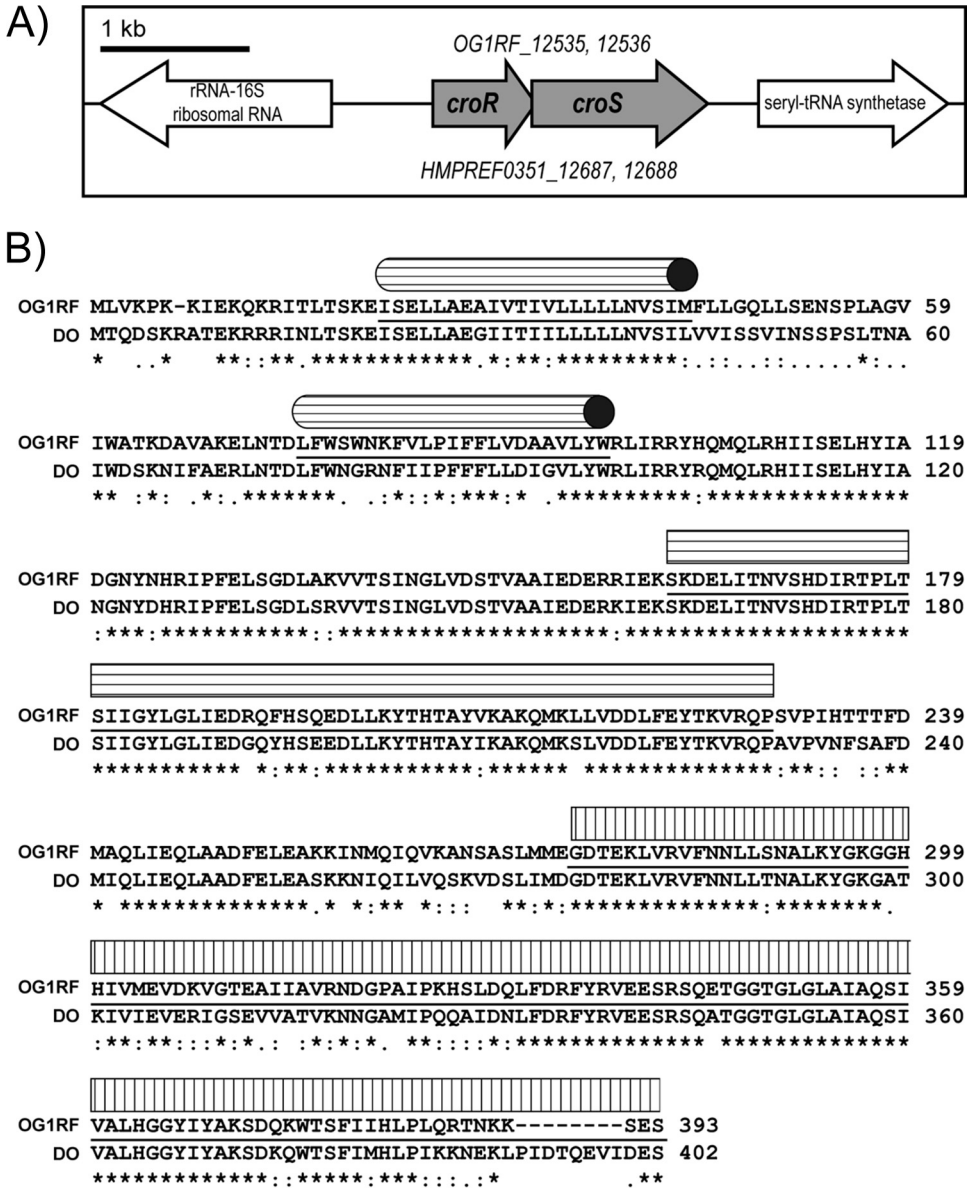

FIG 1 Comparison of the *croRS* loci in *E. faecalis* and *E. faecium*. (A) Genomic organization of the *croRS* locus in the sequenced genomes of *E. faecalis* OG1RF (top locus numbers) and *E. faecium* DO (bottom locus numbers). (B) CLUSTAL W alignment of CroS from *E. faecalis* OG1RF and *E. faecium* DO. Protein domains are underlined as predicted by SMART. Cylinders denote transmembrane domains, the rectangle with horizontal stripes denotes the dimerization and phosphoryl acceptor domain, and the rectangle with vertical stripes denotes the histidine kinase-like ATPase domain.

is 74% identical (87% similar) to $CroS_{Efm}$. Alignment of the CroS protein sequences from *E. faecalis* and *E. faecium* reveal high conservation of key functional domains (e.g., the dimerization and phosphoryl acceptor domain and ATPase domain) in both (Fig. 1B). Collectively, these data led us to hypothesize that $CroRS_{Efm}$ is functionally orthologous to $CroRS_{Efs}$.

We previously identified a second TCS (CisRS), found in a subset of *E. faecalis* strains, that is capable of influencing the activity of $CroRS_{Efs}$ under certain conditions due to mutual overlap in the identity of so-called "specificity" residues of the CroS and CisS kinases that dictate response regulator specificity for TCS kinases (26–28). However, extensive studies indicated that "cross talk" between CisS and $CroR_{Efs}$ only occurs in the absence of $CroS_{Efs}$ and is not physiologically relevant (26). Nevertheless, we searched for homologs of CisRS in sequenced *E. faecium* genomes, but none were found. To

TABLE 1 MICs of various antibiotics for Δ*croRS* mutants

	Median MIC (μg/ml)[a]			
	E. faecium		*E. faecalis*	
Antibiotic	Wild type	Δ*croRS* mutant	Wild type	Δ*croRS* mutant
Cefadroxil (narrow spectrum)	64	32	64	32
Cefuroxime (expanded spectrum)	512	64	32	4
Ceftriaxone (broad spectrum)	64	1	64	8
Cefepime (fourth generation)	512	4	16	8
Ampicillin	1	0.25	1	0.5
Bacitracin	64	8	64	8
Vancomycin	0.5	0.25	2	0.5
Chloramphenicol	4	4	4	4

[a]Median MICs are reported from ≥2 biological replicates. The strains analyzed were wild-type *E. faecalis* OG1, *E. faecalis* Δ*croRS* mutant SB35, wild-type *E. faecium* 1,141,733, and *E. faecium* Δ*croRS* mutant JL537.

probe whether another TCS found in *E. faecium* had the potential to "cross talk" with $CroRS_{Efm}$, we analyzed the specificity residues of all TCS histidine kinases found in the genome of *E. faecium* strain 1,141,733. No kinases were found that possessed substantially similar specificity residues. The closest match to the specificity residues of $CroS_{Efm}$ was with EFSG_00540, which shares only 4 of 9 specificity residues with $CroS_{Efm}$. In comparison, CisS—the only kinase capable of "cross talk" with $CroS_{Efs}$—shares 6 of 9 specificity residues with $CroS_{Efs}$. Moreover, *E. faecalis* encodes three other TCS kinases that share 5, 3, and 3 specificity residues with $CroS_{Efs}$, respectively, and yet none of those are capable of interacting with $CroR_{Efs}$. Given the divergence of specificity residues in TCS kinases of *E. faecium* 1,141,733, we conclude that *E. faecium* 1,141,733 does not possess another TCS kinase that is likely to interact with $CroR_{Efm}$. In this scenario, the phosphorylation state of $CroR_{Efm}$ would therefore be controlled exclusively by $CroS_{Efm}$.

Deletion of $CroRS_{Efm}$ decreases resistance to cell wall-targeting agents. To probe the biological functions of $CroRS_{Efm}$, we made an in-frame deletion of the $croRS_{Efm}$ locus in *E. faecium* 1,141,733 and performed phenotypic analyses of the resulting mutant. As with the *E. faecalis* Δ$croRS_{Efs}$ mutant, the *E. faecium* Δ$croRS_{Efm}$ mutant exhibited substantial loss of resistance to cell wall-targeting agents such as expanded-spectrum to "fourth-generation" cephalosporins and to bacitracin, with modest effects on resistance to ampicillin and vancomycin (Table 1). Resistance to the protein synthesis inhibitor chloramphenicol was unaltered in the absence of CroRS. To test for complementation of the Δ$croRS_{Efm}$ mutation, we produced $CroRS_{Efm}$ from a plasmid with an inducible promoter, revealing an inducer-dependent enhancement of ceftriaxone resistance (Table 2). Although full complementation to wild-type levels of ceftriaxone resistance was not observed, we suspect this is likely due to differences in

TABLE 2 Ceftriaxone resistance of *E. faecium* and *E. faecalis* Δ*croRS* mutants expressing $croRS_{Efm}$

	Median MIC (μg/ml)[b]	
Strain/plasmid[a]	Without cCF10	With cCF10
E. faecium		
Wild type/vector	128	128
Δ$croRS_{Efm}$ mutant/vector	2	2
Δ$croRS_{Efm}$ mutant/$croRS_{Efm}$	2	16
E. faecalis		
Wild type/vector	128	128
Δ$croRS_{Efs}$ mutant/vector	8	8
Δ$croRS_{Efs}$ mutant/$croRS_{Efm}$	8	128

[a]The strains and plasmids analyzed were as follows: wild-type *E. faecium*, 1,141,733; *E. faecium* Δ*croRS* mutant, JL537, wild-type *E. faecalis*, OG1; *E. faecalis* Δ*croRS* mutant, SB35; vector, pJLL105; and $croRS_{Efm}$, pJLL160.

[b]Median MICs are reported from ≥2 biological replicates.

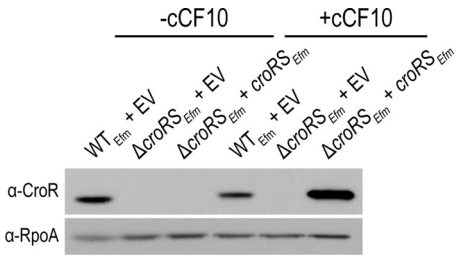

FIG 2 Expression of $CroR_{Efm}$ in the *E. faecium* Δ*croRS* mutant. Standard SDS-PAGE and immunoblot analysis of exponentially growing wild-type (WT_{Efm}) and Δ$croRS_{Efm}$ *E. faecium* 1,141,733 strains. Strains carry empty vector (EV) or *croRS*-expressing cCF10-inducible plasmids. The strains are wild-type *E. faecium* 1,141,733 and JL537 with pJLL105 and pJLL160.

the kinetics or level of $croRS_{Efm}$ expression from the plasmid during the course of the MIC experiment. Consistent with this hypothesis, immunoblot analysis revealed that $CroRS_{Efm}$ levels from the plasmid expression platform were aberrant (elevated) compared to normal chromosomal expression in exponentially growing cells (Fig. 2). It remains unclear precisely how overexpression of $CroRS_{Efm}$ interferes with normal cephalosporin resistance pathway function, but improper localization of $CroRS_{Efm}$ in the membrane, alteration of the kinase/phosphatase balance of $CroRS_{Efm}$, or adverse effects on other membrane proteins might play a role. Regardless of the mechanism, overall, these results indicate that $CroRS_{Efm}$ specifically influences resistance to cell wall-targeting compounds in *E. faecium* in a similar manner as in *E. faecalis*.

To determine whether $CroRS_{Efm}$ initiates signal transduction in response to cell wall-targeting agents, we monitored the phosphorylation state of $CroR_{Efm}$ after exposure of *E. faecium* cells to various antibiotics. Upon sensing their signal, histidine kinases autophosphorylate and subsequently transfer the phosphoryl group to their cognate response regulators. We monitored this process using Phos-Tag SDS-PAGE and immunoblotting for CroR (26). In *E. faecalis*, a slower-mobility isoform of $CroR_{Efs}$ is observed with Phos-Tag SDS-PAGE after treatment of cells with bacitracin and vancomycin (Fig. 3A), reflecting phosphorylation of $CroR_{Efs}$ in response to antibiotic-mediated cell wall stress. Under the conditions tested, robust phosphorylation of $CroR_{Efs}$ is not observed after treatment with ceftriaxone or ampicillin, nor (as expected) after treatment with the ribosomal-targeting antibiotic chloramphenicol. In *E. faecium*, a small but detectable amount of phosphorylated $CroR_{Efm}$ was observed even in the absence of antibiotic stress (Fig. 3B). Phosphorylation of $CroR_{Efm}$ was markedly enhanced after treatment of cells with bacitracin and vancomycin, as observed in *E. faecalis*. Thus, $CroRS_{Efm}$ responds to antibiotic-mediated cell wall stress by enhancing phosphorylation of $CroR_{Efm}$, validating $CroRS_{Efm}$ as functional orthologs of $CroRS_{Efs}$.

The reason a fraction of $CroR_{Efm}$ is phosphorylated in the absence of antibiotic stress is unclear. The population of $CroR_{Efm}$-P vanished upon treatment of cells with chloramphenicol (Fig. 3B). Similar results were obtained with the protein synthesis inhibitor fusidic acid and the DNA gyrase inhibitor norfloxacin (data not shown). Because these agents are expected to quickly arrest cellular growth by inhibiting essential cellular functions, we tested whether small amounts of $CroR_{Efm}$-P accumulated during cell growth itself. Exponentially growing wild-type *E. faecium* 1,141,733 cells were collected by centrifugation and resuspended in either growth medium (Mueller-Hinton broth [MHB], as a control for continued growth) or PBS (to halt growth). As discussed above, cells suspended in growth medium contained a small fraction of $CroR_{Efm}$-P. In contrast, $CroR_{Efm}$-P vanished in cells suspended in PBS (Fig. 4). We conclude that active growth leads to phosphorylation of a small fraction of $CroR_{Efm}$ and that treatment of cells with chloramphenicol (or fusidic acid or norfloxacin) halts growth, leading to loss of this phosphorylated population.

***E. faecium* CroRS can drive resistance in both *E. faecium* and *E. faecalis*.** The molecular nature of the direct signal that triggers signaling through $CroRS_{Efs}$ is un-

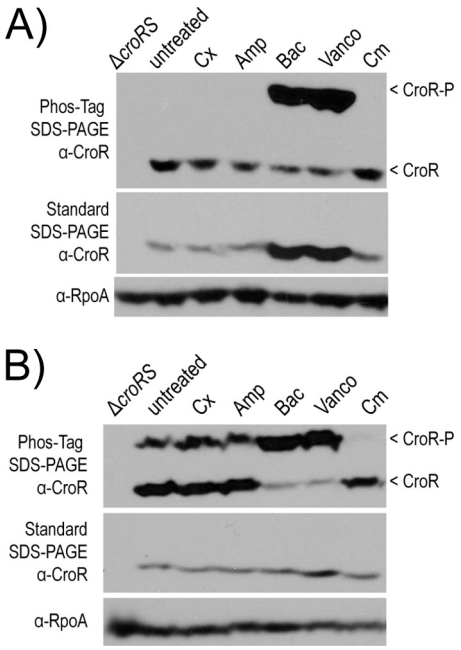

FIG 3 Analysis of CroR phosphorylation in whole-cell lysates. Phos-Tag SDS-PAGE and standard SDS-PAGE was followed by immunoblot analyses in response to various insults in *E. faecalis* OG1 (A) and *E. faecium* 1,141,733 (B). Exponentially growing cells were treated with ceftriaxone (Cx), ampicillin (Amp), bacitracin (Bac), vancomycin (Vanco), or chloramphenicol (Cm) for 30 min. The results are representative of ≥2 experiments. A subunit of RNA polymerase (RpoA) was used as a loading control.

known. Similarly, the identity of the specific output of $CroRS_{Efs}$ signaling that mediates antibiotic resistance (i.e., the antibiotic resistance effectors in the $CroR_{Efs}$ regulon) is unknown. To gain insight into whether $CroRS_{Efm}$ and $CroRS_{Efs}$ sense a similar direct signal and drive common molecular responses we tested for heterologous complementation. To do so, $CroRS_{Efm}$ was produced from an inducible promoter in an *E. faecalis* $croRS_{Efs}$ mutant. We found that $CroRS_{Efm}$ could enhance ceftriaxone resistance of the heterologous host in the presence of inducer (Table 2). This heterologous complementation not only confirms that $CroRS_{Efm}$ is a functional ortholog of $CroRS_{Efs}$, but also suggests that $CroRS_{Efm}$ responds to the same molecular signal and drives a common molecular response in both *E. faecalis* and *E. faecium* upon activation by cell wall-targeting antibiotics.

Expression of Pbp5 is insufficient to restore beta-lactam resistance to the Δ*croRS*$_{Efm}$ mutant. The output of the CroRS TCS that promotes cephalosporin resistance is unknown. To test if enhanced expression of the low-affinity Pbp5 could be an output of CroRS in *E. faecalis*, Comenge et al. constitutively expressed $Pbp5_{Efs}$ in a

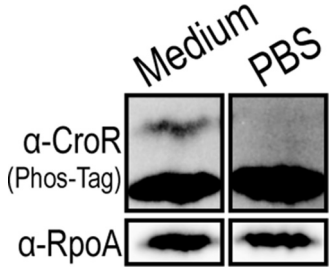

FIG 4 Analysis of CroR phosphorylation after growth arrest. Exponentially growing *E. faecium* 1,141,733 cells were collected by centrifugation and resuspended in either growth medium (for continued growth) or PBS (to halt growth). Phos-Tag SDS-PAGE and immunoblot analysis of whole-cell lysates was used to visualize CroR-P. The results are representative of ≥3 experiments. A subunit of RNA polymerase (RpoA) was used as a loading control.

TABLE 3 Resistance to ceftriaxone and ampicillin of *E. faecium* strains expressing *pbp5* alleles from *E. faecium* strain 1,141,733 or strain 1,231,408

Strain/plasmid[a]	Median MIC (μg/ml)[b]	
	Ceftriaxone	Ampicillin
Wild type/vector	128	4
Wild type/$pbp5_{733}$	1,024	16
Wild type/$pbp5_{408}$	1,024	16
Δ$croRS_{Efm}$ mutant/vector	1	1
Δ$croRS_{Efm}$ mutant/$pbp5_{733}$	1	2
Δ$croRS_{Efm}$ mutant/$pbp5_{408}$	1	2

[a]The strains and plasmids analyzed were as follows: wild-type *E. faecium*, 1,141,733; *E. faecium* Δ*croRS* mutant, JL537; vector, pJH123; $pbp5_{733}$, pSLK252; and $pbp5_{408}$, pSLK253.
[b]Median MICs are reported from ≥2 biological replicates.

Δ$croRS_{Efs}$ mutant strain but observed no change in beta-lactam resistance (i.e., the mutant strain was still susceptible to beta-lactams) (12). Moreover, PBP labeling studies revealed no differences in the pattern or amount of PBP labeling between the wild-type and Δ$croRS_{Efs}$ mutant strains (12), indicating that CroRS impacts resistance via another mechanism. To determine whether $CroRS_{Efm}$ influences cephalosporin resistance through Pbp5 in *E. faecium*, we used Bocillin-FL to label PBPs on exponential-phase wild-type and Δ$croRS_{Efm}$ mutant *E. faecium* strains but found no differences in the pattern or amount of PBP labeling (not shown). In addition, we expressed *E. faecium pbp5* in wild-type and Δ$croRS_{Efm}$ mutant strains. As expected, expression of $pbp5_{Efm}$ derived from *E. faecium* 1,141,733 enhanced ceftriaxone and ampicillin resistance in wild-type *E. faecium* (Table 3). However, as previously observed in *E. faecalis*, production of Pbp5 derived from *E. faecium* 1,141,733 ($Pbp5_{Efm733}$) was essentially unable to enhance beta-lactam resistance in the Δ$croRS_{Efm}$ mutant, suggesting that a $CroRS_{Efm}$-dependent function is required for $Pbp5_{Efm}$ to mediate resistance. To determine whether a variant form of $Pbp5_{Efm}$ associated with enhanced ampicillin resistance could bypass the requirement for $CroRS_{Efm}$, we expressed $pbp5_{Efm}$ from *E. faecium* 1,231,408 in the Δ$croRS_{Efm}$ mutant. $Pbp5_{Efm408}$ carries two amino acid changes (M485A, and insertion of Ser at position 466) that have been found to result in reduced beta-lactam binding by $Pbp5_{Efm}$ and enhanced beta-lactam resistance (17). Despite these changes, production of $Pbp5_{Efm408}$ was similarly unable to enhance beta-lactam resistance in the Δ$croRS_{Efm}$ mutant (Table 3). Collectively, these data indicate that $CroRS_{Efm}$ is essential for Pbp5-mediated beta-lactam resistance in *E. faecium* via a mechanism that is independent of $Pbp5_{Efm}$ expression.

DISCUSSION

An understanding of antibiotic resistance mechanisms used by the opportunistic pathogens *Enterococcus faecalis* and *Enterococcus faecium* will provide an essential foundation for the development of new therapies to treat enterococcal infections or to prevent the expansion of multidrug-resistant enterococcal isolates during antibiotic therapy. With that in mind, we explored the role of the $CroRS_{Efm}$ TCS in intrinsic resistance to cell wall-targeting antibiotics in *E. faecium*. We found that the $CroRS_{Efm}$ signaling pathway is activated in response to cell wall-targeting antibiotics, which is consistent with a model in which $CroRS_{Efm}$ monitors the cell envelope for stress and initiates an adaptive biological response by enhancing the expression of (as-yet-unknown) downstream effector genes when envelope stress is detected. As a sensor kinase embedded in the cytoplasmic membrane, $CroS_{Efm}$ is ideally positioned to respond to insults affecting the integrity of the cell-envelope.

Our data indicate that the $CroRS_{Efm}$ TCS is activated during normal growth (i.e., in the absence of exogenous antimicrobials), albeit at a low level (Fig. 3 and 4). A small fraction of $CroR_{Efm}$ exists in the phosphorylated state in growing cells, and vanishes (presumably due to phosphatase activity of $CroS_{Efm}$) upon diverse treatments that halt growth (e.g., exposure to chloramphenicol, or suspension in PBS). We speculate that this reflects sensing (by $CroS_{Efm}$) of low levels of cell wall stress encountered during the

process of growth and/or cell division. For example, slight imbalances in peptidoglycan synthesis and degradation at sites of nascent peptidoglycan insertion could be perceived by $CroS_{Efm}$ as cell wall stress, leading to kinase activation and phosphorylation of $CroR_{Efm}$. The presence of cell wall-active antimicrobials in the environment would exacerbate the imbalance, enhancing activation of $CroS_{Efm}$ further and leading to robust $CroR_{Efm}$ phosphorylation (Fig. 3).

Consistent with such a model, we found that $CroRS_{Efm}$ function is required for intrinsic resistance of *E. faecium* to a variety of cell wall-targeting antibiotics, including beta-lactams (cephalosporins and ampicillin), bacitracin, and vancomycin. These findings, in concert with our cross-species complementation study, indicate that the CroRS TCS is functionally conserved in both species of enterococci that are of the greatest clinical significance and suggest that CroRS (or its regulon members) could represent viable targets for novel adjunctive therapies to render enterococci susceptible to beta-lactam antibiotics. Moreover, because $CroRS_{Efm}$ is required for beta-lactam resistance even in a strain expressing a variant of Pbp5 bearing mutations that reduce beta-lactam binding affinity and enhances beta-lactam resistance (Table 3), we speculate that any such therapies will prove effective on ampicillin-resistant *E. faecium* clinical isolates.

Although CroRS function is required for resistance to beta-lactam antibiotics such as ceftriaxone and ampicillin in both *E. faecalis* and *E. faecium*, under our conditions ceftriaxone and ampicillin did not lead to robust activation of CroRS signaling (Fig. 3). In contrast, antibiotics that inhibit an earlier step in the peptidoglycan biosynthesis pathway (bacitracin and vancomycin) elicited robust CroRS signaling (Fig. 3). The reason for this apparent disconnect remains unknown. One possibility is that the output of CroRS signaling (i.e., CroR-dependent genes, expected to be the "effectors" of the CroRS regulon) are capable of efficiently mitigating the stress imposed by beta-lactam exposure but not that imposed by inhibitors acting earlier in the peptidoglycan pathway. In this scenario, transient CroRS activation by beta-lactams would lead to expression of effector genes that rapidly mitigate beta-lactam-imposed stress, eliminating the activation signal and leading to dampened CroRS activity. Conversely, inhibition by bacitracin or vancomycin would create a cell wall stress that cannot be efficiently mitigated by CroRS-dependent effectors, leading to sustained and robust formation of CroR-P. Future identification of the CroR regulon is necessary to explore this possibility in more detail. It is perhaps worth noting that Comenge et al. used a different experimental design, with $CroR_{Efs}$-dependent gene expression as a readout and observed activation upon growth in the presence of ceftriaxone, ampicillin, and other beta-lactams (12), indicating that beta-lactams can indeed function as activators of CroRS signaling. We speculate that the transcriptional reporter fusion readout of CroRS signaling is more sensitive than Phos-Tag SDS-PAGE in this case due to the ability of the β-galactosidase reporter to accumulate in cells during growth, whereas Phos-Tag provides only an "instantaneous" snapshot of CroR-P abundance.

How does $CroRS_{Efm}$ influence beta-lactam resistance? As noted above, previous studies indicate that $CroRS_{Efs}$ modulates gene expression in response to stress from cell wall-targeting antibiotics (12, 26), and the functional conservation identified here suggests this is also the case with $CroRS_{Efm}$. Hence, we hypothesize that $CroRS_{Efm}$ enhances expression of genes that are important for resistance upon sensing cell wall stress. However, the "effector" genes under transcriptional control of CroRS that are important for resistance have not been identified in either species. Current evidence indicates that changes in the expression of the PBPs themselves (including the low-affinity Pbp5) are not responsible for enhanced resistance. For example, no changes in labeling of PBPs were observed in Δ*croRS* mutants of *E. faecalis* (12) or *E. faecium* (this study). Moreover, expression of *pbp5* from a constitutive promoter did not significantly enhance resistance of Δ*croRS* mutants in either *E. faecalis* (12) or *E. faecium* (this study). To test the hypothesis that CroRS influences the production of substrates for Pbp5 (peptidoglycan precursors), Comenge et al. analyzed peptidoglycan precursors produced in wild-type and Δ$croRS_{Efs}$ mutant strains of *E. faecalis* (12). However, no

TABLE 4 Strains and plasmids used in this study

Strain	Genotype or description[a]	Source or reference
Strains		
E. coli		
Top10	Routine cloning host	Lab stock
DH5α	Routine cloning host	Lab stock
E. faecalis		
OG1	Wild-type laboratory strain isolated from oral sample (MLST 1)	39
SB35	OG1 Δ(*croR croS*)*3*	26
E. faecium		
1,141,733	Wild-type reference strain, isolated from blood culture of hospitalized patient (MLST 327)	40
JL537	1,141,733 Δ(*croR croS*)*4*	This study
1,231,408	Wild-type reference strain, isolated from blood culture of hospitalized patient (MLST 582)	40
Plasmids		
pJH123	Expression vector, constitutive P_{23S} promoter (Cm^r)	This study
pSLK252	*pbp5* from *E. faecium* 1,141,733 in pJH123	This study
pSLK253	*pbp5* from *E. faecium* 1,231,408 in pJH123; carries M485A and Ser466 insertion	This study
pBK2	cCF10-inducible expression vector with *lacZ* in MCS (Cm^r)	34
pJLL105	pBK2 with *lacZ* removed (Cm^r)	This study
pJLL160	*croRS* from *E. faecium* 1,141,733 in pBK2 (replaces *lacZ*)	This study
pJH086	*E. faecalis* allelic-exchange vector (Cm^r, *repA* V71G, *lacZ pheS**)	This study
pJLL150	Δ($croR_{Efm}$ $croS_{Efm}$)*4* deletion allele in pJH086	This study

[a]Cm^r, chloramphenicol resistance.

differences in relative abundances were detected, suggesting that the beta-lactam sensitivity of the Δ$croRS_{Efs}$ mutant is not due to limitation of the substrates for Pbp5. Therefore, we hypothesize that an as-yet-unknown effector(s) within the CroR regulon influences the enzymatic activity or proper localization of Pbp5 and, in the absence of CroR-mediated expression of this effector, Pbp5 is unable to efficiently mediate peptidoglycan cross-linking. The concept that such a cofactor might be required for Pbp5 function *in vivo* is not without precedent: a specific modification of staphylococcal wall teichoic acid (beta-O-GlcNAcylation) is required for methicillin resistance mediated by the low-affinity Pbp2a (29), and roughly 20 distinct loci in the staphylococcal genome are required for full Pbp2a-mediated methicillin resistance (30–32). We anticipate that ongoing efforts to elucidate the composition of the CroR regulon will reveal new determinants of intrinsic antibiotic resistance that are conserved between *E. faecalis* and *E. faecium*. Analysis of the biological function of these determinants will provide new insights into the mechanisms of antibiotic resistance and cell wall homeostasis with the potential to be exploited for the development of new antimicrobials.

MATERIALS AND METHODS

Bacterial strains, growth media, and chemicals. The bacterial strains and plasmids used in the study are listed in Table 4. *E. faecalis* and *E. faecium* strains were grown in half-strength (0.5×) brain heart infusion medium or MHB for routine maintenance. *Escherichia coli* strains were grown in lysogeny broth. MHB was used in experiments for data collection. Chloramphenicol was used at 10 μg/ml for plasmid selection. cCF10 was used at 0.2 ng/ml for *E. faecalis* and at 10 ng/ml for *E. faecium*. All cultures were grown aerobically with shaking.

Plasmid construction. All plasmids were constructed using Gibson assembly (33) to express native $croRS_{Efm}$ or $pbp5_{Efm}$ alleles. For all recombinant plasmids, sequencing of the full insert was performed to verify the absence of errors. Ectopic expression of $croRS_{Efm}$ was accomplished using the cCF10-inducible promoter in the enterococcal expression vector pBK2 (34). *E. faecium pbp5* alleles were expressed from pJH123, a modified version of the enterococcal expression vector pJRG9 containing the constitutive P23s promoter (35). pJH123 was generated to have a versatile multiple cloning site for which N-terminal or C-terminal hemagglutinin (HA) epitope fusions could be generated if desired. A ribosome-binding site was also included (AGGAGG) for consistency and convenience. The multiple cloning site (MCS) of pJRG9 was removed by restriction digest and the newly constructed MCS was inserted. The resulting MCS contains features in the following order downstream of the P23s promoter: EcoRI site, ribosome binding

site (RBS), PstI site, HA epitope, AgeI site, and XhoI site. The *E. faecium pbp5* alleles were cloned into pJH123 at the PstI and XhoI restriction sites, resulting in no HA fusion.

To improve the temperature-sensitive allelic-exchange vector for *E. faecalis*, pCJK218 (36), RepA was analyzed for hydrophobic residues that may be disrupted to generate a more temperature-sensitive phenotype. The method described by Varadarajan et al. (37, 38) was used to identify V71G as a candidate substitution to provide temperature sensitivity in RepA. Inverse PCR on pCJK218 was used to introduce the V71G substitution into *repA*. During sequence confirmation of the V71G substitution, we discovered that the clone of pCJK218 used did not carry the original temperature-sensitive *repA* substitutions. Therefore, the resulting plasmid, pJH086, carries a *repA* allele with only the V71G substitution but otherwise retains the features of pCJK218 (chloramphenicol resistance [Cm^r], *lacZ*, *pheS**). *E. coli*, *E. faecalis*, and *E. faecium* carrying pJH086 grow at 30°C but are significantly impaired in growth at 42°C, confirming that the V71G substitution provides temperature sensitivity.

Construction of *E. faecium* Δ*croRS* mutant. An in-frame deletion of *croRS* in *E. faecium* was constructed using markerless allelic exchange as previously described for *E. faecalis* (36) with a derivative of pJH086. Although no other genes are expected to be cotranscribed with $croRS_{Efm}$, the deletion allele retains 126 codons at the 5′ end of $croR_{Efm}$ (the entire DNA binding domain of $CroR_{Efm}$ is deleted) and the final 9 codons at the 3′ end of $croS_{Efm}$ in an attempt to avoid perturbing the expression of adjacent genes.

Antibiotic susceptibility determinations. The MICs of antibiotics were determined as described previously (26). Briefly, bacteria from stationary-phase cultures in MHB (plus 10 μg/ml chloramphenicol for plasmid carrying strains) were inoculated at a cell density of ~10^5 CFU/ml into microplate wells containing 2-fold serial dilutions of antibiotic. Plates were incubated in a Bioscreen C plate reader at 37°C for 24 h with brief shaking before each measurement. The optical density at 600 nm (OD_{600}) was read every 15 min, and the lowest concentration of antibiotic that prevented growth was recorded as the MIC.

Phos-Tag SDS-PAGE and immunoblot analysis of CroR. Acrylamide-pendant Phos-Tag is a dinuclear metal complex that is polymerized directly into polyacrylamide gels and acts as a selective phosphate-binding tag to retard the migration of phosphorylated protein isoforms. Analysis of CroR phosphorylation by Phos-Tag-Mn^{2+} SDS-PAGE was performed as described previously (26). Wild-type *E. faecalis* (OG1) and *E. faecium* (strain 1,141,733) were grown to exponential phase (OD_{600} ~ 0.2 in MHB) and treated with 2× the MIC of ampicillin, bacitracin, vancomycin, or chloramphenicol for 30 min. Due to the increase in cephalosporin MIC with high cell density (inoculum effect), 1.5 mg/ml ceftriaxone was used for *E. faecalis* and *E. faecium*. Samples were collected by mixing with an equal volume of cold ethanol-acetone (1:1) mixture to rapidly kill the bacteria and prevent any further signaling events. CroR from *E. faecalis* and *E. faecium* was detected using custom rabbit polyclonal antiserum raised against *E. faecalis* CroR protein.

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