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Virulence Factors and *in Vitro* Adherence of *Enterococcus* strains to Urinary Catheters

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ABSTRACT. The ability to adhere *in vitro* to urinary catheters and the presence of enterococcal virulence factors was determined in 30 *Enterococcus* urinary isolates (12 *E. faecalis*, 12 *E. faecium*, 3 *E. casseliflavus*, 3 *E. gallinarum*). Silicone, siliconized latex and polyvinyl chloride (PVC) were examined by sonication quantitative culture technique and scanning electron microscope. As compared to *E. faecalis* and *E. faecium*, *E. casseliflavus* and *E. gallinarum* displayed lower adhesion to all synthetic materials. All the tests performed showed higher adherence of all tested strains to siliconized latex and silicone than to PVC. Biofilm-forming ability was observed in 5 *E. faecalis* but in none of the remaining strains. The gene coding enterococcal surface protein (Esp) was detected in 7 *E. faecalis* and 6 *E. faecium* strains. Gelatinase was found in 1 *E. faecalis*, 2 *E. faecium* and hemolysins were found in 6 *E. faecalis* and 1 *E. faecium* strains. All *E. casseliflavus* and *E. gallinarum* strainswere negative for these traits. Hydrophobic type of cell surface (measured by its affinity for *n*-hexadecane) was shown in a few isolates. Bacterial adherence was not significantly associated with the above pathogenic factors.

Enterococci are oportunistic organisms forming part of the normal intestinal flora of humans and animals (Facklam *et al.* 1997; Kawalec and Zaręba 1997). In recent years, however, they have become important nosocomial pathogens involved in human infections such as endocarditis (Megran 1992; Moellering 1992; Coque *et al.* 1995; Beneš *et al.* 2002), bacteremia (Vergis *et al.* 2002), urinary tract infections (Zaręba and Hryniewicz 1997) and intra-abdominal infections (Cooper *et al.* 1993). Some of the less pathogenic enterococci (other than *E. faecalis* and *E. faecium*) have been isolated from serious infections as well (Kurup *et al.* 2001). What more, they can survive in a hospital enviroment for an extended period of time (Neely and Maley 2000). Thus, the role of *Enterococcus*, especially in hospital-acquired infections, is still increasing but the understanding of their pathogenesis is not sufficient. Little is known about enterococcal properties which make it possible for the bacteria to adhere and colonize the host tissue (Mundy *et al.* 2000). Among such properties, production of slime (biofilm formation) (Baldassarri *et al.* 2001*a*,*b*), hydrophobic type of cell surface (Toledo-Arana *et al.* 2001), aggregation substances (AS) (Kreft *et al.* 1992; Schlievert *et al.* 1998; Hirth *et al.* 2000; Süßmuth *et al.* 2000) and enterococcal surface protein (Esp) (Hammerum and Jensen 2002; Shankar *et al.* 1999) which contributes to the persistance in the urinary tract (animal models) (Shankar *et al.* 2001), have been intensively studied.

The purpose of this study was to evaluate the ability of different enterococcal species to adhere to urinary catheters *in vitro*. Also, virulence factors such as hydrophobocity, enterococcal surface protein (Esp), hemolysins (Hln), gelatinase (Gel) and biofilm formation were determined in the strains studied.

MATERIALS AND METHODS

Bacterial strains. A total of 30 strains were examined, including 12 *Enterococcus faecalis*, 12 *Enterococcus faecium,* 3 *Enterococcus casseliflavus* and 3 *Enterococcus gallinarum*. All enterococcal isolates were obtained from the midstream urine of patients with urinary tract infection (UTI), hospitalized in the *Medical University Hospitals*, Wrocław (Poland). The strains of *E. faecalis* and *E. faecium* were the single etiological agents of significant bacteriuria ($\geq 10^5$ CFU/mL). The following reference strains were used: *E. faecalis* ATCC 29212, *E. faecium* ATCC 1859, *E. casseliflavus* CCM 2478, *E. faecalis* 23 (*esp*-negative), *E. faecalis* 54 (*esp*-positive), (provided by A. Toledo-Arana, *Instituto de Agrobiotecnologia y Recursos Naturales, Universidad Publica de Navarra*, Pamplona, Spain) and *E. faecalis* OG1X containing pAD1 (Hln^+,

asa1$^+$), *E. faecalis* OG1S containing pCF10 (Gel$^+$, *asa1*$^+$) (provided by K. Waar, *Departments of Medical Microbiology and Surgery, University Hospital Groningen*, The Netherlands).

Characterization of strains. The isolates were identified to species level with API 20 Strep kits (*bioMérieux*, France). The strains of *E. casseliflavus* and *E. gallinarum* were additionaly tested for their motility, production of pigment and growth at 45 °C (Facklam *et al.* 1997; Carvalho *et al.* 1998).

Adherence to catheters and scanning microscopy. For *in vitro* adherence studies, 3 types of urinary catheters were used: polyvinyl chloride, silicone and siliconized latex (*Mærsk Medical*, Denmark). The sonication quantitative culture technique was described by Joyanes *et al.* (2000). For scanning microscopy, fragments of catheters were removed from the medium and placed in individual vials containing PBS buffer (pH 7.4), fixed in 2.5 % glutaraldehyde and postfixed in 1 % OsO_4. After fixation, the specimens were dehydrated in ethanol–acetone series and air-dried. Samples were mounted on a stub with double-sided tape and subsequently coated with silver in the *Hochvakuum* Dresden sputter coater. Observation was carried out with a *Tesla* BS 300 SEM electron microscope at 15 kV.

Biofilm formation. The ability of the strains to produce biofilm was tested in a microtiter assay (Toledo-Arana *et al*. 2001). Briefly, bacterial strains were cultivated overnight in TSB with 0.25 % glucose. The cultures were diluted 1 : 40 in the same medium and 200 μL of the suspension was used to inoculate sterile 96-well microtiter plates (*Nunc*, Denmark). After a 1-d incubation at 37 °C, the wells were washed thrice with phosphate-buffered saline (PBS) and the plates were dried. The biofilm was stained with 1 % crystal violet. The remaining stain was removed with water and the biofilm was fixed in ethanol–acetone (4 : 1, *V*/*V*). Absorbance was measured at 595 nm. The strains of *E. faecalis* 54 and *E. faecalis* 23 were used as positive and negative controls, respectively.

Hemolysin production. For hemolysin (cytolysin) detection, Mueller–Hinton agar (*Biomed*, Poland) supplemented with 5 % human blood was used (Jett *et al.* 1994). The plates were incubated for 1 d at 37 °C and 4 d at 4 °C. Hemolytic activity was considered as a zone of β-hemolysis around the bacterial colonies. *E. faecalis* OG1X was used as a positive control.

Gelatinase production was determined by culturing the strains on Brain-Heart Infusion broth with gelatin (40 g/L) (*Difco*, USA). The bacteria were incubated for 7 d at 37 °C and a positive reaction (medium liquefaction) was observed at 4 °C (Joyanes *et al.* 2000). The results were compared with a positive control of *E. faecalis* OG1S.

Cell surface hydrophobicity was evaluated using the method of Rosenberg *et al.* (1980) as modified by Toledo-Arana *et al.* (2001). The strains were incubated for 1 d at 37 °C in TSB with 0.25 % glucose. The bacteria were washed, suspended in PUM buffer (in g/L: $K_2HPO_4 \cdot 3H_2O$ 22.2, KH_2PO_4 7.26, urea 1.8, $MgSO_4 \cdot 7H_2O$ 0.2) and added to *n*-hexadecane (*Merck*, Germany) in a 10 : 1 ratio. After a 10-min incubation at 37 °C, the tubes were shaken for 1 min. The aqueous phase was removed and absorbance A_{470} was measured. Surface hydrophobicity was expressed as the percentage of bacteria adhering to *n*-hexadecane. The following formula was used for calculation:

$$SH = (1 - A_F/A_I) \times 100$$

where SH is surface hydrophobicity, A_F final absorbance, and A_I initial absorbance.

Detection of esp-*gene.* DNA isolation was performed according to Rademaker *et al.* (1997). Primer sequences used to amplify the 928-bp fragment (5′-TCgTCTTCAATTAgATgAACgAT-3′ and 5′-AgAATC AAAgATgAAATCTTCACC-3′) were designed based on *esp*-gene sequence (AF034779). Oligonucleotides were obtained from *Bionovo Inc*., Legnica (Poland). The reaction mixture for PCR contained PCR buffer (10 mmol/L Tris-HCl, pH 8.8, 2 mmol/L $MgCl_2$, 2 mol/L KCl, 0.1 % Triton X-100 (*Sigma–Aldrich*, USA), 50 ng of template DNA, 0.1 mmol/L of each primer, 1.25 mmol/L of each deoxynucleotide and 1 U of *Taq* polymerase (both *Sigma–Aldrich*) in a total volume of 25 μL. PCR amplification was performed with DNA thermocycler (*Biometra*, Germany). For DNA amplification, the cycles were used as follows: 5 min at 95 °C, pause at 4 °C to add *Taq* polymerase, 90 s at 94 °C, 90 s at 53 °C and 3 min at 72 °C (25 cycles). After the reaction, PCR products (8 μL) were separated in 3 % agarose gels (150 mm long) at 90 V for 45 min. The gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed using the Viller-Lormant System (*Viller-Lormant*, France). Each experiment was repeated at least three times.

RESULTS AND DISCUSSION

Adhesion is the initial step in the process, which may lead to clinical symptoms of infection. Catheterization favors bacterial adherence and the development of the disease (Murga *et al.* 2001). Nosocomial infections are frequently caused by biofilm-forming microorganisms, enterococci being one of the examples

(Donlan 2002; Donlan and Costerton 2002). Among the enterococci isolated from patients with UTI, *E. faecalis* and *E. faecium* were dominant and well defined. Sometimes, however, other eneterococcal species have also been cultured from this type of infection. Thus, it was interesting to compare the adhesion properties as well as other virulence factors of different *Enterococcus* species.

Cultivation of 30 strains of *Enterococcus* spp. with catheter segments resulted in bacterial adhesion (determined by sonication) to the synthetic materials. The individual strains observed adhered to PVC, silicone, and siliconized latex with different intensity. Adhesion to all materials was higher for *E. faecalis* than for *E. faecium* (Table I), but it was unfortunately not clearly confirmed in scanning electron microscopy (Fig. 1). *E. casseliflavus* and *E. gallinarum* (the less frequent agents of infections in humans), as compared to *E. faecalis* and *E. faecium*, adhere to all synthetic materials more weakly (Table I). Similarly, Joyanes *et al.* (1999, 2000) published that the adherence of *E. faecalis* to silicone and siliconized latex was significantly higher than the adherence of *E. faecium.* It may be explained that from all enterococcal UTI asociated with catheters, *E. faecalis* is the most frequently isolated species.

Silicone and siliconized latex were the biomaterials that were colonized by all strains in the shortest time. Adherence occurred after 2 h of incubation and its peak was observed after 1 d.

Joyanes *et al.* (2000) observed no significant difference in the adhesion to silicone and siliconized latex for *E. faecalis* after 1 d. Our findings showed that after 1 d siliconized latex was more efficiently colonized by all strains. After a 2-d incubation of *E. faecalis* and *E. faecium* with siliconized latex, we observed formation of large bacterial aggregates in the inner part of the catheter segment (Fig. 2). No such formation was noted for silicone and PVC catheters.

The formation of biofilm on medical implants may lead to serious complications. Bacterial community structures (biofilm) are very resistant to antibiotics and can be a source of a chronic infection (Donlan 2002; Donlan and Costerton 2002). The biofilm on urinary catheters removed from patients has been well documented, and the organisms involved in this process include both G^- rods and G^+ cocci (Donlan and Costerton 2002). In the report of slime producing enterococci, Baldassarri *et al*. (2001*b*) showed that biofilm production was more common for *E. faecalis* (80 %) than for *E. faecium* (48 %). In our investigations, the ability to form biofilm (slime) was detected in 5 of 12 *E. faecalis* and in none of *E. faecium* strains. Among biofilm-positive *E. faecalis,* the differences in absorbance (A) range from 1.618 to 0.509 (positive control $A = 1.943$ and negative control $A = 0.190$). However, we did not note any difference in adhesion to the 3 types of catheters between biofilm producers and nonbiofilm producers. No biofilm was detected for *E. casseliflavus* ($A \leq 0.190$) and *E. gallinarum* ($A \leq 0.190$).

The factors contributing to the formation of enterococcal biofilm remain insufficiently known (Toledo-Arana *et al.* 2001). Lately, however, enterococcal surface protein (Esp) has been indicated as the agent associated with adherence, colonization and biofilm formation (Toledo-Arana *et al.* 2001; Waar *et al.* 2002*a*,*b*).

Esp has been identified in *E. faecalis* and *E. faecium* (Baldassarri *et al.* 2001*a*; Eaton and Gasson 2002; Waar *et al.* 2002*a*) and many of *E. faecalis* strains isolated from human infections harbor the *esp* gene (Archimbaud *et al.* 2002; Vergis *et al.* 2002; Waar *et al.* 2002*a*). Hammerum and Jensen (2002) found the *esp* gene in 10 of 24 *E. faecalis* and in 10 of 29 *E. faecium* clinical isolates. Some reports showed the high frequency of *esp* gene in *E. faecium* strains cultured from urine, faeces, wound swabs, blood and its lack in isolates from healthy individuals (Eaton and Gasson 2002). In the rarely isolated *Enterococcus*, known as the less pathogenic species, the *esp* gene has not been detected (Shankar *et al*. 1999). Our findings are in agreement with these results: We detected the *esp* gene in *E. faecalis* and *E. faecium* but not in *E. casseliflavus* or *E. gallinarum* (Table II). The *esp*-gene-positive isolates were considered as expressing the Esp protein (Shankar *et al.* 1999; Waar *et al.* 2002*a*). In our study, 7 of 12 *E. faecalis* strains harbored the *esp* gene. Interestingly, 5 of 7 *esp*-positive strains were biofilm producers and 2 were not. Among 12 of *E. faecium* strains, the *esp* gene was present in 5 strains but none of them formed biofilm. Thus we conclude that the Esp protein is not the only agent involved in the process of biofilm formation.

It has been demonstrated that Esp increased the hydrophobicity of bacterial cell surface (Toledo-Arana *et al.* 2001). In our study, more than 50 % affinity for *n*-hexadecane was noted only in 2 *esp*-positive biofilm-producing *E. faecalis.* All *E. faecium* strains negative for biofilm but *esp*-positive presented diverse affinity for the hydrocarbon. *E. casseliflavus* and *E. gallinarum* showed a hydrophilic nature of the cell wall (Table II). We did not note any correlation between the type of bacterial surface, the presence of *esp* gene and biofilm formation, but our study was conducted with a small group of strains and therefore needs confirmation in a statistically significant number of isolates.

Hemolysin (cytolysin), as a virulence factor (which may play a role in human infections) is observed mainly in *E. faecalis* isolates (Elsner *et al.* 2000). We detected hemolysins in 6 of 12 *E. faecalis,* 1 of 12 *E. faecium* and in none of *E. casseliflavus* and *E. gallinarum* strains (Table II).

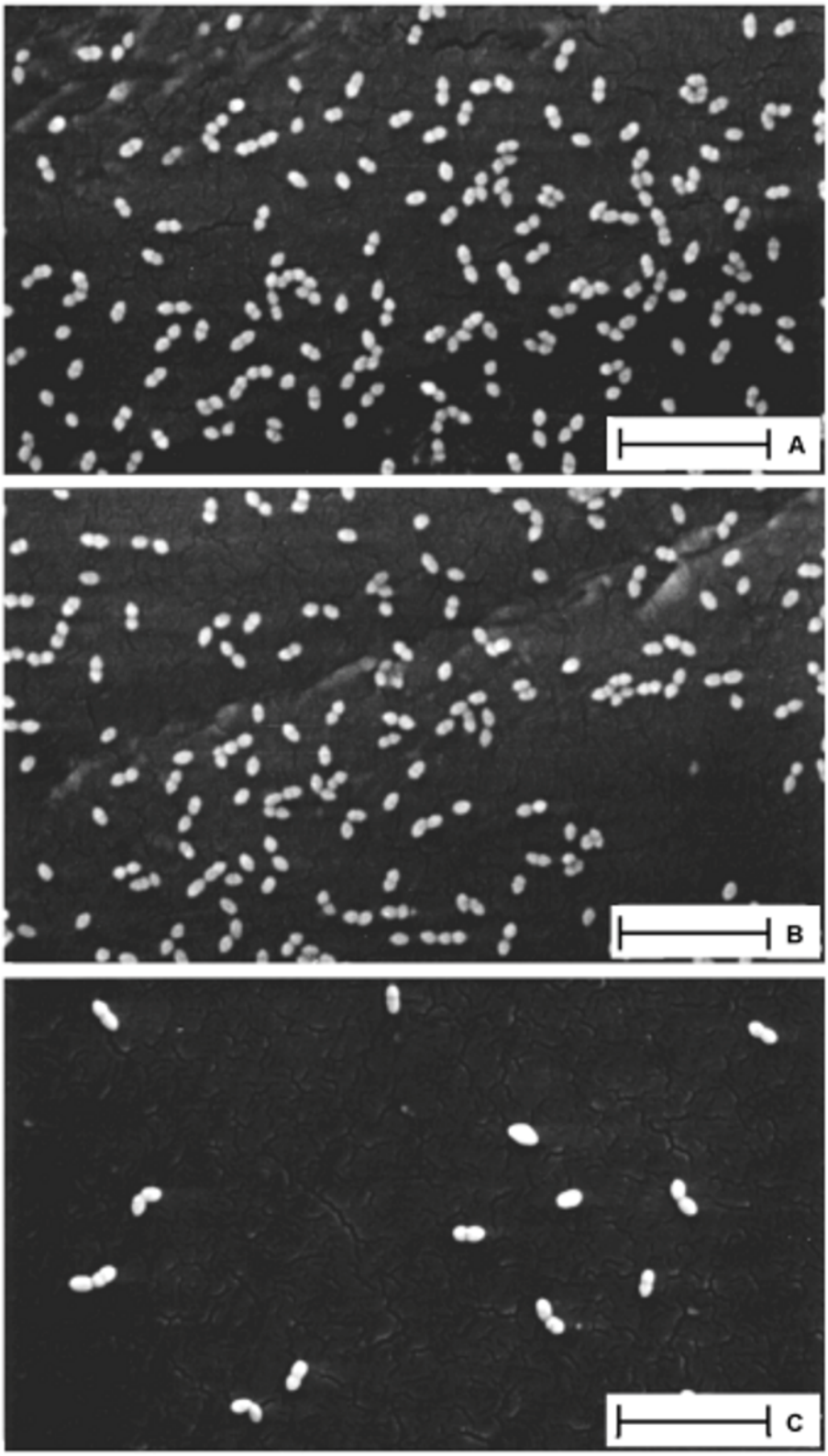
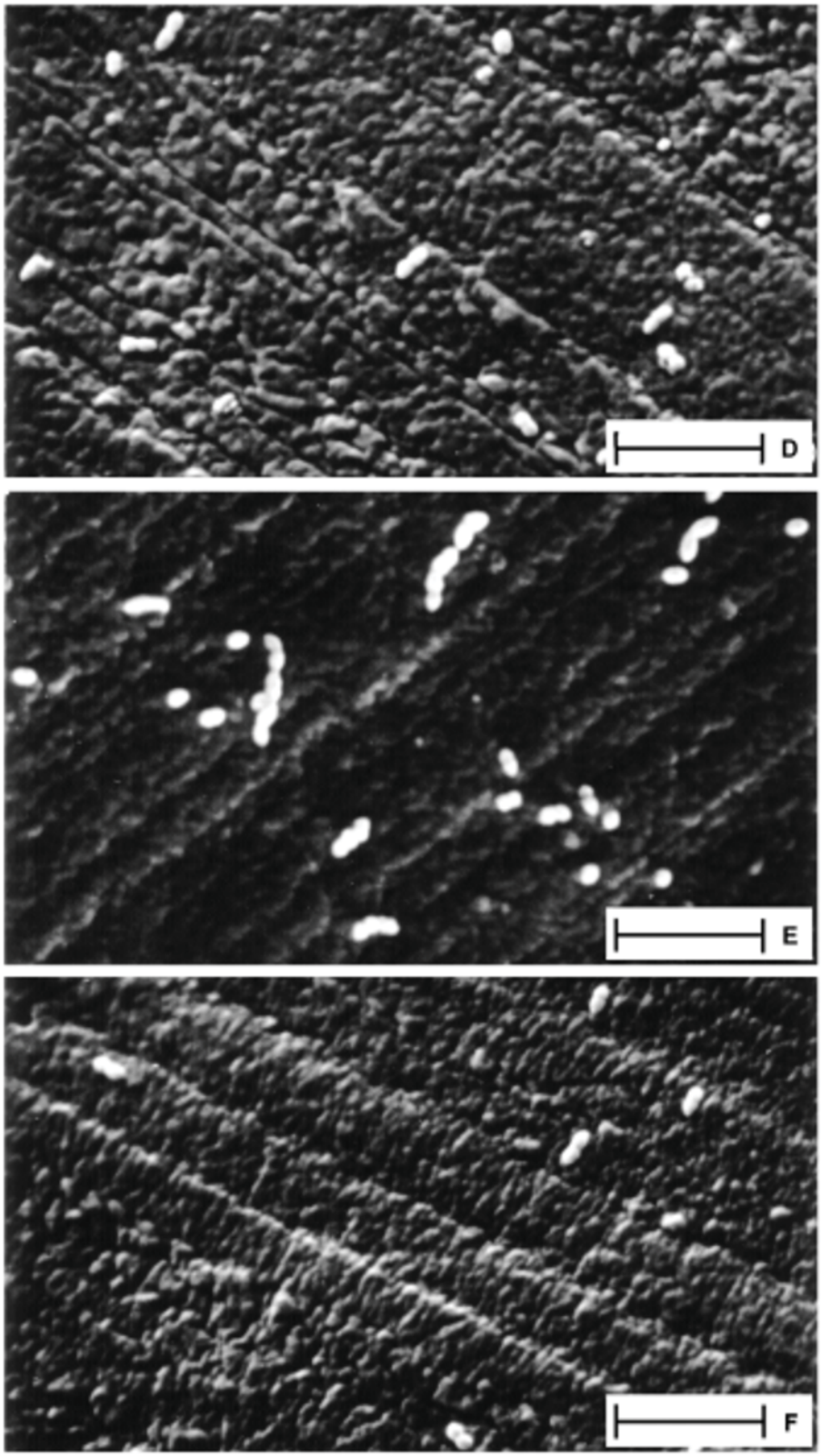


Fig. 1. Scanning electron micrographs of enterococci adhered to silicone (**A**–**C**) and PVC (**D**–**F**) surface (*bars* = 5 μm); A, D – *E. faecalis*, B, E – *E. faecium*, C, F – *E. casseliflavus*.



The contribution of gelatinase (the enzyme hydrolyzing gelatin, collagen, fibrinogen, insulin) to enterococcal pathogenesis is not well defined (Elsner *et al.* 2000). Its presence was observed only in 1 of 12 *E. faecalis* and in 2 of 12 *E. faecium* strains. The remaining species did not produce gelatinase (Table II). Elsner *et al.* (2000) found that 55 % of *E. faecalis* and none of *E. faecium* blood isolates were producers of gelatinase.

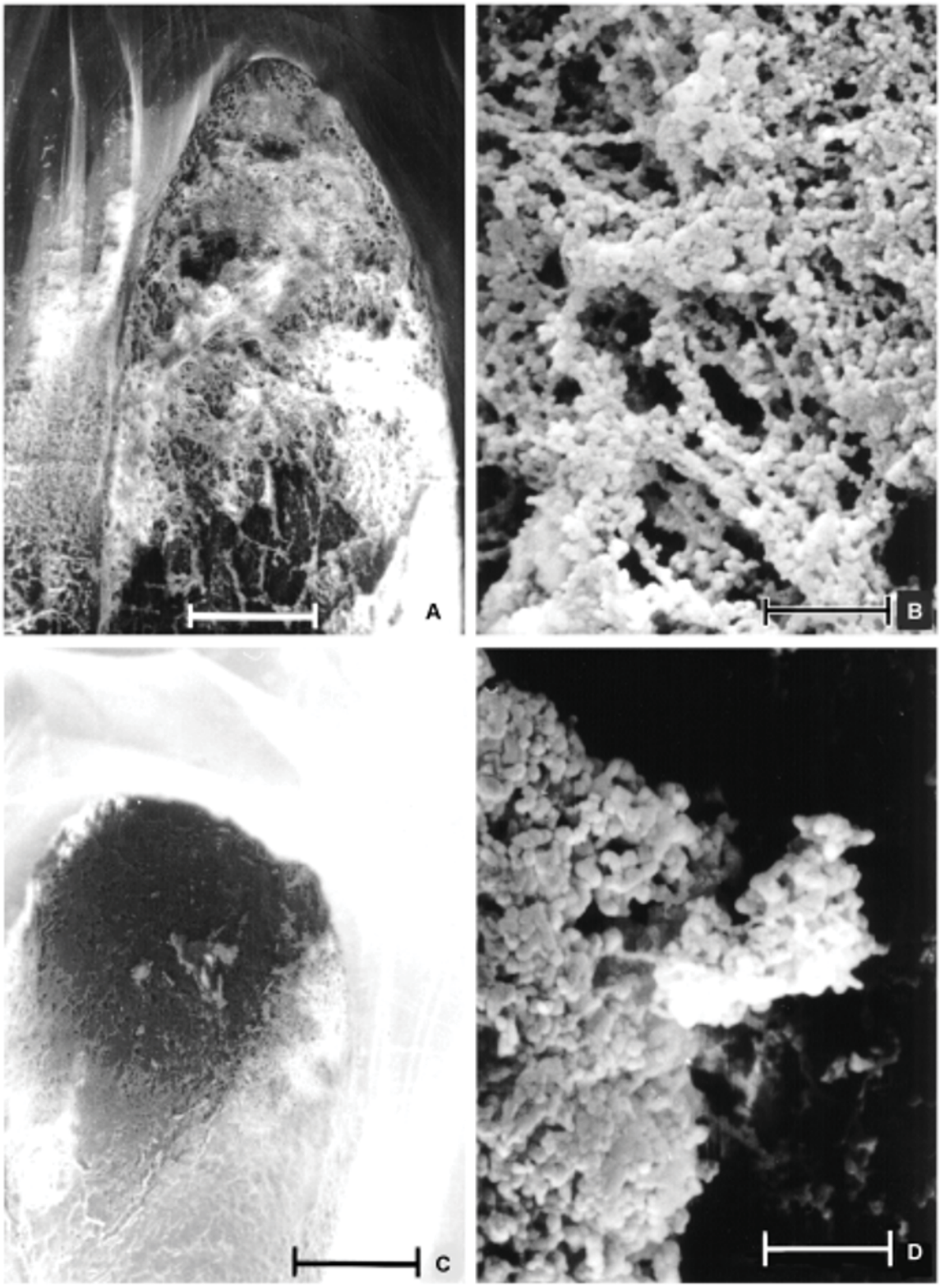


Fig. 2. Scanning electron micrographs of a cross section of siliconized latex showing a biofilm of *E. faecalis* (**A**, **B**) and *E. faecium* (**C**, **D**) on the inner surface; *bars*: A, C – 300 μm, B, D – 5 μm.

Our data indicate that *E. faecalis* and *E. faecium* isolated from the UTI were characterized by a higher virulence potency compared with *E. casseliflavus* and *E. gallinarum.* The lack of virulence factors observed in *E. faecalis* and *E. faecium* may explain to some extent the low adherence of these strains to the

urinary catheters tested. However, some isolates of *E. faecalis* and *E. faecium* which exhibited good adherence ability, lacked the virulence factors studied, suggesting that some other properties are important in the adhesion process.

Table I. Adhesion of *Enterococcus* spp. to 3 types of urinary catheters[a]

Species	siliconized latex	silicone	polyvinyl chloride
E. faecalis	6	5.6	5
E. faecium	5	4	3.5
E. casseliflavus	4	3.7	3.1
E. gallinarum	3.8	3.5	3

[a]log CFU per cm^2 of catheter.

Table II. Potential virulence factors of *Enterococcus* spp.

Species	Number of strains	*esp* gene	Biofilm	Hydrophobicity	Gelatinase	Hemolysin
E. faecalis	12	7	5	2	1	6
E. faecium	12	6	0	0	2	1
E. casseliflavus	3	0	0	0	0	0
E. gallinarum	3	0	0	0	0	0

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