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Analysis of a Gene Cluster of *Enterococcus faecalis* Involved in Polysaccharide Biosynthesis

YI XU,[1,2]† KAVINDRA V. SINGH,[2,3] XIANG QIN,[2,3] BARBARA E. MURRAY,[2,3] AND GEORGE M. WEINSTOCK[1,2]*

Department of Microbiology and Molecular Genetics,[1] Division of Infectious Diseases, Department of Medicine,[3] and Center for the Study of Emerging and Re-emerging Pathogens,[2] University of Texas Medical School, Houston, Texas 77030

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Previously, we described a gene cluster of *Enterococcus faecalis* OG1RF that produced an antigenic polysaccharide when cloned in *Escherichia coli*. The polysaccharide antigen was not detectable in *E. faecalis* strains, however. Here, we show by reverse transcriptase-PCR that the 16 genes in this region are transcribed in OG1RF. Gene disruption of *orfde4*, encoding a putative glycosyl transferase, and *orfde6*, a putative dTDP-rhamnose biosynthesis gene, generated two OG1RF mutants. The mutants showed delayed killing and a higher 50% lethal dose in a mouse peritonitis model. In addition, two mucoid *E. faecalis* isolates from patients with chronic urinary tract infections were found to produce the polysaccharide antigen.

Polysaccharides (PS) of bacterial pathogens play important roles during infection. The capsule of *Streptococcus pneumoniae* is considered its most important virulence factor, since it enables the organism to persist in the host by conferring resistance to phagocytosis (6, 8). Capsular PS from *Staphylococcus aureus* types 5 and 8 were shown to induce cytokine release from human epithelial and endothelial cell lines (22). The serotype f PS (a rhamnose-glucose polymer) of *Streptococcus mutans* was found to stimulate the release of tumor necrosis factor α (23). Furthermore, being the dominant immunogens on the bacterial surface, some PS (e.g., the O antigens of gram-negative bacteria and capsules of streptococci and staphylococci) have been used as the basis for both vaccine development and serological typing of clinical isolates.

Enterococci are a leading cause of nosocomial infections and account for 5 to 15% of infective endocarditis in the United States (14), with most clinical isolates being *Enterococcus faecalis*. The development of multiple antibiotic resistance in enterococci in recent decades has posed a serious threat to effective therapy and raises awareness of the need for a better understanding of the pathogenicity of enterococci. Members of our group previously reported that a cloned gene cluster (*epa*, encoding proteins involved in the biosynthesis of an enterococcal PS antigen) of *E. faecalis* OG1RF produced a PS in *Escherichia coli* (27). The PS reacted with sera from four patients with enterococcal endocarditis. Sequence analysis showed that *epa* genes were similar to those involved in the biosynthesis and export of PS, including genes for rhamnose biosynthesis, glycosyl transferases, and ATP-binding cassette transporters. Insertion mutations in three of the genes, *orfde4*, *orfde5*, and *orfde8*, abolished immunoreactivity of the *E. coli* clone (27). However, we were not able to detect the PS antigen in several *E. faecalis* strains. Early studies of *E. faecalis* showed that some cell wall PS were antigenic and might be used for serological typing (1, 9, 19, 21). Neither the chemical compositions nor the genetic basis of these PS have been elucidated. In addition, Bottone et al. (2) recently described the first isolation of mucoid *E. faecalis* strains from patients with chronic urinary tract infections.

The biosynthesis of PS in many bacteria is regulated. The production of colanic acid in *E. coli* is elevated under certain growth conditions, such as in chemically defined media with high concentrations of phosphate and with high carbon-to-nitrogen ratios and temperatures below 25°C (24, 25). Alginate production in *Pseudomonas aeruginosa* is turned on in the lungs of cystic fibrosis patients (18). Since the synthesis of PS is a costly process, using ATP and sugars that would otherwise provide energy for cell activities, regulation is not surprising. Furthermore, since PS play specific roles in infection, it may also be advantageous to regulate their production during the stages of infection (25). Possibly the production of PS in *E. faecalis* is regulated.

In this study, we demonstrate that even though no antigen is detected, *epa* genes are transcribed in OG1RF with at least three transcriptional start sites. Furthermore, OG1RF mutants with disruptions in two of the genes showed a slightly higher 50% lethal dose (LD_{50}) and a statistically significant delay in killing in a mouse peritonitis model. In addition, two recently reported mucoid *E. faecalis* strains showed positive reactions with specific antibodies against the PS produced by the *E. coli* clone.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown in Luria-Bertani broth or on Luria-Bertani agar with appropriate antibiotics overnight at 37°C. Enterococci were grown in brain heart infusion (BHI) broth or on BHI agar (Difco) overnight at 37°C for routine purposes unless otherwise stated. The following antibiotics for selection of *E. coli* recombinants were used: chloramphenicol (CM) (25 μg/ml) and kanamycin (KM) (50 μg/ml). A concentration of 2,000 μg of KM per ml was used for selection of *E. faecalis* mutants. Serum samples were collected from patients with *E. faecalis* endocarditis infections and had high titers against *E. faecalis* strains (26). E1 and E2 are two mucoid *E. faecalis* isolates kindly provided by Edward J. Bottone at the Division of Infectious Diseases, the Mount Sinai Hospital, New York, N.Y. E1 is constitutively mucoid, while E2 is mucoid after incubation at room temperature but not at 37°C (E. J. Bottone, personal communication).

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas Medical School, 6431 Fannin St., Houston, TX 77030. Phone: (713) 500-6083. Fax: (713) 500-5499. E-mail: georgew@utmmg.med.uth.tmc.edu.

† Present address: Center for Extracellular Matrix Biology, Albert B. Alkek Institute of Biosciences and Technology, Texas A&M University, Houston, TX 77030-3303.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*($r_K^-m_K^+$) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	
TX5159	DH5α(pTEX5159)	27
E. faecalis		
OG1RF		13
TX5179	OG1RF *orfde4*::Km^r	This study
TX5180	OG1RF *orfde6*::Km^r	This study
E1	Mucoid isolate from a patient with chronic urinary tract infection; constitutive mucoid phenotype	E. J. Bottone (2)
E2	Mucoid isolate from a patient with chronic urinary tract infection; mucoid at room temperature	E. J. Bottone (2)
Plasmids and cosmids		
pBeloBAC11	Cosmid vector, F′ replicon, Cm^r	H. Shizuya (26)
pTEX5159	pBeloBAC11 with a 43-kb insert from OG1RF which contains a gene cluster for PS biosynthesis	27
pTEX4577	pBluescript SK(−) with Ωkm2 inserted in the *Sca*I site, Km^r Ap^s	15
pTEX5177	pTEX4577 containing an intragenic fragment of *orfde4* inserted in the *Bam*HI site	This study
pTEX5178	pTEX4577 containing an intragenic fragment of *orfde6* inserted in the *Bam*HI site	This study

DNA manipulations and transformation of *E. coli*. DNA preparation, purification, restriction digestion, agarose gel electrophoresis, and ligation were performed using standard methods (17) or following the manufacturer's instructions, unless otherwise stated. Routine preparation of competent *E. coli* cells and transformation of DNA into *E. coli* were performed by a one-step procedure (5).

Preparation of total RNA. The RNeasy Mini Kit (Qiagen, Santa Clarita, Calif.) was used to extract total RNA from *E. faecalis*, with slight modifications. All the solutions and utensils were treated with diethylpyrocarbonate (DEPC) to eliminate RNase. A single colony was inoculated into 5 ml of BHI broth and incubated at 37°C overnight with shaking. A fresh 5-ml culture was then inoculated

TABLE 2. Primers used for RT-PCR

Primer[a]	Sequence[b]	Position in source sequence[c]
GW301	5′ATGGATCCTATGAGCATGCAAGAAAT	1 of *orfde4*, upper strand[d]
GW302	5′CATTGAATAACACTTGATTATGACC	(3′) 220 of *orfde4*, lower strand
GW303	5′ATGGATCCTATGAAAGGAATTATTTT	1 of *orfde6*, upper strand[d]
GW304	5′CATCTGGGCTTTCTTGTACCGC	(3′) 232 of *orfde6*, lower strand
GW305	5′ATTGAGACAACGCAAAGTCAC	(5′) 49 of *orfde2*, upper strand
GW306	5′CACTCCAAAATGCTGCTAAAG	(3′) 302 of *orfde2*, lower strand
GW307	5′ATGGATCCTATGCCTACAGCAGGAG	1 of *orfde3*, upper strand[d]
GW308	5′CAACGTCACAAAATCTATCCG	(3′) 223 of *orfde3*, lower strand
GW316	5′CGACAACTCATTAAACGACC	(5′) 258 of *orfde8*, upper strand
GW317	5′GAACCCACGCTTTGACTAAC	(3′) 504 of *orfde8*, lower strand
GW318	5′CAAAAGCTACTTTGCCCTGCC	(5′) 15 of *orfde9*, upper strand
GW319	5′ACGTAAACAAGTGTCGCCCC	(3′) 337 of *orfde9*, lower strand
GW320	5′GAGCGGGAAATCGGAAAGTG	(5′) 494 of *orfde10*, upper strand
GW321	5′CAATCAGAATTGCTGAGCCGAC	(3′) 682 of *orfde10*, lower strand
GW322	5′ACATTTTGCGGACGATTGC	(5′) 47 of *orfde10*, upper strand
GW323	5′ATGATTTTAAGGCCAAGTATGC	(5′) 77 of *orfde11*, upper primer
GW324	5′GAATGAAAAACAAGTGGGTG	(3′) 369 of *orfde11*, lower strand
GW325	5′TAGTCCAACCAAAAAATCTTACC	(5′) 90 of *orfde12*, upper strand
GW326	5′CTCTTCCATCTCTTCTCGGG	(3′) 362 of *orfde12*, lower strand
GW329	5′AATGGAAACAGACGAGAGTACC	(5′) 21 of *orfde14*, upper strand
GW330	5′CGGTTTGACTTTTGCTAAGG	(3′) 323 of *orfde14*, lower strand
GW331	5′AGCCTACACGTTAAACATTGA	(5′) 45 of *orfde15*, upper strand
GW332	5′TTCCGCTAAAACTGCCTCC	(3′) 231 of *orfde15*, lower strand
GW333	5′TACAATCACTAAAGAAAGCCC	(5′) 91 of *orfde16*, upper strand
GW334	5′GATGATTGAAAATGAAAAGGG	(3′) 262 of *orfde16*, lower strand
GW337	5′GAGTCAAAGATTAGCGGTAGTC	(5′) 24 of *orfde5*, upper strand
GW338	5′TCAAGGAGCAACAATAATTCAC	(3′) 269 of *orfde5*, lower strand
GW365	5′GAAGGCTACATTTTATCAGAAC	(5′) 295 of *orfde7*, upper strand
GW366	5′CGCTTCAAATTCTTTTAAGG	(3′) 524 of *orfde7*, lower strand
GW367	5′TTAAGAAGCCTGTTTGTGG	(5′) 218 of *orfde13*, upper strand
GW368	5′TTGTTTTGTAGACTAATGGG	(3′) 414 of *orfde13*, lower strand
GW371	5′TAAGAGGGATTGGTAACTTG	(3′) 244 of *orfde5_6*, lower strand

[a] Only the primers that yielded the best results for each ORF are listed.
[b] Underlining indicates a *Bam*HI site which is not present in the template sequence. Sequences run from the 5′ end to the 3′ end.
[c] The position of a primer on the upper strand is the position of the 5′ end of the primer on the upper strand, and the position of a primer on the lower strand is the position of the 3′ end of the primer on the complementary upper strand.
[d] These primers contain altered sequences (ATGGATCC) at the 5′ end.

TABLE 3. Primers used for cloning the intragenic fragments of *orfde4* and *orfde6*

Primer	Length	Sequence[a]	5′ Nucleotide position in corresponding ORF
GW345	26-mer	ATAGGATCC**ATTTATTACTATCCATC**	165 of *orfde4*
GW346	22-mer	ATGGGATCC**ACCGTTTCAAAAG**	671 of *orfde4*, complementary strand
GW347	24-mer	AGAGGATCC**ACCACGTTTTGAAAG**	165 of *orfde6*
GW348	24-mer	TAAGGATCC**GTGTGTGCCTGTATC**	667 of *orfde6*, complementary strand

[a] Underlined letters indicate *Bam*HI site; bold letters indicate sequences from *orfde4* or *orfde6*. Sequences run from the 5′ end to the 3′ end.

with 50 μl of the overnight culture and incubated with shaking for 4 h. The cells were harvested and resuspended in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]) containing 3 mg of lysozyme per ml. After incubation at room temperature for 20 min, 10 μl of 10% sodium dodecyl sulfate (SDS) and 350 μl of buffer RLT (Qiagen) were added and the mixture was vortexed vigorously. Subsequent steps were performed according to the instructions from the kit. After elution with DEPC-treated H_2O, the sample was treated with 5 μl of RNase-free DNase (Promega) at 37°C for 30 min and then boiled for 10 min to inactivate the DNase. After phenol-chloroform extraction and ethanol precipitation, the sample was again resuspended in DEPC-treated H_2O. The RNA concentration was measured in a spectrometer at 260 nm.

RNA RT-PCR. Murine leukemia virus (MuLV) reverse transcriptase (RT) (Perkin-Elmer) was used to synthesize cDNA for PCR. The Perkin-Elmer protocol for reverse transcription, with modifications, was used. A 20-μl reaction mixture contained 4 μl of $MgCl_2$ (25 mM), 2 μl of 10× PCR buffer II (Perkin-Elmer), 2 μl of each dNTP (10 mM), 0.5 μl of RNase inhibitor (20 U/μl), 1 μl of reverse primer (20 μM), 0.5 μl of MuLV RT (50 U/μl), 25 ng of total RNA, and DEPC-treated H_2O. For the DNA control, 5 ng of OG1RF genomic DNA was used instead of total RNA. When MuLV RT was not used, the volume of DEPC-treated H_2O was increased correspondingly. The reaction was carried out at 42°C for 30 to 60 min, and then the mixture was heated at 95°C for 5 min in a Perkin-Elmer 9600 thermal cycler. The subsequent PCR was performed according to the Perkin-Elmer protocol. To each reverse transcription reaction mixture, 4 μl of 25 mM $MgCl_2$, 8 μl of 10× PCR buffer II, 65.5 μl of H_2O, 1 μl of each primer (20 μM), and 0.5 μl of AmpliTaq DNA polymerase (5 U/μl) were added. PCR cycling was done with the following conditions: 24 to 30 cycles of 96°C for 1 min; 94°C for 30 s, 50 to 55°C (based on the melting temperatures of the primers) for 30 s, and 72°C for 30 s, followed by 10 min at 72°C and holding at 4°C. The primers used in RT-PCR are listed in Table 2.

Sequencing of RT-PCR products and sequence comparison. The RT-PCR products were gel purified by using the Qiaquick Gel Extraction kit (Qiagen). After elution with 30 μl of H_2O, 10 μl of each DNA solution was subjected to electrophoresis through an 0.8% agarose gel to check the bands. For sequencing, 3.5 or 7 μl of each DNA solution was used as the template and the upper primers were used as sequencing primers. The sequences were compared to each corresponding open reading frame (ORF) by using the Bestfit program of Genetics Computer Group (Madison, Wis.).

Cloning intragenic DNA fragments of *orfde4* and *orfde6* into plasmid pTEX4577. Plasmid vector pTEX4577 was derived from pBluescript SK(−) containing a KM resistance marker selectable in both *E. coli* and *E. faecalis* (15). Intragenic DNA fragments of *orfde4* and *orfde6* were PCR amplified from chromosomal DNA of OG1RF with primers containing the recognition sequence for *Bam*HI, as follows: GW345, GW346, GW347, and GW348 (Table 3). Each PCR mixture contained 2 μl of OG1RF chromosome DNA (5.6 ng/μl), 10 μl of 10× PCR buffer I (containing 15 mM $MgCl_2$; Perkin-Elmer), 2 μl of each dNTP (100 mM; Perkin-Elmer), 1 μl of each primer (20 μM), 77 μl of H_2O, and 1 μl of AmpliTaq DNA polymerase (5 U/μl; Perkin-Elmer). PCR cycling was performed in a Perkin-Elmer 9600 thermal cycler, as follows: 96°C for 1 min; 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s (30 cycles); 72°C for 10 min; and holding at 4°C.

After amplification, a small aliquot of each reaction mixture was analyzed by agarose gel electrophoresis to determine the size of each product. The remaining reaction mixtures were purified using the Qiaquick PCR purification kit (Qiagen). After *Bam*HI digestion of the purified PCR products and subsequent purification by the Qiaquick PCR purification kit to remove the *Bam*HI restriction enzyme, each fragment was ligated to *Bam*HI-digested pTEX4577 with T4 ligase (New England Biolabs). The ligation mixtures were transformed into DH5α and plated on LB agar-KM (50 μg/ml) with isopropyl-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Four KM-resistant white colonies were picked after overnight incubation at 37°C and analyzed by restriction enzyme digestions. A plasmid containing the intragenic fragment of *orfde4* was designated pTEX5177, and one containing the *orfde6* intragenic fragment was designated pTEX5178. Plasmids pTEX5177 and pTEX5178 were further subjected to DNA sequencing to verify the inserts.

Generation of OG1RF mutants with disruptions in *orfde4* and *orfde6*. DNA from plasmids pTEX5177 and pTEX5178 was prepared from 250 ml of overnight cultures using the standard alkaline-SDS method and purified in a CsCl-ethidium bromide gradient, followed by dialysis against 10 mM Tris, pH 8.0, and precipitation with ethanol (17). The final DNA concentration was adjusted to 2.5 μg/μl with sterile H_2O. Preparation of OG1RF competent cells and electroporation of DNA into OG1RF were carried out as described previously (15), with slight modifications. For each electroporation, 5 μg of DNA and 50 μl of OG1RF competent cells were mixed and added to an ice-cold 0.1-cm gap electrode Gene Pulser/*E. coli* Pulser Cuvette (Bio-Rad). One pulse was given at 1.25 V with 400-Ω resistance and 25-μF capacity. Immediately after the pulse, 1 ml of Todd-Hewitt broth (THB)–0.25 M sucrose was added to the cuvette. After incubation at 37°C for 2 h, the cell mixture was centrifuged and the cell pellet was resuspended in 0.2 ml of THB–0.25 M sucrose and plated onto THB–S0.25 M sucrose agar containing 2,000 μg of KM per ml. Colonies appearing within 36 h of incubation were streaked onto BHI agar with 2,000 μg of KM per ml. Clones that grew up after overnight incubation at 37°C were further analyzed by colony PCR and Southern blot analysis.

Colony PCR of OG1RF mutants. A single colony was picked with a 200-μl sterile pipette tip and resuspended in 100 μl of sterile H_2O. GW378 and GW379 are oligonucleotides directing outward DNA synthesis from the ends of the KM resistance gene of pTEX4577. Their sequences are as follows: GW378, 5′ GTG ATATTCTCATTTTAGCC; and GW379, 5′ GACTTACTGGGGATCAAGCC. GW378 and GW379, and primers to *orfde4* and *orfde6*, GW301 and GW303 (Table 2), and GW345, GW346, GW347, and GW348 (Table 3) were used in different combinations for PCR. PCR mixtures contained 1 μl of the cell suspension, 4 μl of PCR buffer I (Perkin-Elmer), 1 μl of a mixture of the four dNTPs (2.5 mM [each]), 4 μl of each primer (1 μM), 25.5 μl of sterile H_2O, and 0.5 μl of AmpliTaq DNA polymerase (5 U/μl; Perkin-Elmer). PCR cycling was performed in a Perkin-Elmer 9600 thermal cycler with the following conditions: 96°C for 1 min; 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min (30 cycles); 72°C for 10 min; and holding at 4°C. One of each of the colonies showing the expected PCR products was designated TX5179 or TX5180 for interruptions in *orfde4* and *orfde6*, respectively, and was subjected to Southern blot analysis for verification.

Southern blot analysis of OG1RF mutants. Genomic DNA from OG1RF, TX5179, and TX5180 was prepared based on a procedure described for *E. coli* (7). Briefly, enterococcal strains were grown in 5 ml of BHI broth with appropriate antibiotics at 37°C overnight. The cells were harvested, washed once with 5 ml of J buffer (0.1 M Tris-Cl, 0.1 M EDTA, and 0.15 M NaCl [pH 8.0]) and resuspended in 0.16 ml of J buffer. The suspension was then treated with 20 μl of freshly made lysozyme solution (10 mg/ml in 0.25 M Tris-Cl, pH 8.0) at 37°C for 20 min, followed by 1 μl of RNase A (34 mg/ml; Sigma) at 37°C for 10 min and then 70°C for 3 min. After this, 16 μl of 30% Sarkosyl was added and the mixture was incubated at 70°C for 20 min and then at 37°C for 1 h. Then 0.4 mg of proteinase K was added, and the solution was incubated at 37°C for 2 to 4 h. Another 0.4 mg of proteinase K was added at the end of the incubation, and the solution was dialyzed against 0.01 M Tris (pH 8.0)–0.01 M EDTA (pH 8.0)–0.15 M NaCl at 37°C overnight. The DNA solution was extracted once with phenol-chloroform and once with chloroform, and dialyzed against TE for several hours at room temperature. The concentration of DNA was then determined in a fluorometer.

For Southern blot analysis, 3 μg of genomic DNA was digested with *Eco*RI and *Xba*I and subjected to agarose gel electrophoresis. Blotting was performed using Hybond-N^+ nylon membrane (Amersham) and 0.4 N NaOH solution according to the manufacturer's instructions. DNA of plasmids pTEX5177 and pTEX5178 was digested with *Bam*HI, and the insert fragments were purified using the Qiaquick Gel Extraction kit (Qiagen). The Random Primers DNA Labeling System (GIBCO BRL) was used to label the insert fragments with [α-^{32}P]dCTP (Amersham). Hybridization was carried out at 65°C according to manufacturer's instructions.

Determination of growth curves and stability of single crossover disruptions in TX5179 and TX5180. Overnight cultures of TX5179, TX5180, and OG1RF were diluted 100-fold with fresh BHI broth and incubated at 37°C with shaking. Klett units were measured every hour until the stationary phase was reached. Aliquots (100 μl) of each culture were taken every hour, and 100 μl of 10^{-4} to 10^{-8} dilutions of each sample were plated onto BHI agar plates. Colonies were counted after incubation at 37°C for 24 h.

TX5179 and TX5180 were grown in BHI broth without antibiotics at 37°C with shaking. Aliquots (100 μl) of each culture were taken after 24 h of growth, and 10-fold serial dilutions were made. Dilutions (10^{-5}-, 10^{-6}, and 10^{-7}-fold; of each sample in 100-μl volumes) were plated on BHI and BHI-KM (2,000 μg/ml) agar plates and incubated at 37°C overnight. Colonies were counted, and the numbers from BHI agar plates and BHI-KM agar plates were compared.

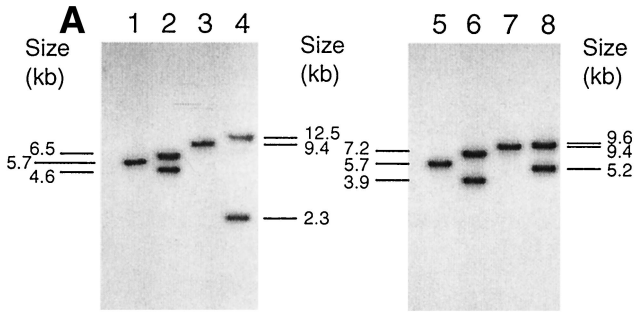
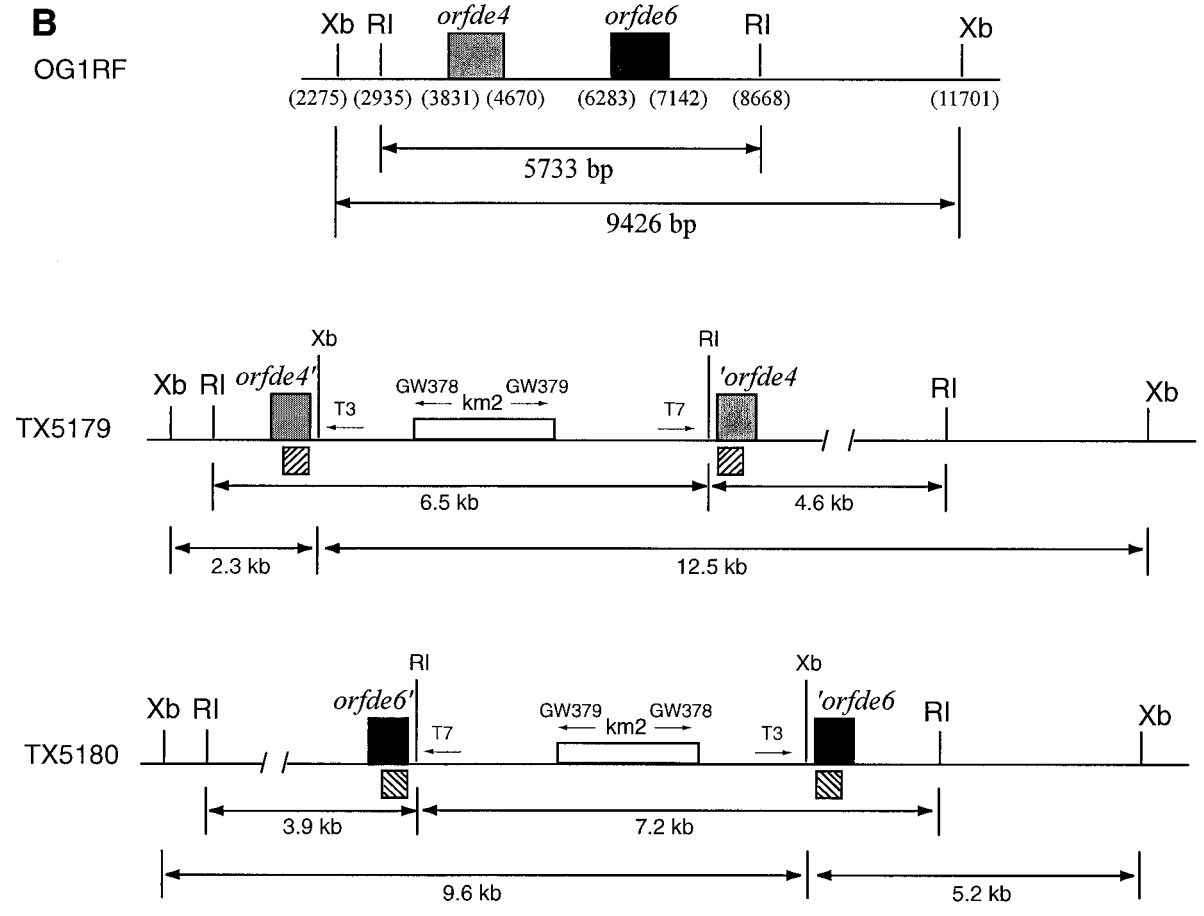


FIG. 1. (A) Southern blot analysis of OG1RF, TX5179, and TX5180. Genomic DNA of OG1RF, TX5179, and TX5180 was digested with *Eco*RI and *Xba*I, separated by electrophoresis in a 0.6% agarose gel and transferred onto a Hybond-N^+ nylon membrane. The insert fragments of pTEX5177 and pTEX5178 were labeled with [^{32}P]dCTP and used as probes. Lanes 1-4 were probed with the insert of pTEX5177, and lanes 5-8 were probed with that of pTEX5178. Hybridization was performed under high-stringency conditions at 65°C. Lane 1, OG1RF plus *Eco*RI; lane 2, TX5179 plus *Eco*RI; lane 3, OG1RF plus *Xba*I; lane 4, TX5179 plus *Xba*I; lane 5, OG1RF plus *Eco*RI; lane 6, TX5180 plus *Eco*RI; lane 7, OG1RF plus *Xba*I; lane 8, TX5180 plus *Xba*I. (B) Illustration of the local organizations of *orfde4*, *orfde6*, and the flanking regions in OG1RF, TX5179, and TX5180. RI, *Eco*RI; Xb, *Xba*I. The numbers in the parentheses indicate the nucleotide positions within the *epa* region in OG1RF. In TX5179, *orfde4* was interrupted by pTEX5177, resulting in two partial copies, while in TX5180, *orfde6* was interrupted by pTEX5178, resulting in two partial copies of *orfde6*. T3 and T7 are promoter regions on pTEX4577, and GW378 and GW379 are primers to the ends of the KM resistance gene. The arrows were not drawn to scale. The striped boxes underneath the disrupted ORFs indicate where the probes hybridized.



Examination of OG1RF mutants in a mouse peritonitis model. Testing of the mutants was performed as described previously (20). OG1RF and the mutants were incubated in BHI broth overnight at 37°C with shaking. The cells were harvested by centrifugation, washed with chilled 0.85% saline solution, and resuspended in 20 ml of ice-cold 0.85% saline to reach a density of $\sim 5 \times 10^{10}$ CFU/ml. A series of twofold dilutions were then made with 50% sterile rat fecal extract to obtain four to five inocula for injection into mice. Meanwhile, 10-fold serial dilutions were made and aliquots of 10^{-6}, 10^{-7}, and 10^{-8} dilutions were plated onto BHI agar plates with proper antibiotics to obtain the actual titer. Outbred ICR female mice, four to six weeks old (Harlan Dawley, Houston, Tex.) were used. Each was injected intraperitoneally with 1 ml of cell suspension with a syringe with a 25-gauge needle. Observations were made every 3 h, and the number of surviving mice was recorded.

The LD_{50} was determined as described by Reed and Muench (16). Survival statistics were computed using the Kaplan-Meier method and compared by log rank using StatView (Abacus Concepts, Inc., Berkeley, Calif.). Spleens were recovered from dead mice injected with the highest inocula, homogenized, and suspended in 3 ml of chilled 0.85% saline. Serial dilutions of each spleen suspension were plated onto BHI agar plates and, for TX5179 and TX5180, also onto BHI agar plates containing 2 mg of KM per ml. Colonies were counted after incubation for 24 h at 37°C.

Elution of antibodies specific to the PS. Antibodies were eluted as described previously (27). Briefly, cell lysates of TX5159 were treated with proteinase K, applied to SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes, and incubated with one of the patient serum samples. Elution was carried out using 100 mM glycine (pH 2.5); the solution was neutralized with a 1/10 volume of 1 M Tris (pH 8.0) and stored at −20°C until used.

Immunoblot analysis of bacterial colonies. Colonies were inoculated onto LB or BHI agar plates containing antibiotics and incubated at 37°C or room temperature for 24 h. NitroBind nitrocellulose transfer membranes (Micron Separations, Inc., Westborough, Mass.) were used to lift the colonies. Subsequent steps were performed as described previously (27).

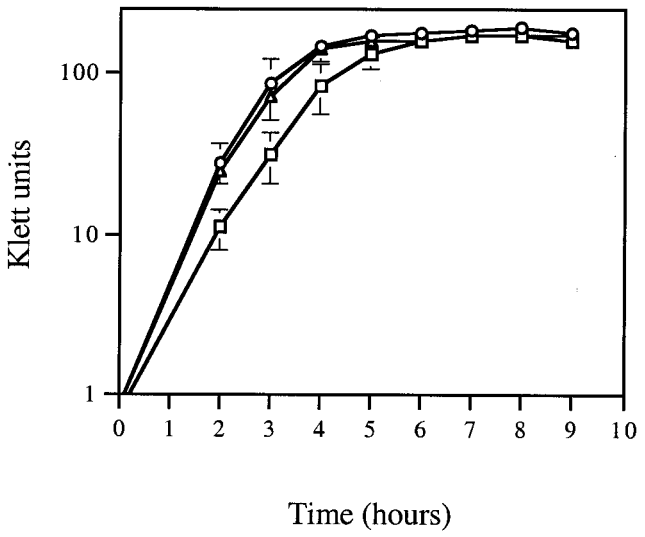


FIG. 2. Growth curves of OG1RF, TX5179, and TX5180. Klett units were measured every hour until stationary phase. Circles, OG1RF; triangles, TX5180; boxes, TX5179.

RESULTS

Detection of mRNA transcripts from *epa* genes in OG1RF. To determine if the ORFs in the *epa* region were transcribed, RT-PCR was performed. In the reactions using RNA as the template, only those reaction mixtures containing both RT and *Taq* polymerase yielded PCR products. Reaction mixtures containing only *Taq* polymerase and no RT (negative controls) did not yield PCR products (data not shown). This confirmed that the PCR products were derived from mRNA templates and not from contaminating DNA. In many cases a single RT-PCR band of the expected size was visualized after agarose gel electrophoresis (*orfde3*, *orfde4*, *orfde5_6*, *orfde8*, *orfde9*, *orfde10*, *orfde12*, *orfde15*, and *orfde16*). However, in some cases a band of the expected size as well as bands of other sizes were observed (*orfde2*, *orfde5*, *orfde6*, *orfde7*, *orfde11*, *orfde13*, and *orfde14*). Varying the RT-PCR conditions and designing new primers to different regions of the ORFs in the latter case improved the results with *orfde5*, *orfde6*, *orfde11*, and *orfde13* but not with *orfde2*, *orfde7*, and *orfde14*. PCR controls using DNA instead of RNA (without RT) yielded only bands of the expected sizes.

To verify that the RT-PCR products observed on agarose gels were the expected products, each band of the correct size from *orfde2* to *orfde16* was gel purified and subjected to DNA sequencing. The DNA sequences were compared with the sequences of each corresponding ORF. The results showed that each product had the correct sequence (data not shown). This indicated that all 16 genes in the *epa* region are transcribed. To examine the nature of the bands of incorrect sizes, some of them were also gel purified and subjected to DNA sequencing. None of the sequences examined showed any homology to the corresponding ORFs, although sequences resembling those of the primers could be found at the ends. Thus, these bands do not represent RNAs derived from an *epa* gene.

Generation of OG1RF mutants. Although PS could not be detected in *E. faecalis*, sera from patients with enterococcal infections contained anti-PS antibodies (27). If the PS was produced only during infection, it may be a virulence factor. To test this, mutations were made in *orfde4* and *orfde6*, both necessary for PS production in *E. coli*. Intragenic fragments of *orfde4* and *orfde6* were cloned in pTEX4577, resulting in plasmids pTEX5177 and pTEX5178, respectively. In pTEX5177, the *orfde4* intragenic fragment was in the same orientation as that of the T7 promoter, while in pTEX5178, the *orfde6* fragment was in the orientation of the T3 promoter.

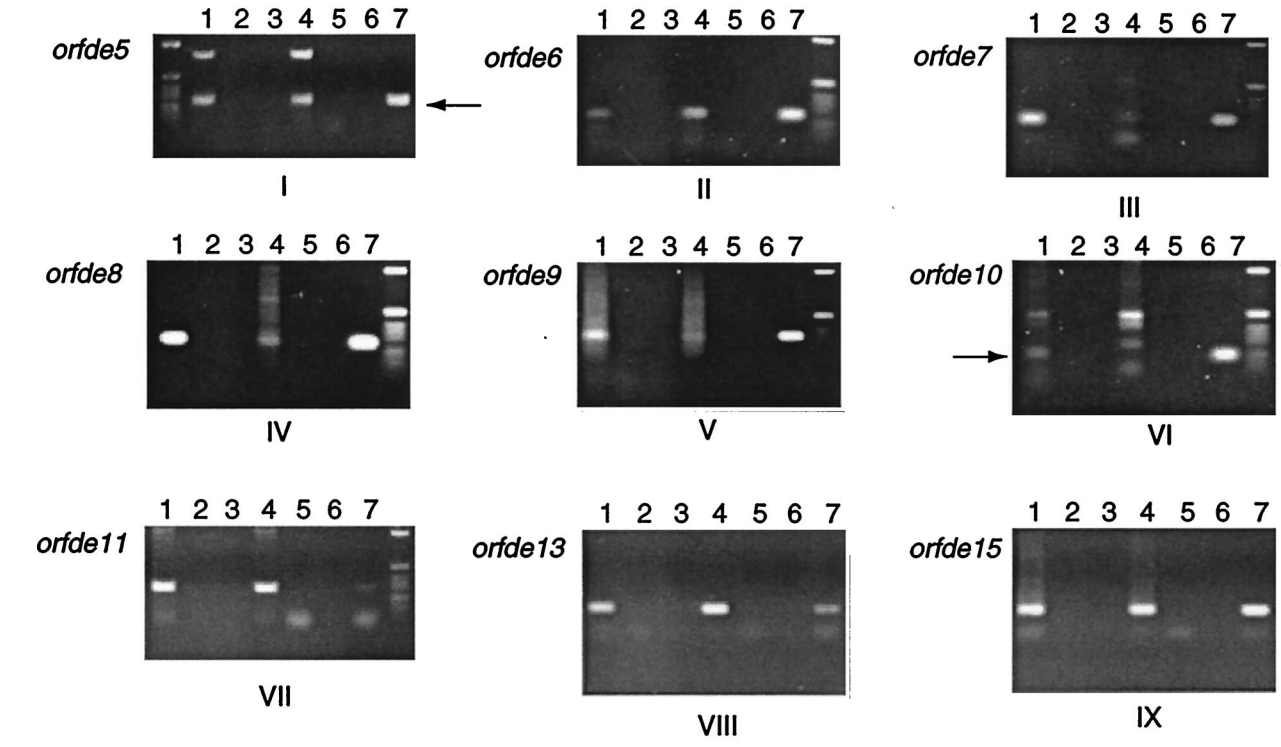
DNA of pTEX5177 and pTEX5178 was electroporated into OG1RF to generate single crossover mutants. Four resistant colonies for *orfde4* and one for *orfde6* were obtained. Colony PCR of these five clones produced bands of the expected sizes (data not shown). To verify the disruptions, one clone for *orfde4* and one for *orfde6*, designated TX5179 and TX5180, respectively, were further analyzed by Southern blot hybridization (Fig. 1A). The results showed that the 5.7-kb *Eco*RI band in OG1RF was split into two bands of about 6.5 and 4.2 kb in TX5179 and into two bands of 7.2 and 3.9 kb in TX5180, while the 9.4-kb *Xba*I band was split into two bands of about 12.5 and 2.3 kb in TX5179 and into two bands of 9.6 and 5.2 kb in TX5180. These sizes are as expected from a single crossover at *orfde4* or *orfde6* (Fig. 1B).

Growth curves of TX5179 and TX5180 and stability of single crossover disruptions. To determine if the growth of mutants TX5179 and TX5180 differed from that of wild-type OG1RF, the optical turbidity (in Klett units) and bacterial titers were measured (Fig. 2). TX5179 and TX5180 grew slightly slower than OG1RF and had slightly lower cell densities at stationary phase. The bacterial titer for OG1RF after growth for 24 h was $(2.20 \pm 0.28) \times 10^9$ CFU/ml, while the titers for TX5179 and TX5180 were $(1.65 \pm 0.13) \times 10^9$ CFU/ml and $(1.80 \pm 0.27) \times 10^9$ CFU/ml, respectively, similar to the turbidity of each culture (Fig. 2). Thus, the insertion mutations did not affect growth.

The numbers of colonies of TX5179 and TX5180 on BHI agar plates were comparable to those on BHI-KM agar plates (1.24×10^9 versus 1.4×10^9 for TX5179 and 1.52×10^9 versus 1.45×10^9 for TX5180) after growth for 24 h in BHI medium, indicating that the insertion mutations in the chromosomes of TX5179 and TX5180 were stable.

Comparison of mRNA transcript levels in OG1RF and TX5180. To determine the effect of gene disruption on the transcription of downstream genes, total RNA was extracted from OG1RF, TX5179, and TX5180 after 4 h of growth in BHI broth. Extraction from OG1RF and TX5180 yielded 300 to 400 ng of RNA per μl, whereas for unknown reasons, no RNA was obtained from TX5179 in two attempts. RT-PCR was performed on total RNA from OG1RF and TX5180, and transcription of a number of ORFs (*orfde5* to *orfde11*, *orfde13*, and *orfde15*) was measured. After RT-PCR, the intensity of each band from TX5180 was compared to that of its corresponding band from OG1RF. The results indicated that the amount of mRNA transcripts of *orfde7* to *orfde10* in TX5180 was greatly reduced, while that of transcripts of *orfde5*, *orfde6*, *orfde11*, *orfde13*, and *orfde15* was not (Fig. 3A). A band was observed for *orfde6* because the region between the primers (GW303 and GW304) used in RT-PCR was not affected by the insertion. This suggests that there is a polar effect of the insertion on transcription of some *epa* genes.

To examine the level of discrimination of RNA concentration in the RT-PCR system, total RNA (25 ng/μl) of OG1RF was diluted in a series of 10-fold dilutions and 1 μl of each dilution was subjected to RT-PCR with reaction and cycling conditions the same as those used for comparing RNAs of TX5180 and OG1RF. Primers GW301 and GW302 for *orfde4* were used (Fig. 3B). The results showed that bands were observed with 25, 2.5, 0.25, and 0.025 ng of total RNA, but not with 2.5 pg of total RNA. The intensity of the bands decreased with the amount of RNA. Thus, under the conditions used, the



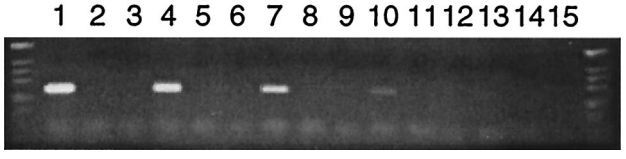


FIG. 3. (A). Comparison of the levels of mRNA transcripts of TX5180 and OG1RF. The target ORF is labeled next to each panel. The primers used for each ORF are as follows: panel I, GW337 and GW338; panel II, GW303 and GW304; panel III, GW365 and GW366; panel IV, GW316 and GW317; panel V, GW318 and GW319; panel VI, GW320 and GW321; panel VII, GW323 and GW324; panel VIII, GW367 and GW368; and panel IX, GW331 and GW332. The arrows next to the *orfde5* and *orfde10* panels indicate the specific bands (of expected sizes). The final concentration of each primer in the reaction was 400 nM for the reverse primers and 200 nM for the forward primers. A total of 25 ng of total RNA or 5 ng of chromosomal DNA was used as the template in each reaction. Lanes 1 to 3, OG1RF RNA; lanes 4 to 6, TX5180 RNA; and lanes 7, OG1RF chromosomal DNA. In addition, AmpliTaq DNA Polymerase (*Taq*) and/or MuLV RT, or neither, was used in each different reaction, as follows: lanes 1 and 4, RT and *Taq*; lanes 2, 5, and 7, *Taq* only; and lanes 3 and 6, neither. Reverse transcription was performed at 42°C for 40 min and then the mixture was heated at 95°C for 5 min. The PCR was carried out in a Perkin-Elmer 9600 thermal cycler using the following conditions: 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s for 30 cycles. (B) RT-PCR of serial dilutions of OG1RF total RNA. Total RNA (25 ng/μl) was diluted 10-, 100-, 1,000-, and 10,000-fold with DEPC-treated H_2O, and 1 μl of each dilution was used as the template for RT-PCR. GW301 and GW302 were used as primers. Lanes 1 to 3, undiluted RNA; lanes 4 to 6, 10-fold dilution; lanes 7 to 9, 100-fold dilution; lanes 10 to 12, 1,000-fold dilution; and lanes 13 to 15, 10,000-fold dilution. AmpliTaq DNA Polymerase (*Taq*) and/or MuLV RT, or neither, was used in each different reaction, as follows: lanes 1, 4, 7, 10, and 13, RT and *Taq*; lanes 2, 5, 8, 11, and 14, *Taq* only; and lanes 3, 6, 9, 12, and 15, neither. Reverse transcription and PCR were carried out using the same conditions as those described for panel A.

system was able to discriminate a 10-fold difference in the amount of total RNA. Furthermore, the difference in the levels of intensity of bands in lanes 1 and 10 of Fig. 3B, indicative of a 1,000-fold difference in the RNA level, appeared to be of the same order of magnitude as the reduction in transcription of *orfde7* to *orfde9* in the mutant TX5180.

Examination of OG1RF mutants in a mouse peritonitis model. To test if the disruptions in *orfde4* and *orfde6* had any effect on the virulence of OG1RF, TX5179, and TX5180 were examined in a mouse peritonitis model. In an initial experiment, a group of six mice was used for each of the four inocula made by twofold serial dilutions of overnight cultures with sterile rat fecal extract. Bacteria were recovered from the spleens of dead mice injected with the highest inocula of

TABLE 4. Statistical significance of the delayed killing of mice by TX5179 and TX5180 compared with OG1RF

Strain	No. of CFU/ml[a]		P^b	No. of CFU/ml[a]		P^b
	OG1RF	TX5179		OG1RF	TX5180	
1	2.3×10^8 (6)	3.3×10^8 (6)	0.0034	2.3×10^8 (6)	3.2×10^8 (6)	0.0178
2	2.3×10^8 (6)	6.6×10^8 (6)	0.0009			
3	3.5×10^8 (6)	4.0×10^8 (16)	<0.0001	3.5×10^8 (6)	5.0×10^8 (16)	0.0002

[a] Size of the inoculum used for injection into the peritoneum of each mouse. Numbers in parentheses are the number of mice used for each inoculation. The time-to-death values for wild-type and mutants are shown in Fig. 4.

[b] The *P* value was calculated in the StatView program using the log rank method. A *P* value of less than 0.05 is statistically significant.

[c] The LD_{50} values calculated for the three strains in this experiment were as follows (in CFU per milliliter): for OG1RF, 2.4×10^8; for TX5179, 6.0×10^8; and for TX5180, 5.0×10^8.

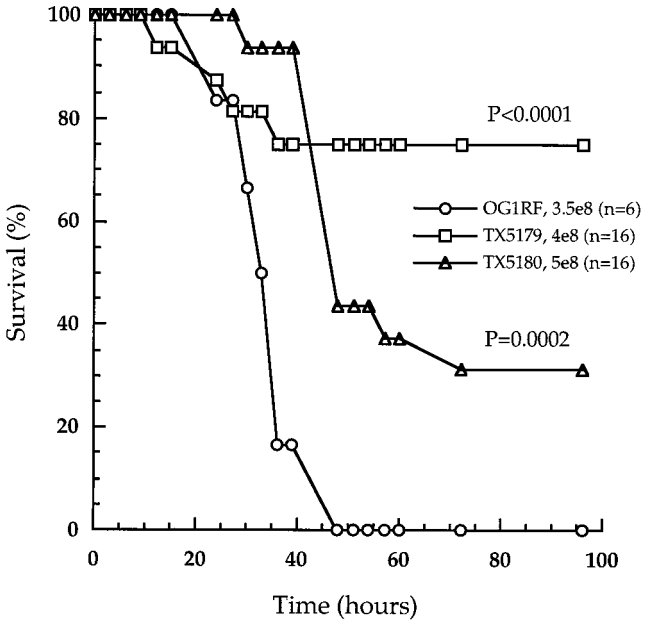


FIG. 4. Percentages of surviving mice injected with OG1RF, TX5179, or TX5180 over time. Circles, OG1RF; squares, TX5179; triangles, TX5180. The inoculum sizes and the number of mice used for each strain were as follows: OG1RF, 3.5×10^8 CFU (6 mice); TX5179, 4.0×10^8 CFU (16 mice): and TX5180, 5.0×10^8 CFU (16 mice). See Table 4 for statistical significance.

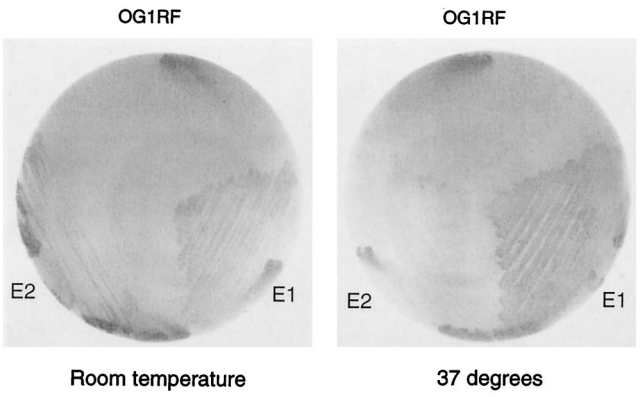


FIG. 5. Immunoblot of two mucoid *E. faecalis* isolates with the eluted antibody. Strains were grown on BHI agar for 24 h at either room temperature or 37°C and then lifted onto nitrocellulose filters. Immunoblotting was carried out with the eluted antibody as described in Materials and Methods.

OG1RF, TX5179, or TX5180, and their titers on BHI agar plates and BHI-KM agar plates were determined. The titers for OG1RF, TX5179, and TX5180 on BHI plates were 3.6×10^8, 1.2×10^8, and 1.6×10^8 CFU/ml, respectively. The numbers of colonies from BHI plates were comparable to those from BHI-KM agar plates for both TX5179 and TX5180, indicating that the disruptions were stable in vivo.

The range of inocula used in this experiment did not allow the calculation of LD_{50}, since the bacterial titers for OG1RF and the two mutants were not closely matched. However, when the data (not shown) were analyzed for the time to killing, TX5179 and TX5180 showed statistically significant delays in killing compared to that of OG1RF (Table 4, rows 1 and 2).

To further examine the effect of the mutations on the survival of mice, sixteen mice were used for each of the two mutants, with inoculum sizes of 4×10^8 CFU for TX5179 and 5×10^8 CFU for TX5180 (Table 4, row 3). Six mice were used for OG1RF at 3.5×10^8 CFU. The survival of the 16 mice injected with TX5179 and TX5180 was compared with that of the six mice injected with OG1RF. The percentage of surviving mice was plotted against time (Fig. 4). The two mutants resulted in a higher percentage of surviving mice and delayed killing compared with that of OG1RF. The *P* values were <0.0001 for TX5179 versus OG1RF and 0.0002 for TX5180 versus OG1RF, consistent with the results obtained in the first experiments.

To compare the LD_{50} values of OG1RF and the mutants, the experiment was repeated using a slightly different range of inocula and six mice for each inoculum. The two mutants showed higher LD_{50} than OG1RF (Table 4). These results demonstrate that genes in the *epa* locus contribute to virulence in the mouse peritonitis model.

Reaction of two mucoid *E. faecalis* isolates with anti-PS antibody. Two mucoid *E. faecalis* isolates from patients with chronic urinary tract infections (2), E1 and E2, were tested for reaction with anti-PS antibody. E1, E2, and OG1RF were streaked on BHI agar plates and incubated at room temperature or 37°C for 24 h. The colonies were lifted onto nitrocellulose membranes for immunoblotting with the antibody eluted from the *E. coli* clone expressing the PS antigen (Fig. 5). The results showed that E1 grown at both room temperature and 37°C reacted, E2 reacted only after incubation at room temperature, and, as found previously, OG1RF did not react at either temperature. The temperature dependence of the immunoreactivity paralleled that of mucoidy, suggesting that the PS being overproduced in the mucoid strains is in fact the antigen.

DISCUSSION

In a previous study, members of our group described a PS antigen biosynthesis gene cluster (*epa*) of *E. faecalis* (27). In this report we demonstrate that although the *epa* antigen is not detectable in laboratory-grown *E. faecalis* cells, the genes within *epa* are transcribed in *E. faecalis* and disruptions in two of the genes (*orfde4* and *orfde6*) caused a statistically significant delay in killing in a mouse peritonitis model.

The *E. coli* clone containing the *epa* gene cluster, TX5159, was previously found to react with sera collected from four endocarditis patients infected with *E. faecalis* in different regions in the United States, suggesting that the PS was produced in all of these patients (27). However, we were able to detect the antigen neither in *E. faecalis* strain OG1RF under different growth conditions (including incubation in the peritonea of mice) nor in several other *E. faecalis* clinical isolates or laboratory strains. To determine if the genes within *epa* were transcribed, we performed RT-PCR analysis of the total RNA of OG1RF grown in BHI medium for 4 h. Each band of the correct size was confirmed by sequencing the purified PCR products. The results indicated that the *orfde2* to *orfde16* genes were transcribed. However, it is not clear whether the amount of mRNA represents a low basal level that must be induced for the production of the PS or whether this is sufficient mRNA for PS production.

To study the effects of mutations in this region, two mutants, TX5179 (in *orfde4*) and TX5180 (in *orfde6*), were generated. Members of our group previously showed that insertions in *orfde4* abolished antigen production in the *E. coli* clone TX5159 (27). Transposon mutagenesis also indicated that one of the putative rhamnose biosynthesis genes, *orfde8*, was re-

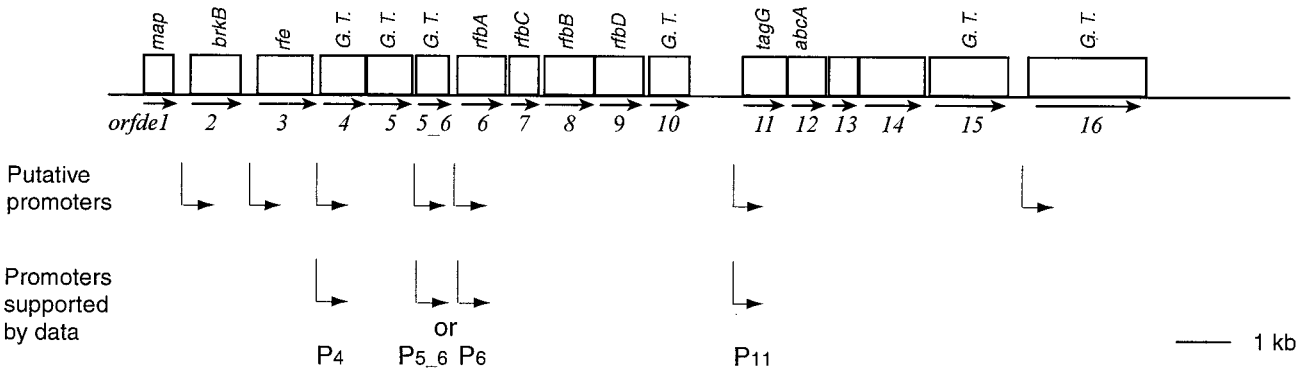


FIG. 6. Hypothetical transcriptional organization of the *epa* region. Line 1, open reading frames in *epa* (open boxes) and their direction of transcription (arrows below the boxes). Line 2, putative promoters identified by homology to the *E. coli* −35 and −10 sequences recognized by σ70 (27); vertical lines indicate the position of each putative promoter, and arrows indicate their directions. Line 3, summary of possible promoters in *epa* that were supported by experimental data. P_{5_6} and P_6 cannot be distinguished based on current data.

quired for the immunoreactivity of TX5159, and *orfde6* is predicted to encode the first enzyme in this rhamnose biosynthesis pathway.

Since the mutants were generated via single crossover events, resulting in two partial copies of *orfde4* or *orfde6*, the stability of the crossovers was determined. The disruptions appeared to be stable, since very few cells became KM sensitive after growth in plain BHI broth for 24 h or incubation in the peritonea of mice for at least 12 h. The mutants grew slightly less well than OG1RF; however, the difference appeared to be minor.

To examine if the disruptions had polar effects on transcription, RT-PCR was performed to measure the mRNA levels from several ORFs. The transcription of *orfde7* to *orfde10* in TX5180 was drastically reduced, while that of *orfde11*, *orfde13*, and *orfde15* was not (Fig. 3A). This suggested that the *orfde6* to *orfde10* genes were in one transcriptional unit and that their transcription was independent of that of *orfde11* and other downstream genes (Fig. 6). This is consistent with the results of DNA sequence analysis, which suggested that a promoter-like sequence was found upstream of *orfde11* (27). In the study previously conducted by members of our group, analysis of Tn*7* transposon mutants indicated that insertions in *orfde3* or between *orfde3* and *orfde4* did not affect the immunoreactivity of TX5159, and an insertion in *orfde4* was complemented by a plasmid, pTEX5175, that contains only *orfde4* and *orfde5*. These results suggested that there should be transcriptional start sites upstream of *orfde4* and *orfde6* (27). Thus, *epa* should contain at least three transcriptional start sites (Fig. 6).

To test if the disruptions had any effect on the virulence of OG1RF, the two mutants were examined in a mouse peritonitis model. TX5179 and TX5180 appeared to be less virulent, as indicated by a high LD_{50} as well as to have delayed killing compared to that of OG1RF. The attenuation in the virulence of TX5179 and TX5180 in the mouse peritonitis model could reflect an involvement of this region in *E. faecalis* virulence. Considering the slightly slower growth of the mutants, the *P* values were calculated using higher initial inocula of the mutants versus OG1RF. In the case of TX5179, for example, a statistically significant *P* value (0.0034) was obtained when results from TX5179 with an inoculum of 6.6×10^8 CFU were compared with those from OG1RF with an inoculum of only 2.3×10^8 CFU. This suggested that the delayed killing was not due to the differences in growth rates.

The recent report of four mucoid encapsulated *E. faecalis* isolates from patients with chronic urinary tract infections by Bottone et al. (2) shed new light on this project. Two of the isolates, E1 and E2, were examined and showed positive reactions with the anti-PS antibody. E1 reacted after incubation at both room temperature and 37°C, while E2 reacted only after incubation at room temperature. These results suggested that the antigenic PS was overproduced in the mucoid strains.

The reasons that no antigenic PS was detected in wild-type *E. faecalis* remain to be determined. One possibility is that it is expressed only under certain conditions. In *P. aeruginosa*, the production of alginate is suppressed by genes in the *muc* locus until the organism invades the lungs of cystic fibrosis patients. The *muc* region was mapped to a separate locus from that of the biosynthesis genes (3, 4, 11, 12, 18). Therefore, it is possible that a negative regulatory region is also present in *E. faecalis* at a different location from *epa* and suppresses the PS production in *E. faecalis*. Under certain conditions, such as chronic infections or attachment to heart valves, the suppression could be turned off. The PS could then be produced, generating antibodies in the patients. By this model, in *E. coli* TX5159 carrying the cloned *epa* locus, the negative control region is absent or not functional and PS production is permitted. Since the *orfde2* to *orfde16* genes were transcribed in OG1RF grown in BHI medium, it is not clear if the level of transcription of these genes reflects a basal level produced under the negative control. Alternatively, the *epa* locus could be posttranscriptionally regulated or the target(s) of regulation could be outside of *epa*.

A second possibility is that some function(s) required for the synthesis or export of the PS is not present in the strains examined. For example, the capsulation gene cluster (*cap*) of *Haemophilus influenzae* type b is on a compound transposon flanked by two insertion element-like (IS*1016*) elements. The type b *cap* locus consists of a duplication of *cap* with an unduplicated small bridge region containing *bexA* between the two large repeats. Recombination between the large repeats can result in the deletion of *bexA*, leading to a capsule-deficient phenotype (10). If this were the case in *E. faecalis*, the lost function(s) must have been complemented in TX5159 by *E. coli* genes outside the *rfb* region (*E. coli rfb* was previously shown not to affect antigen production). A variation of this possibility is that there is a hot spot in this region for transposon or conjugative plasmid integration and/or excision. In support of this hypothesis, comparison of the OG1RF gene cluster with preliminary genome sequences of *E. faecalis* strain V583 recently released by the Institute of Genome Research showed

that all *epa* genes are present in V583 but that the V583 *orfde10* is interrupted by conjugative transposon-like sequences (unpublished data). We think this explanation is less likely, however, since the insertion mutations in the *epa* locus of OG1RF led to reduced virulence, suggesting that the PS functions during infections.

The possibility that the antigenic PS is produced in OG1RF but is different or modified compared to the antigen made in *E. coli* also cannot be excluded. The antibodies generated during infection may be directed against a degradation product of the native molecule. The partial PS may be the antigen made in *E. coli*. In the mucoid strains, the overproduction of the native PS may also provide enough of the partial molecule to be detected, whereas in the other *E. faecalis* strains tested, there would not be enough to detect.

In conclusion, we report here that genes *orfde2* to *orfde16* in the *epa* region were transcribed in OG1RF and that disruptions in *orfde4* and *orfde6* caused statistically significant delayed killing and a higher LD_{50} in a mouse peritonitis model. There are at least three transcriptional start sites hypothesized in *epa* and genes *orfde6* to *orfde10* are in one transcriptional unit. In view of the regulation of the production of many bacterial PS and the fact that E2 reacted only with the eluted antibody after incubation at room temperature, it is possible that *E. faecalis* also has adopted ways of manipulating the production or display of the antigenic PS. Comparisons of the genetic organizations of the *epa* region in OG1RF and in the mucoid *E. faecalis* strains and of levels of gene expression and isolation of the PS material and analysis of its chemical composition will yield information invaluable for addressing many of the issues discussed above. Further study on the involvement of *epa* in virulence should have important implications for prevention and treatment of enterococcal infections.

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REFERENCES

1. **Bleiweis, A. S., and R. M. Krause.** 1965. The cell walls of group D streptococci. I. The immunochemistry of the type 1 carbohydrate. J. Exp. Med. **122:**237–249.
2. **Bottone, E. J., L. Patel, P. Patel, and T. Robin.** 1998. Mucoid encapsulated *Enterococcus faecalis*: an emerging morphotype isolated from patients with urinary tract infections. Diagn. Microbiol. Infect. Dis. **31:**429–430.
3. **Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encoding homologs of the serine protease HtrA. J. Bacteriol. **178:**511–523.
4. **Boucher, J. C., M. J. Schurr, H. Yu, D. W. Rowen, and V. Deretic.** 1997. *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. Microbiology **143:**3473–3480.
5. **Chung, C. T., S. Z. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
6. **DeVelasco, E. A., A. F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. **59:**591–603.
7. **Ebel-Tsipis, J., D. Botstein, and M. S. Fox.** 1972. Generalized transduction by phage P22 in *Salmonella typhimurium*. I. Molecular origin of transducing DNA. J. Mol. Biol. **71:**433–448.
8. **Garcia, E., and R. Lopez.** 1997. Molecular biology of the capsular genes of Streptococcus pneumoniae. FEMS Microbiol. Lett. **149:**1–10.
9. **Jelinkova, J., and J. Rotta.** 1978. Identification and typing of enterococci. Methods Microbiol. **12:**199–220.
10. **Kroll, J. S.** 1992. The genetics of encapsulation in *Haemophilus influenzae*. J. Infect. Dis. **165**(Suppl.)**:**93–96.
11. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. Mol. Microbiol. **9:**497–506.
12. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:** 8377–8381.
13. **Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock.** 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. J. Bacteriol. **175:**5216–5223.
14. **Murray, B. E.** 1990. The life and times of the enterococcus. Clin. Microbiol. Rev. **3:**46–65.
15. **Qin, X., K. V. Singh, Y. Xu, G. M. Weinstock, and B. E. Murray.** 1998. Effect of disruption of a gene encoding an autolysin of *Enterococcus faecalis* OG1RF. Antimicrob. Agents Chemother. **42:**2882–2888.
16. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic.** 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. J. Bacteriol. **176:**3375–3382.
19. **Sharpe, M. E., and P. M. F. Shattock.** 1952. The serological typing of group D streptococci associated with outbreaks of neonatal diarrhoea. J. Gen. Microbiol. **6:**150–165.
20. **Singh, K. V., X. Qin, G. M. Weinstock, and B. E. Murray.** 1998. Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. J. Infect. Dis. **178:**1416–1420.
21. **Smyth, J., H. Matthews, M. K. Halpenny, H. Brandis, and G. Colman.** 1987. Biotyping, serotyping and phage typing of *Streptococcus faecalis* isolated from dental plaque in the human mouth. J. Med. Microbiol. **23:**45–54.
22. **Soell, M., M. Diab, G. Haan-Archipoff, A. Beretz, C. Herbelin, B. Poutrel, and J. Klein.** 1995. Capsular polysaccharide types 5 and 8 of *Staphylococcus aureus* bind specifically to human epithelial (KB) cells, endothelial cells and monocytes and induce release of cytokines. Infect. Immun. **63:**1380–1386.
23. **Soell, M., E. Lett, F. Holveck, M. Scholler, D. Wachsmann, and J. P. Klein.** 1995. Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen and mannan binding protein inhibits TNF-alpha release. J. Immunol. **154:**851–860.
24. **Stout, V.** 1994. Regulation of capsule synthesis includes interactions of the RcsC/RcsB regulatory pair. Res. Microbiol. **145:**389–392.
25. **Whitfield, C., and M. A. Valvano.** 1993. Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. Adv. Microb. Physiol. **35:**135–246.
26. **Xu, Y., L. Jiang, B. E. Murray, and G. M. Weinstock.** 1997. *Enterococcus faecalis* antigens in human infections. Infect. Immun. **65:**4207–4215.
27. **Xu, Y., B. E. Murray, and G. M. Weinstock.** 1998. A cluster of genes involved in polysaccharide biosynthesis from *Enterococcus faecalis* OG1RF. Infect. Immun. **66:**4313–4323.

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