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Survival of enterococcal species in aquatic environments

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Abstract

Analysis of the survival ability of faecal streptococci/enterococci in the environment has almost invariably been conducted using the standard culture method (CFU counts) despite the demonstration that these microorganisms are capable of entering a viable but nonculturable (VBNC) state. In this study we evaluated the fate, in terms of culturability and viability, of different enterococcal species under laboratory stress conditions mimicking those of the aquatic environment. The results indicate that enterococcal species may activate two different survival strategies, namely starvation and the VBNC state, depending on the specific environmental condition. Moreover, the different enterococcal species can be divided into three groups on the basis of the time needed to activate the VBNC state and the resuscitation capability. The differences in activation of the two survival strategies and the different kinetics observed among the enterococcal species reaching the VBNC state should be taken into consideration when the microbiological quality of waters has to be evaluated and because of their role as faecal contamination indicators.

Keywords: Enterococcal species; Culturability; Bacterial survival; Environmental microbiology

1. Introduction

The terms faecal streptococci, enterococci or intestinal enterococci embrace species of the genera *Enterococcus* and *Streptococcus*. These microorganisms are widely accepted as useful indicators of faecal contamination in the aquatic environment in that: (i) they are always present in faeces of warm-blooded animals; (ii) they are unable to multiply in sewage-contaminated waters; and (iii) their persistence rates are similar to those of waterborne pathogenic bacteria [1–3]. However, the role of enterococci as bacterial indicators has also been questioned; for example, it has recently been shown that, in tropical soils, nutrient concentration and temperature allow faecal bacteria to multiply and become a minor population of the biota. For this reason, increasing levels of indicator bacteria, such as faecal coliforms and enterococci, would be detected in water and this may lead to misinterpretation of water quality tests in tropical areas [4–6].

The studies regarding the fate of enterococci in the environment refer to evaluations made by counting colony forming units (CFU/ml) and are thus based on the ability of bacteria to divide and form colonies on culture media. However, it is now clear that in the environment bacteria find adverse conditions, which do not allow their division but do not necessarily cause their death. Bacterial species in general and *Enterococcus faecalis* specifically, can activate several survival strategies including starvation and the viable but nonculturable (VBNC) state [7–10] thus conserving their viability. The VBNC state is defined as a survival mechanism activated by bacteria in response to multiple environmental stresses and allowing microorganisms to conserve their viability despite the loss of their own culturability. Moreover, it has been demonstrated that these bacterial

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forms conserve their pathogenic characteristics [11,12] and can resume division when exposed to optimal conditions [7,13]. There has been a certain amount of controversy regarding studies on the VBNC state: some authors, for example, have postulated that the nonculturable cells of some species are actually hydrogen peroxide-sensitive cells capable of growing in culture media if added with catalase or sodium pyruvate [14]. Others consider that "resuscitation" is probably due to re-growth of a number of still viable cells in the nonculturable population and not to nonculturable cells resuming division [15,16]. In an earlier article [13] we have demonstrated both that VBNC forms of *E. faecalis* cannot growth when hydrogen peroxide inhibitors are added to the culture media and that the use of the most probable number method to calculate resuscitated VBNC cells excludes the possibility that a still-living cell can be responsible for the growth observed in VBNC samples maintained under resuscitation-inducing conditions. On the basis of the considerations described above, as only culture methods are currently used in evaluating the microbiological quality of the environment, we can conclude that the real enterococcal load might be underestimated or undetected unless molecular methods, based on nucleic acid detection, are used [17].

On the basis of the results obtained previously studying the behaviour of *E. faecalis*, *E. faecium* and *E. hirae* maintained in adverse environmental conditions, we have considered, in this study, the possibility that also other enterococcal species could activate survival strategies and have evaluated the fate, in terms of culturability and viability, of enterococcal cells when in oligotrophic aquatic environments. Moreover, because previously studied enterococcal species demonstrated that they are capable of activating different survival mechanisms in dependence of environmental conditions, we have investigated, in this study, the influence of varying environmental parameters on the survival mechanism activated and on the kinetics of activation in 12 different enterococcal species under laboratory stress conditions mimicking those of the aquatic environment.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The different enterococcal species used in this study are listed in Table 1. Bacterial strains were grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI). Cell growth was monitored with an LKB spectrophotometer at 640 nm wavelength (OD_{640}). The colony forming unit (CFU) count of culturable bacteria was done by plating samples in duplicate on tryptic soy agar (TSA, Difco) plates with or without 0.2% sodium pyruvate. When the number of colonies was estimated to be less than 50 CFU/ml, counts were conducted on 10 ml samples of the microcosm which were filtered through a 0.22 μm pore size membrane (Millipore, Co., USA) and the filter was placed face up on TSA plates as described previously [10]. Total cell number was determined as described previously [10] with a ZBI Coulter counter (Coulter Scientific, Krefeld, Germany) equipped with a 30 μm capillary.

Table 1
Enterococcal species used in this study

Enterococcal species	Strain	Source and reference
E. faecalis	56R	Clinical strain [10]
E. faecium	VR6	Clinical strain [13]
E. hirae	ATCC 9790	ATCC collection
E. gallinarum	SS1	[25]
E. avium	AV3	[25]
E. malodoratus	ATCC 1266	ATCC collection
E. durans	ATCC 11576	ATCC collection
E. pseudoavium	PSA2	[25]
E. casseliflavus	ATCC 14436	ATCC collection
E. flavescens	FL1	[25]
E. raffinosus	RAFF4	[25]
E. muntdii	BRNO1	[25]

2.2. Preparation of microcosms

Different laboratory microcosms representing several environmental conditions were used. A microcosm representing freshwater (artificial lake water, ALW) was prepared by filtering water from Garda lake (Verona, Italy) on a 0.22 μm Millipore filter and sterilizing it by autoclaving. The marine environment was reproduced using an artificial sea water (ASW) medium consisting of NaCl 24.7 g, KCl 0.67 g, $CaCl_2 \cdot H_2O$ 1.36 g, $MgCl_2 \cdot 6H_2O$ 4.66 g, $MgSO_4 \cdot 7H_2O$ 6.29 g, $NaHCO_3$ 0.18 g per litre.

Exponentially growing or early stationary enterococcal cell cultures ($OD_{640} = 0.35$ or 1.00) were used to inoculate oligotrophic microcosms (three different microcosm bottles for each condition tested) at a final density of the order of 10^7 cells/ml as described [10]. Inoculated microcosms were maintained at 4 °C or at room temperature with direct or indirect light and monitored for CFU counts every two days as indicated above. It is considered that a population has reached the VBNC state when no colonies are detected after inoculating 10 ml of microcosm in solid culture medium.

2.3. Viability tests

Viability of the enterococcal stressed cells was tested with a modified Kogure direct viable count method [18,19] and consisting of counting elongated enterococcal cells after treatment of stressed cell samples with 1 μg/ml benzylpenicillin or 2 μg/ml of levofloxacin (for