INFECTION AND IMMUNITY, Oct., 1997, p. 4207–4215
0019-9567/97/$04.00+0

Vol. 65, No. 10

Enterococcus faecalis Antigens in Human Infections

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Received 1 April 1997/Returned for modification 18 June 1997/Accepted 5 August 1997

Genomic libraries of two *Enterococcus faecalis* strains, OG1RF and TX52 (an isolate from an endocarditis patient), were constructed in cosmid vectors pBeloBAC11 and pLAFRx, and screened with a serum from a rabbit immunized with surface proteins of an *E. faecalis* endocarditis isolate and sera from four patients with enterococcal endocarditis. Seventy-five cosmid clones reacted with at least two of the sera. Thirty-eight of the 75 immunopositive clones were considered to contain distinct inserts based on their DNA restriction patterns and were chosen for further subcloning into a pBluescript vector. Each sublibrary was screened with one of the five sera, and the DNA sequence of the immunopositive subclones was determined. Analysis of these sequences revealed similarities to a range of proteins, including bacterial virulence factors, transporters, two-component regulators, metabolic enzymes, and membrane or cell surface proteins. Fourteen subclones did not show significant similarity to any sequence in the databases and may contain novel genes. Thirteen of the immunopositive cosmid clones did not yield immunopositive subclones, and one such cosmid clone produced a nonprotein antigen in *Escherichia coli*.

Enterococci are one of the three leading causes of nosocomial infections, and account for 5 to 15% of bacterial endocarditis, a heart disease characterized by infection by the bacteria of heart valves, causing tissue damage and eventually leading to heart failure and death without therapy (58). Eighty to 90% of clinical enterococcal isolates are *Enterococcus faecalis*, while 5 to 15% are *Enterococcus faecium* (58). During the past several decades, enterococci have developed resistance to almost all antimicrobial agents used in hospitals, posing a threat for treatment. In contrast to the large amount of work on the molecular basis of antibiotic resistance in enterococci, the mechanisms of the pathogenicity of these organisms are poorly understood.

Several *E. faecalis* virulence factors have been identified. The hemolysin/bacteriocin lyses a broad spectrum of cells including human, horse, rabbit, and cow erythrocytes and gram-positive organisms (4). The hemolysin/bacteriocin determinant is usually extrachromosomal (33) and enhances the virulence of *E. faecalis* in animal models (10, 34, 35). Aggregation substance is a plasmid-encoded protein mediating cell aggregation in pheromone-induced plasmid transfer between donor cells and pheromone-producing recipient cells. In a study of experimental enterococcal endocarditis in rabbits, hemolysin/bacteriocin increased mortality, while aggregation substance contributed to vegetation weight (10). Aggregation substance has been shown to mediate *E. faecalis* adhesion to cultured pig urinary tract cells (44) and to augment internalization of *E. faecalis* by cultured human intestinal epithelial cells (62). Gelatinase is an extracellular metalloendopeptidase that acts on collagenous materials and certain bioactive peptides such as *E. faecalis* sex pheromone-related peptides, glucagon, or neurotensin (53, 73), suggesting that it may participate in inflammatory processes. A survey that investigated the incidence of the above three virulence factors among enterococcal clinical isolates found that about 40% of the isolates did not have at least one of the three factors (13). Thus, these factors are important in enhancing the virulence of *E. faecalis* but are not essential, implying that there must be other factors that play a role during infection. An *E. faecalis* strain orally fed to mice that had been treated with metronidazole-streptomycin was recovered from mesenteric lymph nodes, livers, and spleens of mice, and structures resembling enterococci were observed adherent to the microvillus border of the epithelium, within vacuoles of intact epithelial cells, and within small vessels in the intestinal tract (78). The exact route and mechanism of this dissemination of enterococci are not clear; however, their ability to translocate across the intestinal barrier may be due to additional virulence factors.

Many genetic approaches for the identification of bacterial virulence factors involve using mutants lacking specific virulence traits or recombinant clones that confer virulence phenotypes. Both of these approaches require some clue as to the virulence function or an assay for some step in infection; such information is not available for *E. faecalis*, an opportunistic pathogen of low virulence. Except for the above-mentioned hemolysin/bacteriocin, aggregation substance, and gelatinase, which are present in only about half of the clinical isolates, there are no obvious virulence-associated phenotypes to target. Methods for the selection of genes that are specifically induced or required during infection or under certain in vivo conditions, such as in vivo expression technology (52), signature-tagged transposon method (28), and differential fluorescence induction (76), have identified virulence genes in *Salmonella* and provided an alternative approach for organisms such as enterococci. The functions identified by these methods are not necessarily involved in host interaction, however, and they may not be appropriate targets for vaccine or therapeutic development.

To overcome these difficulties, we have adopted an approach for isolating *E. faecalis* virulence factors based on their antigenicity during infection. We anticipated that the antigens would be mainly surface or secreted proteins that were ex-

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pressed during infection, recognized by the host immune system, and able to trigger an immune response. Such information should improve our understanding of the infection mechanisms of *E. faecalis* and could lead to the discovery of new virulence factors and the development of serodiagnostic and therapeutic tools. Lowe et al. recently cloned from an *E. faecalis* endocarditis isolate the gene for an antigen (EfaA) showing sequence similarity to several adhesins from oral streptococci (51). This encourages the use of this method. Here we report the further application of this approach by immunoscreening of libraries of two *E. faecalis* strains using sera from patients with enterococcal endocarditis, the sequence analysis of the putative antigen-encoding genes, and the identification of a nonprotein antigen.

MATERIALS AND METHODS

Bacterial stains, plasmids, and culture conditions. *Escherichia coli* DH5α and XL1 Blue MRF′ were the host strains for cloning enterococcal DNA. OG1RF is an *E. faecalis* strain previously described (59). TX52 is an *E. faecalis* endocarditis clinical isolate.

Cosmid vectors pBeloBAC11 and pLAFRx were used to construct genomic libraries. The pBeloBAC11 vector (kindly provided by H. Shizuya and M. Simon, California Institute of Technology, Pasadena) was derived from the bacterial F factor and carries a chloramphenicol resistance marker. It is a modified version of pBAC108L (70) with the polylinker replaced by the *lac*Z-multiple cloning site region of pGEM3Z. The pLAFRx vector (27) is a derivative of pLAFR which contains the *oriT* and tetracycline resistance marker of RK2. pBluescript SK(−) (Stratagene, La Jolla, Calif.) was used as the vector for subcloning DNA from cosmids.

E. coli cells were grown in Luria-Bertani (LB) broth or on LB 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. Antibiotics were used at the following concentrations: chloramphenicol 25 μg/ml; tetracycline 12.5 μg/ml; ampicillin 50 μg/ml. Isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) were used at 0.5 mM and 80 μg/ml, respectively.

Antisera. Sera h1, h11, h17, and h20 were collected from patients from various regions in the United States with enterococcal endocarditis. Serum r17 was collected from a rabbit following immunization with surface proteins of strain TX17 (isolated from the patient from whom serum h17 was collected), grown in BHI broth, prepared with the detergent Zwittergent 3-12 (see "Protein extraction, SDS-PAGE, and Western blotting" below). All sera showed strong reactions with enterococci in enzyme-linked immunosorbent assays (ELISA) (3). Sera used in screening were first absorbed to *E. coli* lysates prepared by sonication (68).

DNA manipulation and transformation of *E. coli*. DNA preparation, purification, restriction digestion, agarose gel electrophoresis, and ligation were done by standard methods (68) unless otherwise stated. Restriction enzymes and T4 ligase were from Promega, Madison, Wis. Preparation of competent cells and transformation of DNA into *E. coli* were performed by the one-step TSS procedure (12).

Construction of genomic libraries. Genomic DNAs from OG1RF and TX52 were prepared by a method previously described (59). About 1 mg of genomic DNA was partially digested with 0.7 and 1.4 U of *Sau*3AI, respectively, for 1 h at 37°C, pooled, and size fractionated in a 10 to 40% sucrose density gradient (68). Fractions containing 35- to 50- and 20- to 35-kb DNA fragments were selected for ligation with vectors pBeloBAC11 and pLAFRx, respectively, which had been previously digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase (United States Biochemical, Cleveland, Ohio). The ligation mixtures were packaged in vitro by using the Gigapack III Gold Packaging Extract Kit (Stratagene) and used to infect *E. coli* DH5α, and antibiotic-resistant transductants were selected. Libraries with titers of 10^4 to 10^5 transductant-forming units/μg of DNA were obtained. Individual colonies from the primary selection plates were picked and stored in 96-well microtiter dishes in LB-antibiotic-glycerol solutions.

Immunoscreening of libraries with antisera. Immunoscreening was performed by a standard method (68) with slight modifications. Clones were inoculated from 96-well microtiter dishes onto LB agar plates with appropriate drugs (pBeloBAC11-OG1RF and pBeloBAC11-TX52 clones on LB-chloramphenicol-IPTG, pLAFRx-OG1RF clones on LB-tetracycline), and the plates were incubated overnight at 37°C. The colonies were lifted onto NitroPlus nitrocellulose transfer membranes (Micron Separations, Inc., Westborough, Mass.). The membranes were exposed to chloroform vapor for 15 min, incubated in lysis buffer overnight at room temperature with gentle shaking, and then washed in TNT buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20 [Sigma Chemicals, St. Louis, Mo.]). After shaking in 3% skim milk in 50 mM Tris-Cl (pH 7.4) for 1 h at room temperature, the membranes were incubated with primary antisera (1:250 to 1:500 dilutions in 1% skim milk in 50 mM Tris-Cl, pH 7.4; preabsorbed with *E. coli* lysates) for 2 h at room temperature or overnight at 4°C and washed in 50 mM Tris-Cl (pH 7.4) with 1% skim milk. Protein A-peroxidase (Sigma) was then used at 0.125 μg/ml in 50 mM Tris-Cl (pH 7.4) with 1% skim milk. The membranes were washed again with 0.05% Tween 20 in 50 mM Tris-Cl (pH 7.4), incubated with the color development mix (10 mM Tris-Cl [pH 7.4], 0.06% 4-chloro-1-naphthol, and 0.01% hydrogen peroxide) for 30 min at room temperature, and then washed thoroughly with distilled water.

Protein extraction, SDS-PAGE and Western blotting. Extraction of surface proteins of *E. faecalis* strains used the detergent Zwittergent 3-12 (37). *E. faecalis* cells were grown in BHI broth overnight at 37°C to a density of about 160 Klett units. The cells were pelleted by centrifugation, washed in phosphate-buffered saline (PBS) (0.14 M NaCl, 1.5 mM KH_2PO_4, 15 mM $Na_2HPO_4 \cdot 7H_2O$, 2.7 mM KCl [pH 7.4]), and resuspended in 1% of the original culture volume in PBS with 0.2% Zwittergent 3-12 (Calbiochem, La Jolla, Calif.). The suspension was shaken for 1 h at room temperature in a roller and centrifuged. The supernatant was dialyzed overnight at 4°C against 50 mM Tris-Cl, pH 7.5, and then stored at −20°C. Protein preparations from about 2 ml of initial culture were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins of recombinant clones were prepared by the Zwittergent method or one of two other procedures. In one procedure for analyzing cell-associated proteins, 0.5 ml of overnight culture was centrifuged, resuspended in 4% SDS sample buffer (25 mM Tris-Cl [pH 6.8], 9% [vol/vol] glycerol, 2.5% [vol/vol] β-mercaptoethanol, 0.0025% bromophenol blue, and 4% SDS), and boiled for 5 min before being applied to gels. In the other method used for preparing supernatant proteins, 1.2 ml of an overnight cell culture was centrifuged and the supernatant was transferred to a new microcentrifuge tube. After addition of 0.125 ml of ice-cold 100% trichloroacetic acid (Mallinckrodt, Paris, Ky.) to the supernatant, the tube was incubated on ice for 5 min and centrifuged for 15 min. The pellet was washed with 1 ml of ice-cold acetone, air dried, and resuspended in 4% SDS sample buffer. The sample was boiled for 5 min before being subjected to SDS-PAGE.

SDS-PAGE was performed by using the Laemmli system (46) with a 5% stacking and 10% separating gel. Protein bands were stained with Coomassie brilliant blue R-250 (Bio-Rad) (68). For Western blots, proteins were electrophoretically transferred from the gel to an Immobilon-P transfer membrane (Millipore Co., Bedford, Mass.) by using the MilliBlot-SDE system (Millipore). The membranes were first incubated with blocking buffer (3% skim milk in 50 mM Tris-Cl, pH 7.4) for 1 h at room temperature and then incubated with the primary antisera (preabsorbed with *E. coli* lysates and then diluted 1:150 to 1:1,000 in 1% skim milk–50 mM Tris-Cl, pH 7.4) overnight at 4°C. Subsequent procedures were the same as those for immunoscreening of libraries.

Proteinase K digestion of antigens. Proteins of recombinant clones were prepared from cells grown overnight in LB broth with appropriate antibiotics. Cells (10 ml of the overnight culture) were centrifuged and resuspended in 0.4 ml PBS. SDS (0.1 ml, 10%) was added, and the mixture was split into two aliquots. The samples were boiled for 5 min and allowed to cool to room temperature. Eighty microliters of proteinase K (16.4 mg/ml; Boehringer Mannheim Corp., Indianapolis, Ind.) was added to one sample, and phenylmethylsulfonyl fluoride (PMSF; EM Science, Gibbstown, N.J.) (100 mM in *N*,*N*-dimethylformamide) was added to a concentration of 1 mM to the other to inhibit proteolysis. Both samples were incubated at 65°C overnight and then subjected to SDS-PAGE and Western blotting.

Subcloning antigen-encoding genes from cosmids. Subclones containing antigen-encoding genes were prepared from immunopositive cosmid clones (containing inserts of 20 to 45 kb) by a procedure based on DNase I digestion (16). Seven micrograms of DNA from a cosmid clone was treated with 0.7 U of RNase-free DNase (Stratagene) in 50 mM Tris-Cl (pH 7.5)–10 mM $MnCl_2$ for 2 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 12 mM. The DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation. The ends of the fragments were blunted with Klenow fragment (Promega), and following extraction with phenol-chloroform and ethanol precipitation, about 200 ng of purified DNA fragments was ligated to 100 ng of pBluescript SK(−) DNA that was previously digested with *Eco*RV and treated with shrimp alkaline phosphatase. One-tenth of each ligation mixture was used to transform DH5α or XL1 Blue MRF′, and the transformation mixtures were plated on LB-ampicillin-IPTG agar plates. The colonies were transferred onto NitroPlus nitrocellulose membranes for immunoscreening. About 800 to 1,000 transformants from each sublibrary were screened with antiserum r17, h1, h11, h17, or h20 (1:500 dilution, preabsorbed with *E. coli* lysates) in the presence of IPTG. Colonies showing any immunoreactivity were picked and rescreened with r17, h1, h11, h17, or h20.

DNA sequencing and sequence analysis. DNAs from immunopositive subclones were prepared for sequencing by BioRobot 9600 (Qiagen Inc., Chatsworth, Calif.) or by using the Qiagen plasmid minikit. DNA sequencing reactions were performed by the *Taq* dye-deoxy terminator method (Applied Biosystems, Foster City, Calif.) using primers for the T3 and/or T7 promoter regions on pBluescript SK(−) and a 373A DNA sequencing system (Applied Biosystems). Subclones with large inserts (≥0.7 kb) were sequenced from both the T3 and the T7 primer sides, while subclones with small inserts (<0.7 kb) were sequenced from only one side. The sequences were analyzed by the BLAST (especially BLASTX and BLASTN) network service at the National Center for Biotechnology Information to search for homologous sequences in the protein database

TABLE 1. GenBank accession numbers

Source of sequence	GenBank no.
YX1	B07874
YX102	B07881,B07882
YX103	B07883,B07864
YX106	B07865,B07866
YX2	B07875,B07876
YX36	B07867,B07868
YX37	B07869,B07870
YX4	B07877,B07878
YX42	B07871,B07872
YX5	B07879,B07880
YX77	B07850
YX78	B07851,B07852
YX79	B07853,B07854
YX80	B07855,B07856
YX81	B07857,B07858
YX82	B07859,B07860
YX84	B07861
YX85	B07872,B07873
YX87	B07884,B07885
YX88	B07886
YX89	B07887,B07888
YX91	B07862,B07863
YX98	B07889,B07890

(nonredundant GenBank coding sequence translations plus Brookhaven Protein Data Bank [PDB] plus SwissProt plus PIR) and the nucleotide database (nonredundant GenBank plus EMBL plus DDBJ plus PDB, no expressed tag sequences). The Bestfit program in the GCG software package (Genetics Computer Group, Madison, Wis.) was used to compare similarities among sequences. Default parameters were used in the analysis.

Nucleotide sequence accession numbers. The accession numbers of the sequences reported in this study are listed in Table 1.

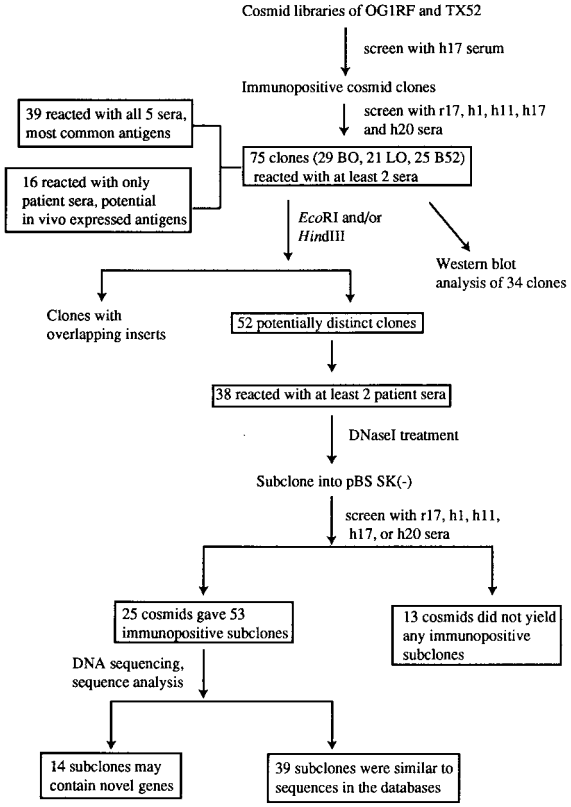

FIG. 1. Immunoscreening and cloning procedure.

RESULTS

Immunoscreening of the genomic libraries. In preliminary experiments, comparison of eleven *E. faecalis* strains including OG1RF and TX52 using the four patient sera h1, h11, h17, and h20 and the rabbit serum r17 in Western blot analysis revealed that multiple common antigen bands, as well as some variable bands, were present in most of these strains (not shown). OG1RF and TX52 were chosen as sources of DNA for construction of genomic libraries. OG1RF has been widely studied, and its physical map has been published (59). TX52 is one of the clinical isolates that showed many common antigen bands.

Several cosmid libraries were constructed, and individual clones were picked into microtiter dish wells. In all, we picked 768 pBeloBAC11-OG1RF (BO) clones, 960 pLAFRx-OG1RF (LO) clones, and 768 pBeloBAC11-TX52 (B52) clones. These 2,496 clones were screened with patient serum h17, and those showing immunoreactivity were rescreened with the r17, h1, h11, h17, and h20 sera. Distinctive morphologies of immunostaining of different clones were seen in the colony immunoblots. For example, clone LO-4E1I (described below) had a very diffuse region of immunostaining, suggesting the presence of secreted antigens in the *E. coli* clone. Seventy-five clones (29 BO clones, 21 LO clones, and 25 B52 clones) showed reactivity with at least two of the sera; 39 of these reacted with all five sera, and 16 reacted only with the patient sera (Fig. 1).

Initial characterization of immunopositive clones. Two types of analysis were carried out, restriction enzyme digestion (RED) and Western blotting.

(i) RED analysis. To determine if clones were unique, cosmid DNAs from the 75 clones were digested with *Eco*RI and/or *Hin*dIII. Clones that had 70% or more bands in common were considered to have overlapping inserts. Fifty-two clones were thus considered to have potentially distinct inserts. Some of these clones may have small overlaps (less than 70% of the fragments) which would have been missed. pBeloBAC11 and pLAFRx clones have different insert sizes (30 to 45 and 20 to 30 kb, respectively), so the pLAFRx clones often gave fewer fragments, and it was difficult to determine if any of the BO clones overlapped with the LO clones. Thus, the actual number of clones with nonoverlapping inserts was lower than 52. Interestingly three BO clones and four B52 clones (the only seven clones that reacted with all four patient sera but not the rabbit serum) showed similar RED patterns, suggesting that they may have come from a conserved region on the chromosomes of the two strains.

(ii) Western blot analysis. Thirty-four clones that showed strong immunoreactivity in the immunoscreens were selected for Western blot analysis with the aim of identifying the antigen(s) they produced. Because the two sera had background reactivity with *E. coli* even after adsorption, only those with bands that were not seen in the *E. coli* controls are discussed. Differences in the number and intensity of antigen bands were observed with protein samples from the same clone but prepared by different methods. Some cosmid clones were obviously positive in the immunoscreens, but did not show any clear antigen bands on the Western blots. Various reasons could account for the last observation; for example, the bands could be masked by *E. coli* bands (which were present even after preadsorption of sera), there could be differential expression in liquid culture and on solid agar, the antigens might be highly hydrophobic and did not dissolve into the solutions

readily, or the antigens were not proteins. Because of these difficulties, the results of Western blot analysis were not considered in choosing cosmid clones for further subcloning.

Several interesting observations were made. The supernatant protein preparation of clone LO-4E1I showed several bands on the Western blot with the rabbit serum r17 (data not shown), suggesting that this clone produces secreted antigens, in agreement with the diffuse immunostain observed in immunoscreening. The three BO clones and four B52 clones that reacted with only the patient sera showed identical but rather unusual Western patterns. No sharp distinct bands were seen, but instead the blots showed a dark smear throughout the lanes (see Fig. 3).

Subcloning of antigen-encoding genes from immunopositive cosmid clones. Thirty-eight cosmid clones which reacted with at least two patient antisera and did not show similar RED patterns were chosen for subcloning. Each cosmid was randomly degraded with DNase I, and the fragments were cloned into the high-copy-number expression vector pBluescript SK(−). Twenty-five of the 38 cosmid clones gave 53 immunopositive subclones, while 13 did not produce any positive subclones (Fig. 1). The 53 immunopositive subclones (YX subclones) were subjected to DNA sequencing.

Analysis of DNA sequences of immunopositive subclones. Fourteen YX subclones did not show any significant homology to any sequences in the databases and may be new genes. Sequences from 39 YX subclones showed similarity to bacterial virulence factors, transporters/binding proteins, proteins involved in the degradation or the integrity of bacterial cell envelope, proteins involved in bacterial metabolism, regulators of gene expression, membrane proteins of unknown function, and hypothetical proteins (Table 2). To determine if these sequences could be expressed in our cloning system, we examined the reading frames in each BLASTX output file and found that 77% of the coding sequences either were in frame with *lacZ* in pBluescript SK(−) or could have their own translational start sites. We also note that the DNase I treatment led to fairly random degradation of cosmid DNA; for example, 15 YX subclones encoding the previously reported *E. faecalis* autolysin were mapped to different regions of the autolysin (Fig. 2).

A number of sequences were similar to those of members of the bacterial ABC (ATP-binding cassette) transporter family. One end of the YX1 (from LO-4E1I) insert showed homology to the N terminus of the glutamine transport ATP-binding protein Q (GlnQ) of *Methanococcus jannaschii* and other ATP-binding proteins of the ABC transporter family. One of the two consensus sequences of the ABC, GXGKST, was found in the sequence. An ATG start codon was found about 130 bp upstream of the consensus site, and a putative ribosome-binding site (GGAGG) was found upstream of the ATG. The other end of YX1 showed low similarity to the envelope protein EnvC, a cytoplasmic membrane lipoprotein of *E. coli* which affects septum formation and membrane permeability (41). A potential stop codon (TAG) for the *envC* homolog was found immediately upstream of the ribosome-binding site for the *glnQ* homolog, with the last G of the stop codon being the first G in the GGAGG sequence. It is likely that the two genes are in an operon.

Sequences from YX87 and YX89 (both from BO-2G8I) showed similarity to the transmembrane protein LPLB of *Bacillus subtilis*. The function of this protein is unknown, but it shows sequence similarity to the integral membrane components of the MalFG family, which belongs to the sugar-binding family of the bacterial ABC transporters.

Sequences from the YX91 T7 end (from B52-8C12) and YX103 T3/T7 ends (from BO-2D12II) showed significant similarity to sequences of members of the bacterial extracellular solute-binding protein family 3 of the bacterial ABC transporter family (74), which binds polar amino acids and opines. The ones with the highest BLASTX scores were the probable amino acid ABC transporter binding protein of *B. subtilis* (64), the high-affinity periplasmic glutamine-binding protein of *Salmonella typhimurium* (42), and FliY from the *fliAZY* operon of *E. coli* (60). Sequences from both ends of YX103 contain GF(DE)(LIV)DLX3(LIVM)(CA)(KE) and P(SA)LX2(KG)X2D(LIVMA)3(SA)(GAS)(LIVM), the two signature sequences of family 3 proteins (74). The relevant DNA sequences from the two ends of YX103 and their corresponding amino acid sequences were compared by using the Bestfit program to see whether the sequences were the same. They were found to be 70% identical and 71% similar at the DNA level and 66% identical and 78% similar at the amino acid sequence level. YX103 has a 2.3-kb insert which has enough room to contain two genes similar in size to *glnH* and *fliY* and maybe a third, small gene in between. It is not certain which part of the YX103 insert (or the whole insert) was responsible for the immunoreactivity of YX103. The region of similarity between YX91T7 and the proteins in family 3 did not overlap with those of YX103.

The T3 end of YX91 showed significant similarity to the hyaluronate synthase of *Streptococcus equisimilis* (47), some oligopeptide-binding proteins in the extracellular solute-binding protein family 5 of the ABC transporters, including the oligopeptide transport system substrate-binding protein of *B. subtilis* (63, 66), and several pheromone-binding proteins of *E. faecalis* (67, 75). The insert of YX91 is about 3 kb, and there may be room for a third gene. As with YX103, it is not certain which gene(s) in the YX91 insert caused its immunoreactivity.

Of the sequences similar to those of the virulence genes listed in Table 2, the YX50 (from BO-1A4I) insert was found to contain a partial coding sequence of the previously identified *E. faecalis* endocarditis antigen EfaA (51), which showed extensive sequence homology with some adhesins from various streptococci including FimA from *S. parasanguis*, SsaB from *S. sanguis*, ScaA from *S. gordonii*, and PsaA from *S. pneumoniae*. The sequence from YX42 (from LO-6H9II) showed significant similarity to those of the 20-kDa proteins (ORF3) encoded by the open reading frames immediately downstream of the streptococcal adhesin genes *fimA*, *ssaB*, *scaA*, and *psaA*. The only bacterial sequence that appeared in the BLASTX output with the sequence from YX5 (from LO-4E1I) was PepM49, the N-terminal 143-amino-acid fragment of the major virulence factor, M protein, of *S. pyogenes* (38).

Fifteen YX subclones from four cosmid clones (LO-6E6II, BO-4G2I, BO-4G1II, and BO-4F3II) contained partial sequences of the previously reported autolysin gene of *E. faecalis* (5), which encodes a cell wall hydrolase (*N*-acetylmuramoyl-L-alanine amidase). The sequences were mapped to different regions of the autolysin gene, mostly in the central and C-terminal regions (Fig. 2). None of the clones had the 5′ sequence, suggesting that the N terminus may be toxic to *E. coli*. Seven YX subclones from three cosmid clones (LO-4E1I, BO-1G2I, and B52-7A8) showed significant homology to the P54 protein of unknown function from *E. faecium* (23).

The sequence from YX84 (from B52-7A8) is similar to those of several response regulators of the two-component regulator family, including PfeR of *Pseudomonas aeruginosa* (15), OmpR of *Campylobacter jejuni*, RstA of *E. coli* (65), and CpxR of *E. coli* (17). The response regulators have three consensus sites around residues that correspond to Asp-13, Asp-57, and Lys-

TABLE 2. BLAST search results

Subclone(s)[a]	Parent cosmid	BLAST hit[b]	Organism	Probability[c]	Reference
Transport/binding					
YX1 (T3)	LO-4E1	Glutamine transport ATP-binding protein Q (*glnQ*)	*Methanococcus jannaschii*	6.7e-06	
YX87 (T7), YX89 (T7)	BO-2G8I	Transmembrane protein LPLB (*lplB*)	*Bacillus subtilis*	6.1e-19	
YX91 (T3)	B52-8C12	Hyaluronate synthase (*has*)	*Streptococcus equisimilis*	6.5e-11	47
YX91 (T7)	B52-8C12	Probable amino acid ABC transporter (*orf1*)	*Bacillus subtilis*	2.3e-06	64
YX103 (T3)	BO-2D12II	High-affinity periplasmic glutamine binding protein	*Salmonella typhimurium*	7.8e-44	42
YX103 (T7)	BO-2D12II	FliY (*fliY*)	*Escherichia coli*	1.2e-27	60
Virulence					
YX50	BO-1A4I	*E. faecalis* endocarditis antigen EfaA (*efaA*)	*Enterococcus faecalis*	1.3e-26	51
YX42	LO-6H9II	20-kDa protein in the SSAB[e] 3′ region (*orf3*)	*Streptococcus sanguis*	2.4e-31	25
YX5	LO-4E1I	PepM49	*Streptococcus pyogenes*	0.997[d]	38
Cell envelope/surface					
YX1 (T7)	LO-4E1I	Envelope protein EnvC (*envC*)	*Escherichia coli*	1.6e-03	41
YX6-12, YX16-17	LO-6E6II	Autolysin	*Enterococcus faecalis*	5.6e-66	5
YX39	BO-4G2I	Autolysin	*Enterococcus faecalis*	6.3e-31	5
YX66, YX74	BO-4G1II	Autolysin	*Enterococcus faecalis*	1.9e-140	5
YX104-105, YX107	BO-4F3II	Autolysin	*Enterococcus faecalis*	1.7e-107	5
YX2, YX3	LO-4E1I	P54	*Enterococcus faecium*	3.2e-26	23
YX36, YX37	BO-1G2I	P54	*Enterococcus faecium*	5.0e-52	23
YX79 (T3), YX81 (T7), YX82 (T7)	B52-7A8	P54	*Enterococcus faecium*	1.2e-12	23
Regulators					
YX84	B52-7A8	PfeR (*pfeR*)	*Pseudomonas aeruginosa*	3.9e-06	15
YX87 (T3)	BO-2G8I	Transcriptional regulator in the ILVO-IBPB intergenic region	*Escherichia coli*	6.2e-06	
Metabolism					
YX77	B52-5F7	Phosphoribosylaminoimidazole carboxylase (*purK*)	*Bacillus subtilis*	2.5e-33	18
YX95	B52-7C9	Dihydrolipoamide acetyltransferase (*pdhC*)	*Enterococcus faecalis*	3.0e-86	1
YX108, YX109	B52-4H1	Dihydrolipoamide acetyltransferase (*pdhC*)	*Enterococcus faecalis*	1.9e-84	1
Hypothetical					
YX4	LO-4E1I	N15OR, N15NR	*Bacillus subtilis*	1.2e-20, 2.6e-07	
YX98 (T7), YX102 (T7)	BO-2B8I	Hypothetical protein 2 (*orf2*)	*Lactobacillus leichmannii*	4.8e-20	
YX106 (T7)	BO-4F3II	Hypothetical protein 2 (*orf2*)	*Lactobacillus leichmannii*	0.29	

[a] Primer end is indicated in parentheses. For subclones without T3 or T7 in parentheses, sequences from both ends matched the same protein or the sequence from one end covers the entire insert.
[b] Sequence with the highest BLASTX score.
[c] If multiple sequences from the same cosmid showed homology to the same gene in the database, only the highest probability value is listed.
[d] See Discussion.
[e] SSAB, *Streptococcus sanguis* adhesin B.

109 in CheY (72). The YX84 sequence contains the conserved regions around the two aspartate residues.

The sequence from YX77 (from B52-5F7) showed significant similarity to that of the phosphoribosylaminoimidazole carboxylase (PurK) of *B. subtilis* (18) and other organisms, which is involved in purine biosynthesis. The clone has a small insert that allows no room for another gene, indicating that the protein is responsible for the immunoreactivity of YX77.

Clones YX95 (from B52-7C9) and YX108 and YX109 (from B52-4H1) encode the previously published dihydrolipoamide led;9qtransferase (E2 chain of the pyruvate dehydrogenase multienzyme complex [PDC]) encoded by *pdhC* of *E. faecalis* (1). The enzyme has two lipoyl domains at the N terminus, a peripheral subunit-binding domain that interacts with other enzymes in the PDC complex, and a C-terminal catalytic domain. All three clones had only the lipoyl domain sequence.

The other database similarities observed were less informative. For example, the function of hypothetical protein 2 of *Lactobacillus leichmannii* is not known. The putative transcriptional regulator in the ILVO-IBPB intergenic region of *E. coli* and N15OR and N15NR of *B. subtilis* were all sequenced in genome sequencing projects.

A nonprotein antigen in clone BO-4B6I. BO-4B6I is one of the seven clones that reacted only with the four patient sera and not the rabbit serum. Because the antigen(s) encoded by cosmid clone BO-4B6I ran anomalously in SDS-PAGE and we

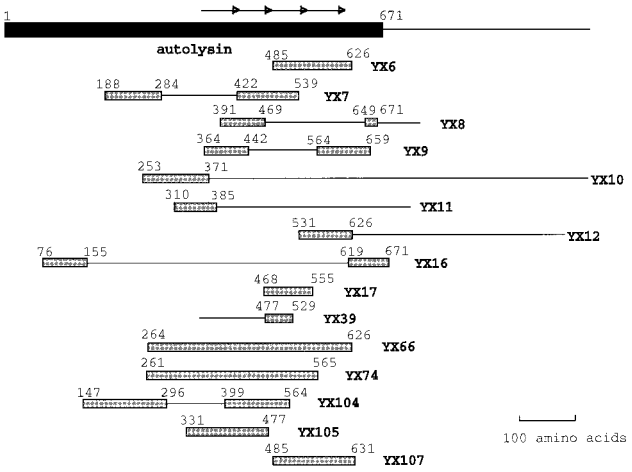

FIG. 2. Mapping of 15 subclones to the *E. faecalis* autolysin gene and downstream region. Arrows, four direct repeats in the C-terminal region of the autolysin; boxes, sequences from the subclones that matched the autolysin gene based on sequence comparison. The positions of amino acids are indicated. Regions of the inserts that have not been sequenced and sequences mapped to regions outside the autolysin gene are represented by lines.

did not obtain immunopositive subclones from the cosmid, it seemed possible that this clone did not encode a protein antigen. To test this, extracts from this cosmid, as well as *E. faecalis* OG1RF and the *E. coli* recombinant clone LO-6E6II, which showed clear antigen bands with serum h17, were treated with proteinase K and subjected to Western blot analysis. As shown in Fig. 3, all antigens were sensitive to proteinase K except the extract from clone BO-4B6I. Given the broad specificity of proteinase K, this clone most likely encodes a nonprotein antigen.

DISCUSSION

Surface components of bacteria play important roles in adherence and colonization, adaptive responses to changes in the environment, resistance to the host immune system, and other interactions with the host. The aims of the approach described here were to identify factors important in enterococcal infections by characterizing antigens detected with sera from enterococcal endocarditis patients. We anticipated that the antigens would be mainly surface and secreted proteins, but they might also be other proteins, for example, intracellular proteins released by cell lysis. Our results showed the latter class to be minor. Critical virulence factors may be among the most ubiquitous antigens, although it is recognized that not all virulence factors are immunogenic. Using the approach we described for auxotrophic mutants (48), it will be possible to construct knockout mutants of *E. faecalis* by disruption of the antigen-encoding genes reported here. These mutants can be used to assess the importance of each function in infection models, cell adherence assays, macrophage susceptibility tests, and other procedures. Probes can be made to intragenic regions of antigen-encoding genes to examine their presence, and immunoassays can be performed to detect their expression in other strains and clinical isolates of *E. faecalis*. The most important factors defined can then be studied in greater detail to understand their role in infection and develop serodiagnostic and therapeutic strategies.

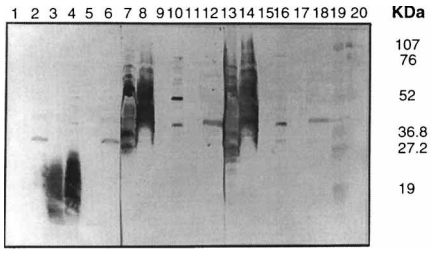

FIG. 3. Proteinase K resistance of BO-4B6I. Extracts were prepared and treated with either proteinase K or PMSF as described in Materials and Methods. Lanes 1 to 6, r17 rabbit serum; lanes 7 to 12, h17 serum; lanes 13 to 18, h1 serum. Lanes 1, 7, and 13, BO-4B6I plus proteinase K; lanes 2, 8, and 14, BO-4B6I plus PMSF; lanes 3, 9, and 15, LO-6E6II plus proteinase K; lanes 4, 10, and 16, LO-6E6II plus PMSF; lanes 5, 11, and 17, *E. faecalis* OG1RF plus proteinase K; lanes 6, 12, and 18, OG1RF plus PMSF. Lanes 19 and 20, molecular weight standards.

Several studies have shown differences between bacteria grown in vivo and in vitro (in laboratory media). Among genes expressed in vivo are those of particular importance to pathogenicity. Besides clones that reacted with the rabbit serum and some or all of the human sera, we identified 16 clones that reacted only with the patient sera and not with the rabbit serum. Because the rabbit serum r17 was raised against a surface protein preparation of TX17 (an *E. faecalis* endocarditis isolate) grown in vitro, these clones may contain an antigen(s) expressed only in vivo. Of the 16 clones, 7 reacted with all four patient sera. Interestingly, these seven clones (from two different *E. faecalis* strains) shared highly similar DNA restriction fragments and showed identical Western blot patterns, indicating that they encode the same antigen(s). We note that since some of the cosmid clones encode multiple antigens, for example LO-4E1I, it is possible that additional in vivo-specific antigens are encoded among the clones that reacted with r17 as well as the patient sera.

DNA sequence analysis of the 53 YX subclones from 25 immunopositive cosmid clones revealed similarities to transporters/binding proteins, bacterial virulence factors, proteins involved in bacterial metabolism, proteins involved in the degradation or the integrity of bacterial cell envelope, regulators of gene expression, membrane proteins of unknown function, as well as hypothetical proteins. Thirteen cosmid clones did not produce any immunopositive subclones. In some cases it was possible that the synthesis of the antigenic material required several genes as in capsule production or the antigen-encoding genes were unstable in a high-copy-number vector.

Some subclones showed similarity to one protein over their entire inserts; for example, the insert of YX50 encodes an region of EfaA only. In this case, it is clear that the sequences giving the BLASTX matches were responsible for the immunoreactivity of the subclones. It was more difficult to draw such a conclusion for the other subclones. For example, YX1, YX91, and YX103 each showed similarity to two membrane or surface proteins, which may all be potential antigens. YX87 encoded sequences similar to that of the transmembrane protein LPLB and a putative transcriptional regulator, it seems more likely that the LPLB homolog was the antigen. YX98 and YX102 were both similar to the hypothetical protein 2 at the T7 end, and no significant homology was found at the T3 end. Thus, the antigen-encoding sequence(s) of these subclones was not clear.

A number of sequences showed similarity to components of the bacterial ABC transporter superfamily (19). ABC transporters play an important role and need not be critical for virulence. However, there is precedent for ABC transport systems to be responsible for the secretion or regulation of virulence factors, for example, the RTX toxins (29, 30). In fact, the first described ABC exporter in gram-positive bacteria was CylB of *E. faecalis* (26). Mutations in CylB prevent the secre-

tion of component L, one of the components of the hemolysin/bacteriocin determinant of *E. faecalis* (26). FimA, the adhesin of *S. parasanguis*, has also been suggested to be a part of an ABC transporter system (21) and may be involved in both adhesion and transport of an unknown substrate.

The bacterial ABC importers require an additional extracellular solute-binding protein (permease). In gram-positive bacteria, the extracellular solute-binding proteins are lipoproteins anchored to the external surface of the cell membrane by their lipid component. Although they have similar structures, the biological effects of their functions involve diverse aspects of bacterial physiology, among which are sporulation, conjugation, chemoreception, virulence, metabolic reactions, and nutrient uptake (74). The involvement in bacterial virulence is of particular interest. In *Listeria monocytogenes*, the ABC arginine permease (Arp) encoded by *arpJ* was found to be induced over a 100-fold when grown inside J774 (macrophage-like) cells. A mutation in *arpJ* resulted in a twofold higher 50% lethal dose than that of the wild-type strain by intravenous injection of mice and reduced bacteria counts in the livers of mice at 48 h after injection (40). In *S. pneumoniae*, screening for the inability to adhere to human cells among mutants with defects in exported proteins resulted in four mutants with an over 50% decrease in adherence to epithelial and endothelial cells (14). Sequence analysis of the four mutants showed that the mutations were in two loci, *plpA* and *ami*, both of which encode peptide permeases, suggesting that peptide permeases modulate adherence either by acting directly as adhesins or by regulating the expression of adhesins in *S. pneumoniae.*

A major antigen associated with *E. faecalis* endocarditis, EfaA, was also identified by our method (YX50). EfaA is strongly homologous with a group of streptococcal adhesins (51), some of which have been shown to be associated with bacterial adhesion (2, 8, 20, 25), suggesting that EfaA can be a potential adhesin of *E. faecalis*. A study using a rat endocarditis model also suggested that FimA may play a role in the initial colonization of damaged heart tissue by *S. parasanguis* and thus may be a major virulence factor for *S. parasanguis* endocarditis (8). The streptococcal adhesin genes are all within operons with similar gene organization and encode ABC transporters (21, 25, 43, 69). The sequence from YX42 showed strong similarity those of the open reading frames 3′ of the adhesin genes which have extensive homology with each other and with the ToxR-activated gene *tagD*. TagD is about 200 bp upstream of and coregulated with the pilus (*tcp*) gene cluster in *Vibrio cholerae* (32). Although their functions are unknown, the physical linkage to the adhesin genes and the similarity of the regulation mechanism to that of genes involved in pilus assembly suggest that they may play a role, possibly in the transport of proteins through bacterial membranes. The two cosmid clones BO-1A4I and LO-6H9II, the sources of YX50 and YX42, respectively, were mapped to different regions on the OG1RF chromosome (data not shown), suggesting that there may be two adhesin operons in OG1RF. It is also possible that the two genes are not physically linked as their homologs in streptococci.

The M protein is the major antiphagocytic factor of *S. pyogenes* and mediates adherence to keratinocytes (61). PepM49 is the N-terminal fragment (143 amino acids) of the M protein after specific proteolysis, has a typical alpha-helix structure, is responsible for the antigenic variation among M proteins, and seems to retain the opsonic antibody epitope of the M protein (39). It was the only bacterial sequence found in the BLAST search. BLASTX found two pairing segments of 23 and 32 amino acids, with similarities of 65 and 59%, respectively. Analysis, using the Bestfit program, of PepM49 and the amino acid sequence of YX5 showed a similarity of 42% over a 57-amino-acid region, and the region corresponding to the first 41 amino acids of PepM49 should start outside the YX5 insert. The low BLASTX probability value could be due in part to the short length of the pairing segment. It could also merely reflect a similar alpha-helical structure; however, because of the interest in this protein as a potential virulence factor, their relation bears further investigation.

A large number of YX subclones were found to encode autolysin, a cell wall hydrolase of *E. faecalis* (5). The autolysin of *E. faecalis*, the muramidase 2 of *Enterococcus hirae* (11), and the muramidase (AcmA) of *Lactococcus lactis* (7) are highly similar in their amino acid sequences, and all contain multiple direct repeats in the C-terminal region. Mutants defective in *acmA* grow in long chains, indicating that AcmA is involved in cell separation (7). Similar repeated units were also found in C-terminal regions of *B. subtilis* ØPZA and Ø29 lysozymes, *Staphylococcus aureus* protein A, and *Listeria monocytogenes* invasion-associated protein (also called the P60 protein, encoded by the *iap* gene) (36). No functional homology among these proteins has been suggested except for P60 of *Listeria monocytogenes*. Mutants impaired in the synthesis of P60 lost the ability to invade nonprofessional phagocytic 3T6 mouse fibroblasts, and the bacteria formed long chains. Treatment of the mutants with P60 led to the disaggregation of the long cell chains and restored the invasiveness of the mutants, while physical disruption of the chains into single cells did not restore their ability to invade (45), suggesting that P60 was involved in both invasion and cell separation.

The role of the *E. faecalis* autolysin in virulence has not been reported previously, but work on the pneumococcal autolysin (*N*-acetylmuramyl-L-alanine amidase) demonstrated that autolysin-negative strains of *S. pneumoniae* had significantly reduced virulence in mice (6). Moreover, immunization of mice with autolysin provided significant protection against challenge with virulent, wild-type *S. pneumoniae* (50). These results suggest the need for the further study of the *E. faecalis* autolysin as a virulence factor and possible target for therapeutic intervention.

P54 was identified in *E. faecium* through its cross-reaction with an antibody against an ATPase of *E. faecium*, and could be detected in the cell wall preparations of *E. faecium* (23). It bears extensive homology to the 45-kDa secreted protein of *Lactococcus lactis* (77) with unknown function. Because of the similarity to the 45-kDa secreted protein, it is possible that the *E. faecalis* P54 homolog is one of the secreted antigens observed in the Western blot analysis of LO-4E1I.

The sequence from YX84 was particularly similar to that of the response regulator PfeR of a two-component regulator system, PfeR/PfeS, of *P. aeruginosa*, which controls the expression of the ferric enterobactin receptor PfeA (15). PfeA is one of the iron-scavenging systems of *P. aeruginosa*. The importance of two-component regulators in bacterial virulence has been reviewed (56, 57). Examples include the *virA*/*virG* system of *Agrobacterium tumefaciens*, the *agr* locus of *Staphylococcus aureus*, and the *bvgA*/*bvgS* system of *Bordetella pertussis*. However, from sequence similarity, it is not clear what particular function the YX84 sequence encodes, and its contribution to virulence needs further study.

The similarities to two metabolic enzymes, PurK and PDC E2, encoded by *purK* and *pdhC* could be due to the release of intracellular proteins in cell lysis. Compared to the large number of clones showing homology to surface or potential surface proteins, these constitute only a small proportion of our pool of antigen clones. In the case of PDC E2, there could be a second explanation. It has been proposed that primary biliary

cirrhosis, a human autoimmune disease, may have a bacterial etiology (9, 22, 24, 31, 71). The autoantibodies from patients with primary biliary cirrhosis react with the lipoyl domains of the *E. coli* PDC E2 (24), in agreement with our finding that the three YX subclones contain sequences for the lipoyl domains of the *E. faecalis* E2. The question of whether the immunoreactivities of the three subclones were due to cross-reactions or are of etiological importance requires further investigation.

Finally, we note the intriguing nonprotein antigen(s) encoded by the seven cosmid clones that reacted only with the four patient sera. The anomalous behavior of the antigen in SDS-PAGE is consistent with a nonprotein antigen such as a carbohydrate. Thus, this clone may contain the gene(s) for synthesis of a nonprotein antigen such as a cell wall polysaccharide or capsular polysaccharide that is expressed during infection. The synthesis of these polysaccharides usually requires several genes either organized in a single operon or scattered around the chromosome. In *Bacillus anthracis*, the genetic region for encapsulation (the Cap region), when cloned in *E. coli*, enabled the *E. coli* recombinants to react with capsule-specific antiserum (54), demonstrating that exogenous gram-positive capsules can be produced in *E. coli*. The Cap region is about 3.2 kb, containing three genes, *capA*, *capB*, and *capC* (55). In *Staphylococcus aureus*, a 14.6-kb region with 13 open reading frames is required for the synthesis of a type 1 capsule (49). This would also be consistent with our inability to subclone the antigen, especially if a gene cluster was required for production of the antigen. This finding is particularly significant in view of the importance of cell surface polysaccharides in gram-positive infections.

The mechanisms of bacterial infections are very complex, involving multiple factors and different levels of regulation. The aim of the method we have described here was to identify the antigens of *E. faecalis* expressed during endocarditis infection. Because of the different natures of antigens, some of them will have been inevitably missed. However, for an organism whose infection mechanisms are not well known, the method has provided us with a good starting point. The antigens identified cover a wide range of categories, from known virulence factors, transporters, two-component regulators, and metabolic enzymes to possible cell surface polysaccharides. Each of these may contribute to the virulence of the organism. Most of the sequences encoding recognizable functions reported here were similar to those of membrane or surface proteins, supporting the premise of this approach. Also, this approach could be easily applied to other organisms for which few genetic techniques have been developed. Further studies on the antigen genes identified and cloned in this study should reveal additional features of the process of *E. faecalis* infection.

ACKNOWLEDGMENTS

This work was supported by grant AI 33516 from the PHS.

We are very grateful to Ronald Christopher Mackenzie for technical support in constructing the genomic and DNase I random libraries, performing DNA sequencing, and advice on sequence analysis; Kavindra V. Singh and Monjula Chidambaram for general technical support; H. Shizuya and Mel Simon at the California Institute of Technology, Pasadena, for providing the cosmid vector pBeloBAC11; and Beto Zuniga for technical support.

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Editor: P. E. Orndorff