Lucilla Baldassarri · Roberto Cecchini · Lucia Bertuccini
Maria G. Amendolia · F. Iosi · Carla R. Arciola
Lucio Montanaro · Roberta Di Rosa · Giovanni Gherardi
Giordano Dicuonzo · Graziella Orefici · Roberta Creti

*Enterococcus* spp. produces slime and survives in rat peritoneal macrophages

Received: 10 September 2001 / Published online: 24 October 2001
© Springer-Verlag 2001

**Abstract** Enterococcal clinical isolates were investigated for the ability to form biofilm on inert surfaces, as a measure of slime production, in an attempt to find new possible virulence factors for these microorganisms. This property was commonly found among *Enterococcus faecalis*. Also *E. faecium* isolates were able to form biofilm, although to a lesser extent; for this species, however, biofilm formation seemed more frequently associated with isolates from infection rather than with environmental strains or isolates from healthy individuals. Biofilm formation was strongly affected by the presence of an additional carbohydrate source in the medium, or by iron deprivation, indicating a role of slime for survival in stressful conditions. Slime-producing *E. faecalis* were able to survive inside peritoneal macrophages for extended periods compared to slime-negative strains or to slime-positive bacteria grown in conditions depressing slime production. In particular, slime-producing and slime-negative cells showed a decrease of 1 and 2 log units, respectively, at 1 h after infection; slime-negative cells were then rapidly killed, with clearance of bacterial cells at 24 h. Slime-producing bacteria persisted up to 48 h, which was the last time point examined, as after that time viability of both infected and non-infected macrophages started to decline. Scanning electron microscopy observations showed the presence of abundant amorphous extracellular material, of possible polysaccharide nature, embedding bacterial cells to form a multilayered biofilm. Even in conditions not supporting biofilm formation, bacterial cells appeared capsulated, suggesting that capsule and slime might represent different structures. Genes belonging to the *epa* locus or to a putative icaA homolog did not seem to be involved in synthesis and export of slime.

**Keywords** Slime · Colonization · Survival in macrophages

**Introduction**

Enterococci are opportunistic pathogens which can cause severe infections in different body districts, particularly among immunocompromised patients. Several characteristics of enterococci have been considered as possible virulence factors such as the hemolysin/bacteriocin [22, 23], the aggregation substance (AS) [31] or gelatinase [32]; however, Berti et al. [7] recently obtained data against a role of the AS in infective endocarditis, and a survey investigating the incidence of the three above-cited virulence factors among enterococcal isolates found that about 40% of the isolates did not have at least one of the three factors [11]. In addition, Elsner et al. [17] evaluated the prevalence of hemolysin, gelatinase, AS, lipase, and hemagglutinin among blood culture isolates and concluded that hemagglutinin and lipase may represent additional virulence factors. All in all, a definitive evidence of the critical importance of one, or of an association of factors, has yet to be found.

Translocation through the intestinal barrier has long been indicated as one of the preferential portal of entry
of enterococci for spreading to distant sites; Gentry-Weeks et al. [19] showed that *E. faecalis* is able to survive within peritoneal macrophages, which may contribute to pathogenesis by allowing translocation of bacteria through the intestinal mucosa and hinder antimicrobial therapy. However, ability to produce gelatinase and cytolysin, evaluated by that group, did not seem to be involved in the survival of *E. faecalis* within macrophages.

For opportunistic pathogens involved in endocarditis and catheter-related infections, such as *Staphylococcus epidermidis*, production of extracellular polysaccharide material (slime) and biofilm formation have been found to have a role in the pathogenic mechanism [9, 20]. As coagulase-negative staphylococci, enterococci are able to survive on inanimate surfaces such as plastic or fabric [26]. We recently noted [16] that enterococci colonizing biliary stents may grow to form a biofilm and that some strains seemed to be able to adhere to plastic surfaces and, possibly, to produce slime.

Weinstock’s group recently described [33, 34] a genetic locus (*epa*), possibly involved in the assembly and export of a polysaccharide antigen in *E. faecalis OGI-RF*; two genes in particular, identified as *orfde4* and *orfde6*, seemed responsible for delayed killing and a higher 50% lethal dose in a mouse peritonitis model.

As far as slime is concerned, it is known that the *ica* locus is the genetic determinant both in *Staphylococcus aureus* [12] and in coagulase-negative staphylococci [2, 21].

In this study, we wanted to phenotypically and genotypically characterize the ability of clinical enterococcal isolates to produce slime, establish the culture conditions affecting this phenomenon, and evaluate its role in supporting survival within rat peritoneal macrophages.

### Materials and methods

**Bacteria, microbiological media, and reagents**

One-hundred eleven enterococcal strains isolated from either clinically relevant infections, healthy subjects (8 isolates: 6 *E. faecalis*, 2 *E. faecium*, or environmental samples (26 isolates from marine or fresh water: 5 *E. faecalis*, 7 *E. faecium*, 14 other species, kindly provided by L. Bonadonna, Rome, Italy) were used in this study. Isolates were identified to species level according to standard biochemical test [15]. Clinical isolates were from urinary tract infections (19 isolates: 12 *E. faecalis*, 7 *E. faecium*), wounds (10 isolates: 9 *E. faecalis*, 1 *E. faecium*), endocarditis (25 isolates: 16 *E. faecalis*, 9 *E. faecium*), and catheter-related infections (23 isolates: 19 *E. faecalis*, 4 *E. faecium*). Species included in the study were: *E. faecalis* (73), *E. faecium* (24), *E. hirae* (7), *E. durans* (2), *E. casseliflavus* (2), *E. gallinarum* (1), *E. raffinosus* (1), *E. avium* (1).

*E. faecalis* OGI-RF, JH2-2, and VS83 were used as reference strains for genetic characterization and *E. faecalis* OGI-RF[pAD1], an *AS*⁺ isolate, kindly provided by Dr. Wirth, University of Regensburg (Germany), was used as control in experiments of survival in macrophages.

Strains, stored in trypticase soy broth (TSB) supplemented with 15% glycerol at −80°C, were routinely maintained on 5% sheep blood agar; for experiments, bacteria were subcultured in TSB, TSB supplemented with 1% glucose (TSBG), brain-heart infusion broth (BHI), or horse serum (HS) (all from Oxoid, Basingstoke, Hampshire, UK). To create iron-limited conditions, TSB was treated with imidazole acid (Sigma-Aldrich, Milan, Italy) [5], and the iron content was determined by atomic absorption spectrophotometry (Perkin Elmer 5100 Z spectrophotometer, equipped with a hollow cathode lamp and using an air acetylene flame). For iron treatment, iron content dropped from 28±0.3 μM to <0.1 μM (three determinations in three different batches of the medium).

**Assay for survival into macrophages**

Rat peritoneal macrophages were harvested by peritoneal lavage (two applications of 5 ml HBSS). Cell viability was determined by trypan blue dye exclusion. Cell suspensions normalized to 150,000 cells/ml in RPMI 1640 supplemented with 1% fetal calf serum (FCS) were dispensed into 24-well plates and incubated for 30 min at 37°C under 5% CO₂. Unattached cells were discarded by washing and macrophages further incubated for 24 h at 37°C with RPMI 1640, 1% FCS, before infection. Overnight bacterial cells grown in TSB or TSBG were washed and suspended into RPMI 1640 supplemented with 5% FCS at final concentration of 1.5×10⁷ (bacterial cell:macrophage ratio 1:100). Macrophages were infected for 1 h at 37°C, thoroughly washed with PBS and further incubated for 3, 24, or 48 h with RPMI 1640 supplemented with 5% FCS, 10 μg/ml gentamicin and 50 μg/ml penicillin (or 150 μg/ml gentamicin and 10 μg/ml vancomycin). At different times, duplicate wells of infected macrophages were washed and lysed with 0.1% Triton X-100 in PBS for 5 min. Dilutions of lysates were made in PBS and plated on TSB agar plates to quantitate viable bacteria. Throughout the experiment, viable macrophages were quantitated by trypan blue dye exclusion and counting with an hemocytometer.

**Biofilm formation**

To test for biofilm formation we used a quantitative adherence assay described previously [4]. Briefly, a 1:100 dilution of overnight cultures in TSB was used to inoculate wells in a microtiter polystyrene plate containing different media. After growth for 18 h at 37°C, plates were gently washed three times with phosphate-buffered saline (PBS), the adherent bacterial film was fixed by air drying at 60°C for 1 h and then stained with Hucker's crystal violet; excess stain was washed off with tap water. The optical density of the biofilm was measured at 570 nm in an automatic spectrophotometer (Novaphath Microplate Reader, Bio-Rad Laboratories, Calif.). For experiments on the effect of iron limitation, the method of calculation and presentation of results were modified as indicated by Deighton and Borland [13] to compensate for the different growth rates observed in broth and in iron-limited broth. In this case, the adherence index was defined as an estimate of the density of the biofilm which would be generated by a culture with an OD₆₀₀ of 0.5 [adherence index = mean density of biofilm (OD₅₇₀) × 0.5/mean growth (OD₆₀₀)].

Results of this test are presented either as “slime production” or “biofilm formation”.

**Polymerase chain reaction**

DNA preparation of enterococci was made by suspending a loop of overnight-grown colonies in a tube containing 500 μl sterile distilled water, boiling for 10 min, and centrifuging at 14,000 g for 5 min. Five microliters of the supernatant were used as template in a final volume of 25 μl PCR mixture containing: 1x PCR buffer (Life Technologies), 200 μM each of dATP, dCTP, dGTP, dTTP (Life Technologies), 400 nM of each primer, and 0.25 U Taq DNA polymerase (Life Technologies). Samples were amplified on a DNA Thermal Cycler (MJ Research, Inc.) by an initial cycle of denaturation (95°C for 5 min), followed by 30 cycles of denaturation...