PART II

Enterococcuss faecalis in Biomaterial related Infections

CHAPTER

Enterococcus faecalis Surface Proteins determine its Adhesion Mechanism to Bile Drain Materials

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Microbiology 2002; 148; 1863-1870

Abstract

An important step in infections associated with biliary drains is adhesion of microorganisms 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.

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 (270). 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 (270). Especially in immune-compromised liver transplant patients the bile may be drained with a T-tube for up to three months and infections pose a major problem (13). In animal experiments, T-tubing of the CBD resulted invariably in contamination of bile by intestinal bacteria within 6 weeks (130). 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 the host defence and the action of antimicrobial agents (45). The initial step in the formation of a microbial biofilm is the transport and adhesion of microorganisms 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 (3, 22, 30). 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 (3, 22, 30). Insight into the involvement of these bacterial surface properties in the 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 (13, 160, 271). Due to the emerging antibiotic resistance of this microorganism, these infections are often difficult to prevent or treat (169). 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 (15, 44, 123, 217). 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 (89) resulting in formation of aggregates of donor and recipient bacteria and transfer of plasmids (41). All sex-pheromone plasmids, except pAM373, contain a homologous DNA region that encodes the 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 (88, 175). Finally, the DNA sequence of *asa373* that was determined proved to be totally different from the DNA sequence of the *asa1* gene (50). The deduced amino-acid sequence of Agg contains two tripeptide arginine-glycine-aspartic acid (RGD) sequences (87). Since RGD is the cell attachment site of a large number of adhesive extracellular matrix, blood, and cell surface proteins (207), 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 (132, 185, 212).

The second surface protein, enterococcal surface protein (Esp), was discovered in an *E.*

faecalis strain that caused multiple infections within a hospital ward (217). 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 (235). Also, the presence of Esp contributed to colonization and persistence of *E. faecalis* in the urinary tract the in an animal model of urinary tract infection (216). A variant *esp* gene was found in vancomycin-resistant *Enterococcus faecium* spreading in hospitals (260). 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.

Materials and Methods

Strains and growth conditions

Four isogenic *E. faecalis* strains were used in this study: the plasmid-free strain OG1X (116); 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) (81, 173); 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 (personal communication with Albrecht B. Muscholl-Silberhorn, Thetis-IBN, Hamburg, Germany) (denoted as OG1X:pWeAsa1) (173) and OG1X containing the plasmid pAM373 which expresses Asa373 after induction with pheromones (denoted as OG1X:pAM373) (42). The four isogenic OG1X derived strains did not contain the *esp* gene as was confirmed by PCR. Expression of the Agg was checked by immunofluorescence with polyclonal antibodies against Asa1 or Asa373. A similar level of Agg expression on 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 (217). MMH594 did not express Agg as was 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 of Todd-Hewitt broth (THB; 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:pWeAsa1 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 10 000 *g* for 5 min at 10 °C and washed twice with demineralized water. Subsequently, bacteria were sonicated on ice for 2 x 10 s to separate cell clusters and counted in a Bürker-Türk counting chamber. The cells were resuspended in phosphate buffered saline (PBS) (10 mM potassiumphosphate and 0.15 M NaCl at pH 7) at a concentration of 3 x 10^8 cells ml^{-1}.
E. faecalis JH2-2 excretes all known sex-pheromones of *E. faecalis* into the growth medium and was used to collect pheromone (120). After 24 h growth at 37 °C in THB, the culture was centrifuged at 10 000 *g* for 10 min at 10 °C and the supernatant containing the pheromones was autoclaved. To induce expression of the 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) (fluoro-ethylene-propylene, FEP) was supplied by Fluorplast 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* x *w* x *h*, 76 x 38 x 0.6 mm) and image analysis system have been described in detail previously (31). Images were taken from the bottom plate (58 x 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 x 15 mm) of the material was affixed centrally into the groove (15 x 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 way that the silicone rubber 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 and demineralized water. Prior to each experiment, all tubes and the flow chamber were filled with PBS, taking care to remove all air bubbles from the system. Once the system was filled, a bacterial suspension of 3 x 10^8 cells ml^{-1} in PBS was allowed to flow through the system at a flow rate of 1.44 ml min^{-1}, corresponding with a shear rate of 10.6 s^{-1}. This shear rate is similar to the shear rate in a bile drain of 2 mm diameter at a bile production of 30 ml h^{-1}. Deposition was observed with a CCD-MXRi camera (High Technology) mounted on a phase-contrast microscope (Olympus BH-2) equipped with a 40 x ultra-long-working distance lens (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyser (TEA, Difa). The bacterial suspension was perfused through the system for 4 h with re-circulation at room temperature, and 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 the growth in presence of the antibiotics erythromycin and chloramphenicol on the adhesion of strain OG1X:pWeAsa1, we tested the adhesion of this strain after the 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 Bonferonni *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 $P \leq 0.05$.

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 from the material with adhering bacteria. The number of adhering bacteria in the images were transformed to bacteria per unit area and least-square-fitted to an exponential curve. The number of bacteria adhering at stationary end point, n at t_∞, could be estimated from this exponential curve (SigmaPlot for Windows, version 5.00, SPSS), as well as a characteristic time constant, τ, describing the approach of the adhesion process to a stationary state (49). The initial increase in the number of adhering bacteria over time was expressed in so-called initial deposition rate, j_0, the increase in the number of adhering bacteria per unit area and time.

Initial deposition rates

In Table 1 the results of the flow chamber adhesion experiments are shown, including initial deposition rates (j_0), characteristic adhesion times (τ) and number of bacteria at stationary end point (n at t_∞). The initial deposition rates (j_0) did not differ for the *E. faecalis* strains with or without surface proteins on FEP and PE. On SR, however, the initial deposition rate of strain MMH594 was significantly higher than of strain OG1X, while in addition the mean initial deposition rates, averaged for all strains, were significantly higher on FEP than on SR ($P \leq 0.05$). The experimentally observed initial deposition rates are all in excess of the theoretical initial deposition rate (j_0*) for deposition in the parallel plate flow chamber under the current experimental conditions according to Von Smoluchowski-Levich, i.e. 692 cm^{-2} s^{-1} (25). Consequently, the mean deposition efficiency (j_0/j_0*) of this collection of enterococci amounts to 2.1 ± 0.4, which indicates that these strains have a high affinity for the hydrophobic substrata used.

Table 1. Adhesion of *E. faecalis* strains to 3 different bile drain materials

Strain	j_0 (10^2 cm^{-2} s^{-1})			τ (10^3 s^{-1})			*n* at t_∞ (10^6 cm^{-2})		
	FEP	PE	SR	FEP	PE	SR	FEP	PE	SR
OG1X (Agg$^-$, Esp$^-$)	16	16	8	3.7	2.9	3.0	6.1	4.9	2.6
OG1XE:pAD1 (Agg$^+$)	17	16	10	8.3*	5.0	11.1*	14.0*	8.0*	11.2*
OG1X:pWeAsa1 (Agg$^+$)	17	16	12	8.3*	8.3*	6.7*	14.9*	13.3*	7.8*
OG1X:pAM373 (Agg$^+$)	16	12	13	7.1*	7.1*	6.3*	11.5*	8.3*	7.9*
MMH594 (Esp$^+$)	17	16	18*	5.9	7.7*	3.8	10.3*	12.6*	7.1*

Numbers show the initial deposition rate (j_0), characteristic adhesion time (τ) and no. of bacteria at the stationary end point (*n* at t_∞). Experiments performed in triplicate with separate bacterial cultures yielded a SD < 30 %. *$P \leq 0.05$ versus OG1X.

Number of bacteria adhering at the stationary end point

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

Characteristic adhesion time constant

The characteristic adhesion time constant τ (= $n_\infty\ j_0^{-1}$) 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 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 (242). 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 for 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 3 different fields of view after 4 h of flow.
Figure 1 compares the spatial arrangements of strains OG1X, OG1XE: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 (224). The strain used in 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 (Figure 1a). (2) *E. faecalis* strains OG1XE:pAD1 and OG1X:pAM373 expressing strong cooperativity, i.e. on the substratum surface distinct aggregates are formed (Figure 1b), in line with an elevated value of $g(r_p)$ (see Table 2). (3) Strain MMH594 adhering exclusively as single cells (Figure 1c), i.e. $g(r_p)$ equals unity, indicating the absence of positive cooperativity (see Table 2).

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 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

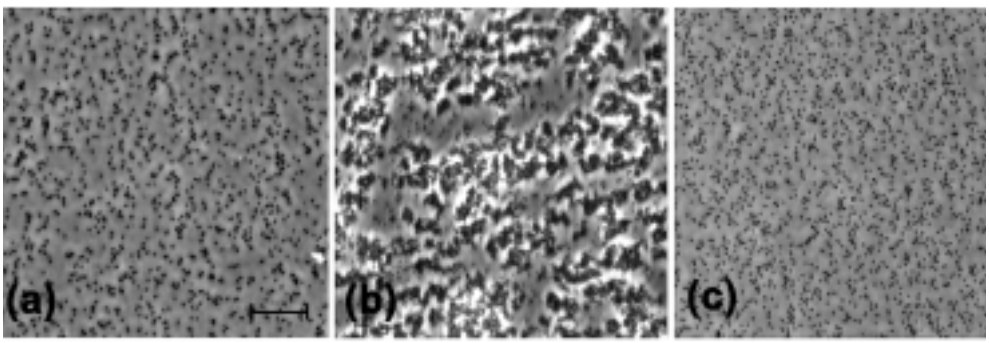

Figure 1. Spatial arrangements of *E. faecalis* after 4 h of flow on FEP for: OG1X (a), OG1XE:pAD1 (b), MMH594 (c). The images show the presence of small aggregates of *E. faecalis* on the surface for OG1X (a), the formation of distinct aggregates if the Agg is expressed (b) and the absence of aggregates 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 OG1XE:pAD1; therefore, these images are not shown. Bar, 15 μm.

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.

Strain	$g(r_p)$			r_p (μm)		
	FEP	PE	SR	FEP	PE	SR
OG1X (Agg^-, Esp^-)	1.4	1.8	2.3	1.2	1.2	1.3
OG1XE:pAD1 (Agg^+)	3.2*	2.7*	2.8	1.8*	1.7	1.8
OG1X:pWeAsa1 (Agg^+)	1.7	1.5	2.2	1.8*	1.9*	1.5
OG1X:pAM373 (Agg^+)	2.0	2.7*	2.6	1.5*	1.3	1.5
MMH594 (Esp^+)	1.0	1.0*	1.1*	2.8*	2.6*	2.3*

Positive cooperativity is concluded from $g(r_p)$ values in excess of 1.4 and r_p indicates the preferential adhesion distance. Experiments performed in triplicate with separate bacterial cultures yielded a SD < 30 %. *$P \leq 0.05$ versus OG1X.

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 microorganism and the substratum surface, without influences of already adhering bacteria (22). 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 e.g. SR with methyl groups on its surface (3).

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 a 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 (58). However, positive cooperative phenomena have also been described for oral streptococci adhering to inert substrata (242). 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 (56, 242).

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 Figure 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 (Figure 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) (Figure 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 (265). The Esp-positive *E. faecalis* strain (MMH594) adheres in high numbers to the substratum, but does not show any interaction between the bacteria (Figure 2c).

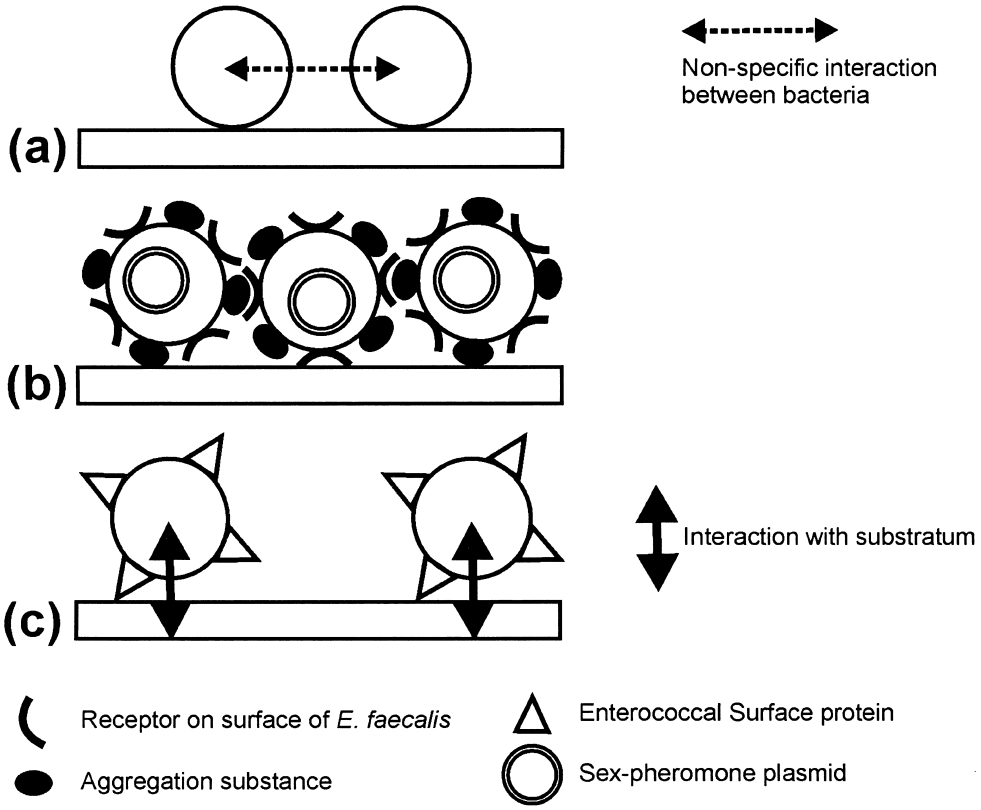

Figure 2. Proposed mechanism of the adhesion of *E. faecalis* with different surface proteins to hydrophobic substrata. (a) Strain OG1X without surface proteins shows weak cooperativity, possibly due to non-specific interactions and adheres in low numbers, (b) *E. faecalis* strains expressing the sex-pheromone plasmids (OG1XE:pAD1 and OG1X:pAM373) adhere in high numbers to the substratum due to specific interaction between the bacteria (positive cooperativity), (c) Esp positive *E. faecalis* strain MMH594 does not show any interaction between the bacteria, but yet adheres in high numbers to the substratum as a result of strong interaction forces with the substratum due to the presence of Esp on the surface.

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 is confirmed by a recent study (235) in which it was shown that Esp increases the number of bacteria adhering to abiotic surfaces by comparing the adhesion with Esp-deficient mutants. The strain OG1X:pWeAsa1 expressing Asa1 but not the sex-pheromone plasmid, does not fit very well into this model. This strain adheres in high numbers, although its cooperativity is similar to that of strain OG1X. Both strain OG1X:pWeAsa1 as well as the strains that express the whole sex-pheromone plasmid showed high numbers of adhering bacteria at stationary end point; this might indicate that not the whole sex-pheromone plasmid, but only the Agg is necessary to reach high numbers of adhering bacteria. On the other hand, 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 OG1XE:pAD1 since the promotor constructed in OG1X:pWeAsa1 is weaker than the promotor constructed in OG1XE: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 (50, 66, 80, 265). 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 hospitalised patient are associated with in-dwelling medical devices, especially bile drains. One way of initiating these biomaterial-centered 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-centered 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 (217). 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 (108, 132). 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 plasmid encoding the 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 utilise 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 drains associated infections.

We thank Albrecht Muscholl-Silberhorn for helpful discussion. We also thank Rudi Tonk and Joop de Vries for reproduction of the figures in this article.