Conservation of Ebp-Type Pilus Genes among Enterococci and Demonstration of Their Role in Adherence of *Enterococcus faecalis* to Human Platelets[∇][†]

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Ebp are endocarditis- and biofilm-associated pili of *Enterococcus faecalis* that are also important in experimental urinary tract infections (UTIs). Our analyses, using available genomes, found that the *ebp* locus is unique to enterococci. In *E. faecalis*, the *ebp* locus is very highly conserved and only 1/473 *E. faecalis* isolates tested lacked *ebpABC*, while only 1.2% had the *bee* pilus locus. No other pilus-encoding operon was identified in 55 available genomes, indicating that the vast majority of *E. faecalis* strains (unlike *Enterococcus faecium* and streptococci) have a single pilus locus. Surface expression studies showed that Ebp pili were produced *in vitro* by 91/91 brain heart infusion (BHI) plus serum-grown *E. faecalis* isolates and that strain OG1RF expressed pili at even higher levels in rat endocarditis vegetations. However, Ebp expression was restricted to 30 to 72% of *E. faecalis* cells, consistent with a bistability mode of expression. We also evaluated *E. faecalis* interactions with human platelets and found that growth of *E. faecalis* in BHI plus serum significantly enhanced adherence to human platelets and that sortase deletion mutants (the ΔsrtA, Δbps, and ΔbpsΔsrtA mutants) were markedly defective. Further studies identified that Ebp pili, but not the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) Ace and Fss2, mediate adherence of *E. faecalis* to platelets. Taken together, our data show that the immunogenic (in human endocarditis patients) and commonly expressed Ebp pili, which are known to be important for experimental endocarditis, are highly conserved and mediate adherence to platelets, suggesting that Ebp pili may be a reasonable immunotherapeutic target for prevention or possibly treatment of endocarditis caused by this species.

Enterococcus faecalis has been recognized as a causative agent of community-acquired infective endocarditis (IE) since the turn of the last century (41, 42), accounting for ~5 to 20% of total cases of IE. Enterococci have also been reported as the second most common cause of health care-associated (HA) endocarditis (14, 17). The recent increase in HA enterococcal infections, especially those caused by multidrug-resistant strains, has created therapeutic problems, thus emphasizing the need for alternative strategies for prevention or therapy, such as immunoprophylaxis. Growing evidence from other Gram-positive pathogens suggests that sortase-assembled pilus subunits may serve as candidates for the development of novel immunotherapies. For example, it has been demonstrated in pneumococci, group A streptococci (GAS), and group B streptococci (GBS) that a combination of pilus subunit proteins elicits antibodies that are capable of inducing complement-dependent opsonophagocytic killing and conferring protective immunity (16, 35, 39).

Our previous efforts to identify surface-exposed virulence factors of *E. faecalis* predicted that 17 LPXTG-type cell wall-associated proteins of *E. faecalis* strain V583 likely encode microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) or pilus subunits (50, 64). Subsequent studies, including our companion paper (48), demonstrated that four of these proteins (the collagen adhesin Ace and fibrinogen adhesins Fss1, Fss2, and Fss3) and Ebp pili (endocarditis- and biofilm-associated pili) mediate adherence to host extracellular matrix (ECM) proteins (46, 50, 61). Further analyses found that both Ace and Ebp pili (which are assembled from three subunits, EbpA, -B, and -C) are ubiquitous among *E. faecalis* isolates (47, 50) and are antigenic during human infections, including IE (47, 50), with little or no expression *in vitro* except under specific growth conditions (e.g., growth medium supplemented with serum), at least by *E. faecalis* strain OG1RF (44, 50). Disruption of genes encoding either Ace or Ebp pili has resulted in attenuation in animal models of IE (50, 67) and urinary tract infection (UTI) (31, 66). While sequence variability and expression of Ace by diverse *E. faecalis* strains have been described (19, 31, 47, 73), no such reports are available on genes encoding Ebp pili.

In addition to adherence of circulating bacteria to ECM proteins likely exposed on the damaged vascular endocardial surface, the presence of platelets has also been shown to facilitate binding of bacteria to vegetations on heart valves, resulting in infective endocarditis, which may then lead to heart failure or to septic emboli, major complications of this disease, as well as death (15, 27, 40). Bacterial interactions with platelets generally occur either directly through a bacterial surface protein or indirectly by a plasma-bridging molecule (15, 27). For example, GspB and Hsa proteins of *Streptococcus gordonii* interact directly with the platelet membrane glycoprotein Ibα (2, 26, 60), while the *Staphylococcus aureus* MSCRAMM ClfA interacts indirectly via fibrinogen with the $\alpha_{IIb}\beta_3$ platelet receptor (34). Although some reports have shown variable platelet aggregation and variable adherence phenotypes (8–10, 24, 56, 59, 74) of *E. faecalis* and *Enterococcus faecium* strains, to our knowledge nothing is known about factors responsible for enterococcal adherence to platelets.

The present study was aimed at testing our hypotheses (i) that *E. faecalis* strains adhere to human platelets, (ii) that either MSCRAMMs or Ebp pili may facilitate adherence to platelets, (iii) that Ebp pili are expressed during experimental IE by OG1RF, and (iv) that the Ebp pili of diverse *E. faecalis* strains are produced under *in vitro* growth conditions that may mimic physiologically relevant environments. We also sought to determine if the Ebp pilus-encoding genes, which are part of the *E. faecalis* core genome, are highly conserved across clonal complexes, as this would have implications for potential immunologically directed strategies in the future.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 518 *E. faecalis* isolates collected from five continents (from Argentina, Belgium, Canada, Chile, China, Colombia, France, Japan, Lebanon, New Zealand, Spain, Thailand, United Kingdom, and the United States) were included in this study. These *E. faecalis* isolates had been recovered (between 1926 and 2009) from human clinical specimens, stools of hospitalized patients, stools of community volunteers, and animals. The sources of clinically derived strains include blood, bile, bone, catheters, cervix, cerebrospinal fluid, placenta, peritoneal fluid, urine, and several types of wounds. *E. faecalis* isolates were identified to the species level by biochemical tests and by hybridization using an intragenic *ace* probe (47). Wild-type *E. faecalis* OG1RF (4), its isogenic deletion mutants, and the complementation construct used for platelet assays are listed in Table S1 in the supplemental material.

E. faecalis isolates were routinely grown in brain heart infusion (BHI) broth/agar (Difco Laboratories, Detroit, MI) and, for expression studies, in BHI broth containing 40% heat-inactivated horse serum (BHI-S) (Sigma, St. Louis, MO). For platelet assays, they were grown in BHI, BHI-S, or tryptic soy broth (Difco) supplemented with 0.25% glucose (TSBG).

Colony hybridization. *E. faecalis* isolates were inoculated onto sterile nylon membranes that had been placed on BHI agar plates and grown overnight. The colonies were lysed, and the filters were hybridized under high-stringency conditions as previously described (65). Intragenic DNA probes were obtained by PCR amplification of *E. faecalis* OG1RF genomic DNA (for *ebpA*, *ebpB*, and *ebpC*) and *E. faecalis* TX0006 genomic DNA (for *bee1*, *bee2*, and *bee3*) by using the appropriate primers and conditions described earlier (50, 72). ^{32}P-labeled DNA probes were prepared using the RadPrime DNA labeling system (Invitrogen, Carlsbad, CA) according to the protocol supplied by the manufacturer.

The *ebp* locus sequence analysis. To study the extent of variability within the genes encoding individual Ebp pilus components, we analyzed the sequence of the *ebp* locus from 54 *E. faecalis* strains (see Table S2 in the supplemental material). Draft genomes of these *E. faecalis* strains (sequenced as part of a collaboration with George M. Weinstock at the Genome Sequencing Center at Washington University School of Medicine [25 isolates] and Baylor College of Medicine [4 isolates] and by other researchers at The Broad Institute [22 isolates] [51] and at the J. Craig Venter Institute [3 isolates]) are available at

http://www.ncbi.nlm.nih.gov/genomes/MICROBES. For the 30 *E. faecalis* strains for which the multilocus sequence type (MLST) was not previously derived, the sequences of 7 housekeeping genes (57) were analyzed in accordance with the international *E. faecalis* MLST database available at http://efaecalis.mlst.net/. The 54 sequenced strains were differentiated into 33 distinct sequence types (STs) by MLST (see Table S2 in the supplemental material), including three new STs. We also analyzed the sequence of the *ebp* locus from 19 *E. faecium*, one *Enterococcus gallinarum*, and three *Enterococcus casseliflavus* genomes available at NCBI.

To identify the nucleotide (nt) variation within the individual *ebp* genes, sequences from different isolates were compared to the corresponding sequences in the well-studied *E. faecalis* strain OG1RF (4). Each *ebp* gene sequence differing by one or more nucleotides was considered a different allele (nucleotide type), and distinct allelic sequences were assigned an arbitrary number. Haplotypes (defined as a combination of *ebpA*, *ebpB*, and *ebpC* types) were determined by allelic variation in the three *ebp* genes.

Phylogenetic analysis and tests for selection and recombination. Multiple sequence alignments of individual *ebp* genes were done by the ClustalW method using the MegAlign program of the DNASTAR software (Lasergene, Madison, WI). Phylogenetic trees (cladograms) based on the matrix of pairwise sequence divergence were constructed using the MegAlign program. Splits graphs were computed from the sequences for each individual gene and also from the complete *ebp* operon. SplitsTree4 (22) (available at http://www.splitstree.org) was used to evaluate split decomposition network methods. Split networks are combinatorial generalizations of phylogenetic trees and are designed to represent incompatibilities within data sets. Nonsynonymous (d_N) and synonymous (d_S) substitutions per (non)synonymous nucleotide site were used to estimate whether the genes encoding pilin subunits are under selection pressure, as described in reference 1. To identify specific amino acids undergoing positive or purifying selection, the rate of nonsynonymous to synonymous polymorphisms (K_a/K_s) for each codon position (70) was performed using an Internet-based selecton server (http://selecton.tau.ac.il/). Potential recombination events between *ebp* sequences were identified using the maximum chi-square test (68), Sawyer's runs test (58), and 1,000 resamplings. Both of these tests are nucleotide substitution distribution methods that test for clustering of polymorphisms along an alignment (54). The START2 (sequence type analysis and recombinational test) program (available at http://pubmlst.org) (23) was used to determine the ratio of d_N/d_S and recombination tests described above.

Whole-cell ELISA. Surface expression of EbpA and EbpC by *E. faecalis* isolates grown for 10 h in BHI-S was detected by a whole-cell enzyme-linked immunosorbent assay (ELISA), as described in the companion paper (48). Of note, anti-rEbpB antibodies that react reasonably well with polymerized pili on Western blots, similar to anti-rEbpC and anti-rEbpA antibodies (50), showed relatively low reactivity with OG1RF cells when analyzed by flow cytometry (as well as by whole-cell ELISA) (25), suggesting that EbpB epitopes may be masked in the native Ebp pilus structure and thus not accessible to antibodies in these assays. Therefore, we did not use these antibodies in surface expression studies with bacteria. To confirm that whole cells of all strains were actually bound to the microtiter plates, antiserum raised against formalin-killed *E. faecalis* strain HH22 whole cells was used as a positive control.

Flow cytometry. (i) Bacteria. For flow cytometry analysis, bacteria grown in BHI-S were blocked with newborn calf serum (NBCS; Sigma) before being incubated at 4°C for 2 h with 20 µg/ml preimmune or affinity-purified anti-rEbpA- or anti-rEbpC-specific antibodies (50) in dilution buffer (phosphate-buffered saline [PBS] containing 20% NBCS and 0.1% bovine serum albumin [BSA]). Bacteria were then labeled with goat anti-rabbit F(ab′)₂ fragment conjugated with R-phycoerythrin (Jackson Immunoresearch, West Grove, PA) at 4°C for 2 h, fixed with 1% paraformaldehyde in PBS, and analyzed using a Coulter EPICS XL AB6064 flow cytometer (Beckman Coulter) and System II software.

(ii) Endocarditis vegetations. Sterile and OG1RF-infected vegetations were produced in rats, as described earlier (50, 67). Vegetations harvested from heart valves 48 h after injection of bacteria were processed by sonication, filtration, and centrifugation to remove host tissue debris, using a method previously described by us (49). Processed samples were labeled with affinity-purified anti-rEbpC antibodies and with immunoglobulins purified from antiserum raised against formalin-killed *E. faecalis* HH22 whole cells (positive control). To assess possible cross-reactivity of anti-rEbpC antibody with host tissue, a noninfected vegetation sample was also probed with anti-rEbpC-specific antibodies. Forward scatter (for analysis of particle sizes in the sample) and side scatter (for analysis of cell granularity or internal complexity) of cells processed from the vegetation were analyzed and compared with *in vitro*-grown *E. faecalis* OG1RF cells.
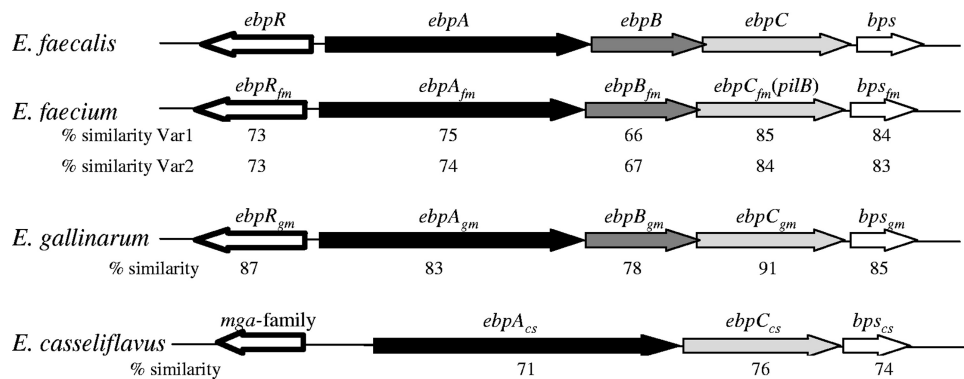
FIG. 1. Ebp-type pilus gene clusters (drawn to scale) identified in the chromosomes of four enterococcal species. Each cluster contains a predicted pilus-specific sortase gene (white arrows with thin borders), genes encoding a major subunit (light gray arrows), one or two minor subunits (black and dark gray arrows), and a gene encoding a cognate regulator (white arrows with thick borders). A second variant (Var 2) of the *ebp* locus found in *E. faecium* had an identical organization to variant one (Var 1). The percent similarity of each protein with its *E. faecalis* homolog is shown.

Quantitative assay for adherence to human platelets. Adherence of *E. faecalis* to human platelets was assessed quantitatively as described previously for *Streptococcus mitis* (37) with minor modifications. In brief, human platelets (prepared using the method described in reference 71) were immobilized in poly-L-lysine-coated tissue culture wells to produce platelet monolayers of 90% confluence. To reduce nonspecific adherence, coated wells were treated for 2 h at room temperature with a casein solution (blocking reagent; Roche Applied Sciences, Indianapolis, IN) in Dulbecco's phosphate-buffered saline (DPBS). After removing the blocking solution, the wells were inoculated with approximately 1×10^8 to 5×10^8 CFU of appropriately grown *E. faecalis* cells suspended in 1 ml of DPBS and incubated at 37°C for 2 h, with gentle rocking to enhance mixing. Unbound bacteria were removed by washing with DPBS, and bound bacteria were then recovered by scraping and resuspension in 1 ml DPBS. The number of organisms bound was determined by plating serial dilutions of the suspension onto BHI agar, and binding was expressed as a percentage of the inoculum. Assays were carried out in triplicate on three to four separate occasions, using platelets from a different human donor on each day. Differences in platelet binding were compared by the unpaired *t* test.

RESULTS AND DISCUSSION

Distribution, organization, and conservation of pilus-encoding genes among diverse *E. faecalis* strains and other enterococci. (i) Distribution in *E. faecalis*. Of the two *E. faecalis* pilus-encoding gene clusters described to date, the *ebp* locus (part of the core genome) was previously shown to be present in all 408 *E. faecalis* isolates tested (50). The second cluster, *bee* (for biofilm enhancer in Enterococcus), encoded on a conjugative plasmid, was restricted to 2 of 40 isolates tested (72). Here, we tested 64 additional isolates for *ebp* and 473 isolates for *bee* by colony hybridization. Our results showed that only a single (fecal) isolate (TX1346) lacked *ebpA*, *ebpB*, and *ebpC*. Consistent with a previous report (72), the *bee* locus (*bee1*, *bee2*, *bee3*) was present in 1% of 359 clinical isolates, 2% of 58 hospital-derived stool isolates, 6% of 33 community-derived stool isolates, and 0% of 23 animal isolates from our collection (1.2% overall). These results (along with genome analysis [see below]) suggest that most *E. faecalis* strains encode only one pilus-encoding locus (*ebp*), similar to GAS FCT (fibronectin- and collagen-binding protein and T antigen) pilus loci (55); however, unlike *ebp*, the FCT locus is highly variable in gene content and organization in different M serotypes (see below). In contrast, many isolates of GBS, *E. faecium*, and corynebacteria possess two or more pilus-encoding loci on variable

genomic islands which, at times, are flanked by transposable elements (28, 36, 69).

(ii) *ebp* locus organization in *E. faecalis*. To study the gene organization and extent of conservation within each gene encoding an Ebp pilus component (see below), we analyzed the sequence of this locus from 54 of 55 *E. faecalis* sequenced strains representing 33 MLSTs (see Table S1 in the supplemental material). The organization of the predicted backbone (*ebpC*) and the two predicted ancillary subunit-encoding genes (*ebpA* and *ebpB*) was *ebpA-ebpB-ebpC-bps* in all isolates tested (Fig. 1). Furthermore, our analysis identified a single pilus locus (*ebp*) in these *E. faecalis* genomes, except strain HIP11704, which also contained a partial *bee* locus in addition to the *ebp* locus. A draft genome of *E. faecalis* strain TX1346 (55th sequenced strain) was found to lack an *ebp*-like locus, confirming our hybridization results. Sequence alignments with the completed genomes of strains OG1RF and V583 enabled us to identify two contigs (Cont1322.1 and Cont123.1) in the TX1346 genome database that encode open reading frames (ORFs) surrounding the deleted *ebp* locus. Sequencing over this region suggested that TX1346 has undergone a 12-kb deletion (12,041 nt versus OG1RF and 12,035 nt versus V583) replaced by a 1,524-nt fragment encoding an IS*1542* transposase, a member of the IS*256* family of insertion sequence elements (12). The deleted region corresponds to 9 complete genes in V583 (*ef1089* to *ef1097*), including the *ebp* locus (*ef1090* to *ef1094*), as well as the 3′ end of *ef1088*.

(iii) Each Ebp subunit-encoding gene of *E. faecalis* is highly conserved. Comparison of the sequences of each individual subunit-encoding gene, *ebpA*, *ebpB*, and *ebpC*, from *E. faecalis* strains representing 33 STs revealed 98.4% to 99.99% identity at the DNA level and 97.5% to 100% identity at the protein level. The polymorphic sites in all three subunit-encoding genes were distributed randomly over the entire genes (data not shown). The numbers of polymorphic nucleotides ranged from 152 (128 point mutations and 24 insertion/deletion changes in *ebpA*) to 66 (*ebpC*) (Table 1). Insertion/deletion changes, which were found only in *ebpA*, were all in-frame changes (an 18-nt deletion in strain E1Sol and a 6-bp insertion in strains T8 and JH2-2). Most point mutations were found to

TABLE 1. Ebp pilin subunit gene characteristics in 54 *E. faecalis* isolates

Gene	Length (nt)	No. of alleles (nt)[a]	No. of amino acid types	% identity[b]	No. of polymorphisms		d_N/d_S ratio[c]
					nt	aa	
ebpA	3,414 to 3,438	32	29	98.8 to 99.99	152[d]	57	0.13
ebpB	1,431	27	24	98.4 to 99.99	67[e]	31	0.27
ebpC	1,884	23	20	98.8 to 99.99	66[e]	25	0.13
7 housekeeping genes	395 to 583[f]	8 to 19	ND[g]	ND	7–20	ND	0 to 0.07

[a] Nucleotide sequence types.

[b] Percent identities between different nucleotide types (alleles).

[c] d_N/d_S, a measure for estimating whether a particular sequence is under selection pressure: $d_N/d_S < 1$, nonsynonymous mutations are under negative (purifying) selection; $d_N/d_S = 1$, all changes are neutral; $d_N/d_S > 1$, substitutions are driven by positive (diversifying) selection.

[d] One hundred twenty-eight point mutations and 24 insertion or deletion changes.

[e] All point mutations.

[f] Region used for MLST analysis.

[g] ND, not determined.

be synonymous (silent) (e.g., EbpC, 41 of 66 mutations were synonymous), and several nonsynonymous mutations caused changes to a similar functional group of amino acids (aa) (e.g., EbpC, 8 of 25 mutations were nonsynonymous). Only one strain (DS5) had a nonsense transversion (C1967A in *ebpA*), which resulted in a premature stop codon (see expression studies below), while *ebpB* and *ebpC* were intact in all strains. The pilin motif and E box, shown to be important for assembly of pilus fibers in corynebacteria (36), were conserved in all strains.

A total of 32 alleles were observed for *ebpA*, 27 for *ebpB*, and 23 for *ebpC*, but the number of amino acid sequence types was slightly lower due to silent mutations; the majority of these

sequence types differed only by 1 or 2 nt or aa (Table 1). Variation between individual amino acid sequence types of all three subunit proteins (Fig. 2) was in the range of 0.1% to 2.4%, which is low compared to that of another, presumably evolving, surface-exposed antigenic protein, Ace. The amino acid variation of Ace in this set of isolates was >7% in the A domain and was much higher for the complete protein due to variation in the number of B domain repeats.

A total of 35 unique Ebp haplotypes (which are likely inherited as a unit) were identified among the 33 MLSTs (see Table S2 in the supplemental material). While most isolates belonging to the same MLST had an identical Ebp haplotype (see Table S1 in the supplemental material), a few had 1 to 2 aa

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EbpA
        00000000000000000000000000000000000000000000000011
        00111111111111111111122222233334445566666677788888889999999901
        24011345555567789124468444627801345668678033467035567902
        59759144567894563627455348928176599837070605750394398654652
EbpA1   ITPELNTEPEKITTEKTAQQGATRG--GKSAPVRPSGNTEDKVRTANTEASDTEHHY
EbpA2   .................S.I......NV..............S..T...KNN.
EbpA3   .................S.I......N..............S..T...KN..
EbpA4   .................S.I......N.......I.................
EbpA20
EbpA21
EbpA5   .S...........D......I..............N..N..S....N.....
EbpA6   .............D....SI..NI.TN.......D.................F
EbpA7   .............D....SI....TN.......D.................F
EbpA8   .................S.I......N.S........N...VS...A..K...
EbpA9   ............R.S.I......N.SI.......VS...A..K...
EbpA10  .................S.I......N.....*..I.................
EbpA11  ..S...........I......N.......D.........A..........
EbpA12  V..K.........D....R..IS....N...............VSA.....
EbpA13  ...............E....I......N.......S......VS.......
EbpA14  ......D..........S.I......N..............I.........
EbpA15  ......------......I......N..............V.....IK...
EbpA16  ............M....R..I...............S.....K...
EbpA17  V................I......N.......................
EbpA18  .................S.I......N........I....I......KN..
EbpA19  ..........N....TI....S.N...H.........S..A..K...
EbpA22  .................S.I......N........I...I..K...KN..
EbpA23  ....I............S.I......N...........S. T...KN..
EbpA24  .................S.I......N.SI.......VS...A..K...
EbpA25
EbpA26  .................I......N......D......V.........F
EbpA27  ......I....D....SI....TN...................
EbpA28  .................S.I......N........K.....S..T...KN..
EbpA29  .................I......N...Q.......A..S.........
EbpA30  ............A....I......N.......I......VS.......
EbpA31  .................S.I......N......................
EbpA32  .................I.S....N..............S.....K...
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EbpB
        00000011111112222222233333334444
        22346800123511445799114577126 6
        13158323166693737457867181622 02
EbpB1   QASQDLAMDISDMTAEAGNTAAAVGIAERMV
EbpB2   .......T....V.........T.F.G...
EbpB3   ..........V..V.......A.F...L.
EbpB4   ............V.........A.F...L.
EbpB21
EbpB5   .......R....V.........T.......
EbpB6   ............V..V.....V.A.F....
EbpB27
EbpB7   .......T.T..VIV.S..A.....F...L.
EbpB8   .......T.T..VIV.SD.A.....F...L.
EbpB9   ..N........V.........A.F...L.
EbpB10  ...K..T....V.............F....
EbpB11  ............V..V....T.T.......
EbpB12  .........T....V...........Q.A
EbpB13  H.......T....V......K.....F...L.
EbpB26
EbpB14  .V.....T....YV..........FV...
EbpB15  .......TN...V.............G...
EbpB16  .......T....V...............L.
EbpB17  ............V.........ARF....
EbpB18  .......T....V.........T......LA
EbpB19  .......T.T...V.............G...
EbpB20  ...N.....L.V..V.......A.F...L.
EbpB22  ......T.....V..V.........F...LA
EbpB23  ............V..V....T.T......L.
EbpB24  H......T.....V........A.F.....
EbpB25  ............V..V.........F...LA
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EbpC
        00000011112222233334555
        11137891468001224036915 66
        01563426470676120400794 78
EbpC1   TILGASATPAVEGSSPTTSDVAPVV
EbpC2   .V...N....T...............
EbpC3   .V................N.......
EbpC4
EbpC5
EbpC6   .V.........I........N......
EbpC17
EbpC7   .V.........I.......NN.....
EbpC8   .V.........I.......N.M....
EbpC9   .V................N.E.M.
EbpC10  AV................N.......
EbpC11  .V.........DR.....N.M....
EbpC12  .V.....I.T.D..T...N.....
EbpC13  AV.............L..N.M....
EbpC14  .V................N.E...
EbpC15  .V.VI.......I.......NN....
EbpC16  AV................N.M....
EbpC18  .V.E..V.TT.......N......
EbpC19  .V................N.M....
EbpC20  AV...N....I.......N.M.S.I
EbpC21  .V.........I..F...IN.M....
EbpC22  .V..S...........N.M.....
EbpC23  .V................M.N.M....
```
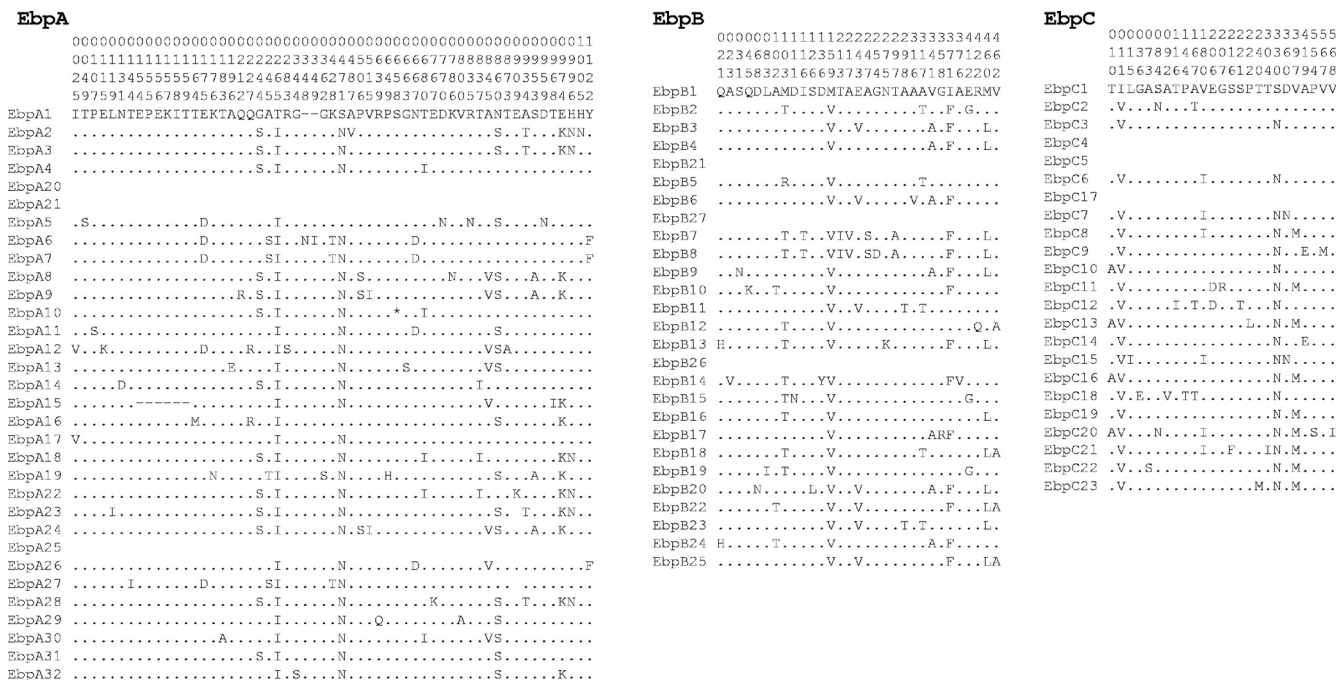
FIG. 2. Amino acid variation in Ebp pilin components of 54 *E. faecalis* strains representing 33 MLSTs. Each *ebp* sequence that differed by ≥1 nt was designated a different allele and assigned an arbitrary number, and the same numbering was retained for each amino acid type. Alleles that differed only by silent mutations and resulted in identical amino acid types are positioned below their identical amino acid type (for example, EbpA20 and EbpA21, which are identical to EbpA4, are placed below EbpA4). Amino acids present in each of the variable sites of *E. faecalis* OG1RF are shown. The position of each variable site within the sequenced fragment is shown by the number above each amino acid, read vertically.

changes in one or more Ebp subunit proteins (details in Table S3 in the supplemental material). Similarly, related MLSTs (ST25 and ST364) had identical Ebp haplotypes. Also, two common Ebp haplotypes were identical in several unrelated STs (see Table S4 in the supplemental material). The incongruent phylogenetic trees of individual *ebp* genes and split decomposition analysis indicate remnants of horizontal gene transfer or recombination. Analysis by the maximum chi-square test on all possible pairwise combinations of alleles (1,000 randomizations; significance value, $P < 0.05$) identified nt 477, 747, 768, and 1053 of *ebpA* as potential recombination sites. The ratio of the rate of nonsynonymous (K_a or d_N) to synonymous (K_s or d_S) polymorphisms (d_N/d_S values are shown in Table 1) suggests that none of the *ebp* pilin subunit genes are under positive selection pressure. Due to the small number of polymorphic sites, reliable prediction of specific amino acids under selection and calculation of associated posterior probabilities were not possible. Taken together, the same amino acid replacements of Ebp proteins that likely occurred independently in different STs may not reflect common ancestry but rather represent parallel, reverse, or convergent changes.

(iv) Ebp-type pili of other enterococcal species. A subsequent search for Ebp-type pilus (defined as >70% aa similarity with all subunit proteins) genes in available genome databases identified that Ebp-type pili are unique to enterococci and present in 19 of 22 *E. faecium* genomes (20, 63), 1 of 1 *E. gallinarum* genome, and 3 of 3 *E. casseliflavus* genomes (Fig. 1). Their genetic organization is highly conserved in each species, but the *E. casseliflavus* isolates lack an *ebpB* homolog. Similar to *E. faecalis*, *E. faecium* and *E. gallinarum* also have *ebpR* (encoding a positive regulator of the *ebp* operon in *E. faecalis* [5, 6]) in the same position (Fig. 1). The gene immediately upstream of the *E. casseliflavus ebp* locus has little similarity to *ebpR*; however, sequence analyses predict that it, like *ebpR*, also codes for a member of the AtxA/Mga regulator family. Interestingly, sequence analysis of the *E. faecium ebp* operon found two variants of this locus with ~90% identities between their subunit-encoding genes; however, both variants had almost similar amino acid similarities with their *E. faecalis* homolog (Fig. 1). As previously reported, a number of *E. faecium* isolates have one or more of three additional pilus loci (*fms11-fms19-fms16*, *fms14-fms17-fms13*, or *fms21* [*pilA*]-*pilE-fms20* [*pilF*]), and each of these loci also encodes a sortase C, predicted to be involved in pilus biogenesis (20, 21, 62).

Taken together, Ebp-type pili of four different species of enterococci have similar gene organizations, including two to three conserved pilin-encoding genes, a single sortase C-encoding gene (*bps*-like) located at the 3′ end of the *ebp* loci (downstream of the major pilin gene *ebpC*), and a divergently transcribed regulatory gene, *ebpR*, at the 5′ start of the *ebp* locus. This is in contrast to the pilus-encoding loci of some other Gram-positive bacteria, such as the FCT locus of GAS, which is highly variable in gene content and organization and possesses ≥1 sortases (28, 36). For example, FCT-3 pili of a GAS serotype M3 strain have a collagen adhesin Cpa as a minor pilin, FCT-1 pili of M6 have a fibronectin adhesin PrtF1, and FCT-4 pili of M12 have both Cpa and PrtF1 (30, 53).

Ebp pilus expression by diverse *E. faecalis* isolates in BHI-S *in vitro* and in experimental IE vegetations. (i) Most *E. faecalis* isolates express Ebp pili after growth in BHI-S. To investigate if the expression of Ebp pili observed with strain OG1RF after growth in BHI-S is a general property of *E. faecalis*, we analyzed Ebp pilus expression of 93 diverse *E. faecalis* isolates, including 25 sequenced strains by whole-cell ELISA using affinity-purified anti-rEbpC- or anti-rEbpA-specific antibodies. These isolates were grouped on the basis of their origin: 20 endocarditis; 20 other, clinical; 20 urine; 19 community, fecal; 12 animal; and 2 of unknown origin. OG1RF was used as a positive control. As shown in Fig. 3, all strains grown in BHI-S, except the one (fecal) isolate, TX1346, that lacks the *ebp* locus, were found to express both EbpA and EbpC on their surface. ST55 strain DS5 (of unknown origin), which has an internal stop codon in *ebpA* (see above), lacked EbpA, while it still expressed EbpC. Individual differences were observed in EbpA and EbpC expression levels (quantified by optical density at 405 nm [OD_{405}] values) of different strains ranging from 0.31 to 2.5 for EbpC and 0.38 to 2.9 for EbpA (excluding TX1346). Statistical analysis, using Pearson's correlation test, confirmed that the amount of surface EbpC correlated strongly ($r = 0.946$; $P < 0.0001$) with the amount of surface EbpA (see Fig. S1 in the supplemental material).

Although expression of Ebp pili after growth in BHI-S was detected in all categories of isolates, a statistically significant difference was observed between the Ebp pilin (both EbpC and EbpA) expression levels of strains from different sources ($P = 0.02$ and $P = 0.0003$ by analysis of variance [ANOVA], respectively). Multiple comparisons with Bonferroni's correction showed significant differences between the endocarditis group versus the community-derived fecal isolate group ($P < 0.05$ and $P < 0.001$, for EbpC and EbpA, respectively) and versus the animal isolate group for EbpA ($P < 0.05$).

(ii) Bistable surface expression of Ebp pili. Using immuno-gold electron microscopy and fluorescence-activated cell sorting (FACS), we previously estimated that only a proportion of OG1RF cells (<10% of BHI-, <20% of TSBG-, and <35% of BHI-S-grown cells) had Ebp pili on their surface *in vitro* (25, 29, 50). Here, we quantified the surface-localized Ebp pilin proteins of 7 diverse endocarditis isolates using FACS and found that 30% (OG1RF) to 72% (TX0001) of cells of the investigated strains expressed EbpA or EbpC after growth in BHI-S (see Fig. S2 in the supplemental material); 6 of 7 strains were inducible (2- to 5-fold) in BHI-S versus in BHI (data not shown). In contrast to Ace (see Fig. S2J and K in the supplemental material), two different peaks (heterogeneity manifested by the bifurcation into two distinct subpopulations) could be distinguished among anti-Ebp-stained versus unstained cells for all strains (see Fig. S2A to H in the supplemental material), suggesting a bistable expression mode for Ebp. Growth in BHI also demonstrated bistable expression, albeit with fewer Ebp-expressing cells. Similar bistable expression was reported for FCT pili of GAS strains (43). While a biological benefit of bistability is currently unknown, it has been proposed to prepare a subpopulation of bacteria for rapidly changing environmental conditions (13, 75).

(iii) *In vivo* expression of Ebp pili during experimental infective endocarditis. To quantify the percentage of Ebp-positive cells *in vivo*, flow cytometry analyses were carried out on extracts directly processed from IE vegetations infected with OG1RF grown in BHI. Processed sterile vegetations from non-infected rats probed with anti-rEbpC-specific antibodies, which
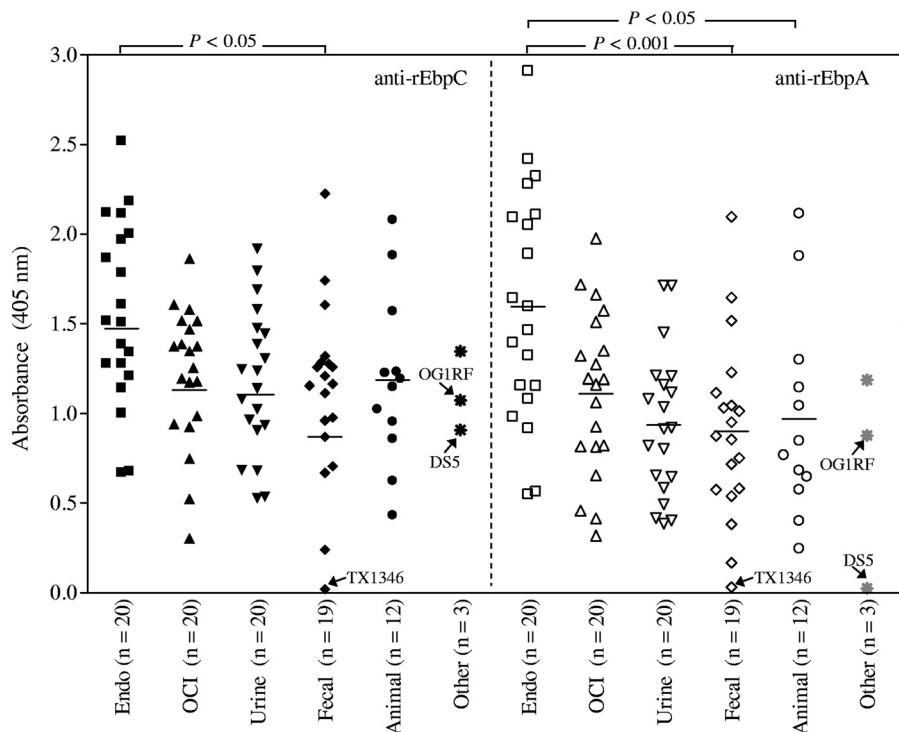
FIG. 3. Determination of EbpC and EbpA surface expression by diverse *E. faecalis* strains grown in BHI-S, grouped by source of isolation. Affinity-purified anti-rEbpC and anti-rEbpA antibodies were used in these whole-cell ELISAs. Each data point represents the mean absorbance at 405 nm ± SD from 9 wells. Results are from three independent experiments. Horizontal bars represent the means (absorbance) of each group of isolates. Endo, strains isolated from patients with *E. faecalis* endocarditis; OCI, strains isolated from *E. faecalis* nonendocarditis clinical infections; urine, strains isolated from urine; fecal, isolates from feces of community healthy volunteers; animal, isolates from animal origin; other, includes isolates of unknown origin and the control OG1RF. TX1346 that lacks the *ebp* locus, DS5, which has an internal stop codon in *ebpA*, and the control, OG1RF, are labeled with arrows. Mean Ebp pilin (both EbpC and EbpA) expression levels of strains from different sources were compared using ANOVA and Bonferroni's posttest; *P* = 0.02 and *P* = 0.0003 for EbpC and EbpA by ANOVA, respectively. Significant differences between individual groups assessed by the *post hoc* test are shown in the figure.

served as a negative control, showed labeling of a minor fraction (<5%) of bacterium-sized particles (Fig. 4A). Immunoglobulins from an antiserum raised against formalin-killed *E. faecalis* strain HH22 whole cells (used as a positive control) bound 85% of bacterium-sized particles of processed infected vegetations, indicating that the majority of these particles were *E. faecalis* cells (Fig. 4A). Affinity-purified anti-EbpC-specific antibodies bound to ~57 to 63% of bacterium-sized particles (~67 to 74% of particles labeled by the general anti-*E. faecalis* HH22 antiserum) from multiple rat endocarditis vegetations infected with OG1RF (Fig. 4A and B). The higher percentage (Fig. 4C) of Ebp-expressing OG1RF cells from vegetations versus ~30% of cells grown in BHI-S demonstrates that Ebp pili are actively expressed in host vegetations during IE. Furthermore, this result is consistent with our previous finding of anti-Ebp pilin antibodies in human endocarditis patient sera infected by diverse *E. faecalis* strains (64).

The role of *E. faecalis* MSCRAMMs and pili on adherence to platelets. Adherence of pathogenic bacteria to platelets is believed to play an important role in the pathogenesis of IE. Numerous endocarditis-associated pathogens have been shown to adhere to platelets directly or indirectly *in vitro*, through a variety of mechanisms (11, 15, 27). Adherence to platelets *in vitro* has been linked to virulence of common endocarditis-

associated species, including *S. aureus*, *S. gordonii*, and *Streptococcus sanguinis* (11, 15, 27).

(i) Effect of growth conditions on adherence of *E. faecalis* strain OG1RF to immobilized human platelets. Since platelets are a major component of IE vegetations and because we found that *E. faecalis* MSCRAMMs and pilus mutants are attenuated in endocarditis (50, 67), we tested whether *E. faecalis* strains were able to adhere to human platelets. Pilot analyses of platelet adherence by *E. faecalis* strain OG1RF, grown in a standard laboratory medium (BHI) to three different growth phases, showed that after 2 h of incubation with platelet monolayers, bacterial adherence ranged from 0.9% to 4.2% of the inoculum (means ± standard deviations [SD] of 3.1% ± 1.0% with log-phase culture, 2.5% ± 0.9% with late-log-phase culture, and 1.1% ± 0.3% with overnight culture) (data not shown). Only 0.2 to 0.7% of the OG1RF inoculum bound to uncoated polylysine plastic wells (negative control), and BHI-grown OG1RF platelet adherence was 2- to 6-fold lower than that by the experimental control *Streptococcus mitis* strain SF100, previously shown to adhere efficiently to platelets (3, 37). Since growth medium has been found to influence the ability of *E. faecalis* strains to form biofilms (e.g., TSBG) (52) and enhance their adherence to several extracellular matrix proteins (e.g., BHI-S) (45), at least in part via increased ex-
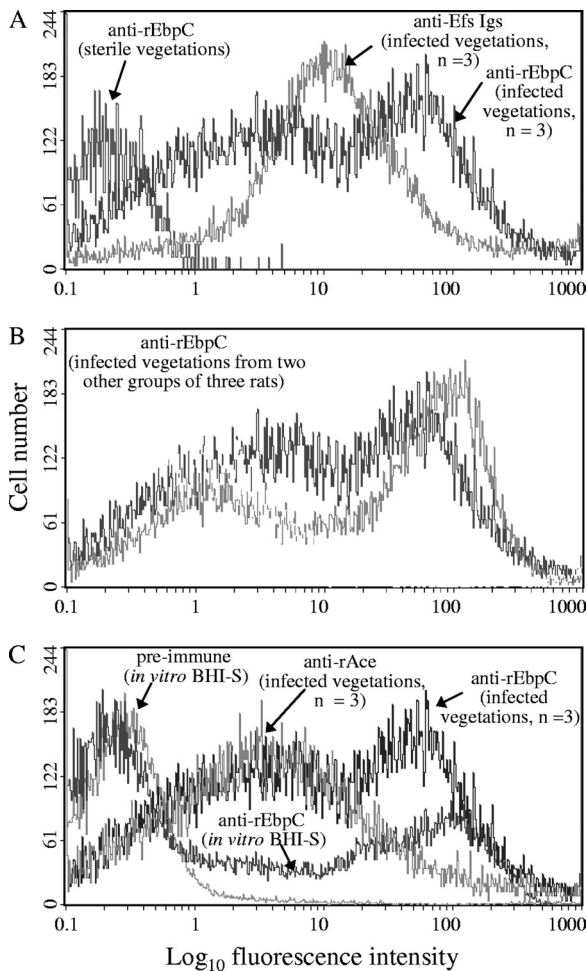
FIG. 4. Flow cytometry analysis of Ebp pilus expression measured by EbpC on *E. faecalis* OG1RF cells derived from vegetations of rat experimental infective endocarditis. (A) Surface expression of Ebp pili by bacterium-sized particles derived from a mixture of vegetations of three rats; (B) expression of Ebp pili by bacterium-sized particles derived from a mixture of vegetations of two other groups of three rats each; (C) for comparison, OG1RF cells grown *in vitro* in BHI-S (the growth condition that exhibited the most *in vitro* Ebp pili expression), stained with preimmune immunoglobulins and anti-rEbpC, as well as surface expression of Ace (a positive control, which we previously reported [67]) by bacterium-sized particles derived from a mixture of vegetations from the three rats from panel A are shown. Vegetations were produced as described earlier (50, 67), and rats were intravenously (i.v.) injected with BHI-grown OG1RF. Processed sterile and infected vegetations were mixed and then divided into aliquots for labeling with (i) immunoglobulins purified from antiserum raised against formalin-killed *E. faecalis* HH22 whole cells (anti-Efs; positive control), (ii) affinity-purified anti-rAce-specific antibodies (positive control), and (iii) affinity-purified anti-rEbpC-specific antibodies, followed by incubation with R-phycoerythrin-conjugated antibodies. Specific binding by anti-rEbpC, anti-Efs, or anti-rAce antibodies is indicated as log fluorescence intensity on the *x* axis. Each histogram represents ~5,000 (noninfected vegetations), ~25,000 (infected vegetations), and 50,000 (*in vitro*-grown cultures) events of bacterium-sized particles.

pression of MSCRAMMs and pili (44, 48, 50), we next tested the adherence of OG1RF, grown to late logarithmic phase in TSBG and BHI-S, to human platelets derived from different donors. As shown in Fig. 5A, adherence to platelets by OG1RF

from different culture conditions increased progressively, starting with cells grown in BHI, TSBG (3.4-fold versus BHI), and BHI-S (5.5-fold versus BHI and 1.6-fold versus TSBG) ($P = 0.007$ by ANOVA and $P < 0.01$ by the *post hoc* test for BHI versus BHI-S). The substantial variability in adherence to platelets from different donors (even cells grown in BHI-S showed no adherence to platelets from 3 of the 7 donors) likely explains the relatively high standard deviations of adherence data from independent experiments (e.g., 19.5 ± 10 for BHI-S); nevertheless, the adherence differences between independent experiments were highly reproducible. Subsequent experiments were carried out using platelets from the donors showing adherence.

We next tested the platelet adherence of two diverse endocarditis isolates. As shown in Fig. 5B, both strains grown in BHI-S were found to adhere to platelets obtained from three different donors, and the adherence percentages varied depending on the donor, similar to OG1RF. For TX0024 (also referred to as MC02152 [47]), the percentage of platelet-adhering cells ranged from 4.6% (donor 2) to 31.75% (donor 3), and that for TX0052 ranged from 6.4% (donor 1) to 30.63% (donor 3). These results indicate that human platelet adherence may be a common phenomenon among *E. faecalis*, albeit with considerable variability between individual *E. faecalis* strains and platelets from different donors. We speculate that the observed specificity of *E. faecalis* to platelets of some individuals may be due to genetic polymorphisms or differential expression of platelet receptors; similar heterogeneity was reported for the binding of *Helicobacter pylori* to human gastric MUC5AC mucin glycoforms (33). This suggests the possibility of a genetic predisposition to enterococcal infections.

(ii) Requirement of sortase activity for platelet adherence. Since cell wall-anchored proteins of other pathogens (11, 27) are known to promote bacterial adhesion to human platelets, we evaluated the global impact of LPXTG-type surface-anchored proteins and pili using mutants of sortases. *E. faecalis* OG1RF contains two sortase genes (housekeeping *srtA* and pilus-associated *bps*) that are ubiquitous (25) among other *E. faecalis* isolates but lacks *ef2524*, a sortase A homolog found in the mobile genetic element *efaC1* (*ef2512* to *ef2546*) (7, 32). As shown in Fig. 6A, Δ*srtA*, Δ*bps*, and Δ*bps*Δ*srtA* mutants grown in BHI-S were defective in adherence to platelets versus that of wild-type OG1RF ($P < 0.0001$ by ANOVA). The mean percentages ± SD of bacteria adhering of the Δ*srtA*, Δ*bps*, and Δ*bps*Δ*srtA* mutants were 9.9% ± 2.5%, 8.4% ± 3.5%, and 2.6% ± 1.2%, respectively, versus 25.8% ± 6.8% for the wild type. The reduction in platelet binding seen with the double-deletion mutant (Δ*bps*Δ*srtA* mutant) versus each of the single-deletion mutants was also significant ($P < 0.001$). These results indicate that binding of OG1RF to platelets is mediated predominantly by one or more surface-anchored sortase targets.

Adhesion to human platelets is directly mediated by Ebp pili of *E. faecalis* OG1RF. The above-mentioned data indicated that the Ebp pili (assembly and cell wall anchoring of which are dependent on Bps and SrtA, respectively) (18, 25, 50) and possibly other SrtA targets (e.g., *ace* encoding a collagen adhesin, *fss2* encoding a fibrinogen adhesin) (46, 61) contribute to adherence to human platelets of BHI-S-grown *E. faecalis* cells. To address this possibility, wild-type OG1RF and the Δ*ace*, Δ*ebpABC*, Δ*ace*Δ*ebpABC*, and Δ*fss2* mutants were tested
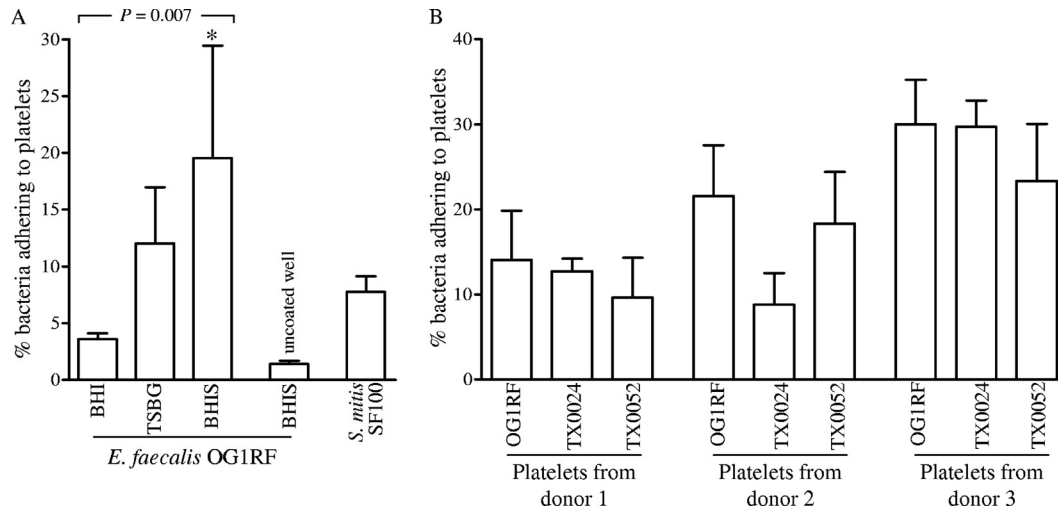
FIG. 5. Adherence of *E. faecalis* strains to immobilized human platelets. (A) Influence of growth conditions on the adherence of *E. faecalis* strain OG1RF; (B) adherence of OG1RF and two endocarditis-derived *E. faecalis* strains grown in BHI-S. Bars represent mean percentages ± SD of bacteria adhering to platelets (from three different donors) coated onto >9 wells from 3 to 4 independent experiments. *S. mitis* SF100, grown in Todd-Hewitt broth, as described in references 37 and 38, was used as a positive control. Means of adherence percentages of different strains were compared using ANOVA and Bonferroni's posttest. ∗, $P < 0.01$ by the *post hoc* test for BHI versus BHI-S.

for this phenotype. As shown in Fig. 6B, OG1RF lacking *ebpABC*, but not *ace* or *fss2* mutants, showed decreased platelet adherence ($P < 0.0001$ by ANOVA). The decrease for the $\Delta ebpABC$ mutant was highly significant compared to that of

the wild type or Δace or $\Delta fss2$ mutants ($P < 0.001$ by the *post hoc* test). This observation was further supported by the values obtained for the $\Delta ace\Delta ebpABC$ mutant, which were similar to that of the $\Delta ebpABC$ mutant and showed 3.5-fold reduction
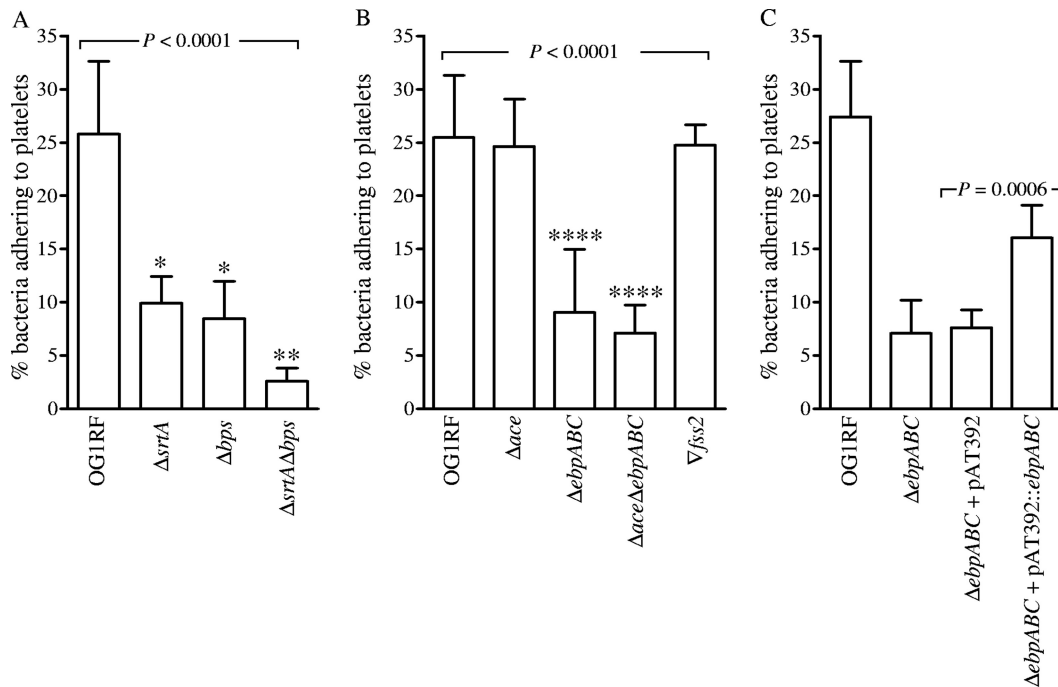
FIG. 6. Effect of deletion of *bps*, *srtA*, *ebpABC*, or MSCRAMM-encoding genes and complementation constructs on the adherence of BHI-S-grown OG1RF to immobilized human platelets. (A) Comparison of platelet adherence of wild-type OG1RF and its sortase mutants ($\Delta srtA$, Δbps, and $\Delta srtA\Delta bps$ mutants); (B) adherence by *ebp*, *ace*, and *fss2* mutants; (C) adherence by complementation derivatives. Bars represent mean percentages ± SD of bacteria adhering to platelets (from three different donors) coated onto 6 to 9 wells from two to three independent experiments. Means of adherence percentages of different strains were compared using ANOVA and Bonferroni's posttest. Means of the complemented strain were compared with the appropriate vector-only control strain using the *t* test. ∗, $P < 0.001$ versus wild-type, $P < 0.05$ versus double deletion, and nonsignificant versus other single deletions; ∗∗, $P < 0.001$ versus wild type and $P < 0.05$ versus both single deletions; ∗∗∗∗, $P < 0.001$ versus wild type and the Δace and $\Delta fss2$ mutants.

versus the wild type or Δ*ace* mutant (*P* < 0.001 by the *post hoc* test). Highly significant enhancement in platelet adherence was observed by the complemented strain (Δ*ebpABC* mutant with pAT392::*ebpABC*) over the empty vector control (Fig. 6C; *P* = 0.0006); however, this restoration was only to 65% of the wild-type level. The higher levels of unpolymerized pilin components leading to shorter, incompletely polymerized pili (see companion paper [48]) could explain the lack of full restoration of the adherence phenotype by the complemented strain. Our preliminary analyses with OG1RF mutants of three additional genes encoding MSCRAMMs (*fss1*, *ef2224*, and *ef1269*) showed no effect on BHI-S-induced platelet adherence (data not shown). Thus, our data demonstrate that Ebp pili contribute to *E. faecalis* OG1RF platelet adherence under the conditions tested. While Ebp pili also mediate fibrinogen adherence, whether these pili interact directly with platelet receptors like GspB and Hsa proteins of *S. gordonii* (2, 26, 60) or indirectly via fibrinogens like *S. aureus* ClfA and FnbpA (26) will be subjects of our future studies. Furthermore, the Ebp expression levels of OG1RF, TX24, and TX52 did not parallel platelet adherence levels, indicating saturation or involvement of additional bacterial factors in this phenotype.

In summary, we have demonstrated that deletion of *ebpABC* results in significant attenuation in the ability of *E. faecalis* to adhere to platelets, which is likely one of the factors contributing to vegetation formation/infection and may help explain our previous experimental evidence that *ebpABC* is important for *E. faecalis* endocarditis (50). We have also shown that Ebp pili are actively expressed by the majority of strains after growth in medium supplemented with serum, a biological cue with *in vivo* relevance, and are also expressed within host vegetations during endocarditis produced by OG1RF. Finally, the highly conserved nature of Ebp pili, in combination with data showing that anti-Ebp antibodies inhibit fibrinogen adherence of *E. faecalis* (companion paper [48]), underscores the possibility of using Ebp pilin components as targets for immunoprophylaxis or possibly in combination with antibiotics for treating *E. faecalis* endocarditis. The observations that anti-rAce antibodies (polyclonal and monoclonal) inhibit adherence of Ace to collagen (19, 46) and to epithelial cell lines (19), in addition to their protective effect in the IE model (50, 67), point toward the prospect of using both Ace and the Ebp pilus components, in combination, as targets for antibody-based therapy against *E. faecalis* infections. Furthermore, since Ebp pilus-like surface structures (with >70% similarities between individual pilin components) and Ace homologs are also found in other enterococci, including the hospital-associated emerging pathogen *E. faecium*, future cross-protection studies may reveal opportunities for the development of immunotherapeutics that may be useful for the prevention and treatment of infective endocarditis caused by different enterococcal species.

REFERENCES

1. **Bambini, S., et al.** 2009. Distribution and genetic variability of three vaccine components in a panel of strains representative of the diversity of serogroup B meningococcus. Vaccine **27:**2794–2803.
2. **Bensing, B. A., B. W. Gibson, and P. M. Sullam.** 2004. The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. J. Bacteriol. **186:**638–645.
3. **Bensing, B. A., C. E. Rubens, and P. M. Sullam.** 2001. Genetic loci of *Streptococcus mitis* that mediate binding to human platelets. Infect. Immun. **69:**1373–1380.
4. **Bourgogne, A., et al.** 2008. Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. Genome Biol. **9:**R110.
5. **Bourgogne, A., et al.** 2007. EbpR is important for biofilm formation by activating expression of the endocarditis and biofilm-associated pilus operon (*ebpABC*) of *Enterococcus faecalis* OG1RF. J. Bacteriol. **189:**6490–6493.
6. **Bourgogne, A., L. C. Thomson, and B. E. Murray.** 2010. Bicarbonate enhances expression of the endocarditis and biofilm associated pilus locus, *ebpR-ebpABC*, in *Enterococcus faecalis.* BMC Microbiol. **10:**17.
7. **Burrus, V., G. Pavlovic, B. Decaris, and G. Guedon.** 2002. The ICE*St1* element of *Streptococcus thermophilus* belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration. Plasmid **48:**77–97.
8. **Clawson, C. C.** 1973. Platelet interaction with bacteria. 3. Ultrastructure. Am. J. Pathol. **70:**449–471.
9. **Clawson, C. C., and J. G. White.** 1971. Platelet interaction with bacteria. I. Reaction phases and effects of inhibitors. Am. J. Pathol. **65:**367–380.
10. **Clawson, C. C., and J. G. White.** 1971. Platelet interaction with bacteria. II. Fate of the bacteria. Am. J. Pathol. **65:**381–397.
11. **Clemetson, K. J.** 2010. Platelets and pathogens. Cell. Mol. Life Sci. **67:**495–498.
12. **Darini, A. L., M. F. Palepou, and N. Woodford.** 1999. Nucleotide sequence of IS*1542*, an insertion sequence identified within VanA glycopeptide resistance elements of enterococci. FEMS Microbiol. Lett. **173:**341–346.
13. **Dubnau, D., and R. Losick.** 2006. Bistability in bacteria. Mol. Microbiol. **61:**564–572.
14. **Fernandez-Guerrero, M. L., C. Verdejo, J. Azofra, and M. de Gorgolas.** 1995. Hospital-acquired infectious endocarditis not associated with cardiac surgery: an emerging problem. Clin. Infect. Dis. **20:**16–23.
15. **Fitzgerald, J. R., T. J. Foster, and D. Cox.** 2006. The interaction of bacterial pathogens with platelets. Nat. Rev. Microbiol. **4:**445–457.
16. **Gianfaldoni, C., et al.** 2007. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. Infect. Immun. **75:**1059–1062.
17. **Giannitsioti, E., et al.** 2007. Nosocomial vs. community-acquired infective endocarditis in Greece: changing epidemiological profile and mortality risk. Clin. Microbiol. Infect. **13:**763–769.
18. **Guiton, P. S., et al.** 2009. Contribution of autolysin and sortase A during *Enterococcus faecalis* DNA-dependent biofilm development. Infect. Immun. **77:**3626–3638.
19. **Hall, A. E., et al.** 2007. Monoclonal antibodies recognizing the *Enterococcus faecalis* collagen-binding MSCRAMM Ace: conditional expression and binding analysis. Microb. Pathog. **43:**55–66.
20. **Hendrickx, A. P., et al.** 2008. Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate. Microbiology **154:**3212–3223.
21. **Hendrickx, A. P., et al.** 2010. Differential PilA pilus assembly by a hospital-acquired and a community-derived *Enterococcus faecium* isolate. Microbiology **156:**2649–2659.
22. **Huson, D. H., and D. Bryant.** 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. **23:**254–267.
23. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
24. **Kahn, R. A., and L. J. Flinton.** 1974. The relationship between platelets and bacteria. Blood **44:**715–721.
25. **Kemp, K. D., K. V. Singh, S. R. Nallapareddy, and B. E. Murray.** 2007. Relative contributions of *Enterococcus faecalis* OG1RF sortase-encoding genes, *srtA* and *bps* (*srtC*), to biofilm formation and a murine model of urinary tract infection. Infect. Immun. **75:**5399–5404.
26. **Kerrigan, S. W., et al.** 2008. Molecular basis for *Staphylococcus aureus*-mediated platelet aggregate formation under arterial shear *in vitro*. Arterioscler. Thromb. Vasc. Biol. **28:**335–340.
27. **Kerrigan, S. W., and D. Cox.** 2010. Platelet-bacterial interactions. Cell. Mol. Life Sci. **67:**513–523.
28. **Kline, K. A., K. W. Dodson, M. G. Caparon, and S. J. Hultgren.** 2010. A tale of two pili: assembly and function of pili in bacteria. Trends Microbiol. **18:**224–232.
29. **Kline, K. A., et al.** 2009. Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in *Enterococcus faecalis.* J. Bacteriol. **191:**3237–3247.
30. **Kratovac, Z., A. Manoharan, F. Luo, S. Lizano, and D. E. Bessen.** 2007.

Population genetics and linkage analysis of loci within the FCT region of *Streptococcus pyogenes*. J. Bacteriol. **189:**1299–1310.

31. Lebreton, F., et al. 2009. *ace*, which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. Infect. Immun. **77:**2832–2839.

32. Lepage, E., et al. 2006. Comparative genomic hybridization analysis of *Enterococcus faecalis*: identification of genes absent from food strains. J. Bacteriol. **188:**6858–6868.

33. Linden, S., et al. 2002. Strain- and blood group-dependent binding of *Helicobacter pylori* to human gastric MUC5AC glycoforms. Gastroenterology **123:**1923–1930.

34. Loughman, A., et al. 2005. Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by *Staphylococcus aureus* clumping factor A. Mol. Microbiol. **57:**804–818.

35. Maione, D., et al. 2005. Identification of a universal group B *Streptococcus* vaccine by multiple genome screen. Science **309:**148–150.

36. Mandlik, A., A. Swierczynski, A. Das, and H. Ton-That. 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol. **16:**33–40.

37. Mitchell, J., I. R. Siboo, D. Takamatsu, H. F. Chambers, and P. M. Sullam. 2007. Mechanism of cell surface expression of the *Streptococcus mitis* platelet binding proteins PblA and PblB. Mol. Microbiol. **64:**844–857.

38. Mitchell, J., and P. M. Sullam. 2009. *Streptococcus mitis* phage-encoded adhesins mediate attachment to {alpha}2-8-linked sialic acid residues on platelet membrane gangliosides. Infect. Immun. **77:**3485–3490.

39. Mora, M., et al. 2005. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc. Natl. Acad. Sci. U. S. A. **102:**15641–15646.

40. Moreillon, P., Y. A. Que, and A. S. Bayer. 2002. Pathogenesis of streptococcal and staphylococcal endocarditis. Infect. Dis. Clin. North Am. **16:**297–318.

41. Murray, B. E. 1998. Enterococci, p. 1723–1730. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. W. B. Saunders Company, Philadelphia, PA.

42. Murray, B. E. 1990. The life and times of the *Enterococcus*. Clin. Microbiol. Rev. **3:**46–65.

43. Nakata, M., et al. 2009. Mode of expression and functional characterization of FCT-3 pilus region-encoded proteins in *Streptococcus pyogenes* serotype M49. Infect. Immun. **77:**32–44.

44. Nallapareddy, S. R., and B. E. Murray. 2006. Ligand-signaled upregulation of *Enterococcus faecalis* ace transcription, a mechanism for modulating host-*E. faecalis* interaction. Infect. Immun. **74:**4982–4989.

45. Nallapareddy, S. R., and B. E. Murray. 2008. Role played by serum, a biological cue, in the adherence of *Enterococcus faecalis* to extracellular matrix proteins, collagen, fibrinogen, and fibronectin. J. Infect. Dis. **197:**1728–1736.

46. Nallapareddy, S. R., X. Qin, G. M. Weinstock, M. Hook, and B. E. Murray. 2000. *Enterococcus faecalis* adhesin, Ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. Infect. Immun. **68:**5218–5224.

47. Nallapareddy, S. R., K. V. Singh, R. W. Duh, G. M. Weinstock, and B. E. Murray. 2000. Diversity of *ace*, a gene encoding a microbial surface component recognizing adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of Ace during human infections. Infect. Immun. **68:**5210–5217.

48. Nallapareddy, S. R., K. V. Singh, J. Sillanpää, M. Zhao, and B. E. Murray. 2011. Relative contributions of Ebp pili and the collagen adhesin Ace to host extracellular matrix protein adherence and experimental urinary tract infection by *Enterococcus faecalis* OG1RF. Infect. Immun. **79:**2901–2910.

49. Nallapareddy, S. R., K. V. Singh, and B. E. Murray. 2008. Contribution of the collagen adhesin Acm to pathogenesis of *Enterococcus faecium* in experimental endocarditis. Infect. Immun. **76:**4120–4128.

50. Nallapareddy, S. R., et al. 2006. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. J. Clin. Invest. **116:**2799–2807.

51. Palmer, K. L., et al. 2010. High-quality draft genome sequences of 28 *Enterococcus* sp. isolates. J. Bacteriol. **192:**2469–2470.

52. Pillai, S. K., et al. 2004. Effects of glucose on *fsr*-mediated biofilm formation in *Enterococcus faecalis*. J. Infect. Dis. **190:**967–970.

53. Podbielski, A. 2007. Flexible architecture of the *Streptococcus pyogenes* FCT genome region: finally the clue for understanding purulent skin diseases and long-term persistence? J. Bacteriol. **189:**1181–1184.

54. Posada, D. 2002. Evaluation of methods for detecting recombination from DNA sequences: empirical data. Mol. Biol. Evol. **19:**708–717.

55. Quigley, B. R., D. Zahner, M. Hatkoff, D. G. Thanassi, and J. R. Scott. 2009. Linkage of T3 and Cpa pilins in the *Streptococcus pyogenes* M3 pilus. Mol. Microbiol. **72:**1379–1394.

56. Rasmussen, M., D. Johansson, S. K. Sobirk, M. Morgelin, and O. Shannon. 2010. Clinical isolates of *Enterococcus faecalis* aggregate human platelets. Microbes Infect. **12:**295–301.

57. Ruiz-Garbajosa, P., et al. 2006. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J. Clin. Microbiol. **44:**2220–2228.

58. Sawyer, S. 1989. Statistical tests for detecting gene conversion. Mol. Biol. Evol. **6:**526–538.

59. Scheld, W. M., O. Zak, K. Vosbeck, and M. A. Sande. 1981. Bacterial adhesion in the pathogenesis of infective endocarditis. Effect of subinhibitory antibiotic concentrations on streptococcal adhesion in vitro and the development of endocarditis in rabbits. J. Clin. Invest. **68:**1381–1384.

60. Siboo, I. R., A. L. Cheung, A. S. Bayer, and P. M. Sullam. 2001. Clumping factor A mediates binding of *Staphylococcus aureus* to human platelets. Infect. Immun. **69:**3120–3127.

61. Sillanpaa, J., et al. 2009. A family of fibrinogen-binding MSCRAMMs from *Enterococcus faecalis*. Microbiology **155:**2390–2400.

62. Sillanpaa, J., et al. 2008. Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*. Microbiology **154:**3199–3211.

63. Sillanpaa, J., et al. 2010. Characterization of the *ebp* pilus-encoding operon of *Enterococcus faecalis* and its role in biofilm formation and virulence in a murine model of urinary tract infection. Virulence **1:**236–246.

64. Sillanpaa, J., Y. Xu, S. R. Nallapareddy, B. E. Murray, and M. Hook. 2004. A family of putative MSCRAMMs from *Enterococcus faecalis*. Microbiology **150:**2069–2078.

65. Singh, K. V., T. M. Coque, G. M. Weinstock, and B. E. Murray. 1998. *In vivo* testing of an *Enterococcus faecalis* efaA mutant and use of efaA homologs for species identification. FEMS Immunol. Med. Microbiol. **21:**323–331.

66. Singh, K. V., S. R. Nallapareddy, and B. E. Murray. 2007. Importance of the *ebp* (endocarditis- and biofilm-associated pilus) locus in the pathogenesis of *Enterococcus faecalis* ascending urinary tract infection. J. Infect. Dis. **195:**1671–1677.

67. Singh, K. V., S. R. Nallapareddy, J. Sillanpaa, and B. E. Murray. 2010. Importance of the collagen adhesin Ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. PLoS Pathog. **6:**e1000716.

68. Smith, J. M. 1992. Analyzing the mosaic structure of genes. J. Mol. Evol. **34:**126–129.

69. Soriani, M., and J. L. Telford. 2010. Relevance of pili in pathogenic streptococci pathogenesis and vaccine development. Future Microbiol. **5:**735–747.

70. Stern, A., et al. 2007. Selecton 2007: advanced models for detecting positive and purifying selection using a Bayesian inference approach. Nucleic Acids Res. **35:**W506–W511.

71. Sullam, P. M., F. H. Valone, and J. Mills. 1987. Mechanisms of platelet aggregation by viridans group streptococci. Infect. Immun. **55:**1743–1750.

72. Tendolkar, P. M., A. S. Baghdayan, and N. Shankar. 2006. Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to *Enterococcus faecalis*. J. Bacteriol. **188:**2063–2072.

73. Tomita, H., and Y. Ike. 2004. Tissue-specific adherent *Enterococcus faecalis* strains that show highly efficient adhesion to human bladder carcinoma T24 cells also adhere to extracellular matrix proteins. Infect. Immun. **72:**5877–5885.

74. Usui, Y., Y. Ichiman, M. Suganuma, and K. Yoshida. 1991. Platelet aggregation by strains of enterococci. Microbiol. Immunol. **35:**933–942.

75. Veening, J. W., W. K. Smits, and O. P. Kuipers. 2008. Bistability, epigenetics, and bet-hedging in bacteria. Annu. Rev. Microbiol. **62:**193–210.