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Tissue-Specific Adherent *Enterococcus faecalis* Strains That Show Highly Efficient Adhesion to Human Bladder Carcinoma T24 Cells Also Adhere to Extracellular Matrix Proteins

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The ability of *Enterococcus faecalis* clinical isolates to adhere to immobilized extracellular matrixes (ECMs) coating the walls of microtiter plates was examined by microscopy. The ECMs consisted of fibronectin, laminin, collagen types I, II, IV, and V, fibrinogen, and lactoferrin. With the exception of fibrinogen, each isolate showed a different level of adherence to each of the ECMs. No significant level of adherence to fibrinogen was observed for any isolate. The tissue-specific adhesive strains AS11, AS12, AS14, AS15, HT11, and HT12, which showed highly efficient adherence to human bladder carcinoma T24 cells and human bladder epithelial cells, showed strong adherence to fibronectin, laminin, and collagen type I, II, IV, and V ECMs, and the levels were greater than 10^4 cells/mm^2 of well surface coated by ECM. None of the isolates that showed little adherence to human bladder carcinoma T24 cells showed efficient adherence to all the ECMs. The levels of adherence of gelatinase-producing isolates to the collagens were lower than the levels of adherence of gelatinase-negative isolates. When tissue-specific adhesive strains that adhered strongly to each ECM were preincubated with fibronectin, the adherence of the strains to fibronectin was inhibited, but the adherence of the strains to collagen type IV was not inhibited. Likewise, preincubation with collagen type IV inhibited adherence to collagen type IV but not adherence to fibronectin. All of the *E. faecalis* isolates were shown to carry the *ace* gene by PCR analysis performed with specific primers for collagen binding domain A of *ace*. The *ace* gene encodes Ace (adhesin of collagen from enterococci). The *prtF* gene of group A streptococci, which encodes the fibronectin binding protein of group A streptococci, was not detected in the tissue-specific adhesive strains by Southern analysis performed with the *prtF* probe of the *Streptococcus pyogenes* JRS4 strain. Mutants with altered collagen binding were isolated by insertion of Tn*916* into the chromosome of tissue-specific adhesive strain AS14. The number of mutant adhesive bacterial cells that adhered to collagen and also to laminin was 1 or 2 orders lower than the number observed for the wild-type strain, but the level of adherence to fibronectin remained the same as that of the wild-type strain.

Enterococci are commensal organisms found in humans and livestock, and they colonize the gastrointestinal tract, vaginal cavity, or oral cavity as part of the normal flora. Enterococci are opportunistic pathogens that cause infections in patients compromised by severe underlying disease. Enterococci are the major organisms that cause hospital-acquired infections, such as urinary tract infections, bloodstream infections, endocardium infections, wound infections, and infections related to the use of implanted devices (14, 15, 19, 21, 24, 25, 26, 43).

Enterococci are recognized as facultative pathogens which possess an intermediate level of virulence compared to streptococci and lactococci (1, 10). Secreted substances or substances (adhesins) in the bacterial cell surface of *Enterococcus faecalis* could act as virulence factors that undermine the commensal relationship in a compromised host. Substances encoded by the *E. faecalis* pheromone-responding plasmids are related to the virulence of *E. faecalis* (2, 3, 4, 6, 10, 17, 21). The pheromone-responsive plasmid pAD1 or pAD1-like plasmids encode a β-homolysin–bacteriocin (cytolysin [Cyl]) mediated by the same genetic determinant (11, 17, 20). The cytolysin is also encoded on the chromosome (16), and it contributes to the pathogenesis of *E. faecalis* infection in animal models (10, 19). In addition to cytolysin, the aggregation substance, which is encoded in pAD1 and is produced on the cell surface by induction of *E. faecalis* carrying pAD1 with pheromone (2, 6, 7, 41), has been shown to enhance adherence to renal tubular cells in a cell culture model, and it has also been shown to enhance internalization by cultured intestinal epithelial cells (23, 28).

Infecting microorganisms adhere to tissues of the host in the initial event of most infectious diseases. Many pathogenic bacteria adhere to extracellular matrixes (ECMs) via adhesins located on the bacterial cell surface (29). The ECMs of mammalian tissues are composed of a variety of macromolecules, including structural glycoproteins (i.e., collagen, laminin, fibronectin, fibrinogen, and lactoferrin) and proteoglycan (39). Collagen, laminin, and fibronectin are constituents of ECMs, and collagen is the major macromolecule and the single most abundant protein among these constituents (39). When present as a commensal organism, *E. faecalis* might possess many factors that are capable of interaction with the mucosal surfaces of host cells. It is highly probable that some of these factors may contribute to, or develop as, factors for adherence

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to the ECM layer of host cells during *E. faecalis* infections. Studies of the adherence of *E. faecalis* to ECMs are limited. In previous studies of the adherence of *E. faecalis* isolates to ECMs, workers have examined the binding of the isolates to soluble or immobilized ECMs (10). Reports of the adherence of *E. faecalis* isolates to each of the different ECMs have shown that there are a variety of phenotypes among the different strains (10). One of the *E. faecalis* adhesins that bind to ECMs, the collagen/laminin binding adhesin, has been identified, and the determinant (*ace*) has been cloned and characterized (27, 30, 40). The *E. faecalis ace* gene usually encodes a putative 31-amino-acid N-terminal signal sequence, followed by a 335-amino-acid nonrepetitive A domain, a B domain composed of various numbers of tandemly repeated 47-residue units with >90% identity, a cell wall-associated domain rich in proline residues that contains the cell wall-anchoring LPXTG consensus sequence, and a hydrophobic transmembrane region consisting of 18 amino acids, followed by a short cytoplasmic tail representing the carboxy-terminal end of the protein. The *ace* gene is present in all of the *E. faecalis* isolates that have been examined (30). The phenotypes for adherence of the isolates to immobilized collagen are different for the individual isolates (27, 40).

It is highly probable that the levels or phenotypes of adherence to the host tissue are different or variable for different *E. faecalis* isolates, and multiple factors might be necessary for tissue-specific adherence by *E. faecalis*. Scanning electron microscopy has been used to quantify the adherence of *E. faecalis* clinical strains, and strains capable of highly efficient adherence to human culture cells have been identified among clinical isolates (34). In this study, strains that show highly efficient adhesion to human culture cells and other clinical isolates were examined for the ability to bind to the ECMs.

MATERIALS AND METHODS

Bacteria and medium. A total of 43 *E. faecalis* clinical strains were used in this study. Some of these 43 strains (strains AS11, AS12, AS14, AS15, HT11, and HT12) are tissue-specific adherent strains that show highly efficient adhesion to human bladder carcinoma T24 cells (34). The other strains are strains that exhibit inefficient adhesion to human bladder carcinoma T24 cells. The laboratory strains used were *E. faecalis* FA2-2 (Rif^r Fus^r) (4), *E. faecalis* OG1S (gelatinase positive) (6), *E. faecalis* OG1X, which is an isogenic gelatinase-negative derivative of OG1S (18), and *Streptococcus pyogenes* strain JRS4 (13, 32). The medium used in this study was Todd-Hewitt broth (Difco).

Preparation of bacterial cells for adherence assay. An *E. faecalis* strain was cultured overnight in Todd-Hewitt broth at 37°C, and the cells were washed three times with phosphate-buffered saline (PBS) and suspended at concentrations of 3×10^8 to 6×10^8 cells per ml in PBS containing 0.1% Tween 80 and 0.1% bovine serum albumin (BSA).

Assay of adherence to ECM proteins. ECMs were immobilized on the surfaces of microtiter dish wells as previously described (40). Each ECM solution contained 20 μg of ECM per ml of PBS. A 50-μl ECM solution was added to a microtiter well to coat the surface with ECM, and the microtiter dish was then incubated at 4°C overnight. The solution was decanted, blocked with 200 μl of 0.2% BSA in PBS at 4°C for 2 h, and washed three times with PBS. A 50-μl bacterial suspension was added to each well and incubated for 2 h at room temperature. The wells were washed three times with PBS containing 0.1% Tween 80 and 0.1% BSA. The number of bacterial cells that adhered to the surface of a well was determined with a microscope (Nikon, Tokyo, Japan). Five fields of vision were chosen at random for microscopic observation of each well surface, and the number of bacteria in each field was determined. Each experiment was performed more than three times.

Generation of Tn*916* insertion mutants with altered adherence to collagen. Derivatives produced by insertion of Tn*916* into *E. faecalis* AS14 were generated as previously described (9). *E. faecalis* CG110 (9) was used as a donor in a 4-h filter mating with the recipient strain *E. faecalis* AS14SS, which is a streptomycin- and spectinomycin-resistant derivative of *E. faecalis* AS14 (34). Transconjugants were selected on Todd-Hewitt agar plates containing 6 μg of tetracycline per ml, 500 μg of streptomycin per ml, and 250 μg of spectinomycin per ml. To avoid selecting derivatives originating from the same mutant and to select mutants in which Tn*916* was inserted into different sites, 6,200 independent mating experiments were performed, and one transconjugant was selected at random from each experiment, which resulted in 6,200 *E. faecalis* AS14SS::Tn*916* derivatives. The adherence of each of the *E. faecalis* AS14SS::Tn*916* derivatives to collagen type IV was examined.

Probe for *prtF* (determinant for fibronectin binding protein of *S. pyogenes*). Plasmids pPTF5 (12) and pPTF54 (33) were kindly provided by M. Caparon and N. Okada. Plasmid pPTF5 contains the 2.9-kb EcoRV fragment of *S. pyogenes* JRS4 chromosomal DNA, which encodes the *prtF* determinant (12, 13). Plasmid pPTF54 contains a 0.6-kbp PCR product which encodes the fibronectin binding domain of *prtF* of *S. pyogenes* JRS4 (33).

Analysis of inhibition of adherence. An *E. faecalis* strain was cultured overnight at 37°C, washed three times with PBS, and suspended in PBS at a concentration of 3×10^8 to 6×10^8 cells per ml. Then 100 μl of ECM protein (100 μg/ml) was added to 100 μl of bacterial cells and incubated for 1 h at 25°C (room temperature). After incubation, the cells were washed three times with PBS and then suspended in PBS containing Tween 80 (0.1%) and BSA (0.1%) at a concentration of 3×10^8 to 6×10^8 cells per ml. Then 50 μl of the bacterial cells was added to a well coated with an ECM and incubated for 2 h at 25°C (room temperature). The well was then washed three times with PBS containing Tween 80 (0.1%) and BSA (0.1%), and then the number of bacterial cells adhering to the well surface was determined with a microscope.

Chemicals. Fibronectin, collagen types I, II, IV, and V, laminin, lactoferrin, and fibrinogen were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Southern hybridization and nucleotide sequence analysis of PCR product. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the procedures described in the manufacturer's manual and standard protocols (30). Nucleotide sequence analysis was carried out as previously described (31, 37, 38). The nucleotide sequences of the pair of primers used for the collagen binding domain of the *ace* gene were as follows: ACEFP1, 5′-GAATTGAGCAAAAGTTCAATCGTT-3′; and ACERP1, 5′-GTCTGTCT TTTCACTTGTTTCTGT-3′. The following PCR primers corresponded to the nucleotide sequences of the external region of the *ace* gene; ACEFP2 (5′-TGG CCTGGGAAACAAAATGAGG-3′) and ACERP2 (5′-GCAGTACTTACCTT ACGTTAGC-3′). These primers were used for analysis of the location of the Tn*916* insertion into the *ace* gene.

Southern hybridization with the *prtF* gene probe, which encodes protein F of the fibronectin binding protein of group A streptococci, was performed under relatively low-stringency conditions. Under the lowest-stringency conditions, an extremely strong hybridization band that hybridized specifically to the *prtF* probe and several nonspecific bands were detected in the chromosomal DNA of *S. pyogenes* JRS4, which was used as the positive reference DNA.

RESULTS

Adherence of *E. faecalis* isolates to immobilized ECMs. The adherence of *E. faecalis* clinical isolates to immobilized ECMs in plastic microtiter wells was examined by microscopy. Typical adherence results are shown in Fig. 1. The adherence of each of the isolates to each ECM and the adherence of each of the isolates to different ECMs are shown in Fig. 2 and Table, respectively. Figure 2 shows the adherence of the 43 clinical isolates examined, and Table 1 shows representative results for 30 of the 43 isolates. The levels of adherence to the ECMs for each isolate varied from no adherence to a high level of adherence (Fig. 2). By using a ×400 lens, the number of cells showing adherence to serum albumin in the absence of ECMs was determined. Either no adherence or a level of adherence that was less than 10^3 cells/mm^2 was observed (Fig. 1A). In this assay, the values for no adherence or adherence of one or a few cells in a microscopic field were approximately 10^2 adherent cells/mm^2 or less. Thus, the results indicated that a binding

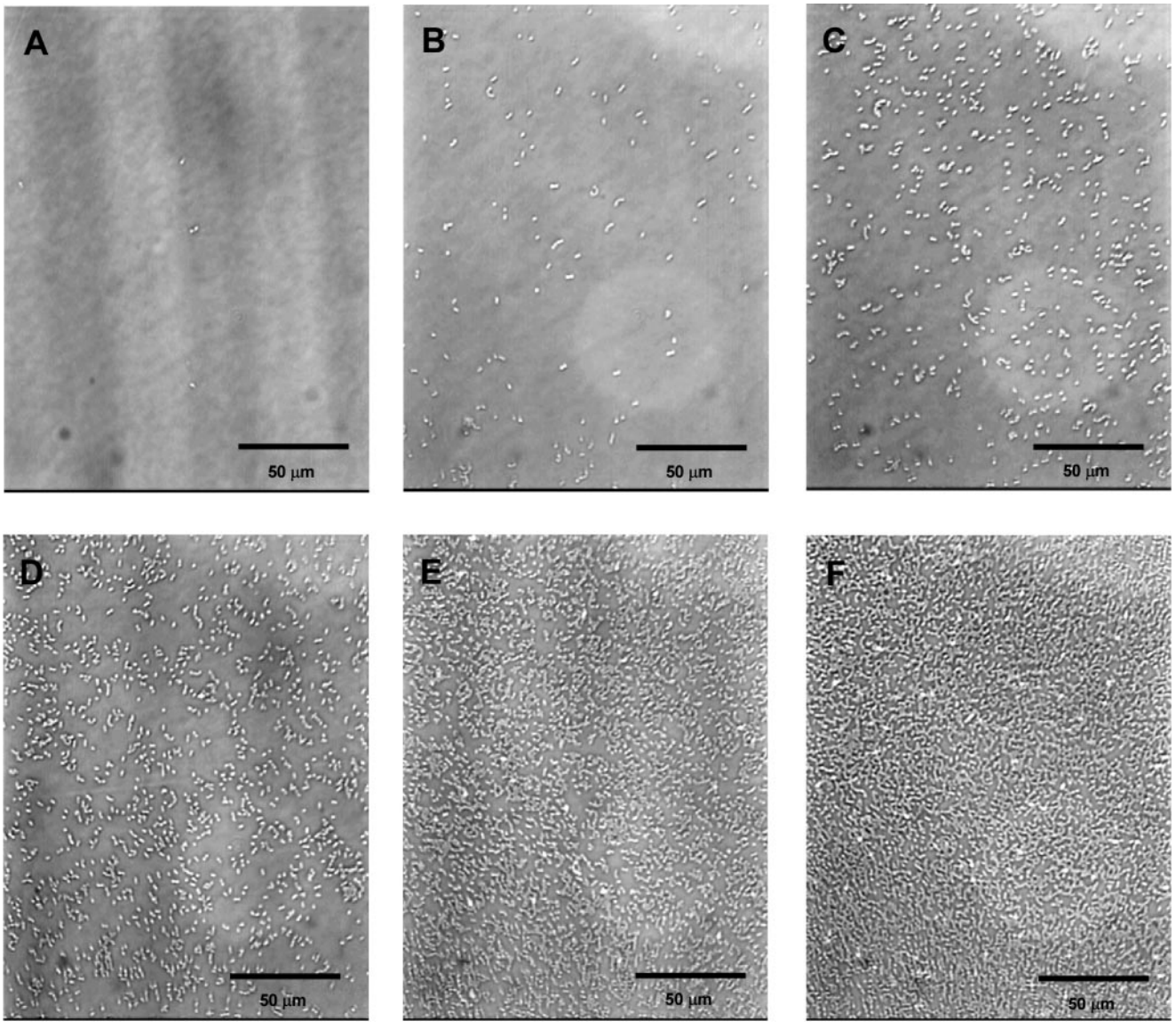

FIG. 1. Typical results for adherence to collagen ECM. (A) No adherence or adherence of a few cells to serum albumin in the absence of ECM. The number of adherent cells was 1.5×10^2 cells/mm^2 ($<10^3$ cells/mm^2). (B to F) Different levels of adherence of the different strains to collagen type IV. The numbers of adherent cells were 4.4×10^3 cells/mm^2 (B), 2.0×10^4 cells/mm^2 (C), 4.5×10^4 cells/mm^2 (D), 1.2×10^5 cells/mm^2 (E), and 1.7×10^5 cells/mm^2 (F).

level of more than 10^3 cells/mm^2 was significant with this assay method.

Strains AS11, AS12, AS14, AS15, HT11, and HT12, which showed strong adhesion to human carcinoma T24 cells, strongly adhered to fibronectin, laminin, and collagen types I, II, IV, and V (Table 1). The level of adherence of each of these strains to each of the ECMs was greater than 10^4 cells/mm^2. These strains showed the highest levels of adherence to collagen type IV; for this compound the levels of adherence were greater than 7×10^4 cells/mm^2.

In general, the strains showing inefficient adherence to human bladder carcinoma T24 cells did not adhere to fibronectin, laminin, and collagens; the only exception was strain U09 (Table 1). However, the level of adherence of U09 to the collagens was lower than the levels observed with the strains that adhered efficiently.

Almost all of the isolates adhered to fibronectin. The numbers of adherent bacterial cells were greater than 10^3 cells/mm^2 (Fig. 2). Many of the isolates adhered to laminin or to collagen type I, II, IV, or V (Fig. 2). The levels of adherence of the gelatinase-producing isolates to the collagens were lower than the levels observed with the gelatinase-negative isolates (Fig. 2). More than one-half of the gelatinase-producing isolates (14 of the 21 gelatinase-producing isolates) did not adhere to laminin. Seven of the gelatinase-producing isolates adhered to collagen type IV at a level of 10^3 cells/mm^2 or less (Fig. 2). The data for these isolates are not included in Table 1. The gelatinase-producing laboratory strain *E. faecalis* OG1S did not

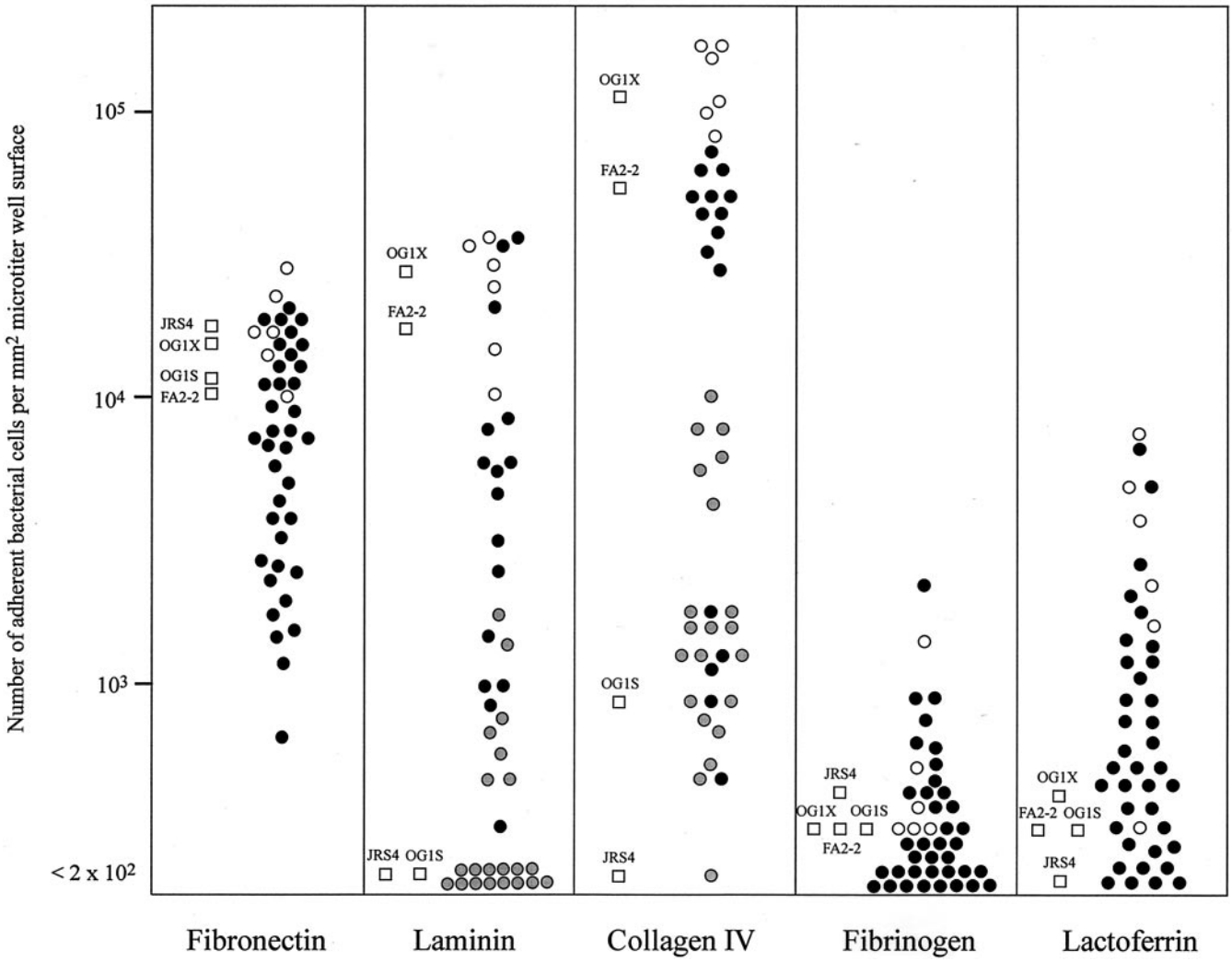

FIG. 2. Adherence of *E. faecalis* strains to each ECM. The open circles represent strains that adhere efficiently to human bladder carcinoma T24 cells. The other circles represent strains that adhere inefficiently to human bladder carcinoma T24 cells. The gray circles for laminin and collagen type IV adherence represent gelatinase-producing strains. The squares represent laboratory strains.

adhere efficiently to collagens or laminin. The gelatinase-negative strain *E. faecalis* OG1X derived from OG1S showed strong adherence to laminin or collagen at a level greater than 10^4 cells/mm^2. These results suggested that gelatinase inhibited the adherence of gelatinase-producing strains to collagens or laminin.

Fifteen (32%) of the 43 isolates adhered to lactoferrin at a level of more than 10^3 cells/mm^2, but the numbers of adherent cells observed for these isolates were less than 10^4 cells/mm^2 (Fig. 2).

With the exception of two isolates, all the isolates showed levels of adherence to fibrinogen of less than 10^3 cells/mm^2 (Fig. 2).

Adherence of *E. faecalis* strains harboring a pheromone-responsive plasmid to immobilized ECMs. In a previous report (34), isolation of the tissue-specific adherent *E. faecalis* strains AS11, AS12, AS13, AS14, and AS15, which show highly efficient adhesion to human bladder carcinoma T24 cells, was described. Two of these strains, AS11 and AS13, harbor the pheromone-responsive plasmid pAS11 (60 kb, Cyl) and the constitutive aggregation plasmid pAS13 (61.4 kb, Tet^r), which encode the aggregation substance for the mating aggregates of the pheromone-responsive plasmid (3, 6). Neither of these plasmids, when transferred into the laboratory strains *E. faecalis* FA2-2 and OG1X, conferred the efficiently adhesive phenotype to human bladder carcinoma T24 cells (34).

The *E. faecalis* FA2-2 or OG1X strain harboring pAS11 was also examined for its adherence to immobilized ECMs. The level of adherence of *E. faecalis* FA2-2(pAS11) or OG1X(pAS11) to each ECM was the same as the level of adherence of host strain *E. faecalis* FA2-2 or OG1X (data not shown). *E. faecalis* FA2-2 or OG1X harboring the constitutive aggregation plasmid pAS13 exhibited constitutive clumping and formed large aggregates in broth culture. Aggregates of each strain detached from the well surface when the well was washed with PBS after the strain had been incubated in a well coated with ECM. Thus, quantitative or reproducible analysis of adherence was difficult with the constitutive clumper (data

TABLE 1. Adherence of *E. faecalis* isolates to ECMs

Strain	Source[a]	Phenotype		Adherence to ECMs[d]							
		Gelatinase[b]	Ace[c]	Fibronectin	Laminin	Collagen type I	Collagen type II	Collagen type IV	Collagen type V	Fibrinogen	Lactoferrin
Strains with efficient adherence to T24 cells											
AS11	Urine	−	+	++++	++	+++	+++	++++	++	−	++
AS12	Urine	−	+	+++	++	+++	+++	++++	++	+/−	+
AS14	Urine	−	+	+++	+++	++++	++++	++++	++	−	+/−
AS15	Urine	−	+	+++	+++	++++	+++	++++	++	+	++
HT11	Urine	−	+	+++	+++	+++	+++	++++	++	+/−	+
HT12		−	+	+++	++	+++	+++	++++	++	++	++
Strains with inefficient adherence to T24 cells											
U09		−	+	+++	+++	++	+++	+++	++	++	++
U35		−	+	+++	++	++	+++	+++	+	+/−	+/−
U66	Urine	−	+	+	+++	+++	+++	+++	+	−	−
U54		−	+	+/−	++	+++	+++	+++	+	+/−	+/−
U55		−	+	+	+++	+++	+++	+++	+/−	+	+
U52	Urine	−	+	++	−	+/−	+	+/−	+	+	+
U19		−	+	+++	+/−	+	+	+/−	−	−	+
U24		−	+	+++	+/−	+	+	+	−	+/−	+/−
U58	Urine	−	+	+/−	+/−	+	+	+	+/−	−	+
U02		−	+	+	−	+/−	−	+/−	−	−	+/−
U01	Urine	+	+	++	−	+/−	+/−	++	+/−	+/−	++
U21	Urine	+	+	++	−	+	+/−	++	+/−	−	+/−
U23	Urine	+	+	+++	−	+	+	++	+/−	−	−
U48	Urine	+	+	++	−	++	+	++	+	−	+/−
U49	Urine	+	+	+++	−	+/−	++	+/−	+/−	+/−	+
U04		+	+	++	−	+	−	+	+/−	+/−	+/−
U14		+	+	++	−	+	+/−	+/−	−	−	+
U30	Urine	+	+	+	−	−	−	+	−	−	−
U32	Urine	+	+	+/−	−	++	+	+	+	+/−	+/−
U33	Urine	+	+	−	−	+	+/−	+	+	−	+/−
U40	Urine	+	+	+	−	++	+	+/−	+	+	+
U43	Urine	+	+	+	−	+	+/−	+	+	−	+/−
U03		+	+	+/−	−	+/−	+/−	+	+/−	+/−	+/−
U11		+	+	+	−	+	+/−	+/−	−	+/−	+
Laboratory strains											
FA2-2		−	+	+++	+	++	+	+++	+/−	+/−	+/−
OG1X		−	+	+++	+++	+++	+++	++++	++	+/−	+
OG1S		+	+	+++	−	+	−	+/−	+/−	+/−	+/−
S. pyogenes JRS4		−	−	+++	−	−	+/−	+/−	−	+	−

[a] No entry indicates a source other than urine.
[b] −, gelatinase negative; +, gelatinase producing.
[c] +, *ace* gene present as determined by PCR analysis; −, *ace* gene not present.
[d] For the number of cells adhering to collagens, fibrinogen, and lactoferrin: − and +/−, less than 10^3 cells/mm^2; +, 10^3 to 4.0×10^3 cells/mm^2; ++, 4.0×10^3 to 2.0×10^4 cells/mm^2; +++, 2.0×10^4 to 7.0×10^4 cells/mm^2; ++++, more than 7.0×10^4 cells/mm^2. For the number of cells adhering to fibronectin: − and +/−, less than 10^3 cells/mm^2; +, 10^3 to 3.0×10^3 cells/mm^2; ++, 3.0×10^3 to 1.0×10^4 cells/mm^2; +++, 1.0×10^4 to 3.0×10^4 cells/mm^2; ++++, more than 3.0×10^4 cells per mm^2.

not shown). These results suggested that the aggregation substance encoded on the plasmids plays little role in efficient adherence to immobilized ECMs.

Inhibition of adherence by fibronectin and collagen. To examine whether fibronectin or collagen inhibits adherence of the strongly adhesive strains, adherence was examined after pretreatment of *E. faecalis* strains with fibronectin or collagen type IV. When *E. faecalis* AS11, AS12, AS14, or AS15 was preincubated with fibronectin, the adherence to fibronectin was inhibited and the number of bacterial cells adhering to fibronectin was about 2×10^{-1} to 4×10^{-1} times the number of bacterial cells that had not been pretreated that adhered to fibronectin, but the adherence to collagen type IV was not inhibited (Fig. 3).

When *E. faecalis* AS11, AS12, AS14, or AS15 was preincubated with collagen type IV, the adherence to this collagen was inhibited, and the number of bacterial cells adhering to collagen type IV was about 10^{-1} times the number of bacterial cells that had not been pretreated that adhered to collagen type IV, but the adherence to fibronectin was not inhibited (Fig. 3).

Analysis of the determinant for collagen adhesin from enterococci (*ace*) and adhesin of the fibronectin binding protein (*prtF*). The *E. faecalis ace* gene encodes Ace (adhesin of collagen from enterococci), which is a collagen binding

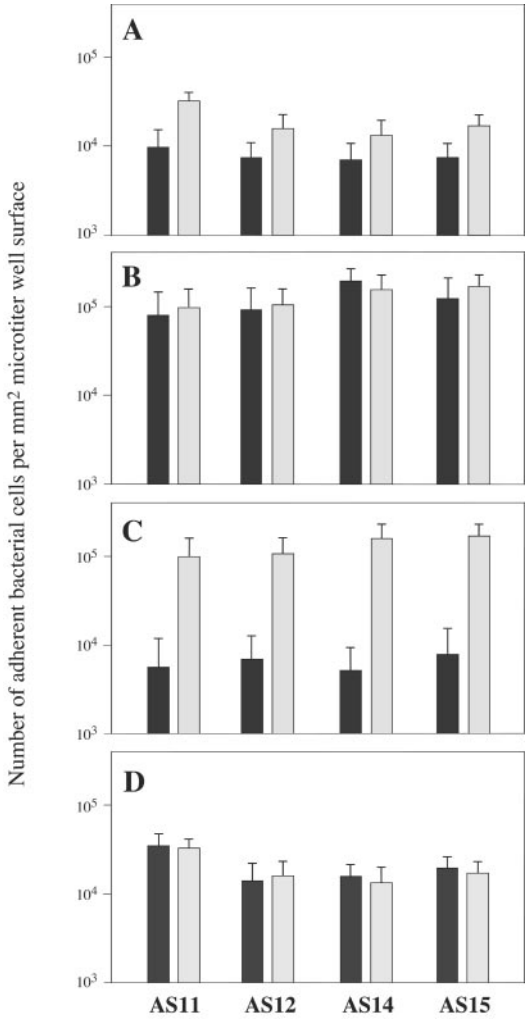

FIG. 3. Adherence of collagen type IV-treated or fibronectin-treated *E. faecalis* strains to ECMs. Solid bars, ECM-treated *E. faecalis* strains; gray bars, *E. faecalis* strains not treated with ECM. The experiments were carried out in triplicate. Each of the strains that adhered efficiently to bladder carcinoma T24 cells was treated with fibronectin (A and B) and then examined for adherence to fibronectin (A) and collagen type IV (B). In addition, each of the strains was treated with collagen (C and D) and then examined for adherence to collagen type IV (C) and fibronectin (D).

MSCRAMM (microbial surface component recognizing adhesive matrix molecules) (29). The DNAs of the *E. faecalis* strains were screened by PCR for the presence of the Ace gene with primers specific for collagen binding domain A of *ace* (30). The DNAs of all the *E. faecalis* strains used in this study gave rise to the expected 864-bp product with the primer specific for the *ace*, indicating that *E. faecalis* strains have the *ace* gene (data not shown) (Table 1) (30).

The adhesins of the fibronectin binding proteins of group A streptococci are believed to mediate attachment to host cells (12, 13, 33). The *prtF* gene of group A streptococcus encodes protein F, which is a fibronectin binding adhesion protein (accession number L10919). Fibronectin binding proteins have been found in other streptococci, and the fibronectin binding domains of these proteins are highly homologous with the fibronectin binding domain of protein F (22). A fibronectin binding protein has not been found in *E. faecalis*. *E. faecalis* strains AS11, AS12, AS14, AS15, HT11, and HT12, which adhere efficiently to human bladder carcinoma T24 cells, fibronectin, and collagens, were studied to determine whether they encode a determinant homologous to *prtF* by using the pPTF5 probe and the pPTF54 probe, which contain the determinant of the fibronectin binding protein and the fibronectin binding domain of *prtF*, respectively (12). The *prtF* probe hybridized to a fragment of HindIII-digested *S. pyogenes* JRS4 DNA and did not hybridize to any HindIII-digested fragment of *E. faecalis* strains, indicating that there is no determinant homologous to *prtF* in the *E. faecalis* strains (data not shown).

Isolation of transposon insertion mutants with altered collagen binding. Mutants with altered collagen binding were isolated by insertion of Tn*916* into the chromosome of the *E. faecalis* AS14 strain as described in Materials and Methods. The AS14SS strain was a streptomycin- and spectinomycin-resistant derivative of the AS14 strain, and AS14SS showed the same adhesive properties with each ECM as the parent strain (data not shown). A total of 6,200 Tn*916* insertion derivatives were obtained in independent experiments, and the derivatives were examined for adherence to collagen type IV. Thirty-eight mutants that did not adhere efficiently to collagen type IV were isolated in independent experiments. The Tn*916* insertions of the mutants were studied by Southern hybridization with the Tn*916* probe. As Tn*916* contains one HindIII site, the Tn*916* probe hybridizes to at least two fragments when DNA from an Tn*916* insertion mutant is digested with HindIII (9). Tn*916* hybridized to two unique HindIII fragments of the HindIII-digested chromosomal DNA of 14 of the 38 Tn*916* insertion mutants, implying that Tn*916* was inserted into a site in the chromosomal DNA of each of these mutants (data not shown). Thirteen of the 14 mutants produced the same hybridization pattern, implying that the transposon was inserted into the same site in all 13 of these mutants. One of the 14 mutants produced a hybridization pattern that was different from that of the other 13 mutants. In the remaining 24 mutants, the Tn*916* probe hybridized to four or more fragments in the HindIII-digested chromosomal DNA, implying that Tn*916* was inserted into several sites of the chromosomal DNA (data not shown). One pair of PCR primers was designed based on the previously published sequences of Tn*916* (5, 8) and the *ace* gene (3a; data bank of The Institute for Genomic Research). The 14 mutants in which Tn*916* hybridized to two unique HindIII fragments were analyzed by PCR performed with the PCR primers described above. The PCR products were detected in the 13 mutants that had the same hybridization pattern. The PCR products were sequenced, and computer analysis of the sequence revealed that Tn*916* was inserted into the N terminus (start codon) of the *ace* gene (data not shown).

Compared with the number of AS14SS cells, the number of mutant AS14-1 cells that adhered to the collagens and laminin was about 1 or 2 orders of magnitude less (Fig. 4). The number of AS14-1 bacterial cells that showed adhesion to fibronectin was identical to the number of cells of parent strain AS14SS that showed adhesion to fibronectin. These results indicated that the adhesin encoded in the *ace* gene mediated adherence to collagens and also to laminin (27) but did not mediate adherence to fibronectin.

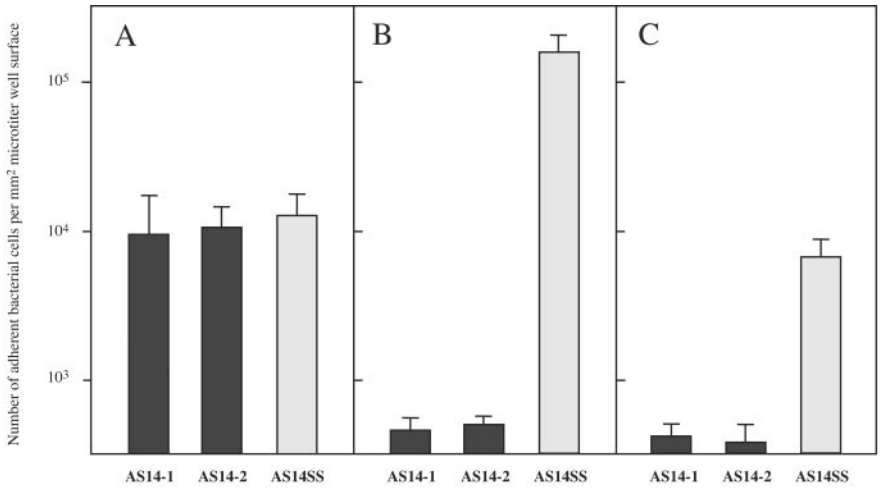

FIG. 4. Adherence to ECMs of mutant *E. faecalis* AS14 strains with the Tn*916* insertion in *ace*. Solid bars, *ace* mutant strains AS14-1 and AS14-2; gray bars, wild-type strain AS14SS. The experiments were carried out in triplicate. (A) Adherence to fibronectin. (B) Adherence to collagen type IV. (C) Adherence to laminin.

DISCUSSION

Microscopy was used in this study for quantitative analysis of the adherence of *E. faecalis* clinical isolates to immobilized ECMs coating the plastic wells of microtiter dishes. This method was sensitive enough to make it possible to quantify the adherence of *E. faecalis* isolates. It has been shown previously that tissue-specific enterococcal strains isolated from urinary tract infections also adhere very efficiently to human bladder carcinoma T24 cells. These strains also strongly adhere to human bladder epithelial cells. The adherence is inhibited by fibronectin, implying that adhesins on the cell surface mediate adherence to fibronectin. The strains that adhered efficiently to human bladder carcinoma T24 cells also adhered strongly to fibronectin, laminin, and collagen types I, II, IV, and V. They showed particularly strong adherence to collagen type IV, and the highest levels were greater than 7×10^4 cells/mm^2. Many other strains adhered to between one and four of the fibronectin, laminin, and collagen type I, II, IV, and V ECMs. These results implied that the ability of *E. faecalis* to bind to fibronectin, laminin, and collagen types I, II, IV, and V plays a role in the efficient adherence to human tissue, although other factors may also play a role in the adherence.

The levels of adherence to each of the ECMs varied from 10^3 to more than 10^4 cells/mm^2. Adherence of *E. faecalis* to a collagen(s) is mediated by the Ace protein, which is encoded by the *ace* gene (30). Ace also mediates the adherence to laminin (27). A polyclonal antibody analysis of domain A of Ace showed that the *E. faecalis* strains that adhere to immobilized collagen express Ace on the cell surface; on the other hand, strains that do not adhere to collagen do not express Ace on the cell surface, despite the presence of the *ace* gene (27, 30). In this study, we showed that the strains that adhered efficiently to immobilized collagen (i.e., strains AS11, AS12, AS14, and AS15) were specifically inhibited by pretreatment of the strains with collagen and that disruption of the *ace* gene of the efficient adhesive strain *E. faecalis* AS14 resulted in a specific decrease in the level of adherence of the mutant strain to immobilized collagen. These data implied that the adherence of these *E. faecalis* strains to collagens was mediated by Ace. The *E. faecalis* isolates used in this study had the *ace* gene, which was identified by PCR by using primers for domain A of *ace* (30). Despite the presence of the *ace* gene in the *E. faecalis* isolates, differences in the levels of adherence to the collagens were observed for each of the isolates, and this could have been due to differences in the expression of Ace on the *E. faecalis* cell surface (40). Although the determinant for adhesion to fibronectin has not been identified yet, the difference in the levels of adherence to fibronectin also might be due to differences in the expression a fibronectin adhesin.

The gelatinase-negative isolates adhered more efficiently to collagens and laminin than the gelatinase-producing isolates. The gelatinase-negative derivative *E. faecalis* OG1X also adhered more efficiently to collagens and laminin than the parent strain of the gelatinase-positive organism *E. faecalis* OG1S. These results implied that the gelatinase could degrade collagens and laminin in addition to gelatin. The gelatinase-positive isolates did not adhere efficiently to the wells coated with collagens or laminin since the ECMs on the surfaces of wells were degraded during incubation of the isolates in the wells used for the adherence assay.

Xiao et al. (40) showed that very few isolates exhibited adherence to collagen types I and IV and mouse laminin after growth of the isolates in standard in vitro physiological conditions (i.e., 37°C). This observation appears to conflict with our results. However, there is essentially no difference between the observations of Xiao et al. and our results. Xiao et al. also showed that two of the eight strains studied bound efficiently to collagen types I and II at 37°C. However, it seems that strains with intermediate levels of adherence to collagen may not be identified by the methods of these workers. This seemingly conflicting observation may be due to the use of direct observation of the adherence by microscopy to score the binding phenotype in our case, which may be a more sensitive method than the method used in binding studies that rely on determining the percentage of bacteria that have bound ECM protein.

There have been reports that *E. faecalis* isolates and *E.*

faecalis laboratory strains bind to fibronectin (35). In other studies workers showed that there were low levels of binding or no binding of *E. faecalis* isolates to fibronectin (13, 36, 40, 42). It has been shown previously that the adherence of an *E. faecalis* strain to human bladder carcinoma T24 cells was inhibited by fibronectin, implying that the *E. faecalis* strain binds to fibronectin (34). *S. pyogenes* (group A streptococcus) encodes adhesin PrtF, which mediates adherence to fibronectin. In this study, *S. pyogenes* JR4 specifically adhered to fibronectin at a level of around 10^4 cells/mm^2. Twenty-one of the 43 isolates and three *E. faecalis* laboratory strains adhered to fibronectin at levels that were the same as or greater than that of the JRS4 strain. The *E. faecalis* AS14 strain adhered to fibronectin, collagens, and laminin at levels that were greater than 10^4 cells/mm^2. The Tn*916* insertion mutant of the AS14 strain for collagen and laminin binding exhibited highly efficient binding to fibronectin, and fibronectin binding was unaffected. Similar results were obtained in the studies of inhibition of adherence by collagen and fibronectin. The adherence of *E. faecalis* AS14 to collagen was inhibited by collagen but was not inhibited by fibronectin, and the adherence of an *E. faecalis* strain to fibronectin was inhibited by fibronectin but was not inhibited by collagen. These results indicate that there was no relationship between the collagen binding adhesin Ace and the fibronectin binding adhesin. Although the specific substance that mediates adherence to fibronectin in *E. faecalis* has not been elucidated yet, these results imply that fibronectin binding could be mediated by a substance on the *E. faecalis* cell surface.

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