Sex Pheromone Response, Clumping, and Slime Production in Enterococcal Strains Isolated from Occluded Biliary Stents

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Bile-resistant bacteria, particularly gram-positive Enterococcus faecalis and Enterococcus faecium, play an important role in biliary stent occlusion, because their sessile mode of growth protects them against host defenses and antimicrobial agents. Twelve E. faecalis and seven E. faecium strains isolated from occluded biliary stents have been investigated for slime production, presence of aggregation substance genes, and ability to adhere to Caco-2 cells. Ten isolates were strong producers of slime, and seven isolates produced clumps when exposed to pheromones of E. faecalis JH2-2 and/or OG1RF. The small E. faecalis clumps differed from the large clumps of E. faecalis and were similar to those of E. faecium LS10(pBRG1) carrying a pheromone response plasmid. After induction with pheromones, the adhesion to Caco-2 cells of clumping-positive strains was found to increase from two- to fourfold. Amplitcons of the expected size were detected in three clumping-positive and three clumping-negative E. faecalis isolates by using primers (agg) internal to a highly conserved region of the E. faecalis pheromone response plasmids pAD1, pPD1, and pCF10 and primers internal to prgB of the E. faecalis plasmid pCF10. The agg/prgB-positive E. faecalis strains were also positive in Southern hybridization experiments with a prgB-specific probe. No PCR products were obtained with the same primers from four clumping-positive isolates (one E. faecalis and three E. faecium strains), which were also Southern hybridization negative. Our results demonstrate that slime production and pheromone response are both present in isolated enterococci, suggesting that clinical strains with these features might have a selective advantage in colonizing biliary stents.

In patients with obstructive jaundice, decompression of an occluded common bile duct can be palliated by using endoscopic stenting, which represents a valid alternative to surgery in inoperable tumors, with a resultant regression of jaundice and perhaps an improvement in the quality of life. Stent insertion presents a low incidence of morbidity and mortality, and perhaps an improvement in the quality of life. Stent insertion presents a low incidence of morbidity and mortality, and perhaps an improvement in the quality of life. Stent insertion presents a low incidence of morbidity and mortality, and perhaps an improvement in the quality of life. Stent insertion presents a low incidence of morbidity and mortality, and perhaps an improvement in the quality of life. Stent insertion presents a low incidence of morbidity and mortality, and perhaps an improvement in the quality of life. 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surface protein, designated “aggregation substance” (AS), which facilitates the initiation of mating pair formation (5). In *E. faecalis*, four plasmids (pAD1, pCF10, pPD1, and pAM373) encoding AS have been described (6). AS-encoding genes have been sequenced, and highly conserved regions have been used to generate primers specific for AS (12). Sex pheromone plasmids have also been described in *E. faecium*, in which they have been found to be associated with vancomycin resistance (15, 16, 23).

Many enterococcal infections are endogenous, originating from the intestinal tract, and several experimental data suggest that AS may be involved in translocation across the intestinal barrier (14, 29). AS promotes enterococcal adhesion to and internalization into cultured human cells (21, 23, 26) either directly or simply by increasing the number of organisms taken up as a clump (C. M. Waters, C. L. Wells, and G. M. Dunny, Abstr. 6th Am. Soc. Microbiol. Conf. Streptococcal Genetics, abstr. 121, 2002). AS also promotes survival of *E. faecalis* in mouse peritoneal macrophages (13). The sequence of the structural gene for the pAD1-encoded AS revealed the presence of two Arg-Gly-Asp motifs (30), recognized by integrins, a family of eukaryotic receptors expressed on leukocytes, thrombocytes, and endothelial and various epithelial cells, including intestinal cells (19).

In this study, *E. faecalis* and *E. faecium* strains isolated from occluded biliary stents removed from patients with obstructive jaundice were investigated for the presence of AS genes and the ability to adhere to Caco-2 cells and to produce biofilms. The susceptibilities of isolates to various antibiotics were also evaluated.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 19 clinical strains of enterococci (12 *E. faecalis* and seven *E. faecium*) were isolated from occluded biliary stents. Two strains of sex pheromone-producing enterococci (*E. faecalis* JH2-2 and *E. faecalis* OG1RF) and two strains containing pheromone-susceptible plasmids (*E. faecalis* OG1RF/pCF10(10) and *E. faecium* LS10/pBRG1(23)) were used as control strains.

**Antibiotics and susceptibility testing.** Antibiotic resistance was detected by the Kirby-Bauer method according to standard procedures approved by the NCCCLS in 2000. Antibiotic disks were obtained from Oxoid.

**Slime production assay.** Biofilm formation was tested as previously described (1). Briefly, bacteria were grown overnight at 37°C with no shaking in 2 ml of TSB containing 1% glucose. The culture was diluted 1:10 in fresh TSB, and 200 μl of the suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Corning Costar, Milan, Italy). After overnight incubation at 37°C in 5% CO₂, the wells were washed three times in phosphate-buffered saline, dried in an inverted position for 1 h at 50 to 60°C, and stained with Hucker’s crystal violet.

After the staining, the optical densities (OD) of the biofilms were read at a wavelength of 570 nm by a spectrophotometer (Novapath Microplate Reader; Bio-Rad Laboratories Inc.). The slime index was defined as an estimate of the density of the biofilm generated by a culture with an OD of 600 nm of 0.5 [slime index = mean OD of the biofilm × (0.5/mean OD growth)].

**Pheromones and clumping assay.** Pheromone-containing filtrates were prepared from strains of *E. faecalis* JH2-2 and OG1RF grown overnight in BHI broth at 37°C with shaking. Late-stationary-phase cultures were obtained by inoculating 1 ml of the overnight culture in 100 ml of fresh BHI broth and incubating it at 37°C with shaking to a final concentration of ~5 x 10⁸ bacteria per ml. The cultures were centrifuged at 7,000 x g for 10 min at 20°C, and the supernatants containing pheromones were then filtered through 0.45- and 0.22-μm-pore-size filters (Millipore, Milan, Italy). The filtrates were autoclaved at 121°C for 15 min and stored at 4°C prior to use. Clumping of clinical enterococci was evaluated by adding 20 μl of stationary-phase culture to 0.5 ml of pheromone-containing filtrate. Negative controls were prepared by replacing pheromone-containing filtrates with BHI broth. Samples were incubated at 37°C for 3 h with shaking, mounted on glass slides, and observed by phase-contrast microscopy.

**Cell line.** Cells from the human colon carcinoma enterocyte-like cell line Caco-2 (ATCC HTB37) were routinely grown in 25-cm² plastic tissue culture flasks (Corning Costar) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, 4 mM L-glutamine, and 3.7 mg of sodium bicarbonate per ml (Euroclone, West York, United Kingdom) with 1% nonessential amino acids supplemented with 10% fetal bovine serum (Euroclone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

**Adhesion to Caco-2 cells.** Confluent cell monolayers were trypsinized, counted, and adjusted at a concentration of ~2.5 x 10⁶ per ml in DMEM. One milliliter of the cell suspension was dispensed into each 22-mm-diameter well of microtiter plates (Corning Costar) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, 4 mM L-glutamine, and 3.7 mg of sodium bicarbonate per ml (Euroclone, West York, United Kingdom) with 1% nonessential amino acids supplemented with 10% fetal bovine serum (Euroclone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

**PCR and restriction analysis of PCR products.** For total DNA extraction, the strains were grown overnight in BHI broth containing 0.5% glycine at 37°C. One milliliter of overnight culture was centrifuged at 2,739 x g for 10 min, and the pellet was resuspended in 1 ml of salt-Tris-EDTA buffer containing 20% saccharose and 2.5 mg of lysozyme/ml. Samples were incubated at 37°C for 30 min and centrifuged at 18,000 x g for 3 min. The pellet was resuspended in 1 ml of lysis buffer and incubated at 60°C for 1 h. The temperature was then raised to 95°C for 10 min for proteinase K inactivation and DNA denaturation.

PCR amplifications were performed using the Gene Amp PCR System 2400 (Perkin-Elmer Cetus, Norwalk, Conn.). Primers (Table 1) included those reported by Eaton and Gasson (12) internal to highly conserved regions in the AS genes of pAD1, pPD1, and pCF10 (agg primers) and the primers internal to the AS structural gene prgB and the regulatory prgX gene of pCF10 (16). EcoRI, DraI, and Scal (Roche Molecular Biochemicals, Mannheim, Germany) restriction enzymes were used in accordance with the manufacturer’s
TABLE 2. Antibiotic resistance, presence of plasmid DNA, slime production, clumping, presence of AS genes, and Southern hybridization with a probe internal to the prgB gene of pCF10 in enterococci isolated from occluded biliary stents

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic resistance</th>
<th>Plasmid DNA</th>
<th>Slime production</th>
<th>Clumping</th>
<th>PCR</th>
<th>Hybridization with prgB</th>
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<td>JH2-2</td>
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<td>prgB</td>
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<td>prgX</td>
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<td>EFS-12</td>
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<tr>
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<td>ND</td>
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<td>EFS-20</td>
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<td>+</td>
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<td>EFS-22</td>
<td>Tc, Em</td>
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<td>EFS-27b</td>
<td>Tc, Gm, Cl</td>
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<td>SP</td>
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<td>ND</td>
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<td>Tc, Em</td>
<td>+</td>
<td>SP</td>
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<td>ND</td>
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<td>EGL-19</td>
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<td>SP</td>
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<td>EFS-38</td>
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<td>ND</td>
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<td>OGISRF(pCF10)</td>
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<td>+</td>
<td>ND</td>
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<td>E. faecium</td>
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<tr>
<td>EFM-13</td>
<td>Tc, Gm</td>
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<td>NP</td>
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<td>NP</td>
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<td>Em, Gm</td>
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<td>NP</td>
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<td>NP</td>
<td>+</td>
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<td>EFM-33</td>
<td>Tc, Em</td>
<td>-</td>
<td>NP</td>
<td>-</td>
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<td>ND</td>
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<tr>
<td>CIUM-22</td>
<td>Tc, Em, Cl, Am</td>
<td>-</td>
<td>NP</td>
<td>-</td>
<td>-</td>
<td>ND</td>
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<tr>
<td>CIUM-34</td>
<td>Tc, Am</td>
<td>-</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>LS10(pBRG1)</td>
<td>Vm</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>ND</td>
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* Am, ampicillin; Cl, chloramphenicol; Em, erythromycin; Gm, gentamicin; Sm, streptomycin; Tc, tetracycline; Vm, vancomycin; Rf, rifampin; Fu, fusidic acid.
* +, present; -, absent.
* SP, strong producers (OD > 0.240); WP, weak producers (0.120 < OD < 0.240); NP, nonproducers (OD < 0.120).
* +, present; -, absent.
* SP, strong producers (OD > 0.240); WP, weak producers (0.120 < OD < 0.240); NP, nonproducers (OD < 0.120).
* a, present; b, absent.
* ND, not determined.

**Results**

**Antibiotic resistance and presence of plasmid DNA.** The clinical isolates of *E. faecalis* and *E. faecium* were evaluated for antibiotic susceptibility to ampicillin, chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline, and vancomycin (Table 2). Most of the isolates exhibited different patterns of antibiotic resistance. The highest percentages of resistant isolates were observed in the presence of chloramphenicol, erythromycin, and tetracycline, while no vancomycin-resistant strains were observed (Table 2). Five strains of *E. faecalis* and one strain of *E. faecium* exhibited multidrug resistance (to four or five antibiotics).

Plasmid DNA was present in 10 of 12 *E. faecalis* strains and in 2 of 7 *E. faecium* strains.

**Slime production.** All clinical enterococcal isolates were tested for slime production. Nine *E. faecalis* strains (EFS-12, EFS-16, EFS-20, EFS-28b, EFS-30d, EFS-32, EFS-35, EFS-30, and EGL-19) and one *E. faecium* strain (CIUM-34) were strong producers (Table 2).

**Enterococcal clumping.** To assess responses to pheromones, the 19 enterococcal strains were microscopically examined for clumping after growth in the presence of pheromone-containing supernatants of *E. faecalis* JH2-2 and *E. faecium* OG1RF. Among all isolates, seven strains (four *E. faecalis* [EFS-27b, EFS-30d, EFS-32, EFS-35, EFS-30, and EFS-16] and EFS-28b, EFS-30, and EGL-19) and one *E. faecium* strain (CIUM-34) were clumping positive. Growth of strains in the presence of pheromones gave rise to different levels of aggregation: some strains elicited a barely detectable effect, other strains generated small aggregates, while others did not aggregate at all (Table 2). Strains also exhibited different aggregation patterns when exposed to pheromone-containing supernatants of strain JH2-2 or OG1RF; moreover, *E. faecium* isolates generated aggregates smaller than those of *E. faecalis* (Fig. 1).

**Adhesion to Caco-2 cells.** All enterococcal isolates were tested in vitro for the ability to adhere to intestinal epithelial cells. In adhesion experiments, *E. faecalis* OG1RF(pCF10) and *E. faecium* LS10(pBRG1) were used as positive controls. The abilities of enterococcal isolates to adhere to Caco-2 cells, expressed as the percentage of adhering bacteria with respect to the initial inoculum, were between 0.18 and 6.4% (Fig. 2). Among *E. faecium* isolates, clumping-positive strains were found to be more adhesive than clumping-negative strains. Moreover, after induction with pheromones, the adhesion of
FIG. 1. Clumping phenomenon in enterococci induced by the presence of pheromone-responsive plasmids produced by *E. faecalis* strain JH2-2 or OG1RF. (a) *E. faecalis* EFS-27b (JH2-2); (b) *E. faecalis* EFS-30d (JH2-2); (c) *E. faecalis* EGL-19 (OG1RF); (d) *E. faecium* CIUM-22 (JH2-2); (e) *E. faecium* CIUM-34 (JH2-2); (f) *E. faecium* LS10(pBRG1) (JH2-2).
Clumping-positive strains was found to increase from two- to fourfold. Particularly after induction with sex pheromones produced by *E. faecalis* strain JH2-2, adhesiveness increased significantly in *E. faecalis* EFS-27b (from 1.1 to 3%) and EFS-30d (from 2 to 8%) and *E. faecium* CIUM-34 (from 6.4 to 15%). In *E. faecalis* EFS-38 (a weakly clumping-positive strain), the percentage of adhesion was doubled after induction with sex pheromones produced by *E. faecalis* OG1RF (Fig. 2).
Presence of AS genes. All enterococcal isolates were screened for the presence of AS genes by using the primers internal to a highly conserved region of the *E. faecalis* pheromone response plasmids pAD1, pPD1, and pCF10 and those internal to prgB and prgX of the *E. faecalis* plasmid pCF10 (Table 1). *E. faecalis* OG1RF(pCF10) and *E. faecium* LS10(pBRG1) were used as controls. Strain OG1RF containing pCF10 was also used to generate a prgB-specific probe. agg primers produced an amplicon of the expected size in six *E. faecalis* strains (three clumping-positive [EFS-27b, EFS-30d, and EGL-19] and three clumping-negative [EFS-12, EFS-20, and EFS-28b] strains) but not in clumping-positive *E. faecium* strains (Table 2). In all agg-positive *E. faecalis* strains, an amplicon of the expected size was also obtained with primers internal to prgB, whereas no PCR products were obtained with primers internal to *prgX*. The agg/prgB-positive *E. faecalis* strains were also positive in Southern hybridization experiments with a prgB-specific probe (Fig. 3). The prgB probe strongly hybridized with Smal-restricted genomic DNA obtained by PFGE from strains EFS12, EGL19, and EFS20 (Fig. 3B, lanes 4, 5, and 6, respectively) and with plasmid DNAs from strains EFS27B, EFS28B, EFS30d, and OG1RF(pCF10) (Fig. 3D, lanes 7, 8, 9, and 11, respectively). In Fig. 3B, hybridizing bands corresponding to the plasmid DNA—in some cases not entering the gel—are also visible (lanes 7, 8, 9, and 11).

PCR amplicons, obtained with agg and *prgB* primers, from the three clumping-positive (EFS-27b, EFS-30d, and EGL-19) and the three clumping-negative (EFS-12, EFS-20, and EFS-28b) *E. faecalis* isolates were digested with DraI, EcoRI, and/or ScaI restriction enzyme, and the restriction profiles were compared with that derived from *E. faecalis* OG1RF(pCF10) after digestion with the same enzymes. Restriction profiles obtained by DraI digestion of 427-bp amplicons obtained with *prgB* primers (Fig. 4) and by ScaI digestion of 1,553-bp amplicons obtained with agg primers (data not shown) were identical in *E. faecalis* clinical isolates and OG1RF(pCF10). The EcoRI restriction profiles of 1,553-bp amplicons obtained with agg primers (Fig. 4) were identical in all clinical isolates but differed from that of OG1RF(pCF10), showing a three-band difference resulting from the disappearance of a fragment of ~1,170 bp and the appearance of two new fragments (~730 and 440 bp).

**DISCUSSION**

Previous studies have indicated that bacterial contamination is a major factor in the pathogenesis of stent occlusion (2, 25, 28). Other authors have reported that stent clogging is significantly associated with polymeric surface irregularities promoting bacterial adherence, biofilm formation, and the accumulation of biliary sludge (24, 32). Furthermore, in vitro and in vivo studies have demonstrated the inhibitory effect of long-term treatment with antibiotics or aspirin on sludge formation, suggesting that both bacteria and mucus glycoproteins play significant roles in the clogging of biliary endoprostheses (22, 27). In this process, bacteria of the genus *Enterococcus*, particularly *E. faecalis* and *E. faecium*, are among the most frequently isolated gram-positive bacteria that translocate from the duodenum to colonize the biliary stent (7). It is well known that enterococcal pheromone-susceptible plasmids carry genes coding for AS, which is expressed as a response to a sex pheromone stimulus produced from strains that lack the specific plasmid (5). In addition to promoting conjugation processes, the presence of AS also increases the ability of enterococci to adhere to host surfaces. The reported increase in uptake of AS-expressing cells into intestinal epithelial cells (20, 26, 31) suggests the possible involvement of AS in the translocation of *E. faecalis* from the intestine to the bloodstream. On the other hand, AS of *E. faecalis* has been implicated as a virulence factor in several model systems (21), and its in vivo induction has recently been demonstrated (17).

In this study, enterococcal strains isolated from occluded biliary stents, all resistant to antibiotics, were studied for slime production and response to sex pheromones produced by *E. faecalis* JH2-2 and *E. faecalis* OG1RF.

As far as slime production is concerned, 9 of 12 *E. faecalis* strains were strong producers compared to 1 of 7 *E. faecium* strains. This finding is particularly interesting because slime production in enterococci has not been extensively described (1). In fact, the production of slime exhibited by most of our *E. faecalis* isolates could play a significant role in the colonization and occlusion of biliary stents. This role in the impairment of indwelling medical devices is well established for staphylococci, for which slime is considered a significant biofilm component and a determining factor involved in the occlusion of intravascular catheters (8).

Our data also demonstrate a pheromone response in enterococcal strains isolated from occluded biliary stents, suggesting that sex pheromone response may play a role in biliary stent occlusion. Actually, clumping assays demonstrated a response to pheromones produced by *E. faecalis* JH2-2 and/or *E. faecalis* OG1RF in 7 (4 *E. faecalis* and 3 *E. faecium*) of 19 strains. Interestingly, clumping-positive *E. faecium* strains generated aggregates that were different (i.e., smaller) from the large aggregates generated by clumping-positive *E. faecalis* strains and similar to those of *E. faecium* LS10 carrying the pheromone-susceptible plasmid pBRG1 (23) and that showed a greater ability to adhere in vitro to cultured intestinal cells (Caco-2) than clumping-negative strains. Adherence to Caco-2 cells by clumping-positive *E. faecalis* and *E. faecium* strains was enhanced after induction with pheromones. These findings are relevant, especially for *E. faecium* species, in which a response to sex pheromones has rarely been described. Moreover, among tested enterococci, PCR products of the expected size were obtained in six *E. faecalis* strains (including three clumping-positive and three clumping-negative strains, all carrying plasmid DNA) using primers internal to AS genes of *E. faecalis*. No PCR products were obtained with the same primers among *E. faecium* isolates, either in clumping-positive or clumping-negative strains, only two of which carried plasmid DNA.

Among agg/prgB-positive *E. faecalis* clinical isolates, restriction profiles obtained by digesting PCR products with DraI and ScaI were identical to those obtained from *E. faecalis* OG1RF(pCF10), whereas EcoRI profiles showed a three-band difference, suggesting the presence of one more EcoRI site. All agg/prgB-positive *E. faecalis* strains were also positive in Southern hybridization experiments with Smal-restricted genomic DNA, using as a probe a DNA fragment, amplified by PCR, internal to *prgB* of *E. faecalis* OG1RF(pCF10). The *prgB* probe...
strongly hybridized with plasmid DNAs from three *E. faecalis* strains, suggesting a plasmid location. These results strongly suggest that these clinical isolates contain AS genes. Considering that *agg* primers amplify a highly conserved 1,553-bp region (12), the slight difference revealed only by EcoRI restriction analysis might be attributed to a minor mutational event.

The *agg/prgB*-positive *E. faecalis* strains were negative for *prgX*, the gene involved in negative regulation of the pheromone response in pCF10; among these, three strains were clumping positive, and their adhesion to Caco-2 cells was enhanced by sex pheromones, suggesting that AS was expressed. Apart from the possible presence of a different gene, an alternative explanation might be found in the occurrence of point mutations in the *prgX* genes of these strains, affecting the affinity of the *prgX* primers for the target gene. The remaining

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**FIG. 3.** PFGE and Southern hybridization of SmaI-digested genomic DNA and plasmid DNA from enterococcal isolates with a psoralen-biotin-labeled probe specific for *prgB*. (A and B) PFGE patterns of genomic DNA (A) and corresponding hybridizing bands (B). (C and D) PFGE profiles of plasmid DNA (C) and corresponding hybridizing bands (D). Lanes: 1, CIUM22; 2, CIUM34; 3, EFM26; 4, EFS12; 5, EGL19; 6, EFS20; 7, EFS27B; 8, EFS28B; 9, EFS30D; 10, EFS38; 11, OG1RF(pCF10); 12, OG1RF; M1, Low Range PFG Marker; M2, Marker II. The arrows indicate chromosomal bands (B, lanes 4, 5, and 6) or plasmid bands (B and D, lanes 7, 8, 9, and 11) hybridizing with the *prgB* probe.
strains were clumping negative, and their adhesion to Caco-2 cells was not enhanced by sex pheromones, suggesting that AS is not expressed. Another significant issue is represented by the four clumping-positive but PCR- and Southern hybridization-negative strains (one *E. faecalis* and three *E. faecium* strains), whose adhesion to Caco-2 cells increased after induction with sex pheromones. As for *E. faecalis*, it can be hypothesized that the AS gene was not recognized by primers, while for *E. faecium*, it is possible that clumping is AS independent, even if the AS gene was not recognized by primers, while for *E. faecium*, it is possible that clumping is AS independent, even if the AS gene was not recognized by primers, while for *E. faecium*, it is possible that clumping is AS independent, even if

Overall, our results emphasize that slime production and/or producing slime have a selective advantage in colonizing biliary stents.

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