Systematic Inactivation and Phenotypic Characterization of Two-Component Signal Transduction Systems of *Enterococcus faecalis* V583†‡

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The ability of enterococci to adapt and respond to different environmental stimuli, including the host environment, led us to investigate the role of two-component signal transduction in the regulation of *Enterococcus faecalis* physiology. Using a bioinformatic approach, we previously identified 17 two-component systems (TCS), consisting of a sensory histidine kinase and the cognate response regulator, as well as an additional orphan response regulator (L. E. Hancock and M. Perego, *J. Bacteriol.* 184:5819–5825, 2002). In an effort to identify the potential function of each TCS in the biology of *E. faecalis* clinical isolate strain V583, we constructed insertion mutations in each of the response regulators. We were able to inactivate 17 of 18 response regulators, the exception being an ortholog of YycF, previously shown to be essential for viability in a variety of gram-positive microorganisms. The biological effects of the remaining mutations were assessed by using a number of assays, including antibiotic resistance, biofilm formation, and environmental stress. We identified TCS related to antibiotic resistance and environmental stress and found one system which controls the initiation of biofilm development by *E. faecalis*.

Generally considered a commensal organism, *Enterococcus faecalis* has emerged as a major nosocomial pathogen (15). Infections caused by *E. faecalis* have been aggravated in the past decade by the acquisition of multiple antibiotic resistances, which has made some enterococci refractory to all antimicrobial regimens. The dual lifestyle of *E. faecalis* as both a commensal and pathogenic organism requires it to be responsive to varying environmental conditions, both in and outside the host. Its ability to sense changing environmental stimuli and respond accordingly is of the utmost importance in its adaptation to these varying conditions. A major question to be answered is how *E. faecalis* accomplishes such a feat. The ability of most bacteria to monitor and adapt to changing conditions is often mediated through signal transduction involving two-component signal transduction systems. Two-component systems (TCS) generally consist of a sensory histidine kinase and a cognate response regulator. The histidine kinase senses the signal and relays the adaptive response through the transfer of a phosphoryl group to the response regulator, which can then act as a transcriptional regulator to modulate gene expression (18). These systems are involved in various cellular processes ranging from sporulation, biofilm formation, chemotaxis, and virulence to antibiotic production and resistance (18).

The best-characterized TCS in *Enterococcus* are the VanRS and VanR_BS_B systems, which regulate VanA and VanB glycopeptide resistance, respectively (1). More recently, studies

dealing with two-component gene regulation in *E. faecalis* have focused attention on the OmpR family of response regulators (21, 33). Teng et al. (33) used the *Bacillus subtilis* PhoP-PhoR sequences to identify 11 putative histidine kinase-response regulator pairs in the *E. faecalis* V583 genome. Seven of the eleven pairs were disrupted in *E. faecalis* strain OG1RF, and one such mutant with a disruption in a locus designated *etaRS* was shown to be significantly attenuated in vivo in a murine peritonitis model and was also shown to be more acid sensitive than the parental strain. In addition it was found that this mutant was more resistant to high temperature than the wild type. A recent study showed that the heat resistance phenotype associated with a disruption in the *etaRS* locus was correlated with an increase in the levels of the heat shock proteins DnaK and GroEL, suggesting that EtaR may serve as a negative regulator of heat shock protein expression (21).

Le Breton et al. (21) used the *Escherichia coli* OmpR sequence to identify 10 different loci encoding putative response regulators in the *E. faecalis* V583 genome. Eight of the ten loci were disrupted by insertion mutagenesis in *E. faecalis* strain JH2-2, and one mutation in the *rr05* response regulator was found to lead to a growth defect and cell morphology alterations. The morphological defects of the mutant were attributed to a lack of expression of *sagA*, previously shown to play an important role in stress resistance to a variety of environmental challenges (22). More recently, this same locus (*hk-rr05*) was found to be required for intrinsic β -lactam resistance in *E. faecalis* strain JH2-2 and was redesignated *croRS* for “ceftriaxone resistance” (7).

In an effort to gain a more comprehensive view of the role of two-component signal transduction pathways in the biology of *E. faecalis*, we targeted each of the 18 response regulators previously identified in *E. faecalis* strain V583 (14) for insertion mutagenesis. Strain V583 possesses two TCS (*vncSR* [*hk-*

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TABLE 1. Strains used in this study

<i>E. faecalis</i> strain	Description	Origin ^a or reference
V583	Clinical isolate, Vn ^r , TIGR ^b sequence strain	28
RR01	<i>rr01</i> insertion mutant of V583, Tet ^r	pML01→V583
RR02	<i>rr02</i> insertion mutant of V583, Tet ^r	pML02→V583
RR03	<i>rr03</i> insertion mutant of V583, Tet ^r	pML03→V583
RR04	<i>rr04</i> insertion mutant of V583, Tet ^r	pML04→V583
RR05	<i>rr05</i> insertion mutant of V583, Tet ^r	pML05→V583
RR06	<i>rr06</i> insertion mutant of V583, Tet ^r	pML06→V583
RR08	<i>rr08</i> insertion mutant of V583, Tet ^r	pML08→V583
RR09	<i>rr09</i> insertion mutant of V583, Tet ^r	pML09→V583
RR10	<i>rr10</i> insertion mutant of V583, Tet ^r	pML10→V583
RR11	<i>rr11</i> insertion mutant of V583, Tet ^r	pML11→V583
RR12	<i>rr12</i> insertion mutant of V583, Tet ^r	pML12→V583
RR13	<i>rr13</i> insertion mutant of V583, Tet ^r	pML13→V583
RR14	<i>rr14</i> insertion mutant of V583, Tet ^r	pML14→V583
RR15	<i>rr15</i> insertion mutant of V583, Tet ^r	pML15→V583
RR16	<i>rr16</i> insertion mutant of V583, Tet ^r	pML16→V583
RR17	<i>rr17</i> insertion mutant of V583, Tet ^r	pML17→V583
RR18	<i>rr18</i> insertion mutant of V583, Tet ^r	pML18→V583
V583T	Insertion mutant of V583 with p3TET inserted between <i>hk02</i> and the adjacent open reading frame encoding a putative lipoprotein	pML19→V583

^a → denotes construction by transformation followed by single-crossover integration.

^b TIGR, The Institute for Genomic Research.

rr08] and *kdpDE* [*hk-rr12*]) that have recently been shown to reside within mobile elements or pathogenicity islands (25, 30). Unlike the plasmid-free *E. faecalis* strains OG1RF and JH2-2, strain V583 possesses acquired resistance to a variety of antimicrobials, including vancomycin (28). We therefore examined the resistance profile of the response regulator insertion mutants against a panel of anti-infectives and identified several loci related to antibiotic resistance. We also tested each of the mutants against a variety of environmental stresses and found one mutant which was compromised in growth at elevated temperature and in the presence of sodium dodecyl sulfate (SDS). Finally, we examined each of the mutants for biofilm formation and showed that the *ftr* signal transduction system plays an important role in this developmental process (16).

MATERIALS AND METHODS

Bacterial strains and culture conditions. All relevant bacterial strains and plasmid constructions used in this study are listed in Tables 1 and 2. *E. coli* strain DH5 α was used for propagating plasmid constructions, and clones were cultured aerobically in Luria-Bertani broth (29). *E. faecalis* strains were routinely cultured in brain heart infusion (BHI), M17, or Todd-Hewitt broth (THB) at 37°C without aeration. Tetracycline was used at 15 μ g/ml for both *E. coli* and *E. faecalis*. Electrotransformations of *E. coli* and *E. faecalis* were performed as previously described (8, 9).

Construction of insertion mutations. The insertional inactivation vector p3TET was constructed from p3ERM (5) by replacing the erythromycin resistance determinant with the *tetM* gene, originally from plasmid pFW16 (26). To facilitate cloning, the *tetM* gene first was cloned as an *AvrII* fragment into *EcoRV*-digested pBluescript (Stratagene, La Jolla, Calif.) to create pJM133 (M. Perego, unpublished results). To obtain p3TET, p3ERM was digested with *MfeI* and *NaeI* and ligated to the *tetM* fragment from pJM133 obtained by digestion with *EcoRI* and *HincII*. To clone internal fragments of each response regulator gene, primers (see Table S1 in the supplemental material) were designed to amplify regions of the *E. faecalis* V583 genome encompassing each response regulator gene and an internal gene fragment for each response regulator was recovered from restriction digests of the corresponding PCR products. The internal fragments were cloned into p3TET, and the resulting constructs (Table 2) were verified by restriction analysis. Plasmid constructs were electroporated into *E. faecalis* strain

V583, and transformants resistant to tetracycline were screened by colony PCR to confirm insertional inactivation of the intended target, using a primer whose sequence hybridized to a region external to the cloned fragment and a primer hybridizing to the vector (Fig. 1). The growth curves and stabilities of single-crossover insertions were determined as previously described (36).

Antibiotic susceptibility test. MICs were determined in THB using twofold serial dilutions with the following antibiotics: chloramphenicol, ampicillin, penicillin, vancomycin, gentamicin, and erythromycin. Approximately 5×10^4 CFU were inoculated into each well of a 96-well microtiter plate and incubated at 37°C for 24 h prior to evaluation of the MIC. The lowest concentration of antibiotic still inhibiting bacterial growth was marked as the MIC. Susceptibility to a panel of antibiotics by the disk diffusion method was also performed. Cultures were grown in BHI broth to a MacFarland standard of 0.5 to 1.0 and then swabbed onto BHI agar. Antibiotic disks (6 mm in diameter; Becton Dickinson Laboratories) were then placed on the agar plates. After overnight incubation at 37°C, the diameters of the zones of bacterial growth inhibition were measured. The antibiotics assayed under these conditions included ampicillin (10 μ g), bacitracin (10 U), cefotaxime (30 μ g), cefuroxime (30 μ g), cephalothin (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), clindamycin (2 μ g), imipenem (10 μ g), nalidixic acid (30 μ g), nitrofurantoin (300 μ g), novobiocin (30 μ g), rifampin (5 μ g), and vancomycin (30 μ g).

Growth curves determined in the presence of high-level erythromycin and with environmental stresses. *E. faecalis* strains were evaluated for growth in THB containing 256 μ g of erythromycin/ml. This concentration is not inhibitory to any of the tested strains, as strain V583 and mutant derivatives are resistant to greater than 1,000 μ g/ml. Growth curves for each strain tested were determined

TABLE 2. Plasmids used in this study

Plasmid	Description ^a
p3TET	Suicide vector for insertional mutagenesis, Tet ^r
pML01	p3TET derivative containing a 903-bp internal fragment of <i>rr01</i> (<i>XbaI</i> / <i>SphI</i>)
pML02	p3TET derivative containing a 486-bp internal fragment of <i>rr02</i> (<i>BanII</i> / <i>AccI</i>)
pML03	p3TET derivative containing a 551-bp internal fragment of <i>rr03</i> (<i>AvrII</i>)
pML04	p3TET derivative containing a 567-bp internal fragment of <i>rr04</i> (<i>EcoRI</i> / <i>BamHI</i> *)
pML05	p3TET derivative containing a 540-bp internal fragment of <i>rr05</i> (<i>BspI061</i>)
pML06	p3TET derivative containing a 568-bp internal fragment of <i>rr06</i> (<i>SfaNI</i>)
pML07	p3TET derivative containing a 556-bp internal fragment of <i>rr07</i> (<i>Sau3AI</i> / <i>RsaI</i>)
pML08	p3TET derivative containing a 476-bp internal fragment of <i>rr08</i> (<i>EcoRI</i> / <i>BamHI</i> *)
pML09	p3TET derivative containing a 518-bp internal fragment of <i>rr09</i> (<i>BspHI</i> / <i>PmlI</i>)
pML10	p3TET derivative containing a 537-bp internal fragment of <i>rr10</i> (<i>RsaI</i>)
pML11	p3TET derivative containing a 520-bp internal fragment of <i>rr11</i> (<i>EcoRI</i> / <i>NdeI</i>)
pML12	p3TET derivative containing a 575-bp internal fragment of <i>rr12</i> (<i>Sau3AI</i>)
pML13	p3TET derivative containing a 483-bp internal fragment of <i>rr13</i> (<i>DraI</i> / <i>HinFI</i>)
pML14	p3TET derivative containing a 499-bp internal fragment of <i>rr14</i> (<i>AccI</i> / <i>XbaI</i>)
pML15	p3TET derivative containing a 622-bp internal fragment of <i>rr15</i> (<i>HincII</i> / <i>RsaI</i>)
pML16	p3TET derivative containing a 465-bp internal fragment of <i>rr16</i> (<i>HincII</i>)
pML17	p3TET derivative containing a 460-bp internal fragment of <i>rr17</i> (<i>BsmAI</i> / <i>BsiWI</i>)
pML18	p3TET derivative containing a 554-bp internal fragment of <i>rr18</i> (<i>SfaNI</i>)
pML19	p3TET derivative containing a 1,408-bp fragment encompassing 5' region of <i>hk02</i> , intergenic region, and 3' region of an open reading frame encoding a putative lipoprotein (<i>EcoRI</i> / <i>SphI</i>)

^a *, sites artificially introduced by oligonucleotides used in PCR.

FIG. 1. Schematic of insertional mutagenesis of *E. faecalis* V583 response regulators. (A) Construction of p3TET insertion vector. Vector p3ERM was restricted with MfeI and NaeI, and the larger 1.85-kb fragment was ligated to the *tetM* gene (EcoRI/HincII) from pJM133. (B) Internal gene fragments from each response regulator were cloned into p3TET, and the resulting constructs were used to transform *E. faecalis* V583. Insertions were confirmed by colony PCR using primers flanking the insertion site along with primers corresponding to the insertion vector.

by optical density at 600 nm (OD_{600}) every hour beginning 2 h after diluting an overnight culture 1:100 into fresh THB.

Experiments were performed to identify MICs under several environmental stresses, i.e., NaCl, H_2O_2 , bile salt (deoxycholate), acidic pH, and SDS. The concentration of each agent that still permitted growth was used in growth curve

experiments in THB at 37°C. These included 5% NaCl, 2 mM H_2O_2 , 0.1% deoxycholate, pH 5.5 (adjusted with lactic acid), and 0.003% SDS. Growth at 46°C was also evaluated. Growth was monitored at 550 nm in 96-well microtiter plates using a V_{max} microtiter plate reader (Molecular Devices, Dade, Fla.). For growth in SDS, a Hitachi U-2000 spectrophotometer was employed.

FIG. 2. Growth curves of *E. faecalis* strains. Cells were grown in THB at 37°C with growth monitored by OD₆₀₀. Filled diamonds, V583T; filled triangles, RR04; filled squares, RR14. Error bars denote standard deviations.

Biofilm formation. The ability of *E. faecalis* strains to form biofilms on polystyrene was quantified essentially as described elsewhere (16, 35). Each assay was performed in triplicate and repeated twice.

RESULTS

Construction of insertion mutants and growth rate analyses.

An internal gene fragment from each response regulator was cloned into the integration vector p3TET, which confers tetracycline resistance (*tetM*). The resulting plasmid constructs were electroporated into *E. faecalis* V583, and transformants were selected on THB-tetracycline plates (Fig. 1). As a control strain, we constructed a V583 derivative with p3TET inserted between *hk02* and an adjacent gene, encoding a putative lipoprotein. This strain, designated V583T (tetracycline resistant), had the vector insertion placed between two genes without gene disruption and thus served as a tetracycline-resistant parent strain to ensure that observed phenotypic effects in the mutants were not the result of vector insertion. Insertion mutants were obtained for all but one of the response regulators, the exception being *rr07* (*vicR*), an ortholog of the essential gram-positive response regulator *yycF* (12). Repeated attempts to inactivate this gene failed to result in a viable mutant, suggesting that, as observed for other low-GC gram-positive bacteria, this gene product is essential for cell viability in *E. faecalis*.

The growth rates of V583T and the 17 viable response regulator mutants in THB at 37°C were examined. The results showed that nearly all the mutants had similar doubling times, with the exception of RR04 and RR14, which displayed reproducibly slower doubling times in exponential phase than V583T (Fig. 2). All mutants had similar cell densities at stationary phase. Colony counts on THB and THB-tetracycline plates were comparable for all mutants, indicating that the insertion mutants were stable under the conditions examined (data not shown).

Antibiotic resistance. Microtiter serial dilutions and disk diffusion were employed to assess antibiotic resistance of V583T and the 17 response regulator mutants. The results shown in Table 3 illustrate the resistance patterns against a broad panel of antibiotics. Mutant RR05 displayed the most dramatic change with respect to antibiotic resistance. RR05 showed increased

sensitivity to bacitracin (17 versus 14 mm), cefotaxime (16 versus 6 mm), cefuroxime (14 mm versus 6 mm), and vancomycin (13 versus 8 mm; 32 versus 128 µg/ml). Surprisingly, RR05 was not altered in its sensitivity to other cell wall-active agents in the panel, such as ampicillin, imipenem, and cephalothin, a first-generation narrow-spectrum cephalosporin. The other mutants displaying increased susceptibility to cell wall-active agents were RR01 and RR03, with increased susceptibility to bacitracin (19 versus 14 mm). Furthermore, the mutant lacking VanR_B (RR11) showed increased susceptibility to vancomycin (17 versus 8 mm; <2 versus 128 µg/ml), as expected from previous work (2, 11, 17). The sensitivities of the mutants to the remaining panel of antibiotics were not significantly different from those of V583T.

Growth curves determined in the presence of erythromycin.

Preliminary observations in conducting serial dilutions with erythromycin suggested that some mutants were capable of a more rapid doubling time compared to V583T. To address this phenotype more quantitatively, we determined growth curves in the presence of a subinhibitory concentration of erythromycin (256 µg/ml). This concentration is more than fourfold below the erythromycin MIC for the strains examined (Table 3). Interestingly, six mutants, RR02, RR11 (*vanR_B*), RR14, RR15 (*fsrA*), RR16, and RR18 grew with a faster doubling time when compared to V583T (Fig. 3). This observation suggests that these mutants are affected in drug uptake with this class of antibiotics, because growth curves in the presence of high-level gentamicin (1,024 µg/ml) were not significantly different among V583T and these response regulator mutants (data not shown).

Response to environmental stress. Among the environmental stresses examined, including osmolarity (NaCl), oxidative stress (H₂O₂), low pH (5.5), heat (46°C), and detergents (deoxycholate and SDS), the only significant response we observed was to heat and SDS by mutant RR06 compared to V583T (Fig. 4A and B). The remaining mutants and conditions were not significantly different (data not shown). RR06 displayed a longer doubling time both at high temperature and in the presence of SDS (0.003%), suggesting a possible effect on membrane architecture leading to a delayed growth rate under the conditions examined. Compared to unstressed conditions,

TABLE 3. Antibiotic resistance of *E. faecalis* response regulator mutants^c

Strain	Amp ^a	Amp ^b	Bac ^a	Cef ^a	Cxm ^a	Ctx ^a	Cam ^a	Cam ^b	Cip ^a	Cli ^a	Ery ^b	Gen ^b	Imi ^a	Nal ^a	Nit ^a	Nov ^a	Rif ^a	Van ^a	Van ^b
V583T	27	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	20	16	16	8	128
RR01	26	4	19	14	6	6	18	8	20	6	>1,000	>2,000	24	6	21	17	16	8	128
RR02	27	4	13	14	6	6	18	8	19	6	>1,000	>2,000	23	6	20	17	16	8	128
RR03	26	4	19	14	6	6	18	8	19	6	>1,000	>2,000	24	6	21	16	17	8	128
RR04	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	20	16	16	8	128
RR05	27	4	17	14	14	16	18	8	20	6	>1,000	>2,000	24	6	20	16	16	13	32
RR06	26	4	14	14	6	6	18	8	19	6	>1,000	>2,000	24	6	20	16	16	8	128
RR08	26	4	13	14	6	6	18	8	20	6	>1,000	>2,000	24	6	20	16	16	8	128
RR09	26	4	14	14	6	6	18	8	19	6	>1,000	>2,000	24	6	20	16	17	8	128
RR10	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	20	16	16	8	128
RR11	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	21	16	16	17	<2
RR12	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	20	17	17	8	128
RR13	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	21	16	16	8	128
RR14	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	24	6	21	17	17	8	128
RR15	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	23	6	20	16	16	8	128
RR16	26	4	14	14	6	6	18	8	19	6	>1,000	>2,000	23	6	21	16	16	8	128
RR17	26	4	14	14	6	6	18	8	19	6	>1,000	>2,000	24	6	21	17	16	8	128
RR18	26	4	14	14	6	6	18	8	20	6	>1,000	>2,000	23	6	21	16	16	8	128

^a Determined by the disk diffusion method. The values reported are the diameters of the zones of inhibition in millimeters. The diameter of each disk is 6 mm, and for some antibiotics no inhibition zone was observed.
^b Determined by the microtiter twofold serial dilution method. The values reported are the MICs in micrograms per milliliter.
^c Antibiotic resistance abbreviated as follows: Amp, ampicillin; Bac, bacitracin; Cef, cephalothin (first-generation cephalosporin); Cxm, cefuroxime (second-generation cephalosporin); Ctx, cefotaxime (third-generation cephalosporin); Cam, chloramphenicol; Cip, ciprofloxacin; Cli, clindamycin; Ery, erythromycin; Gen, gentamicin; Imi, imipenem; Nal, nalidixic acid; Nit, nitrofurantoin; Nov, novobiocin; Rif, rifampin; Van, vancomycin. Boldface values indicate significant differences between mutants and the parental strain.

parental and mutant strains showed similar delayed growth rates under all the assay conditions tested.

Biofilm formation. Examination of biofilm formation on a solid surface by V583T and the response regulator mutants identified RR15 (*fsrA*) as defective in biofilm formation on polystyrene (Fig. 5), whereas biofilm formation by the other response regulator mutants was not significantly different from that of the parental strain. The role of FsrA in biofilm formation was further attributed to its role in transcriptional control of the *gelE* gene, encoding the zinc metalloprotease gelatinase, as described by Hancock and Perego (16).

DISCUSSION

E. faecalis is highly adaptable to a number of harsh environmental conditions, is capable of growth over a wide temperature range and in the presence of detergents and bile salts, and is tolerant to acidic and alkaline conditions. Over the past several decades, enterococci have also distinguished themselves as among the most antibiotic-resistant microorganisms affecting human health. The physiological features that allow *E. faecalis* to survive these environmental insults are not well understood. Two-component signal transduction is a primary means by which bacteria sense environmental changes and respond with the appropriate expression of gene products that allow them to adapt to a given environment. In this study, we examined the full complement of TCS present in the *E. faecalis* multidrug-resistant strain V583. This strain possesses 17 TCS that consist of a paired histidine kinase and response regulator, as well as one additional orphan regulator (14).

We generated insertion mutations in each response regulator gene, with the exception of *rr07*, whose product shares extensive sequence similarity with the essential response regulator YycF/VicR from a number of low-GC gram-positive bacteria. Our inability to generate an insertion mutation in this gene is in good agreement with other studies published to date

(21, 33). We also tested whether the histidine kinase gene *hk07* (*vicK*) is also essential for cell viability in *E. faecalis*. However, multiple attempts to inactivate the *yycG* homologue gene of *E. faecalis* by means of single-crossover integration of plasmid p3TET carrying an 850-bp internal fragment never resulted in any transformant, suggesting that YycG is also essential for growth in this organism (unpublished data). In *B. subtilis* and *Staphylococcus aureus*, YycG and YycF are both essential (12, 23), and genes controlled by this system have recently been identified (10, 19). In contrast, only the response regulator VicR is essential in *Streptococcus pneumoniae* (34). Using in vitro biochemical assays, Clausen et al. (6) showed that VanS_B

FIG. 3. Growth curves of *E. faecalis* strains in the presence of erythromycin. Cells were grown in THB with erythromycin (256 µg/ml) at 37°C with growth monitored by OD₆₀₀. Filled circles, V583T; open squares, RR02; filled triangles, RR11; filled squares, RR14; filled diamonds, RR15; open circles, RR16; open triangles, RR18. Error bars denote standard deviations.

FIG. 4. Growth curves of *E. faecalis* strains with environmental stress. (A) Growth of *E. faecalis* strains at 46°C were monitored at OD₅₅₀ using a microtiter plate reader. Filled circles, V583T; filled squares, RR06. (B) Growth curves of *E. faecalis* strains in THB with SDS (0.003%) at 37°C with growth monitored at OD₆₀₀. Filled circles, V583T; filled squares, RR06. Error bars denote standard deviations.

from *E. faecalis* could phosphorylate VicR from *S. pneumoniae* and raised the possibility that other histidine kinases could phosphorylate VicR by in vivo cross talk. Our results suggest that this is probably not the case in *E. faecalis*.

Examination of growth curves determined with standard media for all the viable mutants identified mutants RR04 and RR14 as being compromised in growth compared to the parent strain. The product of *rr04* is most similar to PhoP from *B. subtilis* (32), sharing 54% sequence identity. In *B. subtilis*, this system responds to phosphate limitation by coordinating gene expression to adapt to this environmental condition (31). Why a mutation in this locus in *E. faecalis* would affect growth in a rich medium, presumably where phosphate is not limiting, is rather puzzling and suggests that it may be involved in responding to some essential nutrient that is found in trace amounts in rich media. Interestingly, this growth defect was not observed in the two other strains of *E. faecalis*, OG1RF and JH2-2, that have been examined to date (21, 33). It is conceivable that the observed growth defect of RR04 may be strain and/or medium dependent. In addition, we also observed a growth defect in RR14 compared to the parental strain. The product of gene *rr14* appears to be most similar to the CitB/CitT family of response regulators (13). The strongest similarity was found with putative orthologs in *Streptococcus pyogenes* and *Streptococcus agalactiae*. The CitAB family of TCS senses and responds to changes in citrate and/or C4 dicarboxylate levels (13). It is unclear at present what role a mutation in this locus has on the overall fitness of *E. faecalis*.

We identified four response regulator mutants that were rendered more susceptible to a variety of cell wall-active agents, including bacitracin, second- and third-generation cephalosporins, and vancomycin. An insertion in *rr05* resulted in the most dramatic mutant observed. It was more susceptible to the ceph-

FIG. 5. Biofilm formation by *E. faecalis* V583T and response regulator mutants. Shown are OD₅₅₀ values of solubilized crystal violet from a microtiter plate assay at 24 h for the strains listed. All strains are derivatives of V583, and the nomenclature for each response regulator insertion mutant corresponds to the designations assigned by Hancock and Perego (14). Error bars denote standard deviations.

alosporins (second and third generation), bacitracin, and vancomycin than the parental control. In a recent study, Comenge et al. (7) identified a mutant of strain JH2-2 that was 4,000-fold more susceptible to ceftriaxone than the parental strain. The locus responsible for this phenotype consisted of a histidine kinase and a response regulator and was designated *croRS* (ceftriaxone resistance). The *croRS* deletion mutant was also fourfold more susceptible to ampicillin. The RR05 response regulator corresponds to CroR. We observed that an *rr05* mutant was very susceptible to second- and third-generation cephalosporins but not to the more-narrow-spectrum first-generation cephalosporin (cephalothin). This is interesting from a clinical standpoint because the use of third-generation cephalosporins has been linked as a predisposing factor to colonization by multidrug-resistant enterococci, presumably because these drugs lack antienterococcal activity. We observed that a V583 *rr05* (*croR*) mutant was also more susceptible to vancomycin, despite the functional presence of the VanB resistance phenotype in strain V583. While the resistance levels were much lower for RR11 (*vanR_B*) than RR05 (*croR*) (<2 versus 32 µg/ml), it is intriguing that the pathway controlled by RR05 (CroR) also plays a role in vancomycin resistance. In addition, the V583 *rr05* (*croR*) mutant was also more susceptible to bacitracin, although to a lesser extent than the other cell wall-active agents. Somewhat surprisingly, we did not observe a resistance defect with two other cell wall-active antibiotics in our panel, namely ampicillin and imipenem. It appears that RR05 (CroR) is involved in resistance to a wide range of cell wall-active agents, indicating that this system may have a role in regulating cell wall synthesis. To that end, it was observed by Le Breton et al. that a *croR* mutation in *E. faecalis* JH2-2 led to significant growth defects and cell morphology alterations (21). The observed defects were hypothesized to be due to a dysregulation of *sagA*, as a similar phenotype was observed for a *sagA* mutant (22). However, we did not observe a growth defect in a V583 *rr05* (*croR*) mutant, and no mention of growth defect was made in the analysis by Comenge et al. (7) of the role of *croRS* mutants in intrinsic β-lactam resistance of strain JH2-2. Perhaps different growth conditions are accountable for these discrepancies.

Two additional TCS mutants, RR01 and RR03, displayed increased sensitivity to bacitracin. These mutants possess insertions in response regulators that are not members of the OmpR family; RR01 possesses a DNA-binding domain similar to that of the AraC family, while RR03 belongs to the NarL family. It was recently noted that in *B. subtilis*, *yvqC*, a highly conserved ortholog of *rr03*, is upregulated in response to bacitracin (24). Similarly, Kuroda et al. found that the product of the *S. aureus* *vraR* gene, an ortholog of YvqC and RR03, plays an important role in resistance to a variety of cell wall-active antibiotics, including bacitracin (20).

We also observed that strains RR02, RR11, RR14, RR15, RR16, and RR18 displayed a faster doubling time compared to V583 in the presence of erythromycin. This observation suggests that such mutants possess alterations in the uptake of this class of antibiotic.

Our phenotypic analysis also included an autolysis assay previously used by Brunskill and Bayles (4) to show the involvement of the LytS/LytR system of *S. aureus* in cell wall integrity. Regardless of the strong similarity between the his-

tidine kinase-RR02 system of *E. faecalis* and the LytS/LytR of *S. aureus*, we did not observe any change in autolysis rate in the *rr02* mutant or in any of the other mutants tested (data not shown).

As an opportunistic pathogen, *E. faecalis* causes a variety of infections in a hospital setting, many of which are related to indwelling devices. The ability to colonize these foreign bodies in the form of microbial biofilms likely enhances the in vivo survival of *E. faecalis*. In the present analysis, we identified the *fsrA* mutant as the only one being affected in biofilm formation among the 17 analyzed. *fsrA* is part of a quorum-sensing locus which regulates the production of two secreted proteases, gelatinase and serine protease (27). Related to this regulation, we recently demonstrated that the *E. faecalis* gelatinase, under the control of the *fsr* signal transduction system, is directly responsible for biofilm formation (16). As the biofilm or slime phenotype has been linked to survival of *E. faecalis* within macrophages (3), it is likely that biofilm development allows persistence of the organism at sites of infection.

In terms of environmental challenges, the RR06 mutant displayed a retarded growth rate in the presence of subinhibitory levels of SDS and at 46°C compared to the wild type. It is unclear at present how these processes may relate, but it is tempting to speculate that the observed phenotypes may relate to membrane architecture, as both conditions are capable of stressing the cell in this compartment. With respect to other environmental stresses, we did not observe any significant changes between the response regulator mutants and the parental strain. This was somewhat surprising because both Teng et al. (33) and Le Breton et al. (21) observed an acid-sensitive phenotype with an *rr10* (*etaR*) mutant. Given the fact that enterococci are highly promiscuous as it relates to acquisition of so-called foreign or mobile DNA (25), it is not surprising that some strains may have acquired a backup system to deal with the common stresses faced by these organisms. *E. faecalis* V583 possesses three TCS (HK-RR08, HK-RR11, and HK-RR12) that are linked to mobile DNA elements (25, 30).

It is clear from this study that two-component signal transduction systems govern important biological parameters of this organism ranging from environmental persistence and antibiotic resistance to biofilm formation. Disrupting the organism's ability to utilize these systems should prove valuable in a search for more effective ways to treat infections caused by enterococci, as well as in limiting its persistence in the hospital setting.

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