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EbpR Is Important for Biofilm Formation by Activating Expression of the Endocarditis and Biofilm-Associated Pilus Operon (*ebpABC*) of *Enterococcus faecalis* OG1RF[▽]

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We identify ef1090 (renamed *ebpR*) and show its importance for the transcriptional regulation of expression of the *Enterococcus faecalis* pilus operon, *ebpABC*. An *ebpR* deletion (Δ*ebpR*) mutant was found to have reduced *ebpABC* expression with loss of pilus production and a defect in primary adherence with, as a consequence, reduced biofilm formation.

Enterococcus faecalis is a gram-positive bacterium that is part of the normal intestinal flora of most humans but is also a major cause of opportunistic infection (9). A major regulator of virulence in *E. faecalis* OG1RF is the Fsr system (15). In a previous microarray analysis, we identified several Fsr-regulated genes encoding putative regulators, one of which was ef1090 (2). EF1090 contains two putative helix-turn-helix DNA-binding domains and shares 42% similarity with AcpB from *Bacillus anthracis*, a member of the AtxA/MgaA family (4). Upstream and in the opposite orientation of ef1090 is the *ebpABC* operon, which encodes pilus components shown to be important for virulence in the rat endocarditis model and the mouse urinary tract infection model (13, 17).

To test whether the putative EF1090 regulator was involved in the regulation of the pili genes, we first performed semiquantitative reverse transcription-PCR (qRT-PCR) (2). The *ebpA*, *ebpB*, and *ebpC* transcripts were strongly reduced in an ef1090 transposon insertion mutant (5) compared to the parent strain (data not shown). Based on these results, ef1090 was renamed *ebpR* for its role as an endocarditis- and biofilm-associated pilus regulator. To prevent any effects caused by the transposon insertion from complicating our analysis, we then created an unmarked in-frame *ebpR* deletion mutant (Table 1) of OG1RF by using the recently published PheS* system (7). The Δ*ebpR* mutant had no growth defects compared to OG1RF in BHI, TSBG (biofilm medium), or BHI-40% horse serum (binding assay medium).

To assess transcriptional differences between OG1RF and the Δ*ebpR* mutant, we used microarray analysis with slide preparation (1), probe labeling, hybridization, data acquisition, and statistical analysis performed as described previously (2). RNA was extracted at late log growth phase from TSBG-grown cultures (2). Within the level of detection of the microarray analysis, the *ebpABC* transcript was undetectable in the Δ*ebpR* mutant compared to OG1RF, indicating that *ebpR* is important for *ebpABC* expression at the transcriptional level. *srtC* was not significantly altered in these conditions. We confirmed these results by using qRT-PCR (12). The amount of transcript for each gene of interest was normalized against *gyrB* transcripts. qRT-PCR showed that *ebpA* transcripts levels were decreased 119-fold ($P = 0.0018$) in the *ebpR* mutant versus the wild type, whereas *srtC* transcripts were only 1.7-fold decreased ($P = 0.028$) (Fig. 1A). The slight effect of *ebpR* on *srtC* is likely related to the presence of an independent *srtC* promoter and the low level of readthrough from the *ebpA* promoter described previously (13). Since basal *ebpABC* expression was detected in our *ebpR* mutant, our results indicate that *ebpR*, although important, is not essential for *ebpABC* expression.

To begin examining how *epbR* is regulated, we mapped the transcriptional start site of OG1RF. Using the RACE (for rapid amplification of cDNA ends) kit, only one start site for *ebpR* was found at −21 bp. Looking at the promoter area, no canonical −10 and −35 bp sequences were detected, indicating that the expression of *ebpR* is likely sigma A independent. We next looked at possible *ebpR* autoregulation by inserting a 301-bp fragment overlapping the entire 209-bp region separating *ebpA* from *ebpR* into pTCV-*lacZ* to create a transcriptional *lacZ* fusion. The β-galactosidase activity of this construct in an *ebpR* background compared to the wild type was measured (6). No β-galactosidase activity was detected using the P_{ebpR}::*lacZ* fusion in the *ebpR* mutant, whereas it was detectable in the wild type (Fig. 1B). The results indicate that *ebpR* is autoactivated.

In addition to examining transcript levels, we examined pilus production in the wild type (OG1RF), the Δ*ebpR* mutant (TX5514), and a complemented strain (TX5582) in which *ebpR* was cloned under the control of the nisin promoter in pMSP3535 (3). As determined by RT-PCR, the expression of *ebpR* in TX5582 was detected even without nisin (data not shown). In order to detect the pili, mutanolysin extracts were made from OG1RF as described previously (13). As seen before, a ladder profile was detected using antibodies to EbpA (Fig. 2, lane 1). It was not observed in extracts from the *ebpR* mutant (lane 2) or in extracts from the mutant carrying the

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TABLE 1. Strains, plasmids, and primers used in this study

Strain or plasmid	Relevant characteristics or sequence[a]	Source or reference
Strains		
E. coli		
TG1	*E. coli* general cloning host	16
EC1000	*E. coli* cloning host for *repA*-dependent plasmids; carries constitutive *repA* allele on chromosome; Kan^r	8
E. faecalis		
OG1RF	*E. faecalis* Fus^r Rif^r; *p*-Cl-Phe^r	11
CK111/pCF10-101	*E. faecalis* conjugative donor strain; carries constitutive *repA* allele on chromosome and nontransferable pCF10 derivative; Spc^r Tet^r	7
DAGF27	Tn*917* transposon insertion at +581 bp from the *ebpR*[a] ATG in OG1RF; Erm^r Fus^r Rif^r	5
TX5475	*ebpA* allelic replacement *aph(3′)-IIIa* mutant of OG1RF; Fus^r Kan^r Rif^r	13
TX5460	*ebpB* insertion disruption mutant of OG1RF; Fus^r Kan^r Rif^r	13
TX5448	*ebpC* insertion disruption mutant of OG1RF; Fus^r Kan^r Rif^r	13
TX5514	*ebpR* in-frame deletion from bp −5 to +1337 from the ATG of *ebpR* of OG1RF; Fus^r Rif^r; *p*-Cl-Phe^r	This study
TX5584	TX5514(pMSP3535); Erm^r Fus^r Rif^r; *p*-Cl-Phe^r	This study
TX5582	TX5514(pTEX5515); *ebpR* mutant containing *ebpR* gene inserted into pMSP3535; Erm^r Fus^r Rif^r; *p*-Cl-Phe^r	This study
TX5590	OG1RF(pTEX5586); OG1RF containing the *ebpR* promoter fused to *lacZ*; Erm^r Fos^r Rif^r	This study
TX5591	TX5514(pTEX5586); *ebpR* mutant containing the *ebpR* promoter fused to *lacZ*; Erm^r	This study
Plasmids		
pMSP3535	Nisin-inducible expression shuttle vector with pAMβ1 and ColE1 replicons; Erm^r	3
pTCV-*lacZ*	Shuttle vector containing promoterless *lacZ*; Erm^r	14
pTEX5515	pMSP3535 with *ebpR* from bp −20 to +1561 from the ATG; this *ebpR* fragment contains the full ORF and the RBS of *ebpR*; Erm^r	This study
pTEX5586	pTCV-*lacZ* containing 301 bp from bp 248 upstream to bp 53 downstream of the start codon of *ebpR*; Erm^r	This study
pTEX5269	pTCV-*lacZ* containing 103 bp from bp −110 to −8 of the start codon of *fsrB*; Erm^r	26
Primers		
ebpR deletion	Upstream fragment was amplified using GCTCTAGACAGGACCCACACTTTTCA with CGGGATCCTTTCCTCCCA AGTCCTTT, and the downstream fragment was amplified using CGGGATCCTTGAAACCAGTGTTCCAAG with GGAATTCTGGTCAGCAAACGATTTT	
ebpR complementation	The primers CCGAGCTCGGACTTGGGAGGAAACAATA and CGGGATCCAAGTCATTTCGACTTATGTCCT were used to amplify *ebpR* from 20 bp upstream of the start codon to 138 bp downstream of the stop codon	
ebpR promoter fusion	The primers GGAATTCAAGACTACGCCGAAAACC and CGGGATCCACACGAATGATTTCTTCCA were used to amplify from 248 bp upstream to 53 bp downstream of the start codon of *ebpR*.	
Quantitative RT-PCR primers	*gyrB*, ACCAACACCGTGCAAGCC and CAAGCCAAAACAGGTCGCC; *ebpA*, AAAAATGATTCGGCTCCAGAA and TGCCAGATTCGCTCTCAAAG; *srtC*, ACACATGCGGTCATTTCAGG and GCGTCTTCCCATTGACTTCG	

[a] ORF, open reading frame; RBS, ribosome binding site. *ebpR* = ef1090. Kan^r, kanamycin resistance; Fus^r, fusidic acid resistance; Rif^r, rifampin resistance; *p*-cl-Phe^r, *p*-chloro-phenylalanine resistance; Spc^r, spectinomycin resistance; Tet^r, tetracycline resistance; Erm^r, erythromycin resistance. Restriction enzyme recognition sites are underlined.

empty vector (lane 3). However, it was detected in the complemented strain (Fig. 2, lane 4). A similar result was observed using EbpC antibodies (data not shown).

Nallapareddy et al. reported that only 10 to 20% of OG1RF cells had observable pili by electron microscopy (13). To evaluate this observation using another methodology, intact bacteria fixed with 4.4% (wt/vol) paraformaldehyde were probed with anti-EbpA or anti-EbpC rabbit antibodies by indirect im-

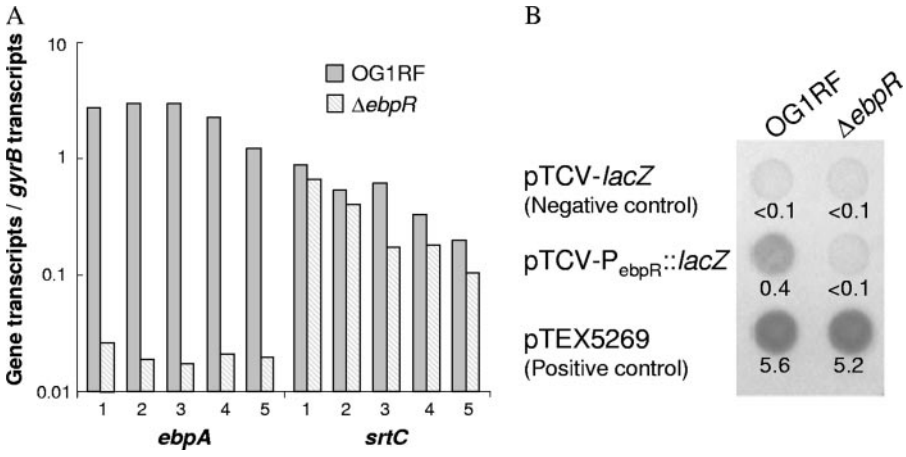

FIG. 1. *ebpA*, *srtC*, and *ebpR* expression. (A) Real-time transcript levels of *ebpA* and *srtC* in OG1RF and TX5514 (Δ*ebpR*). The numbers below the *x* axis correspond to the five independent cultures used for this assay. (B) pTCV-P_{ebpR}::*lacZ* in OG1RF and in TX5514 streaked onto TSBG plates containing 10 μg of erythromycin/ml and 200 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml and incubated for 48 h at 37°C. The negative control plasmid is pTCV-*lacZ*, and the positive control is pTEX5269. The β-galactosidase assay results (expressed as the optical density at 420 nm/mg of protein/ml) located under the spots correspond to a representative experiment performed with samples collected at entry into the stationary growth phase of cultures grown in TSBG (performed in triplicate).

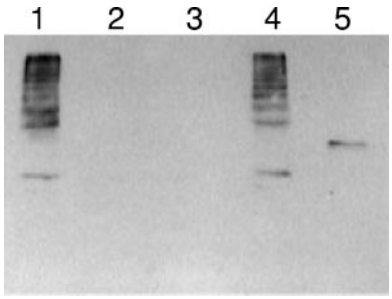

FIG. 2. Western blots of OG1RF and its Δ*ebpR* mutant (TX5514). Mutanolysin surface extract preparations were probed with anti-EbpA rabbit polyclonal immune serum. Lanes: 1, OG1RF; 2, Δ*ebpR* mutant (TX5514); 3, Δ*ebpR*(pMSP3535) (TX5584); 4, Δ*ebpR*(pTEX5515) (TX5582); 5, EbpA recombinant protein with a molecular mass of 113 kDa.

munofluorescence. Binding of anti-EbpA or anti-EbpC antibodies to the surface-exposed epitopes were detected using fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibodies (Sigma, St. Louis, MO). First, we tested OG1RF and the *ebpA* deletion mutant for the presence of pili in cells grown in TSBG and collected in stationary phase. Using anti-EbpA, FITC-labeled cells were detected in the OG1RF population (Fig. 3A) but not in the *ebpA* mutant population. The fluorescence was present on the entire surface of the cells except at the septum. Since EbpA was shown to be expressed and detectable as a monomer by Western blotting in *ebpB* and *ebpC* insertion mutants (13), these two strains were also assessed by immunofluorescence. As shown in Fig. 3A, a fluorescent dot (marked by an arrow and shown at a higher resolution in the insert) was visible on a few of the *ebpB* insertion mutant cells, whereas no fluorescence was detected at the surface of the *ebpC* insertion mutant. In a subsequent experiment, OG1RF, the *ebpR* deletion mutant, and the complemented strain were grown under the same conditions (TSBG, stationary phase, no nisin induction). In the OG1RF population, 15% of the cells were labeled with FITC, whereas no fluorescence was detected on the surface of the *ebpR* mutant cells (Fig. 3B). With the complemented strain, 17% of the population was FITC labeled (Fig. 3B, boxed numbers). We can postulate that *ebpR* and/or *ebpABC* are influenced by and/or may influence other

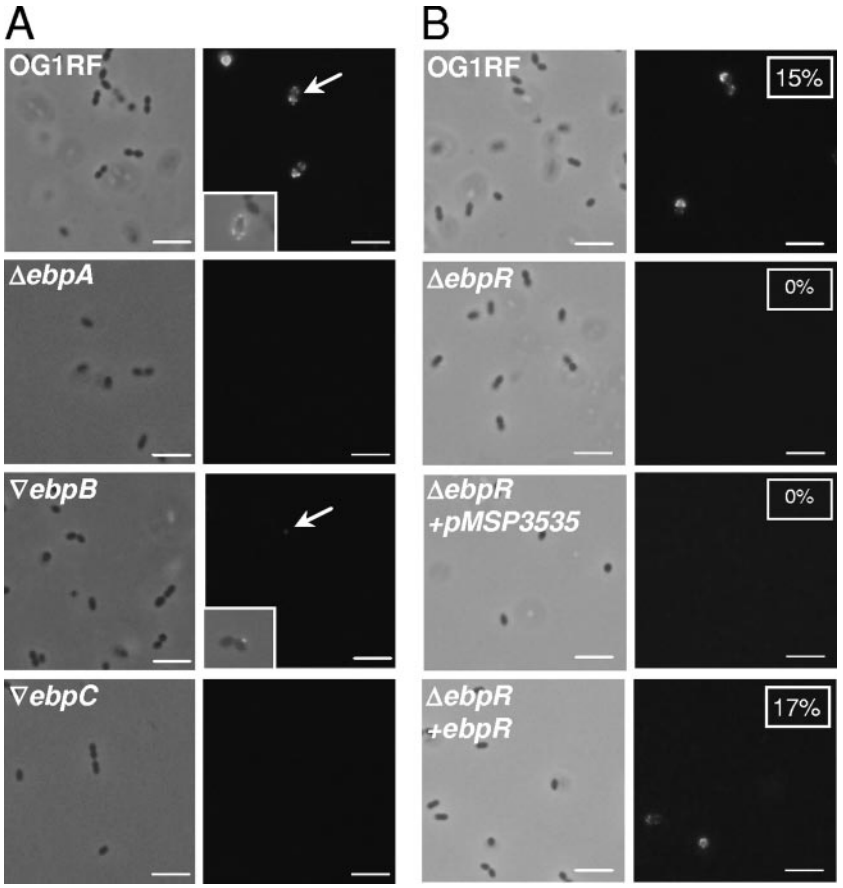

FIG. 3. Immunofluorescence analysis showing surface localization of EbpA. Cells were collected at stationary growth phase from a TSBG culture and assayed using anti-EbpA rabbit serum. The left panel is the phase-contrast image of the field visualized with the FITC filter (right panel). (A) Parental OG1RF. TX5475 is the Δ*ebpA* mutant, TX5460 is the ∇*ebpB* mutant (∇, insertion mutant), and TX5448 is the ∇*ebpC* mutant. The area indicated with an arrow is shown in a box as an overlay between phase contrast and FITC visualization. (B) Parental OG1RF. TX5514 is the Δ*ebpR* mutant; the Δ*ebpR* mutant electroporated with vector only (pMSP3535) served as a control. The Δ*ebpR* mutant electroporated with pMSP3535::*ebpR* is the complemented strain. The boxed numbers correspond to the percentage of FITC-labeled cells from at least 250 cells counted. Bar, 10 μm.

TABLE 2. Biofilm and primary adherence assays

Strain	Median (interquartile range)		
	OD_{570} for biofilm assay[a]	Primary adherence[b] at:	
		30 min	2 h
OG1RF	1.257 (1.184–1.313)	267 (238–316)	649 (614–722)
TX5475 (Δ*ebpA*)	0.817 (0.766–0.882)	97 (89–123)	203 (168–229)
TX5515 (Δ*ebpR*)	0.667 (0.644–0.701)	151 (114–231)	314 (230–385)
TX5584 (Δ*ebpR*)/ pMSP3535	0.814 (0.750–0.891)	166 (81–202)	293 (260–429)
TX5582 (Δ*ebpR* + *ebpR*)	1.534 (1.487–1.674)	221 (181–290)	692 (527–750)

[a] The optical density at 570 nm (OD_{570}) readings for the biofilm assays are from three independent experiments (total of 24 wells).

[b] Primary adherence was assessed after incubation for 30 min and 2 h. Bacteria in four fields from two independent plates from at least two independent experiments were subjected to light microscopy and counted.

regulatory pathways, since even in the presence of a functional *ebpR*, the majority of the cells are not producing pili at their surfaces. However, when cloned *ebpR* was transferred into the wild type, the level of FITC-labeled cells increased to 70% (preliminary data), whereas only 17% were labeled when the same plasmid was introduced into the Δ*ebpR* strain. These results indicate that the level of *ebpR* expression affects *ebpABC* expression or that the presence of the plasmid is titrating out a negative regulator.

Strains containing mutations in the *ebpABC* operon were previously shown to have reduced biofilm formation (13). Since the *ebpR* mutant displays greatly reduced expression of the *ebpABC* operon, it was also predicted to be defective in biofilm formation. Biofilm formation and adherence to a polystyrene surface were assessed quantitatively as described previously (10). As shown in Table 2, the *ebpR* mutant displayed a significant reduction in biofilm formation (47% reduction, $P < 0.01$) (Table 2). Of interest, the complemented strain presented a statistically significant increase in biofilm formation compared to OG1RF (22% more biofilm formation, $P < 0.01$). The biofilm defect in the *ebpA* and *ebpR* mutants was consistent with reductions in primary adherence of 43 and 64% at 30 min and 53 and 69% reduction at 2 h, respectively ($P < 0.01$) (Table 2).

In vivo, it has been shown that the *ebpA* mutant was significantly attenuated in the rat endocarditis model (13) and the murine urinary tract infection model (17). We attempted to quantify gene expression in vivo by using the rat endocarditis model. Preliminary data from vegetations collected at 48 h indicate that *ebpA* expression was 100 times lower in the *ebpR* deletion mutant, whereas *srtC* expression was not appreciably affected (preliminary results, data not shown). This experiment demonstrates the feasibility of detecting and quantifying gene expression in the rat endocarditis model, opening new avenues for research directed at understanding bacterial endocarditis.

In conclusion, we identified an *E. faecalis* transcriptional regulator, EbpR, which positively affects the expression of the endocarditis-associated pilus operon, thereby influencing biofilm formation. It will be of interest in future studies to address the signals to which *ebpR* is responding and whether these signals affect pilus production both in vitro and in vivo.

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