Enterococcus faecalis Antigens in Human Infections

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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-
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 XLI1 Blue MRF were the host strains for cloning enterococcal DNA. OGI1RF is an *E. faecalis* strain previously described (59). TX2 is an *E. faecalis* endocarditis clinical isolate.

Cosmid vectors pbeloBAC11 and pLAFlR 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 containing the oriT and tetracycline resistance marker of RK2. pBlueScript SK(−) (Strategen, 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; isopropyl-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were used at 0.5 mM and 80 μM, respectively.

**Antiserum.** 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.

**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 OGI1RF and TX2 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 Sau3AI, 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 pLAFlR, respectively, which had been previously digested with BamHI 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 (Strategen) and used to infect *E. coli* DH5α, and antibiotic-resistant transformants were selected. Libraries with titers of 107 to 108 transformant-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 antibiotic selection (pEleoBAC11 and plasmids OGI1RF and pbeloBAC11). 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 lys 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* lyssates) 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-agarose beads (Sigma) was then used at 0.125 μl/g 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 in 1% of the original culture supernatant (100 μl of the supernatant, the tube was incubated on ice for 5 h 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-sD system (Millipore). The blots were incubated with blocking buffer for 2 h at room temperature and then incubated with the primary antiserum (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 immunoblotting.

**Protease 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 protease K (16.4 mg/ml; Boehringer Mannheim Corp., Indianapolis, Ind.) was added to one sample, and phenylmethylsulfonyl fluoride (PMSF; Sigma) (100 mM in 1 ml of PBS, pH 8.0) 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 6 and Western blotting.

**Screening 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 DNAse I (16) in 20 ml of 50 mM Tris-Cl, pH 7.4, for 2 h at 37°C. The reaction was stopped by adding EDTA to a final concentration of 10 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 EcoRV and treated with shrimp alkaline phosphatase. One-tenth of each ligation mixture was used to transform DH5α or XLI 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 1000 transformants from each sublibrary were screened with antisera 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-decay terminator method (Applied Biosystems, Foster City, Calif.) using primers for the T3 and/or T7 promoter regions on pBluescript SK(−) DNA sequencing templates) were performed.

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 BLASTN and BLASTX) network service at the National Center for Biotechnology Information to search for homologous sequences in the protein database.

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**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 cosmids 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 sera 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-4E11 (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 *Hind*III. 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.

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readily, or the antigens were not proteins. Because of these
difficulties, the results of Western blot analysis were not con-
sidered in choosing cosmids clones for further subcloning.

Several interesting observations were made. The superna-
tant protein preparation of clone LO-4E11 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 immu-
noscreening. 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 bands showed a dark smear throughout the lanes
(see Fig. 3).

Subcloning of antigen-encoding genes from immunopositive
cosmid clones. Thirty-eight cosmids clones which reacted with
at least two patient antisera and did not show similar RED
patterns were chosen for subcloning. Each cosmid was ran-
domly degraded with DNase I, and the fragments were cloned
into the high-copy-number expression vector pBluescript
SK(−). Twenty-five of the 38 cosmids clones gave 53 immu-
nopositive subclones, while 13 did not produce any positive
subclones (Fig. 1). The 53 immunopositive subclones (YX sub-
clones) 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 bacte-
rial 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 exam-
ined 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 transla-
tional 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-4E11) 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 ribo-
some-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 Bac-
illus subtilis. The function of this protein is unknown, but it
shows sequence similarity to the integral membrane compo-
nents 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-2D12I) showed significant similar-
ity to sequences of members of the bacterial extracellular
solute-binding protein family 3 of the bacterial ABC trans-
porter family (74), which binds polar amino acids and oligos.
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 Sal-
monella typhimurium (42), and FliY from the flaAZY operon of
E. coli (60). Sequences from both ends of YX103 contain
GDF(DE)(LIV)DLX3(LIVM)(CA)(KE) and P(SA)LX2(KG)
X2D(LIVMA)3(SA)(GAS)(LIVM), the two signature se-
quences of family 3 proteins (74). The relevant DNA se-
quences 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 glnH1 and fliY1 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 hy-
larionate synthase of Streptococcus equisimilis (47), some
oligopeptide-binding proteins in the extracellular solute-bind-
ing protein family 5 of the ABC transporters, including the
oligopeptide transport system substrate-binding protein of B.
subtilis (63, 66), and several periplasmic-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 identi-
fied F. enterolitica 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 strep-
tococcal 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 PepMP49, the
N-terminal 143-amino-acid fragment of the major virulence
factor, M protein, of S. pyogenes (38).

Fifteen YX subclones from four cosmids clones (LO-6E6I,
BO-4G2I, BO-4G1II, and BO-4F3II) contained partial se-
quences 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 (Fig. 2).

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 glnH1 and fliY1 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 hylarionate 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 periplasmic-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 F. enterolitica 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 PepMP49, the N-terminal 143-amino-acid fragment of the major virulence factor, M protein, of S. pyogenes (38).

Fifteen YX subclones from four cosmids clones (LO-6E6I, 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 S sequence, suggesting that the N terminus may be toxic to E. coli. Seven YX subclones from three cosmids 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 PleR 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-

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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 dehydrogenase (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.

### Table 2. BLAST search results

<table>
<thead>
<tr>
<th>Subclone(s)</th>
<th>Parent cosmid</th>
<th>BLAST hit</th>
<th>Organism</th>
<th>Probability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport/binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YX1 (T3)</td>
<td>LO-4E1I</td>
<td>Glutamine transport ATP-binding protein Q (glnQ)</td>
<td>Methanococcus jannaschii</td>
<td>6.7e-06</td>
<td></td>
</tr>
<tr>
<td>YX87 (T7), YX89 (T7)</td>
<td>BO-2G8I</td>
<td>Transmembrane protein LPLB (lpLB)</td>
<td>Bacillus subtilis</td>
<td>6.1e-19</td>
<td></td>
</tr>
<tr>
<td>YX91 (T3)</td>
<td>BS2-8C12</td>
<td>Hyaluronate synthase (has)</td>
<td>Streptococcus equisimilis</td>
<td>6.5e-11</td>
<td>47</td>
</tr>
<tr>
<td>YX91 (T7)</td>
<td>BS2-8C12</td>
<td>Probable amino acid ABC transporter (orf1)</td>
<td>Bacillus subtilis</td>
<td>2.3e-06</td>
<td>64</td>
</tr>
<tr>
<td>YX103 (T3)</td>
<td>BO-2D12II</td>
<td>High-affinity periplasmic glutamine binding protein</td>
<td>Salmonella typhimurium</td>
<td>7.8e-44</td>
<td>42</td>
</tr>
<tr>
<td>YX103 (T7)</td>
<td>BO-2D12II</td>
<td>FliY (fliY)</td>
<td>Escherichia coli</td>
<td>1.2e-27</td>
<td>60</td>
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<td>Virulence</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>YX50</td>
<td>BO-1A4I</td>
<td>E. faecalis endocarditis antigen</td>
<td>Enterococcus faecalis</td>
<td>1.3e-26</td>
<td>51</td>
</tr>
<tr>
<td>YX42</td>
<td>LO-6H9II</td>
<td>20-kDa protein in the SSAB region (orf3)</td>
<td>Streptococcus sanguis</td>
<td>2.4e-31</td>
<td>25</td>
</tr>
<tr>
<td>YX5</td>
<td>LO-4E1I</td>
<td>PepM49</td>
<td>Streptococcus pyogenes</td>
<td>0.997</td>
<td>38</td>
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<tr>
<td>Cell envelope/surface</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YX1 (T7)</td>
<td>LO-4E1I</td>
<td>Envelope protein EnvC (envC)</td>
<td>Escherichia coli</td>
<td>1.6e-03</td>
<td>41</td>
</tr>
<tr>
<td>YX6-12, YX16-17</td>
<td>LO-6E6II</td>
<td>Autolysin</td>
<td>Enterococcus faecalis</td>
<td>5.6e-66</td>
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<tr>
<td>YX39</td>
<td>BO-4G2I</td>
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<td>Enterococcus faecalis</td>
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<td>YX66, YX74</td>
<td>BO-4G1H</td>
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<td>YX104-105, YX107</td>
<td>BO-4F3II</td>
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<td>Enterococcus faecalis</td>
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<tr>
<td>YX2, YX3</td>
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<td>PS4</td>
<td>Enterococcus faecium</td>
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<tr>
<td>YX36, YX37</td>
<td>BO-1G2II</td>
<td>PS4</td>
<td>Enterococcus faecium</td>
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</tr>
<tr>
<td>YX79 (T3), YX81 (T7), YX82 (T7)</td>
<td>BS2-7A8</td>
<td>PS4</td>
<td>Enterococcus faecium</td>
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<td>Regulators</td>
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</tr>
<tr>
<td>YX84</td>
<td>BS2-7A8</td>
<td>PfeR (pfeR)</td>
<td>Pseudomonas aeruginosa</td>
<td>3.9e-06</td>
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</tr>
<tr>
<td>YX87 (T3)</td>
<td>BO-2G8I</td>
<td>Transcriptional regulator in the ILVO-IBPB intergenic region</td>
<td>Escherichia coli</td>
<td>6.2e-06</td>
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<td>Metabolism</td>
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<td></td>
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<tr>
<td>YX77</td>
<td>BS2-5F7</td>
<td>Phosphoribosylaminomimidazole carboxylase (purK)</td>
<td>Bacillus subtilis</td>
<td>2.5e-33</td>
<td>18</td>
</tr>
<tr>
<td>YX95</td>
<td>BS2-7C9</td>
<td>Dihydrolipoamide acetyltransferase (pdhC)</td>
<td>Enterococcus faecalis</td>
<td>3.0e-86</td>
<td>1</td>
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<tr>
<td>YX108, YX109</td>
<td>BS2-4H1</td>
<td>Dihydrolipoamide acetyltransferase (pdhC)</td>
<td>Enterococcus faecalis</td>
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<td>Hypothetical</td>
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<tr>
<td>YX4</td>
<td>LO-4E1I</td>
<td>N15OR, N15NR</td>
<td>Bacillus subtilis</td>
<td>1.2e-20</td>
<td>2.6e-07</td>
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<tr>
<td>YX98 (T7), YX102 (T7)</td>
<td>BO-2B8I</td>
<td>Hypothetical protein 2 (orf2)</td>
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<tr>
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<td>Hypothetical protein 2 (orf2)</td>
<td>Lactobacillus leichmannii</td>
<td>0.29</td>
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</tr>
</tbody>
</table>

* 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.

* Sequence with the highest BLASTX score.

* If multiple sequences from the same cosmid showed homology to the same gene in the database, only the highest probability value is listed.

* See Discussion.

* SSAB, Streptococcus sanguis adhesin B.
did not obtain immunopositive subclones from the cosmids, it
seemed possible that this clone did not encode a protein anti-
gen. 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 pro-
teinase K, this clone most likely encodes a nonprotein antigen.

DISCUSSION

Surface components of bacteria play important roles in ad-
herence 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 infec-
tions by characterizing antigens detected with sera from en-
terococcal endocarditis patients. We anticipated that the anti-
gen(s) expressed only in vivo. Of the 16 clones, 7 reacted with
test sera and not with the rabbit serum. Because the rabbit serum r17 was raised against
a surface protein preparation of TX17 (an E. faecalis endocar-
ditis isolate) grown in vitro, these clones may contain an anti-
gen(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 pat-
tterns, indicating that they encode the same antigen(s). We note
that since some of the cosmids 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 cosmids clones revealed similarities to trans-
porters(binding proteins, bacterial virulence factors, proteins
involved in bacterial metabolism, proteins involved in the de-
gradation or the integrity of bacterial cell envelope, regulators
of gene expression, membrane proteins of unknown function,
as well as hypothetical proteins. Thirteen cosmids 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 immu-
noreactivity 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(s) of these subclones was not clear.

A number of sequences showed similarity to components of
the bacterial ABC transporter superfamily (19). ABC trans-
porters play an important role and need not be critical for
virulence. However, there is precedent for ABC transport sys-
tems to be responsible for the secretion or regulation of viru-
ulence 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-1A41 and LO-6H9III, 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 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-4EII.

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 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 OPZA and O29 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.
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circosis, 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 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 in separate genetic regions. The synthesis of a nonprotein antigen such as a cell wall polysaccharide or capsular polysaccharide that is expressed during infection is usually the result of a genetic region for encapsulation (the Cap region), when several genes are essential for both initiation and maintenance of the polysaccharide chain. 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 virulence 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 antigen identified covers 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.

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