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# Mutations in the β Subunit of RNA Polymerase Alter Intrinsic Cephalosporin Resistance in Enterococci

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**As major causes of hospital-acquired infections, antibiotic-resistant enterococci are a serious public health concern. Enterococci are intrinsically resistant to many cephalosporin antibiotics, a trait that enables proliferation in patients undergoing cephalosporin therapy. Although a few genetic determinants of cephalosporin resistance in enterococci have been described, overall, many questions remain about the underlying genetic and biochemical basis for cephalosporin resistance. Here we describe an unexpected effect of specific mutations in the β subunit of RNA polymerase (RNAP) on intrinsic cephalosporin resistance in enterococci. We found that RNAP mutants, selected initially on the basis of their ability to provide resistance to rifampin, resulted in allele-specific alterations of the intrinsic resistance of enterococci toward expanded- and broad-spectrum cephalosporins. These mutations did not affect resistance toward a diverse collection of other antibiotics that target a range of alternative cellular processes. We propose that the RNAP mutations identified here lead to alterations in transcription of as-yet-unknown genes that are critical for cellular adaption to cephalosporin stress.**

As major causes of hospital-acquired infections, antibiotic-resistant enterococci are a serious public health concern (14). Enterococci normally inhabit the gastrointestinal tract of many insects and animals, including humans (1, 34). They are adept at sharing mobile genetic elements carrying antibiotic resistance determinants, contributing to the emergence of multiresistant clones in the hospital setting (13). In addition, enterococci are intrinsically resistant to broad-spectrum cephalosporins (27), antibiotics that belong to the β-lactam family and interfere with cell wall (peptidoglycan) biosynthesis by inhibiting the penicillin-binding proteins (PBPs) that cross-link peptidoglycan (35, 38). Enterococci proliferate and achieve an abnormally high population size in patients undergoing cephalosporin therapy (8), presumably due to this cephalosporin resistance trait. This population bloom likely contributes to the emergence of enterococcal infections by facilitating dissemination of the bacteria to other sites. Intrinsic cephalosporin resistance is a trait exhibited by enterococci belonging to both of the species that account for nearly all hospital-acquired enterococcal infections, *Enterococcus faecalis* and *Enterococcus faecium*.

Despite the importance of intrinsic cephalosporin resistance, only a few genetic determinants specifying resistance in enterococci have been described. These include the following: (i) *pbp5*, which encodes a so-called "low-affinity" penicillin-binding protein that is thought to mediate peptidoglycan cross-linking in the presence of cephalosporins due to its reduced affinity for the drugs (4, 33); (ii) *ireK* (formerly *prkC*), which encodes a transmembrane Ser/Thr kinase whose kinase activity is critical for cephalosporin resistance (19, 21), although its targets remain unknown; and (iii) a locus encoding a two-component signal transduction system (CroRS), mutations in which render *E. faecalis* markedly susceptible to cephalosporins (6, 12). The response regulator component of the CroRS signaling system (CroR) contains a functional DNA-binding domain, suggesting that transcriptional remodeling is necessary for adaptation to the stress imposed by cephalosporins. However, thus far, only a few genes controlled directly by CroR have been identified. These include *salB*, encoding a secreted protein that does not contribute to cephalosporin resistance (26), *croRS* itself (26), and a locus of genes encoding a putative glutamine transporter (22) with no obvious connection to cephalosporin resistance.

Here we describe an unexpected effect of specific mutations in the β subunit of RNA polymerase (RNAP) on intrinsic cephalosporin resistance in enterococci. We found that RNAP mutants, selected initially on the basis of their ability to provide resistance to the antibiotic rifampin (Rif$^r$ mutants), resulted in allele-specific alterations of the intrinsic resistance of enterococci toward expanded- and broad-spectrum cephalosporins. This effect was observed in divergent genetic lineages of *E. faecalis*. Similarly, a specific mutation in RNAP from 2 lineages of *E. faecium* led to enhanced cephalosporin resistance. In contrast, these RNAP mutations did not affect resistance toward a diverse collection of other antibiotics that target a range of alternative cellular processes. We propose that the RNAP mutations identified here lead to alterations in transcription of as-yet-unknown genes that are critical for cellular adaption to cephalosporin stress.

## MATERIALS AND METHODS

**Bacterial strains, growth media, and chemicals.** The strains used in this study are listed in Table 1. Brain heart infusion medium (BHI) and Mueller-Hinton broth (MHB) were prepared as described by the manufacturer (Becton Dickinson). Bacteria were stored at −80°C in BHI supplemented with 30% glycerol. Antibiotics and other chemicals were obtained from Sigma unless otherwise indicated. Erythromycin (Em) was used at 10 μg/ml for growth of resistant *E. faecalis*.

**Isolation and sequencing of enterococcal Rif$^r$ mutants.** The MIC of *E. faecalis* OG1 for rifampin (Rif) under our conditions (see below) was determined to be 8 μg/ml. To isolate Rif$^r$ mutants, independent cultures

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**TABLE 1** Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or description[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. faecalis* | | |
| OG1 | Wild-type, original unmarked isolate (MLST 1) | 11 |
| CK135 | OG1 *rpoB* H486Y (spontaneous Rif$^r$ derivative) | This work |
| JL206 | OG1 Δ*ireK2* | This work |
| JL339 | OG1 Δ*pbp5-2* | This work |
| JL209 | OG1 *rpoB* H486Y (spontaneous Rif$^r$ derivative, independent isolate) | This work |
| JL213 | OG1 *rpoB* H486D (spontaneous Rif$^r$ derivative) | This work |
| JL211 | OG1 *rpoB* Q473K (spontaneous Rif$^r$ derivative) | This work |
| JL308 | OG1 *rpoB* H486Y Δ*pbp5-2* | This work |
| JL235 | OG1 *rpoB* H486Y Δ*ireK2* (spontaneous Rif$^r$ derivative of JL206) | This work |
| T1 (SS498) | Wild-type (MLST 21), CDC reference strain | 23 |
| JL220 | T1 *rpoB* H486Y (spontaneous Rif$^r$ derivative) | This work |
| JL219 | T1 *rpoB* H486D (spontaneous Rif$^r$ derivative) | This work |
| JL224 | T1 *rpoB* Q473K (spontaneous Rif$^r$ derivative) | This work |
| *E. faecium* | | |
| Com12 | Fecal isolate | 25 |
| JL282 | Com12 *rpoB* H486Y (spontaneous Rif$^r$ derivative) | This work |
| 1,231,501 | Clinical isolate | 30 |
| JL277 | 1,231,501 *rpoB* H486Y (spontaneous Rif$^r$ derivative) | This work |
| Plasmids | | |
| pJRG8 | *E. faecalis* expression vector, constitutive $P_{23}$ promoter (Em$^r$) | 19 |
| pJLL52 | *rpoB* H486Y cloned into pJRG8 | This work |
| pJLL50 | *rpoB* H486D cloned into pJRG8 | This work |
| pJLL21 | Δ*pbp5-2* allele in the allelic exchange vector pJRG32 | 35a |
| pCJK74 | Δ*ireK2* allele in the allelic exchange vector pCJK47 | 21 |

[a] MLST, multilocus sequence type.

inoculated from isolated colonies on BHI agar plates were incubated overnight at 37°C in BHI with agitation. Bacteria were concentrated approximately 20-fold by centrifugation and resuspension in BHI, followed by plating on BHI agar supplemented with rifampin at 200 μg/ml and incubation at 37°C. Rif$^r$ colonies typically appeared within ~24 h. Colonies that arose were streaked for purification on BHI agar and retested to ensure they retained the Rif$^r$ phenotype. To identify the mutation responsible for resistance to Rif, a segment of the *rpoB* gene that included Rif clusters I to III was amplified by PCR from genomic DNA of the Rif$^r$ mutants and the amplicon was subjected to DNA sequencing. The numbering scheme of Ozawa and coworkers (29) for *E. faecalis* RpoB was used to designate amino acids that had undergone substitutions in the mutants.

**Construction of *E. faecalis rpoB* H486Y mutants lacking *ireK* or *pbp5*.** Unmarked, in-frame deletions of the genes encoding IreK or Pbp5 were constructed as previously described (21, 35a) using the markerless exchange system described by Kristich et al. (18). Briefly, an allelic exchange plasmid carrying the desired deletion allele (pJLL21 or pCJK74) was transferred to the corresponding native location in the *E. faecalis* chromosome using pVE6007 as a helper plasmid to facilitate recombination via published methods (20). Successful isolation of deletion mutants was achieved by plating on counterselection plates and incubating at 30°C for ~2 to 3 days. A mutant (the JL308 strain) carrying the *rpoB* H486Y allele in combination with the *pbp5* deletion was constructed by allelic exchange with pJLL21 in the CK135 strain. A mutant (the JL235 strain) carrying the *rpoB* H486Y allele in combination with the *ireK* deletion was constructed by selecting spontaneous Rif$^r$ mutants in the Δ*ireK* mutant (JL206) background and then sequencing *rpoB* to verify the allele.

**Construction of plasmids.** Plasmids to express alleles of *rpoB* in *E. faecalis* were constructed by amplifying the full-length *rpoB* open reading frame (ORF) from the genomic DNA of the appropriate strain (CK135 or JL213) and cloning the amplicons with primer-specified restriction sites into SpeI/XhoI-digested pJRG8, an *E. faecalis* expression vector, creating pJLL52 and pJLL50, respectively. Because *E. faecalis rpoB* contains an internal SpeI restriction site, we used the enzyme BsaI to generate SpeI-compatible ends on the *rpoB* PCR amplicons for cloning.

**Tests of antibiotic sensitivity.** MICs for antibiotics were determined in aerobic liquid cultures using a microtiter plate serial dilution method in a Bioscreen C plate reader (Oy Growth Curves Ab, Ltd.). Two-fold dilutions of antibiotics in MHB were prepared in the wells of a 100-well honeycomb microtiter plate. Bacteria from stationary-phase cultures in MHB were inoculated into each well at a density of ~$10^5$ CFU/ml. Plates were incubated at 37°C for 24 h, with brief shaking and measurement of the optical density at 600 nm ($OD_{600}$) at 15-min intervals. The lowest concentration of antibiotic that prevented growth was recorded as the MIC. In some cases, antibiotic susceptibility was also assessed by preparing serial 10-fold dilutions of stationary-phase cultures and inoculating aliquots onto the surface of agar plates supplemented with antibiotics.

## RESULTS

We initially sought to generate a derivative of wild-type *E. faecalis* OG1 that was marked with a stable genetic lesion conferring an antibiotic resistance phenotype. One such phenotype that has historically been used to mark susceptible strains of *E. faecalis* is resistance to the antibiotic rifampin (5, 9), which binds to bacterial RNA polymerase and prevents initiation of transcription (37). Spontaneous Rif$^r$ mutants can readily be obtained by plating a population of bacteria in the presence of inhibitory concentrations of Rif. Previous studies of numerous species of bacteria established that such Rif$^r$ mutants carry missense mutations in specific segments of the β subunit of RNA polymerase (encoded by the *rpoB* gene), the most common of which is known as "Rif cluster I" (3, 10, 16, 17, 24, 28, 36). These mutations cluster in or near the Rif-binding site on RNAP and prevent Rif from binding efficiently (reviewed in reference 10).

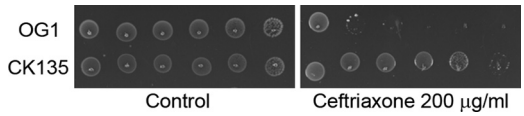


**FIG 1** An *E. faecalis* Rif$^r$ mutant carrying *rpoB* H486Y exhibits enhanced resistance to ceftriaxone. Cultures were subjected to 10-fold serial dilutions and inoculated (left to right, least to most dilute) on MHB agar supplemented with ceftriaxone. The strains used were OG1, the wild-type *E. faecalis* strain, and CK135, OG1 carrying the *rpoB* H486Y allele. Results are representative of a minimum of 3 independent experiments.

**Rif$^r$ mutants of *E. faecalis* exhibit altered resistance to cephalosporins.** After obtaining a spontaneous Rif$^r$ mutant of *E. faecalis* OG1 (strain CK135) on selection plates containing 200 $\mu$g/ml rifampin, we serendipitously observed that the mutant exhibited enhanced resistance to ceftriaxone (a broad-spectrum cephalosporin antibiotic) relative to that of its otherwise isogenic parent (Fig. 1 and Table 2). To explore this observation in more detail, we isolated additional spontaneous Rif$^r$ mutants from multiple independent cultures of *E. faecalis* OG1. Rif$^r$ mutants arose at a frequency of $\sim 10^{-8}$ per viable CFU, comparable to that of previously reported selections in *E. coli* (17). The mutations responsible for resistance to Rif were identified by sequencing the region of *E. faecalis rpoB* corresponding to Rif clusters I to III of *E. coli rpoB*. All *E. faecalis* Rif$^r$ mutants we isolated carried missense mutations in Rif cluster I. Three *rpoB* alleles were identified, all of which have been reported to confer Rif$^r$ in other bacterial species: *rpoB* H486Y (isolated in multiple independent mutants), *rpoB* H486D, and *rpoB* Q473K. To determine if these mutations conferred substantial defects in fitness, we measured generation times for exponentially growing cells, including two independently derived *rpoB* H486Y mutants. Mutants with substitutions at H486 exhibited growth rates similar to those of the wild type (generation times of 69 $\pm$ 4 min for wild-type OG1, 74 $\pm$ 4 min for *rpoB* H486Y, and 70 $\pm$ 4 min for *rpoB* H486D). Only the *rpoB* Q473K mutation had an obvious effect on growth rate, leading to a minor increase in generation time (generation time of 79 $\pm$ 4 min). A similar trend was previously reported for an analogous set of RpoB mutants in *Bacillus subtilis* (24).

In light of the initial observation that the Rif$^r$ strain CK135 exhibited enhanced cephalosporin resistance, we measured the relative susceptibilities (MICs) of the Rif$^r$ mutants toward a panel of antibiotics that target a spectrum of diverse cellular processes (Table 2). We found that susceptibility to expanded-spectrum (cefuroxime) and broad-spectrum (ceftriaxone and ceftazidime) cephalosporins was substantially altered in an allele-specific manner. Both *rpoB* H486Y mutants exhibited markedly enhanced resistance to these drugs, whereas a mutant carrying a different substitution at the same site (*rpoB* H486D) or a substitution at a nearby site (*rpoB* Q473K) exhibited reduced resistance to the expanded- and broad-spectrum and cephalosporins. Changes in susceptibility to a narrow-spectrum cephalosporin (cefadroxil) and to the other $\beta$-lactam antibiotic tested (ampicillin) followed a similar trend in most cases but were modest in magnitude (2-fold), and 2-fold changes in MICs determined via the broth microdilution method are typically not considered reliable. In contrast to the susceptibility changes described above, none of the Rif$^r$ mutants exhibited differences in susceptibility toward antibiotics that target other aspects of cell wall biosynthesis (vancomycin, bacitracin, D-cycloserine) or toward antibiotics with non-cell-wall targets (chloramphenicol, kanamycin, norfloxacin). Thus, specific alleles of *rpoB* exert unique effects on susceptibility of *E. faecalis* to expanded- and broad-spectrum cephalosporins.

To determine if this phenomenon was unique to the OG1 genetic background, we isolated spontaneous Rif$^r$ mutants in a divergent lineage of *E. faecalis* (T1). Spontaneous *E. faecalis* T1 Rif$^r$ derivatives containing equivalent sets of *rpoB* alleles were obtained from multiple independent cultures and verified by sequencing of Rif cluster I of *rpoB*. Susceptibility tests of the T1 mutants revealed that, as was observed in the OG1 genetic background, the *rpoB* H486Y mutant exhibited markedly enhanced resistance to cephalosporins (Table 3). No change in susceptibility toward norfloxacin was observed for any of the mutants, confirming the specificity of the effect for cephalosporins. In contrast to what was observed

**TABLE 2** Median MICs for *E. faecalis* OG1 and its derivatives[a]

| Antibiotic | MIC ($\mu$g/ml) of each *E. faecalis* strain (*rpoB* allele) | | | | |
|---|---|---|---|---|---|
| | OG1 (wild type) | CK135 (H486Y) | JL209 (H486Y) | JL213 (H486D) | JL211 (Q473K) |
| Cephalosporins | | | | | |
| Ceftriaxone (broad spectrum) | 64 | 512 | 1,024 | 16 | 8 |
| Ceftazidime (broad spectrum) | 512 | 2,048 | 2,048 | 128 | 32 |
| Cefuroxime (expanded spectrum) | 64 | 512 | 512 | 16 | 8 |
| Cefadroxil (narrow spectrum) | 64 | 128 | 128 | 64 | 64 |
| Others that target cell wall synthesis | | | | | |
| Ampicillin | 1 | 2 | 1 | 0.5 | 0.5 |
| Vancomycin | 2 | 2 | 2 | 2 | 2 |
| Bacitracin | 64 | 64 | 64 | 64 | 64 |
| D-Cycloserine | 128 | 128 | 128 | 128 | 128 |
| Others that do not target cell wall synthesis | | | | | |
| Chloramphenicol | 8 | 8 | 8 | 8 | 8 |
| Kanamycin | 64 | 64 | 64 | 64 | 32 |
| Norfloxacin | 4 | 4 | 4 | 4 | 4 |

[a] MICs were determined in MHB after 24 h at 37°C from a minimum of 3 independent experiments.

**TABLE 3** Median MICs for enterococci of other genetic lineages[a]

| Antibiotic | MIC (μg/ml) of each strain and *rpoB* allele[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *E. faecalis* T1 | | | | *E. faecium* Com12 | | *E. faecium* 1,231,501 | |
| | WT | H486Y | H486D | Q473K | WT | H486Y | WT | H486Y |
| Ceftriaxone | 16 | 128 | 16 | 16 | 128 | 1,024 | 256 | 1,024 |
| Ceftazidime | 64 | 512 | 128 | 32 | ND | ND | ND | ND |
| Norfloxacin | 2 | 2 | 2 | 2 | ND | ND | ND | ND |

[a] MICs were determined in MHB after 24 h at 37°C from a minimum of 3 independent experiments.
[b] WT, wild-type *rpoB* allele (H486) (for all 3 strains); ND, not determined.

in the OG1 genetic background, the *rpoB* H486D and *rpoB* Q473K mutants did not exhibit a reduction in cephalosporin resistance in the *E. faecalis* T1 genetic background. The reasons for this difference remain unclear.

**Genetic linkage of *rpoB* H486Y with enhanced cephalosporin resistance.** The observation that multiple independently isolated, single-step *rpoB* H486Y mutations in divergent genetic lineages all conferred enhanced cephalosporin resistance to *E. faecalis* was a strong indication that the H486Y substitution was indeed responsible for this phenotype. To formally exclude the possibility that a (unknown) secondary mutation(s) elsewhere in the genome was responsible for enhanced cephalosporin resistance of the *rpoB* mutants, we tested for linkage between the *rpoB* H486Y allele and the enhanced cephalosporin resistance phenotype. Unfortunately, genetic tools that would enable efficient backcrossing of the *rpoB* mutations to a clean genetic background are not available for *E. faecalis*. Technical challenges associated with using available allelic exchange methods to introduce mutations into the large and essential *rpoB* gene *de novo* prompted us to address this question by simply cloning the *rpoB* genes from selected *rpoB* mutants into an *E. faecalis* expression vector. Recombinant expression vectors carrying either *rpoB* H486Y (from the CK135 strain) or *rpoB* H486D (from the JL213 strain) were introduced into the Rif-susceptible (Rif$^s$) parental strain (OG1) to create merodiploid strains carrying both wild-type (chromosomal) and mutant copies of *rpoB*. Susceptibility analyses of the resulting strains revealed that, as expected, expression of both the *rpoB* H486Y and *rpoB* H486D alleles conferred rifampin resistance (Table 4). While both plasmid-borne *rpoB* alleles conferred resistance to rifampin, expression of the *rpoB* H486Y allele enhanced cephalosporin resistance of the parental strain whereas expression of the *rpoB* H486D allele did not (Table 4), confirming that *rpoB* H486Y is indeed responsible for enhanced cephalosporin resistance in the original spontaneous Rif$^r$ mutants.

**TABLE 4** Median MICs for *E. faecalis* OG1 strain with plasmid-borne *rpoB* alleles[a]

| Antibiotic | MIC (μg/ml) of each plasmid-carrying OG1 strain (*rpoB* allele carried on plasmid) | | |
|---|---|---|---|
| | OG1(pJRG8) (empty vector) | OG1(pJLL52) (H486Y) | OG1(pJLL50) (H486D) |
| Rifampin | 8 | >256 | >256 |
| Ceftriaxone | 64 | 256 | 64 |
| Ceftazidime | 256 | 1,024 | 256 |
| Cefuroxime | 128 | 256 | 128 |
| Cefadroxil | 64 | 64 | 64 |

[a] MICs were determined in MHB supplemented with Em after 24 h at 37°C from a minimum of 3 independent experiments.

**RNA polymerase mutations do not bypass IreK or Pbp5.** Previous studies established that deletions of the genes encoding the Ser/Thr kinase IreK or the penicillin-binding protein Pbp5 render *E. faecalis* markedly susceptible to cephalosporins (2, 21). To probe the relationship of *rpoB* H486Y-mediated cephalosporin resistance to these previously described resistance determinants, we sought to determine if *rpoB* H486Y-mediated enhanced cephalosporin resistance could overcome the effect of these deletions. To do so, we performed epistasis experiments by constructing strains carrying the *rpoB* H486Y allele in combination with a deletion in either *ireK* or *pbp5*. Susceptibility tests on the resulting strains revealed that both deletions eliminated the hyperresistant phenotype conferred by *rpoB* H486Y and rendered *E. faecalis* susceptible to ceftriaxone. In side-by-side experiments, ceftriaxone MICs were determined to be 1 μg/ml for *pbp5* deletion mutants that were either Rif$^s$ (the JL339 strain) or Rif$^r$ (due to *rpoB* H486Y [the JL308 strain]). Similarly, ceftriaxone MICs were determined to be 4 μg/ml for the Rif$^s$ *ireK* deletion mutant (the JL206 strain) and 8 μg/ml for the Rif$^r$ *rpoB* H486Y *ireK* deletion mutant (the JL235 strain), indicating that *rpoB* H486Y cannot bypass the requirements for IreK or Pbp5 to confer high-level intrinsic cephalosporin resistance in *E. faecalis*.

**Rif$^r$ mutations in *E. faecium* enhance cephalosporin resistance.** Given that we observed *rpoB* H486Y-mediated enhancement of cephalosporin resistance in divergent genetic lineages of *E. faecalis*, we asked if a similar phenomenon could also be observed in other enterococcal species. To test this, we selected spontaneous Rif$^r$ mutants derived from two independent lineages of *E. faecium* (*E. faecium* Com12 and 1,231,501), an enterococcal species that also exhibits Pbp5-dependent intrinsic resistance to cephalosporins (4). Targeted sequencing of the *E. faecium rpoB* Rif regions from a collection of Rif$^r$ mutants revealed isolates containing *rpoB* H486Y alleles, which were subjected to cephalosporin susceptibility tests. The results revealed that *rpoB* H486Y mutants from both *E. faecium* lineages exhibit enhanced resistance to ceftriaxone (Table 3), indicating that this phenomenon is not restricted to the species *E. faecalis*.

## DISCUSSION

This work describes an unexpected effect of specific mutations in the β subunit of RNAP on intrinsic cephalosporin resistance in enterococci. The principal findings of this study can be summarized as follows.

(i) Spontaneous Rif$^r$ mutants derived from two divergent lineages of *E. faecalis* were obtained. Each of the mutants carried a

mutation in *rpoB*, encoding the β subunit of RNAP, and each mutation we obtained corresponds to an *rpoB* allele that is known to confer resistance to rifampin in other species of bacteria. We confirmed that two such alleles of *E. faecalis rpoB* (*rpoB* H486Y and *rpoB* H486D) are indeed responsible for resistance to Rif by expressing them in an otherwise susceptible *E. faecalis* strain (Table 4). Although our selections yielded mutants with two different substitutions at H486 of RpoB, we did not obtain the *rpoB* H486R allele that is responsible for Rif$^r$ in the widely used laboratory strain of *E. faecalis*, OG1RF (29). The reasons for this are unknown but may be related to the specific environmental conditions used in our Rif$^r$ selections. Previous studies in *Bacillus subtilis* have established that environmental conditions can influence the spectrum of Rif$^r$ mutations obtained in a given selection (28).

(ii) The Rif$^r$ mutations in *rpoB* altered enterococcal intrinsic resistance to cephalosporins in an allele-specific manner (Tables 2 and 3). In particular, the *rpoB* H486Y allele markedly enhanced resistance to expanded- and broad-spectrum cephalosporins, while other Rif$^r$ alleles—including a different substitution at the same site in *rpoB* (*rpoB* H486D)—either conferred a reduced level of resistance (in the OG1 genetic background) or did not substantially affect resistance (in the T1 genetic background). In addition, the *rpoB* H486Y mutations conferred enhanced resistance to cephalosporins not only in two lineages of *E. faecalis* but also in two lineages of a distinct species of enterococci, *E. faecium*. Thus, it seems likely that the underlying mechanism leading to enhanced cephalosporin resistance in strains carrying the *rpoB* H486Y allele is widely conserved among enterococci.

(iii) The effects of *rpoB* alleles on resistance were specific to expanded- and broad-spectrum cephalosporins (Tables 2 and 3). Little to no effects of the *rpoB* mutations were observed for other antibiotics that target cell wall synthesis or for antibiotics with targets unrelated to cell wall synthesis, strongly suggesting that enhanced cephalosporin resistance observed with *rpoB* H486Y is not the result of a nonspecific general change in integrity of the cell wall or an enhanced general stress response but rather an alteration in a specific cellular process that promotes resistance to cephalosporins.

(iv) The *rpoB* H486Y allele cannot confer high-level cephalosporin resistance to *E. faecalis* in the absence of either IreK kinase or Pbp5. This observation is perhaps unsurprising in the case of Pbp5, as Pbp5 is thought to perform the critical final peptidoglycan cross-linking step during cell wall synthesis in the presence of cephalosporins. For IreK, interpretation of this result is more complicated, as the signaling network controlled by IreK has not yet been defined.

Rif$^r$ alleles of RNAP confer a variety of phenotypic effects in other bacteria. In *B. subtilis*, extensive analysis of the effects of a variety of Rif$^r$ mutations in *rpoB* revealed allele-specific differences in genetic competence (24), sporulation (24), and carbohydrate utilization (32). It seems likely that global aberrations in coordinated transcriptional remodeling due to Rif$^r$ mutant polymerases may have an adverse impact on these phenotypes. Other studies point to a potential role of Rif$^r$ mutations in directly modulating transcription as a means of perturbing phenotype. For example, the RpoB H482Y mutant of *B. subtilis* RNAP (equivalent to H486Y of *E. faecalis*) appeared to inhibit Rho function, leading to enhanced *rho* autoregulation (16). In addition, a Rif$^r$ mutation in cluster I of *B. subtilis* RNAP led to enhanced transcription from *sigA*-dependent promoters (15), and several Rif$^r$ mutations in cluster I of *Escherichia coli* RNAP led to changes in promoter usage that mimic the stringent response (39). Although the latter two examples involve mutations at residues in Rif cluster I that are distinct from the equivalent of *E. faecalis rpoB* H486, they illustrate the point that Rif$^r$-conferring mutations in the β subunit of RNAP can confer allele-specific changes in transcriptional profile and, by extension, phenotype. Additionally, a recent report (7) describes a mutation in *rpoB* of *Staphylococcus aureus* that, although it does not confer resistance to Rif, enhances resistance to daptomycin and vancomycin and leads to global changes in gene expression.

In light of these observations, we propose that specific Rif$^r$ mutations in the β subunit of *E. faecalis* RNAP result in altered transcription, particularly in genes that promote cephalosporin resistance. We predict that the *rpoB* H486Y allele leads to enhanced transcription of such genes, while the other Rif$^r$ alleles may lead to reduced transcription of the same genes. At this time, few specific genes are known to be required for cephalosporin resistance in *E. faecalis*. One of those genes is *croR*, which encodes the response regulator of a two-component signal transduction system that is required for normal cephalosporin resistance in *E. faecalis* (6, 12). CroR can regulate transcription of its target genes (22, 26), suggesting a possible mechanism in which mutations in RNAP may influence CroR-directed transcription of genes that are critical for cephalosporin resistance. Thus far, only a few CroR-dependent genes have been identified, none of which are known to play a role in cephalosporin resistance.

To probe for an impact of RNAP mutations on other known cephalosporin resistance determinants in *E. faecalis*, we assessed the abundance of IreK kinase in *rpoB* H486Y mutants via immunoblotting, but we did not observe any significant differences from that of the wild type. Similarly, a whole-cell penicillin-binding assay to probe levels of Pbp5 in *rpoB* H486Y mutants did not reveal any differences from that of the wild-type (data not shown). Therefore, it seems likely that as-yet-unknown genes that promote cephalosporin resistance exist. We are optimistic that ongoing study of the *rpoB* H486Y mutant will reveal new insights into the identity of those genes and the underlying mechanisms of cephalosporin resistance in *E. faecalis*.

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