MAJOR ARTICLE

A Putative Sugar-Binding Transcriptional Regulator in a Novel Gene Locus in *Enterococcus faecalis* Contributes to Production of Biofilm and Prolonged Bacteremia in Mice

Markus Hufnagel,[1,3,a] Stefanie Koch,[1,a] Roberta Creti,[2] Lucilla Baldassarri,[2] and Johannes Huebner[1]

[1]Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; [2]Istituto Superiore di Sanita, Rome, Italy; [3]University Children's Hospital Kiel, Kiel, Germany

A biofilm-negative transposon mutant was created from an *Enterococcus faecalis* strain that produces a lot of biofilm. The transposon had been inserted in the second gene of a locus consisting of 4 open-reading frames, designated *bop* (biofilm on plastic surfaces). A nonpolar deletion of this gene and of parts of the 2 flanking genes was created; production of biofilm by this deletion mutant was significantly enhanced, compared with that by the wild-type strain. Expression of a downstream gene was significantly lower in the transposon mutant than in the wild-type strain and the biofilm-enhanced deletion mutant. Transformation of this gene into the transposon mutant partially restored production of biofilm. Mice challenged by intravenous injection with the biofilm-negative mutant strain showed significantly reduced numbers of colony-forming units in the blood, compared with mice challenged with the biofilm-enhanced deletion mutant and the wild-type. These results indicate that *bop* is involved in production of biofilm and probably regulates expression of biofilm in the *E. faecalis* strain tested.

Enterococci are one of the leading causes of infections in hospitalized patients and the third-most-common cause of nosocomial bloodstream infections [1]. Because of their intrinsic resistance to many clinically available antibiotics, these pathogens are associated with significant morbidity and mortality, especially among immunocompromised patients [2]. Production of biofilm is recognized as a virulence factor in many pathogens [3], and several authors have described the ability of enterococci to produce biofilm [4–8].

Native-valve endocarditis is a well documented biofilm process, and biofilms formed on heart valves are described by the medical community as vegetations [9]. Biofilms also play important roles in enterococcal infections of dental root canals [10], in the obstruction or blocking of urethral catheters and ureteral stents [11, 12], and in ocular infections [13].

Relatively little is known about the molecular mechanisms that control production and maintenance of biofilm in enterococci [3]. Baldassarri et al. [5] noted that production of biofilm by enterococci is influenced by either the presence of additional carbohydrates or the depletion of iron in growth media. Toledo-Arana et al. [4] have shown that the gene encoding enterococcal surface protein (Esp), *esp*, is involved in the primary attachment of enterococci to abiotic surfaces. However, their finding that the inactivation of *esp* has no effect on the production of biofilm by the *E. faecalis* strain analyzed that produced the most biofilm indi-

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[a] M.H. and S.K. contributed equally to this work.

Reprints or correspondence: Dr. Johannes Huebner, Channing Laboratory, Brigham and Women's Hospital, 181 Longwood Ave., Boston, MA 02115 (jhuebner@channing.harvard.edu).

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cates that additional mechanisms might be involved. The present study was performed to gain a better understanding of the molecular mechanisms involved in the establishment of enterococcal biofilms and to clarify their role in pathogenicity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are shown in table 1. Enterococci were grown without agitation at 37°C in Todd-Hewitt broth (THB; Becton Dickinson) or tryptic soy broth (TSB; Becton Dickinson), with the addition of 1% glucose as indicated (THBG or TSBG, respectively). *Escherichia coli* strains DH5α and TOP 10 were cultured aerobically in Luria-Bertani broth on a rotor rack at 37°C. Erythromycin (10 μg/mL) was added for *E. faecalis,* kanamycin was added for enterococci (2000 μg/mL) and *E. coli* (50 μg/mL), and spectinomycin was added for enterococci (500 μg/mL) and *E. coli* (250 μg/mL) (all from Sigma Chemical).

Biofilm plate assay. Enterococci were tested for production of biofilm according to the protocol described by Baldassarri et al. [5]. In brief, bacteria were grown in TSBG overnight at 37°C. Polystyrene 96-well tissue-culture plates (Costar) were filled with 180 μL of fresh TSBG, and 20 μL of the culture grown overnight was added to each well. The plates were incubated for either 8 or 18 h at 37°C and read in an ELISA reader (BIO-TEK Instruments) at an optical density at 595 nm, to assure homogeneous growth in all wells. The culture medium was discarded, and the wells were washed carefully 3 times with 200 μL of PBS without disturbing the biofilm on the bottom of the wells. The plates were dried for 1 h at 60°C and were stained with 2% Hucker's crystal violet for 2 min. Excess stain was removed by rinsing the plates under tap water, and plates were dried for 10 min at 60°C. The optical density at 595 nm was determined in an ELISA reader. Each assay was performed at least in triplicate and repeated at least twice. The optical density values were analyzed by analysis of variance (ANOVA) with Newman-Keuls multiple comparisons test. Bartlett's test for equal variances was done to ensure that variances between groups did not differ significantly (PRISM software, version 3; GraphPad).

DNA manipulations. Chromosomal DNA from enterococci was prepared by use of the Qiagen DNeasy Tissue Kit, according to the manufacturer's instructions. Plasmid DNA was prepared from enterococci and *E. coli* by use of the Wizard Plus Mini or Midipreps kit (Promega). DNA was purified from agarose gels and from polymerase chain reactions (PCRs) by use of the QIAquick Gel Extraction Kit or the PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Restriction enzymes and modifying enzymes were obtained from Invitrogen or New England Biolabs. Custom primers were ordered from Invitrogen. Electrocompetent enterococci were prepared according to the method of Fiedler and Wirth [14], and electroporation was performed in a Bio-Rad Gene Pulser II by use of the parameters given by Fiedler and Wirth [14]. Southern hybridization was performed by use of the ECL direct nucleic acid labeling and detection system (Amersham). All other methods (DNA ligations, electrophoresis, and transformation of competent *E. coli*) were performed by use of standard techniques [15].

Table 1. ***Enterococcus faecalis*** **strains and plasmids.**

Strain or plasmid	Characterization	Reference
Strain		
E. faecalis type 9	Strong biofilm-producing strain	[17]
E. faecalis 12030	Strong biofilm-producing strain	[29]
E. faecalis 10D5	Biofilm-negative transposon mutant of *E. faecalis* type 9	This study
E. faecalis 10D5-pEU327	*E. faecalis* 10D5 containing gram-positive expression vector pEU327 without insert	This study
E. faecalis 10D5-pSBR	*E. faecalis* 10D5 containing pEU327 with insertion of *bopD*	This study
E. faecalis T9-TDM	*E. faecalis* type 9 with deletion of part of *bopA,* complete deletion of *bopB,* and partial deletion of *bopC*	This study
Escherichia coli DH5α and TOP 10	Gram-negative hosts for cloning	
Staphylococcus aureus ATCC 35556	Strong biofilm-producing *S. aureus* strain	[28]
Plasmid		
pTV1-OK	Transposon-mutagenesis vector containing Tn917	[16]
pTEX4577	Suicide vector for targeted mutagenesis	[20]
pEU327	Gram-positive expression vector	[22]
pCRII-TOPO	Cloning vector	Invitrogen
pTDM	pTEX4577 with insert carrying deletion in 3 ORFs	This study
pSBR	pEU327 with insertion of the *bopD* gene	This study

NOTE. ORFs, open-reading frames.

Transposon mutagenesis. Plasmid pTV1-OK carrying transposon Tn*917* was used for transposon mutagenesis [16]. The plasmid was electroporated into *E. faecalis* type 9 [17], and the bacteria were grown with kanamycin and erythromycin at 30°C to maintain the plasmid. After a temperature shift to the nonpermissive temperature (42°C), the plasmid was cured, and the transposon was forced to integrate into the chromosomal DNA. Bacteria were replica-plated onto erythromycin-containing Todd-Hewitt agar (THA) plates with or without kanamycin.

Only erythromycin-resistant colonies that had lost kanamycin resistance were processed. Approximately 1500 colonies were analyzed in a biofilm plate assay, as described above. The presence of a single transposon insertion was verified by Southern hybridization, using Tn*917* as a probe, in 6 mutants with significantly reduced production of biofilm. One mutant, designated *E. faecalis* 10D5, was chosen for further analysis.

The gene interrupted by the transposon insertion was identified by use of a modified single-primer PCR protocol [18]. Primer 1 (table 2) was used, and the PCR product was sequenced by use of primer 2. A homology search was performed in the National Center for Biotechnology Information database and the TIGR database (available at: http://www.tigr.org) using BLAST (available at: http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of a nonpolar deletion mutant. A nonpolar deletion of the *bopB* gene and parts of the 2 neighboring genes, *bopA* and *bopC,* was created by use of the method of Qin et al. [19, 20]. Primers 3 and 4 were used to amplify a 623-bp fragment in the *bopA* gene, and primers 5 and 6 yielded a 622-bp fragment in the *bopC* gene (figure 1). Primers 4 and 6 contain a 21-bp complementary sequence (underlined in table 2). Overlap-extension PCR was used to create a PCR product consisting of the 5′ part of the *bopA* gene attached to the 3′ part of the *bopC* gene linked with the 21-bp overlap. This fragment was cloned into pCRII (Invitrogen) and cut with the restriction enzymes *Xho*I and *Bam*HI. The construct was inserted into the mutagenesis vector pTEX4577 [20]. The resulting plasmid, pTDM, was transformed into *E. faecalis* type 9 by electroporation, and integrants were selected on THA plates with kanamycin. A single colony was picked, and the insertion of plasmid pTDM into the chromosome was confirmed by PCR.

The integrant was passaged 10 times in liquid culture without antibiotics, and colonies were replica-plated to screen for loss of kanamycin resistance. The excision of the plasmid pTDM creates either a reconstituted wild-type strain or leads to an allelic replacement with the interrupted sequence in the chromosome [21]. The deletion mutant created was designated *E. faecalis* T9-TDM and was confirmed by PCR by use of primers 7 and 8 (table 2) and by automated sequencing of the PCR product.

Complementation of the transposon mutant. Expression vector pEU327 [22] contains a xylose promoter that has been shown to be expressed constitutively in *E. faecalis* [23]. The putative sugar-binding transcriptional regulator *bopD* gene was amplified by use of primers 9 and 10 containing *Hind*III linkers (underlined in table 2). The resulting PCR product was digested with *Hind*III and ligated into pEU327, which had been digested with *Hind*III and dephosphorylated. The chimeric plasmid pSBR was transformed into *E. coli* DH5α, and the correct insert and orientation were confirmed by automated sequencing. pEU327 (without insert) and pSBR were transformed into *E. faecalis* 10D5 by electroporation, and transformants were selected on THA plates containing spectinomycin. The presence of the plasmid in *E. faecalis* was confirmed by plasmid preparations and subsequent restriction digestion.

Preparation of RNA. Bacteria were inoculated from a fresh plate in 10 mL of TSBG and incubated for 12 h at 37°C (*E. faecalis* type 9, *E. faecalis* 10D5, and *E. faecalis* T9-TDM were

Table 2. Oligonucleotide primers used to sequence *Enterococcus faecalis* strains in the present study.

Primer	Description	Sequence, 5′→3′
1	SP-PCR primer Tn917 left	AGAGAGATCACCGTCAAG
2	SP-PCR sequencing primer	AATGTACAAAATAACAGCGAA
3	*bopA* left	CGGGGAAATTTTGAAGAACA
4	*bopA* right	ACTAGCGCGGCCGCTTGCTCCAGCCAAGGGCTGTTGTGTAT
5	*bopC* right	CCCCACTGAGGATGATGAAC
6	*bopC* left	GGAGCAAGCGGCCGCGCTAGTGAGCCCTTTGAAAAAAAGGA
7	*bopA* left-2	TCCAAATTGATCCTTGGAAAA
8	*bopC* right-2	CCGTTTGGTAGGTTTGCTTT
9	*bopD Hind*III left	CCCAAGCTTAATGGCAATTACAGTAAAAGATG
10	*bopD Hind*III right	CCCAAGCTTTTAATGAATAGGAATAACTGTTTC
11	*bopD* left	ACGGCACGGAATTTGGGTAAAC
12	*recA* left	GCAACGAAATGGTGGAACAG
13	*bopD* right	GGCTTCCTCGTTGATGGCTTC
14	*recA* right	AAGGCATCGGCAATCTCTAAG
15	*bopD* right-2	TCACGCTTAATTAATTTGTGAGGA

NOTE. Underlined nucleotides in primers 4 and 6 represent the 21-bp overlap used in the overlap-extension single-primer (SP) polymerase chain reaction (PCR). Underlined nucleotides in primers 9 and 10 represent the *Hind*III site introduced into the *bopD* gene.

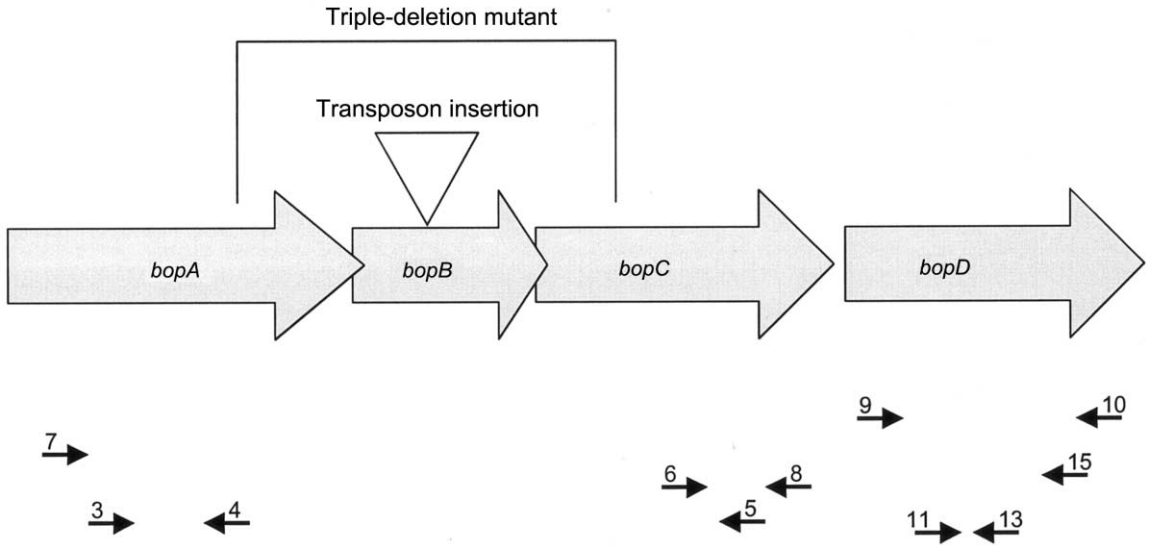

Figure 1. Schematic representation of the gene locus in *Enterococcus faecalis* involved in production of biofilm. All 4 genes are expressed on a single mRNA transcript. The insertion of the transposon in *E. faecalis* 10D5 is indicated by a triangle, and the gene deletion of *E. faecalis* T9-TDM is shown by the bracket. *Arrows,* primers; nos. correspond to the nos. used in table 2. Primers 1, 2, 12, and 14 are not shown.

grown in TSBG without antibiotics; *E. faecalis* 10D5-pEU and *E. faecalis* 10D5-pSBR were grown with 500 μg/mL spectinomycin). A volume of 400 μL of this culture was used to inoculate 20 mL of TSBG with or without the above-mentioned antibiotics and was incubated for 3 h at 37°C without aeration. Cells (5×10^9; calculated after measurement of optical density at 650 nm) were mixed with a 2× volume of RNAprotect Bacteria reagent (Qiagen), and RNA was isolated by use of the RNeasy Midi Kit (Qiagen), according to the manufacturer's recommendations, which included digestion with DNase I on the spin column. The concentration of RNA was calculated after measurement of optical density at 260 nm, and equal amounts were used as starting material in subsequent experiments. To remove any remaining DNA contaminations, a second DNase I treatment (Invitrogen) of each RNA sample was performed according to the manufacturer's instructions. The enzyme was inactivated at 65°C in the presence of EDTA. To verify the absence of residual genomic DNA in each sample, the RNA was amplified in a PCR with *Taq* DNA polymerase and the respective primers in the presence and absence of SuperScript II Reverse Transciptase (Invitrogen). RNA samples were used for cDNA synthesis by use of the iScriptcDNA synthesis kit (Biorad), according to the manufacturer's recommendations.

Quantitative real-time PCR. Quantitative real-time PCR was performed in an iCycler iQ (Bio-Rad) by use of the QuantiTect SYBR green PCR kit (Qiagen), according to the manufacturer's recommendations. The *bopD* transcripts were amplified with the primers 11 and 13 (table 2). The constitutively expressed housekeeping gene *recA* was amplified with the primers 12 and 14 (table 2) as an internal control to normalize RNA concentrations. To monitor the specificity of the reactions, final PCR products were analyzed by melting curves showing that all specific primers resulted in only 1 specific product. The standard curves of dilutions of starting material, amplification efficiencies, and expression ratios between *bopD* and *recA* were calculated by use of Microsoft Excel. The values used for comparison of gene expression between the different strains were the numbers of PCR cycles required to reach the threshold cycle (C_t), which was set at 10× the SD of the fluorescence of the first 10 cycles. The amount of *bopD* transcript relative to the control gene *recA* was calculated by use of the standard-curve method, as described elsewhere [24, 25], and was expressed as the percentage of the amount of transcripts of the wild-type *E. faecalis* type 9.

Reverse-transcription (RT) PCR. RT-PCR was performed with the RNA samples, to detect cotranscription of all 4 *bop* genes by use of the SuperScript One-Step RT-PCR with Platinum Taq Kit (Invitrogen), according to the manufacturer's recommendations, by use of the primers 7 and 15 (table 2).

Scanning electron microscopy. Bacteria were grown in TSB or TSBG in 24-well plates containing polystyrene segments. After a 12-h incubation at 37°C, polystyrene segments were rinsed twice in PBS and once in 0.1 mol/L cacodylate buffer and fixed as described elsewhere [5], to preserve extracellular polysaccharide. In brief, cells were first fixed for 20 min at room temperature with 0.1 mol/L cacodylate-buffered 2.5% glutaraldehyde containing 0.075% (wt/vol) ruthenium red (Glut-RR; Merck) and 75 mmol/L lysine. They were then fixed with Glut-RR without lysine for 2 h and finally fixed with 1% OsO_4 plus ruthenium red for an additional 1 h. Samples were

dehydrated by exposure to a graded series of ethanol, critical-point dried, gold sputtered, and examined with a Cambridge SE360 scanning electron microscope.

Catheter adherence assay. The ability of bacteria to adhere to polyethylene catheters was tested by use of the method described by Muller et al. [26] with modifications. In brief, bacteria were inoculated in THB and incubated at 37°C until an OD_{650} of 0.4 was reached and diluted to ~4×10^7 cfu/mL. Sections of polyethylene catheter tubing (Intramedic PE tubing, diameter 0.61 mm; Becton Dickinson) were gas sterilized. Sections of 20 mm were dipped into the broth with the bacterial inoculum for 30 min at 37°C. The catheter pieces were subsequently washed 3 times for 15 seconds in PBS and vigorously rolled over tryptic soy agar (TSA) plates. The plates were incubated at 37°C overnight, and colonies were counted.

Mouse sepsis model. The animal studies described in the present study were reviewed and approved by the Institutional Animal Care and Use Committee at Harvard University. Female BALB/c mice were inoculated intravenously in the tail vein with 5×10^8 cfu of either wild-type *E. faecalis* type 9, biofilm-negative transposon mutant *E. faecalis* 10D5, or the biofilm-enhanced triple-gene deletion mutant *E. faecalis* T9-TDM. Bacteria were grown in THB overnight, centrifuged, and resuspended in sterile saline. Aliquots were shock-frozen and stored at −80°C. The concentration of the stock was verified by dilutions and viable counts on TSA, and these numbers were used to calculate the appropriate dilutions for the desired inoculum. For the experiments, aliquots were thawed, diluted in sterile saline, and injected into the tail vein; the actual diluted inoculum was once more verified by viable counts. After 3 days, the mice were killed, blood was obtained by cardiac puncture, and 100 μL was plated on TSA.

Statistical analysis. Statistical analysis was done by ANOVA with Newman-Keuls multiple comparison tests, and Bartlett's test for equal variances was used to assure that variances did not differ significantly between groups (PRISM, version 3; GraphPad Software).

RESULTS

Clinical *E. faecalis* strains and laboratory isolates were tested for production of biofilm [27]. One strain, *E. faecalis* type 9 [17], showed a very high degree of production of biofilm (OD_{595} of >2.5) after an 18-h incubation period. This value corresponded to the results obtained with a strain of *Staphylococcus aureus* (ATCC 35556) that produces a lot of biofilm [28].

Transposon mutagenesis of *E. faecalis* type 9 was performed by use of plasmid pTV1-OK [16]. After 3 subpassages at the nonpermissive temperature, ~1500 erythromycin-resistant colonies were picked with sterile toothpicks and transferred to 96-well tissue-culture plates. A biofilm plate assay was performed, and, after 18 h, 1 strain (*E. faecalis* 10D5) showed a level of production of biofilm that was 16% of that of the wild-type strain. This strain was selected for further studies. The presence of a single insertion of the transposon was confirmed by Southern blot, with Tn*917* used as a probe (data not shown). A comparison of the growth curves over a 7-h period until the stationary phase showed no difference between the wild-type and mutant 10D5 strains (data not shown). Scanning electron microscopy of *E. faecalis* type 9 and *E. faecalis* 10D5 showed that the wild-type strain grows in multiple layers on polystyrene, whereas the mutant strain grows in only 1 plane, without the piling up observed with the wild-type strain (figure 2).

The DNA sequence interrupted by the transposon was amplified and sequenced according to a modified single-primer PCR protocol [18]. The transposon insertion was mapped to an open-reading frame (ORF) by a homology search within the TIGR database by use of the sequence data for *E. faecalis* V583. The function putatively assigned to this gene by TIGR is a β-phosphoglucomutase. This ORF was designated as *bopB.* It is surrounded upstream by a putative glycosyltransferase *(bopA)* and downstream by a putative aldose-1-epimerase *(bopC)* and a sugar-binding transcriptional regulator *(bopD).* All 4 ORFs are predicted to be transcribed in the same direction, and some of the genes overlap (figure 1). The presence of all 4 ORFs on a single mRNA transcript in *E. faecalis* type 9 wild-type strain was confirmed by RT-PCR with primers 7 and 15 (figure 1). A putative ρ-independent transcriptional terminator is located downstream from *bopD,* and the next 6 ORFs are predicted to be transcribed in the opposite direction.

A nonpolar deletion mutant was constructed by removing part of the 3′ end of the *bopA,* all of *bopB,* and part of the 5′ end of *bopC* (figure 1). This deletion mutant was created by targeted mutagenesis with the suicide plasmid pTEX4577. The deletion was verified by performing PCR of the region with primers outside of the overlap-extension construct. Sequencing of the respective loci was performed to confirm the deletion. Production of biofilm of the deletion mutant *E. faecalis* T9-TDM was compared with that of the wild-type strain (figure 3), showing significantly enhanced production of biofilm (122% that of the wild-type strain; $P < .05$). For this experiment, a shorter incubation period (8 h) was used, because 18-h readings for the deletion mutant exceeded the maximum values obtained in the photometer.

Insertional inactivation of the *bopD* gene was unsuccessful in *E. faecalis* type 9 and in a different biofilm-producing *E. faecalis* strain, *E. faecalis* 12030 [29], which possesses the *bopD* gene, suggesting that the *bopD* gene is essential for production of biofilm under the growth conditions used. Using the same conditions, we were able to create deletion mutants in 2 different genes.

To test the hypothesis that the biofilm-negative phenotype

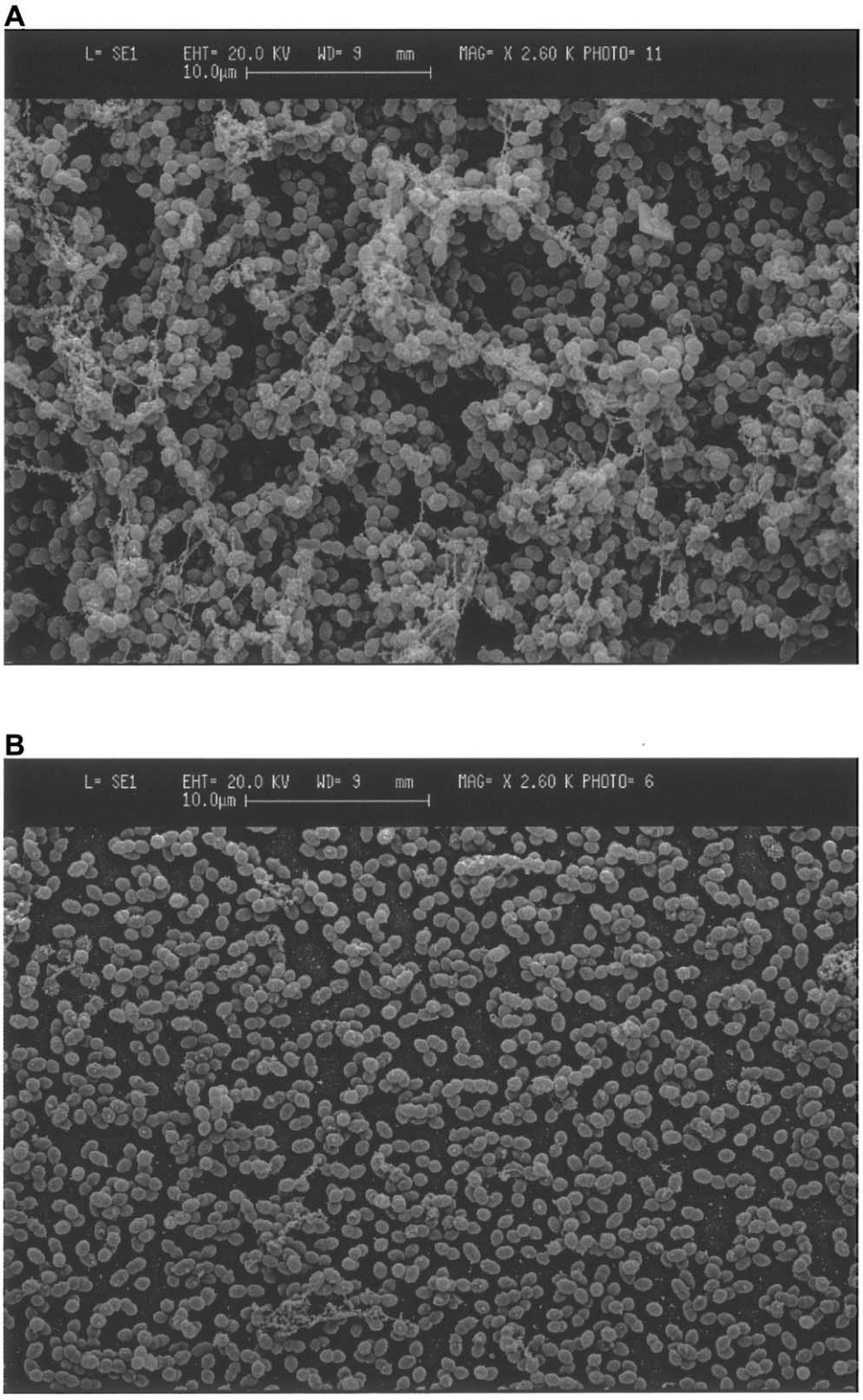

Figure 2. Scanning electron micrograph of wild-type *Enterococcus faecalis* type 9 and the mutant strain *E. faecalis* 10D5. *A,* The wild-type strain shows a multilayered growth pattern, whereas the biofilm-negative mutant grows mostly in a single layer *(B).*

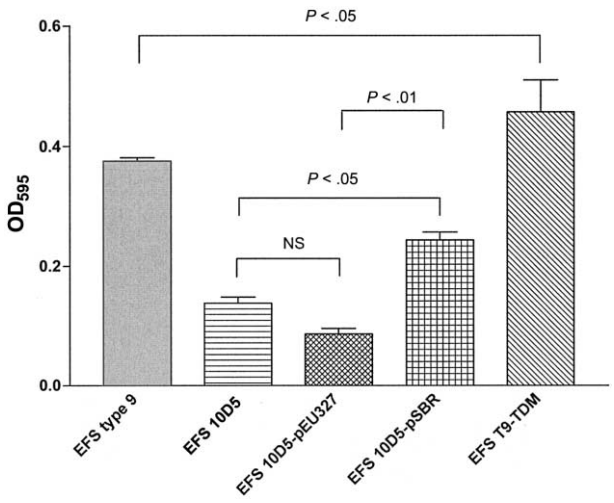

Figure 3. Comparison of the biofilm production in wild-type *Enterococcus faecalis* (EFS) type 9, biofilm-negative transposon mutant EFS 10D5, transposon mutant reconstituted with plasmid pEU327 without insert, transposon mutant reconstituted with the *bopD* gene, and the biofilm-enhanced triple-deletion mutations EFS T9-TDM. The biofilm-negative transposon mutant 10D5 produces significantly less biofilm than does the wild-type strain and the biofilm-enhanced mutant EFS T9-TDM ($P < .001$). Plasmid pEU327 does not affect the production of biofilm by the mutant. However, expression of the *bopD* gene by reconstitution of the gene *in trans* restores production of biofilm in the transposon mutant strain to 65% of the wild-type levels ($P < .05$, EFS 10D5 vs. EFS 10D5-pSBR). The triple-gene deletion mutant EFS T9-TDM produces significantly more biofilm (122%) than does the wild-type EFS type 9 ($P < .05$). NS, not significant.

of the transposon mutant 10D5 was due to a polar effect of the transposon insertion on a gene downstream of the *bopB* gene, we cloned the *bopD* gene into the gram-positive expression vector pEU327 [22]. The construct pSBR was electroporated into the transposon mutant *E. faecalis* 10D5. The vector pEU327 without insert was used as a control. After 8 h, the amount of biofilm produced by *E. faecalis* 10D5 with pEU327 but without the *bopD* gene was identical to that produced by the transposon mutant. After an 8-h incubation period, *E. faecalis* 10D5 displayed 36.8% of the biofilm produced by the wild-type *E. faecalis* type 9, compared with 16% after a 18-h incubation period. However, *E. faecalis* 10D5 with the substitution of the *bopD* gene produced 176% of that produced by the biofilm-negative transposon mutant *E. faecalis* 10D5 ($P < .05$) and 65% of that produced by the wild-type strain *E. faecalis* type 9 (figure 3). Overexpression of the other ORFs (i.e., *bopA–bopC*) did not result in increased production of biofilm, compared with the transposon mutant carrying the plasmid without insert (data not shown). These results indicate that the insertion of this gene leads to at least a partial reconstitution of the biofilm-producing phenotype in the transposon mutant *E. faecalis* 10D5.

We used quantitative analysis of mRNA transcripts by real-time PCR to investigate whether the decrease in biofilm production observed in the transposon mutant 10D5 was indeed caused by a polar effect of the transposon on the transcription of the downstream *bopD* gene. Isolation of total RNA was performed under 2 different conditions: the wild-type, the transposon mutant, and the triple-deletion mutant were grown without antibiotics; and the transposon mutant supplemented with the expression vector pEU327—with and without the insertion of the *bopD* gene—was grown in the presence of the selective antibiotic spectinomycin. The samples were DNase treated, reverse transcribed, and amplified with primers inside the *bopD* gene (primers 11 and 13; table 2 and figure 1) using quantitative real-time PCR. The levels of expression of *bopD* were normalized to the expression of the constitutively expressed housekeeping gene *recA*, which showed similar levels for all strains (C_t range, 16.43–17.1), using the standard-curve method [24, 25]. The calculated amounts of transcripts of the sugar-binding transcriptional regulator *bopD* showed that expression of *bopD* mRNA in the transposon mutant and the transposon mutant with pEU327 was significantly reduced, to 8.5% and 11.2%, respectively, compared with the expression in *E. faecalis* type 9, which was assumed to be 100%. The amount of *bopD* mRNA transcripts expressed by the biofilm-enhanced deletion mutant *E. faecalis* T9-TDM was 161%; the transposon mutant reconstituted with the *bopD* gene showed an expression of 237.7%, compared with that of the wild-type strain (table 3).

The ability of *E. faecalis* type 9 to adhere to plastic catheters was compared with that of the biofilm-negative transposon mutant. After a 30-min incubation period, no difference in adherence could be observed between the wild-type and the transposon mutant ($P = .4082$, Mann-Whitney U test; figure 4).

We studied the role of biofilm as a pathogenicity factor in a mouse bacteremia model. BALB/c mice were inoculated intravenously in the tail vein with 5×10^8 cfu of either the wild-type strain ($n = 10$), the biofilm-enhanced triple-gene deletion mutant *E. faecalis* T9-TDM ($n = 8$), or the biofilm-negative transposon mutant strain *E. faecalis* 10D5 ($n = 8$). In mice that received the wild-type strain *E. faecalis* type 9, significantly less

Table 3. Threshold cycles (C_t) for *Enterococcus faecalis bopD* transcripts, comparing the wild-type strain, the biofilm-negative transposon mutant, the biofilm-enhanced deletion mutant, and the reconstituted strain.

E. faecalis strain	C_t, mean (95% CI)
Type 9	15.93 (15.68–16.18)
10D5	19.48 (19.33–19.62)
T9-TDM	15.09 (15.02–15.16)
10D5-pEU327	18.6 (18.27–18.29)
10D5-pSBR	14.28 (13.68–14.88)

NOTE. CI, confidence interval.

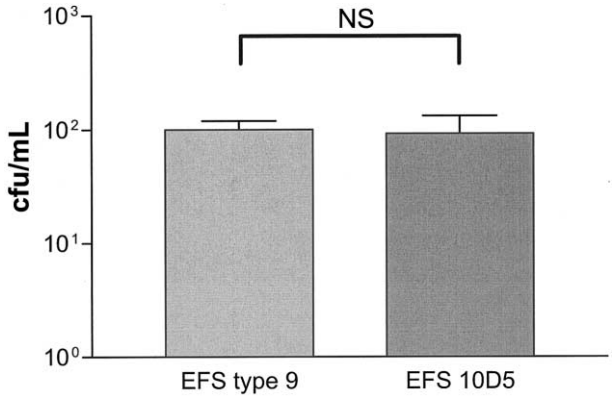

Figure 4. Catheter adherence of *Enterococcus faecalis* (EFS) type 9 and the biofilm-negative mutant EFS 10D5. No statistically significant difference was observed. Error bars represent SEM. NS, not significant.

bacteria growth was seen ($P < .05$) than in mice challenged with the biofilm-enhanced triple-gene deletion mutant *E. faecalis* T9-TDM, and significantly more bacteria growth was seen ($P < .05$) than in mice challenged with the biofilm-negative transposon mutant *E. faecalis* 10D5 (figure 5).

DISCUSSION

Production of biofilm plays a major role in the pathogenesis of many clinically important pathogens [9]. The occurrence of biofilm in enterococcal infections has frequently been reported [10, 12, 13, 30, 31]; however, only a few studies have attempted to elucidate the underlying molecular mechanisms [4, 5].

We have described a novel gene locus, designated as *bop,* that is involved in production of biofilm. The observed phenotype in the triple-gene deletion mutant and the complementation studies indicate that the biofilm-negative phenotype of the transposon mutant is due to a polar effect on a downstream gene (i.e., *bopD*), whereas the enhanced production of biofilm in the triple-gene-deletion mutant correlates with an increased amount of *bopD* mRNA. This result may be due to the closer proximity of the ORF to the promoter and/or to an increased half-life of the mRNA transcript. Alternative explanations include the creation of a new promoter that is secondary to the deletion or the possible lack of negative feedback of 1 or all of the first 3 gene products of the transcripts. From the results of the present study, we conclude that production of biofilm may be a virulence factor in enterococci that leads to prolonged bacteremia. The ability of the strain tested to produce biofilm in vitro is correlated with its persistence in the mouse bacteremia model in vivo. A reduction or increase in production of biofilm by the 2 mutants leads to decreased or increased bacteremia, respectively. Possible mechanisms leading to increased bacterial colony counts in the blood of the biofilm-enhanced mutant are resistance to natural surfactants [32] and chemical biocides [33], resistance to phagocytes or better survival in macrophages [5, 9, 34, 35], or resistance to antibodies [9, 33].

Shankar et al. [36] described Esp, which has homology to the α-C and rib proteins in streptococci. This protein has been shown to be associated with the colonization of the urinary tract [37], and Willems et al. [38] and Baldassarri et al. [39] discovered that epidemic enterococcal isolates causing nosocomial infections carried the *esp* gene more frequently than did fecal isolates.

Toledo-Arana et al. [4] examined 200 strains of enterococci and found a high correlation between the presence of the *esp* gene and the ability of a given strain to produce a biofilm. Deletion mutants in the *esp* gene were created in several biofilm-producing *E. faecalis* strains; 2 strains lost the ability to form biofilm, whereas, in 1 strain that produce a lot of biofilm, disruption of the *esp* gene did not result in a significant decrease in production of biofilm. Toledo-Arana et al. [4] concluded that *esp* is responsible for the primary attachment of enterococci to abiotic surfaces but that additional surface adhesins were involved in this process, especially in strains that produce a lot of biofilm. Because *E. faecalis* type 9, the strain used in the present study, is an extremely potent producer of biofilm, it seems likely that different mechanisms exist for greater biofilm production, compared with the establishment of a weak-to-moderate biofilm.

Toledo-Arana et al. [4] reported that 93.5% of the biofilm-producing strains possessed and expressed the *esp* gene, whereas none of the biofilm-negative strains possessed the *esp* gene. However, we could not verify this strong association between the

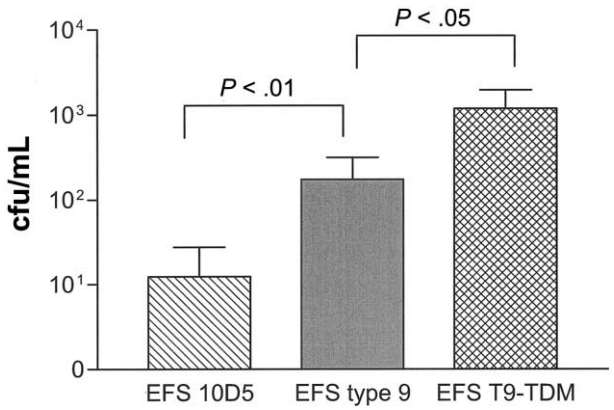

Figure 5. *Enterococcus faecalis* (EFS) in mice challenged with the wild-type strain (EFS type 9), biofilm-negative mutant EFS 10D5, and the biofilm-enhanced mutant EFS T9-TDM. The no. of cfu in blood was significantly lower in mice challenged with EFS 10D5 than in those challenged with the wild-type strain EFS type 9 ($P < .05$). The no. of bacteria recovered from blood of mice challenged with the biofilm-enhanced triple-gene deletion mutant (EFS T9-TDM) was significantly higher, compared with mice inoculated with the wild-type strain ($P < .05$). Bars show average and SE.

production of biofilm and the presence of *esp* in the clinical *E. faecalis* isolates tested [5]. Thirty-five (44%) of 79 strains tested negative for the presence of *esp* by PCR, but were nevertheless able to produce a lot of biofilm (authors' unpublished data). Although this phenomenon may be related to different methods to test production of biofilm, it may also represent different characteristics in the strain collections analyzed. Toledo-Arana et al. [4] used an assay adapted by O'Toole et al. [40] for the study of production of biofilm by *Pseudomonas fluorescens*. In that assay, the crystal-violet is dissolved in ethanol-acetone before optical density is measured, whereas the method used in the present study [41] uses readings of the dried microtiter plates. In the present study, the ethanol-acetone method produced less-consistent results than did the method used by Christensen et al. [41]. Because the procedure proposed by Christensen et al. [41] was developed for gram-positive bacteria (i.e., *Staphylococcus epidermidis*) and has been used successfully by many researchers to study the production of biofilm, we feel confident that this method is also applicable to enterococci. Differences between the biofilm assays exist also with regard to the concentration of glucose in the medium used (0.25–0.5% used by Toledo-Arana et al. [4] vs. 1% used in the present study). No systematic comparison that may explain the conflicting results between the 2 different methods has been performed.

The development of biofilm is a multistep procedure [42], with phase 1 involving the primary attachment of planktonic microorganisms to biotic and abiotic surfaces. Toledo-Arana et al. [4] speculate that Esp is one of the mediators of primary attachment in enterococci. Phase 2 of production of biofilm is the molecule-mediated secondary attachment of microorganisms to surfaces. Adhesins, such as the polysaccharide intercellular adhesin in staphylococci [43], mediate the interaction between the microorganisms and the surface and between individual bacterial cells. This phase is characterized by the formation of microcolonies [33]. Phase 3 involves the maturation of the biofilm. The microorganisms change their metabolic state, produce different extracellular products (such as exopolysaccharides) to form a glycocalyx, and divide within the biofilm. Phase 4 is marked by the detachment of planctonic cells from the biofilm to colonize further surfaces. On the basis of findings from scanning electron microscopy studies, we hypothesize that our transposon mutant is defective in phase 2 of production of biofilm. The transposon mutant grew in a monolayer on polystyrene segments, whereas the wild-type strain and the biofilm-enhanced triple deletion mutant grew in a multilayer, with a 3-dimensional pile-up and the formation of microcolonies, which were noticeably absent in the transposon mutant. The monolayer represents the primary attachment, whereas the formation of a multilayer with microcolonies requires the machinery of the secondary attachment. Comparison of the initial attachment in a catheter adherence model [26] showed no difference in the attachment to a polyethylene catheter of the transposon mutant and the wild-type strain. We therefore assume that the secondary attachment, and not the primary attachment, is impaired in our transposon mutant. On the other hand, Toledo-Arana et al. [4] speculate that *esp* is involved in the initial adherence of the bacteria to abiotic surfaces. This may explain the results by Toleda-Arana et al. [4] that strains that produce a lot of biofilm were not affected in their adherence by the inactivation of *esp*. Another possibility might be that *bopD* up-regulates expression of *esp* and possibly other virulence factors involved in adhesion of the bacteria to surfaces. The residual production of biofilm by our transposon mutant may also be related to the intact primary attachment of the bacteria mediated by *esp* that is not affected by the reduction of *bopD* expression.

We are aware that the creation of a deletion mutant in the *bopD* gene would be the definitive proof that the *bopD* gene causes the observed phenotype in the transposon mutant. Despite numerous attempts with different suicide vectors, flanking regions, and bacterial strains (data not shown), we were unable to delete the *bopD* gene by targeted mutagenesis. One possible explanation is that *bopD* is essential in the biofilm-positive strains tested. We screened a collection of 18 unrelated *E. faecalis* strains by use of PCR for the presence of the *bopD* gene and found that all strains carried the *bopD* gene, supporting the hypothesis that the gene is essential (data not shown). Furthermore, this hypothesis is supported by the fact that the transposon insertion did not completely inactivate *bopD* and that the mutant strain still expresses a residual amount of *bopD* mRNA (8.5% of the wild type). Depending on the incubation period, the amount of biofilm produced by the mutant is 16%–36% of that produced by the wild-type, suggesting a close relationship between the expression of *bopD* and production of biofilm.

Baldassarri et al. [5] found that, of 73 strains of clinical *E. faecalis* isolates analyzed, 66% were strong producers of biofilm, 14% were weak producers, and 20% did not produce any biofilm. The formation of biofilm in these strains was strongly affected by the presence of additional carbohydrates in the growth medium.

The dependence of production of biofilm on the presence of specific carbohydrate sources in the growth medium has been described by a number of investigators [3–5, 9]. The attributed function (sugar-binding transcriptional regulator) of the *bopD*-encoded protein responsible for the biofilm-negative phenotype in the present study might explain this observation. This gene shows significant sequence homology (29% identity and 50% similarity) with the *ccpA* gene of *E. faecalis* [44], which is involved in carbohydrate metabolism. The gene also shows significant homology with the *ccpA* gene of *Listeria innocua*

(30% identity and 55% similarity), *Listeria monocytogenes* (30% identity and 54% similarity), *Lactococcus lactis* (30% identity and 54% similarity), *Bacillus subtilis* (29% identity and 54% similarity), *Streptococcus pyogenes* (28% identity and 51% similarity), *Streptococcus pneumoniae* (29% identity and 52% similarity), and with a putative maltose operon transcriptional repressor in *S. pyogenes* (45% identity and 65% similarity) and *S. aureus* (31% identity and 53% similarity).

O'Toole et al. [45] found that a gene with a similar function to *bopD* (i.e., carbon catabolite regulation), the global carbon metabolism regulator *crc,* is part of a signal transduction pathway required for production of biofilm by *Pseudomonas aeruginosa.* A *crc* mutant created by O'Toole et al. [45] showed, in scanning electron microscopy, a morphologic pattern similar to that of our mutant, growing only as a dispersed monolayer. The phenotype of their strains was associated with a defective type IV pilus–mediated twitching motility caused by decreased *pilA* transcription.

Carbon catabolite repression proteins, such as CcpA, are known to regulate transcription of hundreds of promoters [46] and might therefore be involved in the regulation of different genes probably associated with production of biofilm, such as *esp,* aggregation substance, and others. Further studies are under way to identify proteins that are expressed under the control of the *bopD* gene in our prototype strain.

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