**Enterococcus faecalis** surface proteins determine its adhesion mechanism to bile drain materials

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An important step in infections associated with biliary drains is adhesion of micro-organisms to the surface. In this study the role of three surface proteins of *Enterococcus faecalis* (enterococcal surface protein, aggregation substances 1 and 373) in the adhesion to silicone rubber, fluoro-ethylene-propylene and polyethylene was examined. Four isogenic *E. faecalis* strains with and without aggregation substances and one strain expressing enterococcal surface protein were used. The kinetics of enterococcal adhesion to the materials was measured in situ in a parallel plate flow chamber. Initial deposition rates were similar for all strains, whereas the presence of surface proteins increased the total number of adhering bacteria. Nearest neighbour analysis demonstrated that enterococci expressing the whole sex-pheromone plasmid encoding aggregation substances 1 or 373 adhered in higher numbers through mechanisms of positive cooperativity, which means that adhesion of bacteria enhances the probability of adhesion of other bacteria near these bacteria. Enterococci with the enterococcal surface protein did not adhere through this mechanism. These findings indicate that the surface proteins of *E. faecalis* play a key role in the adhesion to bile drains and bile drain associated infections.

Keywords: bacterial adhesion, positive cooperativity, infection, biliary drains, biomaterials

**INTRODUCTION**

After an operation involving the common bile duct (CBD), for instance a liver transplantation, the bile is often diverted for prolonged times using a bile drain (T-tube). Infections are a frequent complication of T-tubing of the CBD (Yu et al., 1996a). Normally, the biliary tract is sterile, but bacterial colonization occurs in up to 75% of patients within several weeks after insertion of an in-dwelling foreign body (Yu et al., 1996a). Especially in immune-compromised liver transplant patients the bile may be drained with a T-tube for up to 3 months and infections pose a major problem (Ben-Ari et al., 1998). In animal experiments, T-tubing of the CBD resulted invariably in contamination of bile by intestinal bacteria within 6 weeks (Koivusalo et al., 1996).

After insertion of the bile drain in the CBD, bile-colonizing bacteria may form a biofilm that can be a source of persistent infection, because the biofilm mode of growth protects the adhering bacteria against host defences and the action of antimicrobial agents (Costerton et al., 1999). The initial step in the formation of a microbial biofilm is the transport and adhesion of micro-organisms to the surface of the in-dwelling foreign body. This adhesion is often determined by the presence of a host-derived coating of the foreign body and different bacterial cell surface structures, like fimbriae, fibrillae or specific surface proteins (An & Friedman, 1998; Bos et al., 1999; Busscher et al., 2000). In a physico-chemical approach, these specific structural and chemical cell surface properties are included in overall cell surface properties, as hydrophobicity and zeta potential (An & Friedman, 1998; Bos et al., 1999; Busscher et al., 2000). Insight into the involvement of
these bacterial surface properties in adhesion to biomaterials, as an important initial step in biofilm formation, may lead to strategies to reduce or prevent biomaterial-centred infections.

Enterococcus faecalis is one of the Gram-positive microorganisms commonly found in infections associated with foreign bodies in the bile duct (Ben-Ari et al., 1998; Molinari et al., 1996; Yu et al., 1996b). Due to the emerging antibiotic resistance of this microorganism, these infections are often difficult to prevent or treat (Murray, 1990). Some surface proteins of E. faecalis are found more frequently in enterococci causing infections and are therefore reported to be associated with enterococcal adhesion and infection (Berti et al., 1998; Coque et al., 1995; Jett et al., 1994; Shankar et al., 1999). One of these proteins, aggregation substance (Agg), is encoded by sex-pheromone-responsive plasmids. Agg is expressed in response to peptide pheromone induction as a sticky ‘hair-like’ structure on the cell wall (Galli et al., 1989), resulting in formation of aggregates of donor and recipient bacteria and transfer of plasmids (Clewell, 1993). All sex-pheromone plasmids, except pAM373, contain a homologous DNA region that encodes Agg (e.g. Asa1 encoded on pAD1). The Agg encoded on plasmid pAM373 (Asa373) does not fit the overall homology as no similarities could be detected by Southern or Western blots using Asa1-specific DNA probes or antibodies (Galli & Wirth, 1991; Muscholl-Silberhorn, 1999). Finally, the DNA sequence of asa373 proved to be totally different from the DNA sequence of asa1 (De Boever et al., 2000). The deduced amino acid sequence of Agg contains two tripeptide arginine-glycine-aspartic acid (RGD) sequences (Galli et al., 1990). Since RGD is the cell attachment site of a large number of adhesive extracellular matrix, blood and cell surface proteins (Ruoslatti, 1996), it has been suggested that Agg might play a role in the adherence to, and the colonization of, host tissues by E. faecalis. This has been confirmed by different groups, showing that Agg is correlated with the binding of E. faecalis to renal tubular cells and intestinal epithelial cells (Kreft et al., 1992; Olmsted et al., 1994; Sartingen et al., 2000).

The second surface protein, enterococcal surface protein (Esp), was discovered in an E. faecalis strain that caused multiple infections within a hospital ward (Shankar et al., 1999). Recently, it was shown that the presence of the esp gene was highly associated with the capacity of E. faecalis to form a biofilm on a polystyrene surface (Toledo-Arana et al., 2001). Also, the presence of Esp contributed to colonization and persistence of E. faecalis in the urinary tract in an animal model of urinary tract infection (Shankar et al., 2001). A variant esp gene was found in vancomycin-resistant Enterococcus faecium spreading in hospitals (Willems et al., 2001). The fact that surface proteins Agg and Esp are associated with E. faecalis causing infections or epidemics suggests that these proteins may be associated with the ability of this microorganism to adhere to and subsequently infect host tissue, or to spread within the hospital.

The purpose of this study was to investigate whether surface proteins Agg (Asa1 and Asa373), encoded on sex-pheromone-responsive plasmids, and Esp of E. faecalis influence its adhesion to different bile drain materials. To this end, we compared the adhesion of five E. faecalis strains with different surface proteins to fluoro-ethylene-propylene (FEP), polyethylene (PE) and silicone rubber (SR) under flow conditions similar to those in a bile drain.

**METHODS**

**Strains and growth conditions.** Four isogenic E. faecalis strains were used in this study: the plasmid-free strain OG1X (Ike et al., 1983); OG1X containing the sex-pheromone-responsive plasmid pAD1, encoding the Agg Asa1, with a positive regulator gene inserted that induces constitutive expression of this plasmid (denoted as OG1XE:pAD1; E indicates the positive regulator gene) (Franke & Clewell, 1981; Muscholl et al., 1993); OG1X containing the plasmid pW-e-Asa1, a derivative of the Escherichia coli–Enterococcus faecalis shuttle vector pWM401 which contains the asa1 gene and constitutively expresses Asa1 but no other proteins encoded on pAD1 nor any proteins that might be encoded on the vector (Albrecht B. Muscholl-Silberhorn, Thetis-IBN, Hamburg, Germany, personal communication) (denoted as OG1X:pWWeAsa1) (Muscholl et al., 1993); and OG1X containing the plasmid pAM373 which expresses Asa373 after induction with pheromones (denoted as OG1X:pAM373) (Clewell et al., 1985). The four isogenic OG1X-derived strains did not contain the esp gene, as confirmed by PCR. Expression of Agg was checked by immunofluorescence with polyclonal antibodies against Asa1 or Asa373. A similar level of Agg expression in all three Agg-positive strains was detected (data not shown). A. B. Muscholl-Silberhorn kindly provided OG1X strains and antibodies. V. Shankar (University of Oklahoma Health Sciences Centre, Oklahoma City, USA) provided MMH594, the Esp-expressing strain (Shankar et al., 1999). MMH594 did not express Agg, as confirmed by immunofluorescence.

The strains were streaked and grown overnight at 37 °C from a frozen stock on blood agar plates. The plate was then kept at 4 °C, but never longer than 4 weeks. Several colonies were used to inoculate 3 ml Todd–Hewitt broth (Oxoid) that was incubated at 37 °C in ambient air for 24 h. From this preculture, 2 ml was used to inoculate a second culture of 200 ml THB that was grown for 18 h. E. faecalis OG1X:pWWeAsa1 was grown in THB with 20 µg erythromycin ml⁻1 and 20 µg chloramphenicol ml⁻1, and MMH594 was grown in THB with 500 µg gentamicin ml⁻1. Bacteria from the second culture were harvested by centrifugation at 10000 g for 10 min at 4 °C and washed twice with demineralized water. Subsequently, bacteria were sonicated on ice for 2 × 10 s to separate cell clusters and counted in a Bürker-Türk counting chamber. The cells were resuspended in PBS (10 mM potassium phosphate, 0.15 M NaCl, pH 7) at a concentration of 3 × 10⁹ cells ml⁻¹.

E. faecalis JH12-2 excretes all known sex-pheromones of E. faecalis into the growth medium and was used to collect pheromone (Jacob & Hobbs, 1974). After 24 h growth at 37 °C in THB, the culture was centrifuged at 10000 g for 10 min at 4 °C and the supernatant containing the pheromones was autoclaved. To induce expression of Agg in strain OG1X:pAM373, cells must be cultivated in the presence of pheromone, therefore the second culture of strain OG1X:pAM373 consisted of 100 ml fresh THB and 100 ml pheromone-containing THB supernatant.
**Biomaterials.** Implant-grade SR was obtained from Medin. Poly(tetrafluoroethylene-co-hexafluoropropylene) (fluoroethylene-propylene, FEP) was supplied by Fluorolast and low density PE from Goodfellow. To ensure biomaterial surfaces were clean, they were sonicated for 3 min in a surfactant solution (2% RBS 35 in water; Omniclean), rinsed thoroughly with water and then washed with methanol and demineralized water before use.

**Parallel plate flow chamber, image analysis and adhesion.** The flow chamber (internal dimensions: l × w × h, 76 × 38 × 0.6 mm) and image analysis system have been described in detail previously (Busscher & Van der Mei, 1995). Images were taken from the bottom plate (58 × 38 mm) of the parallel plate flow chamber. The Perspex bottom plate was completely covered with the material under study for FEP and PE. For SR a thin square (15 × 15 mm) of the material was affixed centrally into the groove (15 × 15 mm) of a thicker (2/0 mm) Perspex plate. The depth of the groove was adapted to the thickness of the biomaterial in such a way that the SR surface was at the same height as the surface of the Perspex plate. The top plate of the chamber was always made of glass. The flow chamber was cleaned with Extran (Merck) and thoroughly rinsed with water before use.

**RESULTS**

To measure the adhesion of *E. faecalis* strains to different bile drain materials, a bacterial suspension was perfused through a flow chamber system for 4 h during which images were taken at different time intervals and analysed. All adhesion experiments were performed in triplicate with separately cultured bacteria. To exclude the influence of growth in the presence of the antibiotics erythromycin and chloramphenicol on the adhesion of strain OG1X:pWeAsa1, we tested the adhesion of this strain after growth in medium without antibiotics and the results fell within the range found for adhesion after growth in the presence of antibiotics (data not shown). However, as the vector used is very unstable if the bacteria are grown without antibiotics, strain OG1X:pWeAsa1 was grown in the presence of antibiotics for better reproducibility.

**Statistical analysis.** Data were analysed with one-way analysis of variance followed by the Bonferroni t-test for pairwise multiple comparisons. The Kruskal–Wallis test followed by the Mann–Whitney test were used for non-parametric data. Significance was defined as *P* ≤ 0.05.

The number of bacteria at the stationary end point (n at *t*ₜₜ) in the flow chamber was significantly higher for *E. faecalis* with surface proteins compared to OG1X without surface proteins (Table 1). The total number of bacteria for all strains on FEP was significantly higher than on SR (*P* ≤ 0.05).

**Characteristic adhesion time constant**

The characteristic adhesion time constant τ describes the way in which the adhesion process approaches a stationary state. For *E. faecalis* strains with surface proteins, τ is higher than for OG1X without surface proteins and for most strains this difference is significant, indicating that it takes longer for these bacteria to attain stationary state adhesion. No significant difference could be detected between the different substrata averaged over all strains.

**Positive cooperativity**

Positive cooperativity in microbial adhesion to surfaces is defined as the ability of one adhering organism to stimulate the adhesion of other organisms in its immediate vicinity. Originally, positive cooperativity was
Table 1. Adhesion of *E. faecalis* strains to three different bile drain materials

Numbers show the initial deposition rate (*j*), characteristic adhesion time (*τ*) and no. of bacteria at the stationary end point (*n* at *t*ₜ). Experiments performed in triplicate with separate bacterial cultures yielded an SD < 30%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>10⁻²×<em>j</em>₀ (cm⁻² s⁻¹)</th>
<th>10⁻³×<em>τ</em> (s⁻¹)</th>
<th>10⁻⁴×<em>n</em> at <em>t</em>ₜ (cm⁻²)</th>
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<tbody>
<tr>
<td></td>
<td>FEP</td>
<td>PE</td>
<td>SR</td>
</tr>
<tr>
<td>OG1X (Agg⁺, Esp⁻)</td>
<td>16</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>OG1X:E:pAD1 (Agg⁺)</td>
<td>17</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>OG1X:pWeAsa1 (Agg⁺)</td>
<td>17</td>
<td>16</td>
<td>12</td>
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<tr>
<td>OG1X:pAM373 (Agg⁺)</td>
<td>16</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>MMH594 (Esp⁺)</td>
<td>17</td>
<td>16</td>
<td>18⁺</td>
</tr>
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*⁺ P ≤ 0.05 versus OG1X.

Fig. 1. Spatial arrangements of *E. faecalis* after 4 h of flow on FEP for OG1X (a), OG1X:E:pAD1 (b) and MMH594 (c). The images show the presence of small aggregates of *E. faecalis* on the surface for OG1X (a), the formation of distinct aggregates if Agg is expressed (b) and the absence of aggregates if Esp is expressed (c). The distribution of OG1X:pWeAsa1 was very similar to OG1X, while the distribution of OG1X:pAM373 was very similar to OG1X:E:pAD1; therefore, these images are not shown. Bar, 15 µm.

indirectly inferred from Scatchard plots of bound over unbound organisms. However, the Scatchard analysis involves several assumptions that are not always met for microbial adhesion (Van der Mei *et al.*, 1993). Sjollema & Busscher (1990) have pointed out that positive cooperativity is directly reflected in the spatial arrangement of adhering organisms over a substratum surface, provided the spatial arrangement is fully preserved as existing during adhesion. Positive cooperativity is then concluded from high local relative densities around a given adhering organism. The spatial arrangement of adhering enterococci over a substratum surface was analysed by radial pair distribution functions. Each adhering bacterium was taken once as a centre point and local densities of adhering enterococci were determined in circular shells at a distance *r* from this centre point. These local densities were normalized with respect to the mean density of adhering bacteria, resulting in the radial pair distribution function *g*(*r*). When enterococci are randomly distributed over the entire substratum surface, *g*(*r*) = 1. However, if there is preferential adhesion at a given separation distance *r* between adhering bacteria, then *g*(*r*) > 1. The maximum value of *g*(*r*ₚ) indicates the preference for bacterial deposition near an already adhering bacterium and is a direct measure of positive cooperativity. The distance at which this maximum occurs (*r*ₚ) indicates the preferential adhesion distance of the bacteria. The maximum value of *g*(*r*ₚ) was calculated for three different fields of view after 4 h of flow.

Fig. 1 compares the spatial arrangements of strains OG1X, OG1X:E:pAD1 and MMH594 on FEP after 4 h of flow and in Table 2 the accessory values for *g*(*r*ₚ) and *r*ₚ are shown. Previously, for inert polystyrene particles *g*(*r*ₚ) values ranging from 1.2 to 1.4 were reported, which provides a criterion for the absence of positive cooperativity (Sjollema & Busscher, 1990). The strain used in
adhering exclusively as single cells (Fig. 1c), i.e. elevated value of distinct aggregates are formed (Fig. 1b), in line with an strong cooperativity, i.e. on the substratum surface strains OG1XE:pAD1 and OG1X:pAM373 expressing with some small aggregates (Fig. 1a). (2) strain OG1X shows mainly singly adhering organisms non-specific cooperativity, as the spatial arrangement of OG1X and OG1X:pWeAsa1 expressing weak, possibly this study can be divided into three groups with regard to their strength of cooperativity. (1) E. faecalis strains OG1X and OG1X:pWeAsa1 expressing weak, possibly non-specific cooperativity, as the spatial arrangement of strain OG1X shows mainly singly adhering organisms with some small aggregates (Fig. 1a). (2) E. faecalis strains OG1XE:pAD1 and OG1X:pAM373 expressing strong cooperativity, i.e. on the substratum surface distinct aggregates are formed (Fig. 1b), in line with an elevated value of $g(r_p)$ (see Table 2). (3) Strain MMH594 adhering exclusively as single cells (Fig. 1c), i.e. $g(r_p)$ equals unity, indicating the absence of positive cooperativity (see Table 2).

<table>
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<th>Strain</th>
<th>$g(r_p)$</th>
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<tr>
<td></td>
<td>FEP</td>
<td>PE</td>
</tr>
<tr>
<td>OG1X (Agg⁺, Esp⁻)</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>OG1XE:pAD1 (Agg⁺)</td>
<td>3.2⁺</td>
<td>2.7⁺</td>
</tr>
<tr>
<td>OG1X:pWeAsa1 (Agg⁻)</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>OG1X:pAM373 (Agg⁻)</td>
<td>2.0</td>
<td>2.7⁺</td>
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<tr>
<td>MMH594 (Esp⁺)</td>
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* $P \leq 0.05$ versus OG1X.

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### DISCUSSION

This study addressed the question whether different surface proteins on E. faecalis influence its adhesion to bile drain materials. Surface proteins do not influence the initial deposition of E. faecalis on different hydrophobic materials. However, the total number of bacteria adhering at the stationary end point was significantly higher for E. faecalis expressing surface proteins. The surface proteins enhance the total number of adhering bacteria via a different mechanism. The expression of Agg (Asa1 and Asa373) and other gene products encoded on the sex-pheromone plasmids, but not solely Agg (Asa1), invokes positive cooperativity. This means that adhering individual enterococci stimulate adhesion of more enterococci in the more advanced stages of the adhesion process, whereas the expression of Esp or solely Agg without the sex-pheromone plasmid enhances the number of adhering enterococci through direct interactions with the material and not via positive cooperativity.

The initial deposition rates reflect the direct interaction between the micro-organism and the substratum surface, without influences of already adhering bacteria (Bos et al., 1999). The initial contact between the bacterium and the substratum is determined by the presence of hydrophobic or electrostatic groups. When surface proteins are only scarcely present on the bacterial cell surface, these physico-chemical interaction forces will be influenced little by the presence of the surface proteins. Whereas the initial deposition rates vary little among the different strains, deposition rates of enterococci on FEP were higher than on PE and SR. This difference is likely to be a result of a combination of the high surface hydrophobicity of FEP and a different chemical composition. FEP is a very inert material with only fluorine groups on its surface, in contrast to, for example, SR with methyl groups on its surface (An & Friedman, 1998).

In contrast to the initial deposition, the total number of bacteria at the stationary end point was increased by the presence of surface proteins on E. faecalis. The number of bacteria at the stationary end point is influenced not only by fundamental interaction forces originating from the substratum surface, but also by more specific interactions between the bacteria. Positive cooperativity is one of the mechanisms that might play a role in the increase in total number of bacteria if surface proteins are present. Adhesion of bacteria gives rise to new adhesion sites or diminishes the influence of antagonistic sites, and newly depositing bacteria will adhere preferably in the neighbourhood of already adhering ones. As a corollary of positive cooperativity, organisms adhere close to each other and appear not randomly distributed. Positive cooperativity on saliva-coated substrata has been explained predominantly by the capacity of adhering cells to induce conformational changes in the pellicle or on the surfaces of approaching cells (Doyle et al., 1982). However, positive cooperative phenomena have also been described for oral streptococci adhering to inert substrata (Van der Mei et al., 1993). Proposed explanations are based on the hypothesis that the magnitude of the interaction forces between adhering cells is higher than the interaction forces between adhering cells and their substrata or between cells in suspension (Doyle, 1991; Van der Mei et al., 1993).

A model for the adhesion of the different E. faecalis strains to an inert substratum surface, accounting for the role of surface proteins, is proposed in Fig. 2. The model shows three different mechanisms of adhesion to the substratum and interactions between bacteria, as found for the different strains in this study. Strain OG1X, without any surface proteins, shows a weak cooperativity between adhering enterococci that might be due to non-specific interactions (Fig. 2a). The E. faecalis strains expressing the sex-pheromone plasmids (OG1XE:pAD1 and OG1X:pAM373) adhere in high numbers to the substratum due to specific interaction between the bacteria (positive cooperativity) (Fig. 2b). This high cooperativity between sex-pheromone-plasmid-expressing bacteria might be explained by the fact that the plasmid plays a role in contact between bacteria for its own transfer (Wirth, 1994). The Esp-positive E. faecalis strain (MMH594) adheres in high numbers to the

Table 2. Degree of positive cooperativity involved in the adhesion of E. faecalis strains to FEP, PE and SR, as derived from radial distribution functions

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* $P \leq 0.05$ versus OG1X.
substratum, but does not show any interaction between the bacteria (Fig. 2c). Consequently, the interaction force with the substratum of this strain must be strong, possibly due to the presence of Esp on the surface. This result has been confirmed by a recent study (Toledo-Arana et al., 2001) in which it was shown that Esp increases the number of bacteria adhering to abiotic surfaces by comparing the adhesion with Esp-deficient mutants. Strain OG1X:pWeAsa1 expressing Esp, does not show any interaction between the bacteria (negative cooperativity). The lower cooperativity of strain OG1X:pWeAsa1 compared to the strains with the whole sex-pheromone plasmid indicates that other factors than Agg encoded on the sex-pheromone plasmid play a role in positive cooperativity. The difference in cooperativity could also be explained by differences in the expression level of Agg between OG1X:pWeAsa1 and OG1X:pAD1, since the promoter constructed in OG1X:pWeAsa1 is weaker than the promoter constructed in OG1X:pAD1 (Albrecht B. Muscholl-Silberhorn, personal communication). However, a similar level of expression was seen in the immunofluorescence assay, which indicates that the difference in expression might not be very high. The sex-pheromone plasmid transfer system is very complicated with many factors involved in its control. The functions of some genes on the plasmids are still not completely understood today (De Boever et al., 2000; Dunny et al., 1995; Francia et al., 2001; Wirth, 1994). The question of which of these factors is involved in positive cooperativity on the surface is intriguing and still needs to be answered more fully.

The surface proteins described in this paper are possible virulence factors associated with infections in humans. However, the exact role of these proteins in the pathogenesis of infections is still not known. Many of the infections in hospitalized patients are associated with in-dwelling medical devices, especially bile drains. One way of initiating these biomaterial-centred infections is by adhesion to the device. In this study we found that the surface proteins of *E. faecalis* are possible virulence factors because they enhance the number of bacteria adhering to bile drain materials. Higher total numbers of bacteria adhering to in-dwelling medical devices may lead to more colonization and biomaterial-centred infections. In addition, the question might arise whether the surface proteins studied under *in vitro* conditions will be expressed in patients. Esp is always expressed on the surface if the bacterium contains the *esp* gene, therefore it is likely to be expressed in vivo (Shankar et al., 1999). The in *vivo* expression of Agg is more difficult to describe. To our knowledge until now no research has been published on the expression of Agg in bile or the human gut. However, it has been shown that factors other than pheromone (e.g. serum) can induce expression of Agg in *vivo* (Hirt et al., 2000; Kreft et al., 1992). Therefore, we think that it is reasonable to assume that Agg is expressed in vivo.

In summary, enterococci expressing Esp or Agg adhere in higher numbers to hydrophobic bile drain materials. However, *E. faecalis* with different surface proteins adheres to the substratum through different mechanisms. Enterococci expressing the sex-pheromone plas-
mid encoding Asa1 or Asa373 adhere in high numbers through positive cooperativity between adhering bacteria. Enterococci positive for Esp also adhere in high numbers, but do not utilize positive cooperative mechanisms in their adhesion to these materials. These findings indicate that interfering with the positive cooperativity between adhering enterococci, as resulting from the expression of sex-pheromone plasmids, or interfering with the interaction between surface proteins and materials, might yield a way to prevent bile-drain-associated infections.

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K. Waar and others


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