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Short communication

Adherence and intracellular survival within human macrophages of *Enterococcus faecalis* isolates from coastal marine sediment

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Abstract

Enterococcus faecalis is part of the human intestinal microbiota and an important nosocomial pathogen. It can be found in the marine environment, where it is also employed as a fecal indicator. To assess the pathogenic potential of marine *E. faecalis*, four strains isolated from marine sediment were analyzed for their ability to survive in human macrophages. *Escherichia coli* DH5α was used as a negative control. The number of adherent and intracellular bacteria was determined 2.5 h after the infection (T_0) and after further 24h (T_{24}) by CFU and qPCR counts. At T_{24} adherent and intracellular enterococcal CFU counts were increased for all strains, the increment in intracellular bacteria being particularly marked. No CFU of *E. coli* DH5α were detected. In contrast, qPCR counts of intracellular enterococcal and *E. coli* bacteria were similar at both time points. These findings suggest that whereas *E. coli* was killed within macrophages (no CFU, positive qPCR), the *E. faecalis* isolates not only escaped killing, but actually multiplied, as demonstrated by the increase in the viable cell population. These findings support earlier data by our group, further documenting that marine sediment can be a reservoir of pathogenic enterococci.

Keywords: *Enterococcus faecalis*; Macrophages; Adhesion; Internalization

1. Introduction

Enterococci are gram-positive bacteria and commensal residents of the intestinal microbiota of warm-blooded animals, including humans [1]. They are also important nosocomial pathogens capable of inducing a wide range of infections, including sepsis and endocarditis [2]. The success of *Enterococcus faecalis* as an infectious agent is due to the ability of specific clones to acquire and express a number of virulence traits that facilitate the infection process by allowing host adhesion and colonization, resistance to macrophage killing, and immune evasion [3]. Among these traits, *gelE* (gelatinase) has been associated with biofilm production and virulence in different animal models [4], and *efaA* (endocarditis-specific antigen) has been associated with endocarditis and increased mortality, even though its role in the pathogenesis of enterococcal infections has yet to be determined [5].

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Once inside the human host enterococci, regardless of their origin (human, animal, or environmental), are engulfed by macrophages and transported through the body, causing systemic infection [6]. The ability of human *E. faecalis* isolates to survive within macrophages has been reported by different studies using in vivo and in vitro macrophage infection models [7,8] and has been suggested to be strain background-dependent [6].

Enterococci are widespread in the environment and have been recovered from fresh and seawater, marine sediment, beach sand, and macrophytes [1]. Strains isolated from marine water and sediment have recently been described and found to carry a variety of virulence factors [9]. These potentially pathogenic bacteria can affect humans both through the food chain and through recreational activities. The aims of this study were: *i)* to investigate the ability of enterococci from marine sediment to survive within human macrophages; *ii)* to develop a qPCR assay capable of detecting the total number (i.e. live and dead) of adherent and intracellular bacteria; and *iii)* to compare the value of the information provided by qPCR and the traditional culture-based approach in exposing macrophage survival dynamics.

2. Materials and methods

2.1. Bacterial strains

Four *E. faecalis* strains carrying virulence factors, isolated from marine sediment and characterized in a previous study [9], were used in macrophage infection experiments. The negative control was *Escherichia coli* DH5α, a laboratory strain found to be susceptible to killing by mouse peritoneal macrophages in an earlier study testing the survival of intracellular *E. faecalis* isolates, where it was used as a negative control [10]. The *E. faecalis* strains included the strong biofilm producer *E. faecalis* 14e15 and the weak biofilm producers *E. faecalis* 15e20, *E. faecalis* 16e35, *E. faecalis* 16e40. All carried *gelE* and *efaA*, they showed gelatinase activity, and were sensitive to ampicillin and gentamicin.

2.2. Human macrophage culture and adhesion and intracellular survival assays

Human macrophages were obtained from healthy donor buffy coats by separation on Ficoll Histopaque solution (Sigma Aldrich, St. Louis, MI, USA) as described previously [11]. Mature cells were seeded in a 24-well culture plate (Sarstedt, Nümbrecht, Germany) at approximately 10^4 cells/well and maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (Lonza) at 37 °C in 5% CO_2. Adhesion and survival assays were performed as described by Süssmuth et al. [12], with some modifications. Briefly, 10 μl of bacterial culture grown to 0.1 OD_{650} were used to infect macrophages (multiplicity of infection, 100). After 1 h incubation at 37 °C in 5% CO_2, cells were washed 3 times in PBS and incubated for 1.5 h in RPMI 1640 supplemented with 10% FCS without (adhesion test) or with (internalization test) gentamicin (100 μg/ml) and ampicillin (20 μg/ml). After two PBS washes, cells were lysed by adding 0.01% Triton X-100 (Fluka Analytical, Sigma–Aldrich, Buchs, Switzerland) in 0.5 ml PBS, either immediately (T_0) or after a further 24 h incubation in RPMI 1640 with 10% FCS (T_{24}). The lysates were serially diluted, 10 μl of each dilution was spotted on Brain-Heart Infusion Agar (BHIA) plates and the plates were incubated for 24 h at 37 °C. Bacterial counts were expressed as Colony Forming Units (CFU)/10^4 macrophages. The Adhesion Index (AI) was calculated as percent adherent/inoculated bacteria, and the Internalization Index (II) as percent intracellular/inoculated bacteria. Each experiment was performed in triplicate.

2.3. qPCR

Bacterial DNA was extracted from macrophage lysates as described by Hynes et al. [13]. The enterococcal cells were counted by qPCR assays as described by Di Cesare et al. [14]; the standard curve is reported in Fig. S1. The abundance of *E. coli* DH5α was determined by qPCR using the species-specific primer pair described by Srivnsann et al. [15] and the protocol used for enterococci, except that the annealing temperature was 58 °C instead of 60 °C. To assess the difference between experimental data and actual bacterial concentrations, the accuracy of qPCR was evaluated by spiking experiments using 10^6 and 10^4 enterococcal cells as described by Luna et al. [16]. A bacteria-free, macrophage (10^4) lysate was the negative control. The absence of PCR inhibitors in the eukaryotic cell lysate was confirmed as described previously [14].

2.4. Data analysis

Counts of adherent and intracellular bacteria at T_0 and T_{24} were compared by the unpaired *t* test. Differences were considered significant at P values <0.05.

3. Results

3.1. E. faecalis *adherence and survival inside primary human macrophages*

To gain information on their pathogenic potential, four *E. faecalis* strains isolated from marine sediment and carrying virulence traits were tested for their ability to adhere to and survive in human macrophages. The number of adherent bacteria was calculated by CFU and qPCR counts. A similar procedure was applied to calculate internalized bacteria (bacteria within macrophages), except that for this count macrophages were incubated in antibiotic-containing medium after the infection. Adherent and intracellular bacterial counts were performed 2.5 h after the infection (T_0) and after further 24 h (T_{24}) incubation with macrophages.