Persistence of *Enterococcus faecalis* in Aquatic Environments via Surface Interactions with Copepods

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Several human pathogens and fecal-pollution indicators may persist as viable organisms in natural environments, owing to their ability to activate different types of survival strategies. These strategies include adhesion on both abiotic and biotic surfaces and the entrance to the so-called viable but nonculturable (VBNC) state. In an 18-month survey for the detection of enterococci in both lake water and seawater, C. Signoretto et al. (Appl. Environ. Microbiol. 70:6892–6896, 2004) have shown that *Enterococcus faecalis* was detected mostly bound to plankton and in the VBNC state. In the present study, we show that in vitro adhesion of *E. faecalis* to copepods accelerated the entry of cells into the VBNC state relative to that of planktonic bacteria. VBNC *E. faecalis* cells maintained their adhesive properties to copepods and chitin (the main component of the copepod carapace), though to a reduced extent in comparison with growing cells. Sugar competition experiments showed interference with adhesion to both copepods and chitin by GlcNAc and only to copepods by D-mannose. Four enterococcal cell wall proteins present in both growing and VBNC cells and lipoteichoic acid were shown to be capable of binding chitin. The results indicate that copepods may represent an additional environmental reservoir of enterococci, thus suggesting the advisability of redesigning the protocols currently used for microbial detection during the evaluation of the microbiological quality of environmental samples.

Several human pathogens and fecal-pollution indicators may persist as viable organisms in natural environments because of their ability to activate different types of survival strategies. These strategies include adhesion, possibly the formation of biofilm on both abiotic and biotic surfaces (5, 13–15, 23, 34), and the entry into the so-called viable but nonculturable (VBNC) state. Bacteria in the VBNC state are characterized by loss of culturability on conventional growth media, but cells maintain viability, pathogenicity factors, and the potential ability to reinfect humans on resuscitation to the culturable state (2, 8–10, 17, 20, 21, 26).

Among human pathogens, the role of the adhesion of *Vibrio cholerae*, the etiological agent of a severe waterborne diarrheal disease (cholera), as well as of other vibrios, was extensively investigated, and it has been suggested that altered forms of *V. cholerae* in specific association with plankton organisms are the most plausible reservoirs of fully virulent strains during interepidemic periods (9). Bacterial binding to various surfaces involves several forces, ranging from hydrophobic and ionic bonds to the lectin-like interactions between the bacterial ligand(s) and complementary receptor(s) displayed by the substrate. A few examples of specific interactions between human-pathogenic bacteria and chitin-containing surfaces are known, but they are entirely restricted to the *Vibrio* genera. Lectins with specificity for *N*-acetylglucosamine (GlcNAc), the sugar component of chitin, have been demonstrated to occur in *V. cholerae*, *Vibrio harveyi*, *Vibrio damsela*, and *Vibrio furnissii* (24, 29, 36). In addition, specific chitin-binding proteins (CBPs) are displayed on the surfaces of *V. cholerae* (29, 35), *Vibrio alginolyticus* (5, 27), and *V. harveyi* (24) and have been shown to be directly correlated to the binding ability of the microorganisms to various substrates.

In an 18-month survey for the detection of enterococci in both lake water and seawater, Signoretto et al. (32) have shown that *Enterococcus faecalis* was bound mostly to plankton when present. In addition, *E. faecalis*, either in bound or in free form, was most frequently detected in the VBNC state. We believe that this unexpected result deserves particular attention because it constitutes new evidence which, together with the vibrio model, indicates that this may be the main mode of persistence of medically important bacteria in surface waters. The creation of an unexpected pathogen reservoir might possibly invalidate the culture methods currently used to assess the microbiological quality of surface waters in that zooplankton, by removing bacteria from water, may concentrate and move bacteria with currents and tides.

In this work, we analyze the ability of enterococci to bind lake zooplankton in vitro in both the growing and VBNC states and try to identify the possible enterococcal ligand(s) involved. The data presented are the means of results of three distinct experiments. The standard deviation (SD) is indicated in each table or figure. Data were analyzed for significance using Student's *t* test. Differences were considered significant at a *P* of ≤0.05.

Bacterial strains and copepods. *E. faecalis* 56R (33) and *E. faecalis* JH2-2 (16) were used. Strain 56R is a clinical isolate and is a producer of the enzyme chitinase, while JH2-2 is a laboratory strain and a nonproducer of chitinase as evaluated by the hydrolysis of ethylene glycol chitin, as described by Connell et al. (11; C. Pruzzo, unpublished observation). Enterococcal strains were grown in brain heart infusion (BHI)

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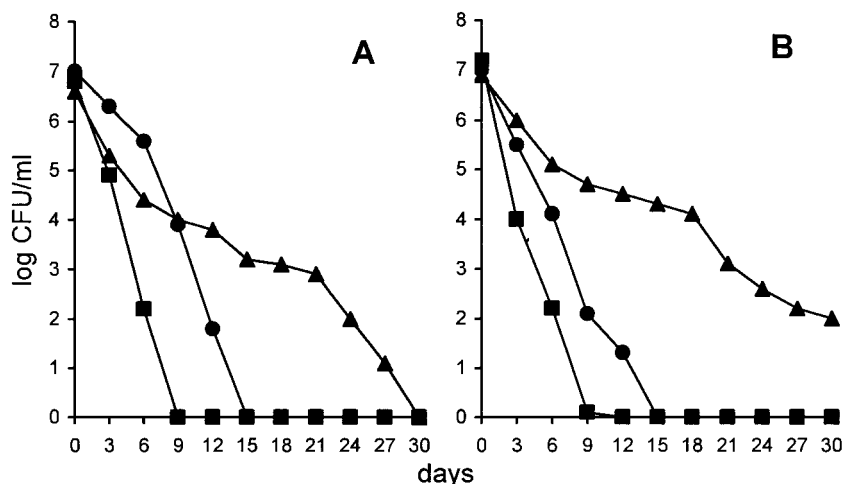
FIG. 1. Decline of *E. faecalis* 56R (A) and JH2-2 (B) when inoculated at 4°C in lake water in free form (●), adherent to copepods (■), and adherent to chitin particles (▲). Each point is the mean from triplicate samples. The standard error averaged 15% of the value indicated when the number of CFU/ml was greater than 50.

broth or BHI agar (BHIA) (Difco) at 37°C. Cell growth in liquid media was monitored by reading optical density at a 640-nm wavelength (OD_{640}) with a Beckman model DU 530 spectrophotometer.

Copepods used in this study were collected by horizontal dragging at 1 m below the surface of Lake Garda (Italy) with a 100-μm net (32). Copepods were checked, before use, for the absence of *E. faecalis* by PCR amplification of a DNA tract within the *pbp5* gene, as previously described (22, 32). Only copepod lots that revealed no amplification band were used in this study.

Evaluation of the time needed to enter the VBNC state by *E. faecalis* cells in different microcosms. Three different laboratory microcosms were created: (i) enterococci resuspended in autoclaved water collected from Lake Garda, (ii) enterococci adherent to copepods and resuspended in sterile lake water, and (iii) enterococci bound to purified chitin particles and resuspended in sterile lake water. To attach enterococci to copepods, 500 copepods per ml (10-ml final volume) were placed in contact with 1×10^9 bacteria per ml (10 ml) and allowed to stand for 1 h at room temperature (RT). Copepods were collected by filtration onto a piece of 64-μm net, washed thoroughly with sterile lake water, and finally resuspended in lake water. To attach enterococci to chitin particles, 150 mg per ml of sterilized chitin purified from crab shell (Sigma) (10 ml) was placed in contact with 1×10^8 enterococci per ml (10 ml), processed, and collected as copepods, except that chitin particles were collected on 8-μm membrane filters. Microcosms were maintained at 4 ± 0.5°C under illumination in a static state. Every 3 days, samples were withdrawn aseptically from the microcosms, and culturable cell numbers were evaluated as CFU on BHIA plates with suitable dilutions of the samples. For counts of enterococci adherent to copepods and chitin particles, before CFU determination, bacteria were detached from the respective substrates by 1 min of sonication in a water bath cleaner (Branson model 1210). This procedure allowed detachment of all bacteria from the zooplankton but had no effect on bacterial viability or culturability. When the

culturable cell count was close to 0, 10-ml samples from the microcosms were filtered onto 0.22-μm Millipore filters, which were placed face up on BHIA plates. Cells were considered as having entered the VBNC state when the culturable cells numbered <0.1/ml.

Previous results have indicated that *E. faecalis*, resuspended in lake water, enters the VBNC state in about 2 weeks (20, 21). In the first series of experiments, we analyzed culturability over time in a population of *E. faecalis* organisms which adhered to copepods or to chitin particles (the main component of the copepod carapace). Figure 1A shows that *E. faecalis* 56R became totally nonculturable in 9 days when it was adherent to copepods, as opposed to 15 days when it was resuspended in lake water ($P < 0.05$). Surprisingly, when the same bacteria were bound to chitin particles, a much longer time period (30 days) was needed to reach the nonculturable state (P was <0.01 in comparisons to bacteria that were both adherent to copepods and in water). This behavior could be easily explained by the fact that the 56R strain is a chitinase producer, which allows polymer degradation with subsequent cell growth or, at least, the maintenance of cell culturability, as a result of nutrient availability (1). To test this possibility, we used an *E. faecalis* strain (JH2-2) which is a nonproducer of chitin-degrading enzyme. Figure 1B shows that this strain behaved like 56R but that, in addition, after 30 days, 10^2 cells were still culturable when cells adhered to chitin particles. To explain this discrepancy, the presence of an additional chitin-degrading enzyme(s) should be postulated; alternatively, an amount of chitinase should be present in JH2-2 cells but at an undetectable level when ethylene glycol chitin is used as the substrate in the test performed in this study (11). The major discrepancy between enterococci adherent to copepods and those attached to chitin particles may be explained by the fact that the surface of the copepod carapace is layered with a proteinaceous cuticle that prevents access to the lower chitin layer. Alternatively, a signal triggered by adhesion to the living surface might induce the rapid entry of enterococci into the VBNC state. The ease of its entry into this state when adherent to planktonic organisms

TABLE 1. Effect of growth phase on *E. faecalis* 56R interactions with copepod surface or chitin particles

Growth phase or state	Mean no. of enterococci (10^5)/copepod ($\pm$SD) at:			Mean no. of enterococci (10^6)/2.5 mg of chitin ($\pm$SD) at:		
	30 min	60 min	120 min	30 min	60 min	120 min
Exponential	1.8 ± 0.1	2.2 ± 0.3	2.1 ± 0.2	1.3 ± 0.2	2.4 ± 0.2	2.5 ± 0.3
Stationary	5.4 ± 0.4	7.9 ± 0.6	7.5 ± 0.7	4.6 ± 0.4	7.1 ± 0.5	6.8 ± 0.5
VBNC	0.61 ± 0.01	0.89 ± 0.02	0.84 ± 0.2	1.7 ± 0.2	2.9 ± 0.1	3.0 ± 0.2
UV killed[a]	ND[b]	0.0076 ± 0.0002	ND	ND	0.032 ± 0.0001	ND

[a] Exposed to a UV lamp for 15 min and subsequently incubated for 15 days at 4°C, as previously described (33).
[b] ND, not determined.

might explain why *E. faecalis* was found in both lake water and seawater adherent to copepods and mainly in the VBNC state (32).

To provide evidence that the reduction in CFU counts was really due to bacteria entering the nonculturable state rather than the simple loss of the microorganisms, we enumerated enterococci (56R strain) adherent to copepods or chitin particles at the time of entering the nonculturable state (i.e., 9 and 30 days later) by means of a quantitative PCR method. The *E. faecalis pbp5* gene was used as the amplification target. The competitive-PCR protocol used in this study was the one previously described (22, 32). The same cell numbers or slight reductions (though invariably less than 10%) were detected in all samples.

The cell viability of *E. faecalis* was determined with the Live/Dead Baclight bacterial viability kit (Molecular Probes), which evaluates cell membrane integrity (31). Data revealed that 84% $\pm$ 4% to 93% $\pm$ 4% of the cells were still fluorescent green (viable) when *E. faecalis* cells became totally nonculturable. This result provides evidence that the loss of culturability was really due to entry of the majority of the cells into the VBNC state.

Evaluation of binding ability to copepods and chitin particles by *E. faecalis* 56R in different states. Since it is impossible to evaluate the VBNC cells adherent to copepods or chitin particles with the culture method, we were forced to apply the strategy of using radiolabeled bacteria. Radioactive bacteria were obtained as previously described (28) by external labeling of bacteria with *N*-succinimidyl [2,3-^{3}H]propionate. This procedure allowed the binding of the same amount of radioactivity (ca. 13,500 cpm/1×10^7 bacteria), whether these cells were exponentially growing, stationary, or in the VBNC state.

Bacterial attachment to chitin particles was evaluated as previously described by Tarsi and Pruzzo (35). Briefly, 1 volume of labeled bacterial suspension (about 1×10^8 bacteria/ml) was added to 1 volume of phosphate-buffered 3% NaCl solution (pH 8) containing chitin (2.5 mg/ml), and the mixture was incubated at 20°C with shaking. A control sample without chitin was also prepared. At timed intervals, three replicate samples from each treatment were filtered onto 8-μm-pore-size filters (25-mm polycarbonate membranes; Bio-Rad Laboratories), which were then rinsed with phosphate-buffered 3% NaCl solution (pH 8) and radioassayed with an LS 7000 scintillation counter (Beckman Instruments, Inc.). The total number of bacteria attached to chitin particles was calculated using a cell labeling efficiency method. To evaluate background counts due to bacterial attachment to filtration membranes, triplicate samples for each treatment were incubated without

chitin and filtered to correct for unattached cells left on the filter. The values from these control filters were subtracted from the sample values.

To evaluate the adherence of *E. faecalis* to copepods, 1 ml of radiolabeled bacterial suspension (about 2×10^8 bacteria/ml) was added to 1 ml of lake water containing 100 copepods and incubated at 20°C. Three replicates of each treatment were prepared. At timed intervals, copepods were collected and gently washed three times to remove nonadherent bacteria. Copepod-bound radioactivity was evaluated as described above. The total number of bacteria per copepod was calculated using the cell labeling efficiency method (35).

Table 1 shows the time course of attachment to chitin particles and adherence to copepods of enterococci harvested in different growth phases and states (exponentially growing [$OD_{640} = 0.35$], stationary [48 h old], and VBNC cells). The best binding efficiency was obtained at 60 min for both copepods and chitin particles. In addition, it was found that bacteria in the stationary growth phase interacted more efficiently with both copepods and chitin particles than exponentially growing and VBNC cells. VBNC cells of *E. faecalis*, though reduced in numbers, still maintained their binding capability. A control consisting of UV-killed cells of *E. faecalis* and incubated at 4°C in lake water for 15 days was used to evaluate the residual binding ability to both copepods and chitin particles. Table 1 shows that the binding ability of UV-killed cells was drastically impaired, as shown by a roughly 3-log decrease, in comparison with the binding ability of stationary-phase enterococci. This clearly indicates that only live enterococci are capable of binding copepods or chitin particles.

In order to establish that the evaluation of the radioactivity bound to chitin or copepods corresponded to the real determination of the adherent bacterial count, the number of bacteria bound to chitin particles was determined in one experiment by CFU counting on BHIA plates. This experiment was performed only for exponentially growing and stationary cells. The same numbers as those reported in Table 1 were obtained when a cell number evaluation was performed by counting CFU. Although we are unable, at present, to completely rule out the possibility that the labeling process has no effect on the cell's ability to attach to the various substrates, it must be stressed that this is not the case for bacteria in the exponential and stationary phases.

Table 2 shows the effects of ions and sugars on the binding efficiency of *E. faecalis* to copepods or chitin particles. The following salts at the concentrations indicated were separately added to the lake water: NaCl, 0.9%; MgCl$_2$, 1 mM; MgCl$_2$, 30 mM; CaCl$_2$, 1 mM; and CaCl$_2$, 30 mM. When sugar competi-

TABLE 2. Effect of ions and sugars on enterococcal interactions with copepod surface and chitin particles

Substance added	Mean no. ($\pm$SD) of enterococci (10^5)/copepod[a]			Mean no. ($\pm$SD) of enterococci (10^6)/2.5 mg of chitin		
	Exponential	Stationary	VBNC	Exponential	Stationary	VBNC
None[b]	2.20 $\pm$ 0.3 (100)	7.90 $\pm$ 0.6 (100)	0.89 $\pm$ 0.02 (100)	2.40 $\pm$ 0.2 (100)	7.10 $\pm$ 0.5 (100)	2.90 $\pm$ 0.1 (100)
NaCl (0.9%)	2.86 $\pm$ 0.3 (131)	6.79 $\pm$ 0.6 (86)	0.79 $\pm$ 0.01 (89)	3.00 $\pm$ 0.2 (125)	6.46 $\pm$ 0.4 (91)	2.49 $\pm$ 0.2 (86)
Mg^{2+} (1 mM)	3.30 $\pm$ 0.4 (150)	6.47 $\pm$ 0.5 (82)	1.08 $\pm$ 0.02 (122)	2.88 $\pm$ 0.3 (120)	5.68 $\pm$ 0.4 (80)	2.96 $\pm$ 0.2 (102)
Mg^{2+} (30 mM)	9.46 $\pm$ 0.8 (430)	7.29 $\pm$ 0.6 (92)	1.15 $\pm$ 0.03 (129)	7.82 $\pm$ 0.5 (326)	6.03 $\pm$ 0.4 (85)	2.95 $\pm$ 0.1 (102)
Ca^{2+} (1 mM)	2.53 $\pm$ 0.4 (115)	7.26 $\pm$ 0.5 (92)	0.83 $\pm$ 0.01 (93)	2.61 $\pm$ 0.2 (109)	6.46 $\pm$ 0.5 (91)	2.46 $\pm$ 0.2 (85)
Ca^{2+} (30 mM)	8.60 $\pm$ 0.7 (391)	7.28 $\pm$ 0.6 (92)	0.80 $\pm$ 0.02 (90)	5.76 $\pm$ 0.4 (240)	6.11 $\pm$ 0.5 (86)	2.44 $\pm$ 02 (84)
GlcNAc	1.28 $\pm$ 0.2 (58)	3.79 $\pm$ 0.3 (58)	0.27 $\pm$ 0.009 (30)	1.20 $\pm$ 0.27 (53)	3.34 $\pm$ 0.2 (57)	0.81 $\pm$ 0.01 (28)
D-(+)-Glucose	2.55 $\pm$ 03 (116)	8.29 $\pm$ 0.6 (105)	0.83 $\pm$ 0.02 (93)	2.09 $\pm$ 0.3 (87)	6.82 $\pm$ 0.5 (96)	2.58 $\pm$ 0.1 (89)
D-Fructose	2.09 $\pm$ 0.4 (95)	7.26 $\pm$ 0.6 (92)	0.87 $\pm$ 0.1 (98)	2.15 $\pm$ 0.2 (88)	6.46 $\pm$ 0.5 (91)	2.48 $\pm$ 0.1 (86)
D-Mannose	1.36 $\pm$ 0.1 (62)	1.66 $\pm$ 0.2 (21)	0.11 $\pm$ 0.004 (12)	2.73 $\pm$ 0.3 (114)	8.94 $\pm$ 0.6 (126)	3.13 $\pm$ 0.2 (108)

[a] Values in parentheses are percentages of the values for the respective controls.
[b] Lake water.

tion was evaluated, *N*-acetylglucosamine, D-glucose, D-fructose, and D-mannose were used, each at the concentration of 10 mg/ml. As far as ions are concerned, no relevant effects were observed on the adhesion of either stationary or VBNC cells. On the other hand, both bivalent cations at the concentration of 30 mM substantially improved the adhesion of exponentially growing cells of *E. faecalis* 56R to both copepods and chitin, thus increasing the absolute cell numbers of adherent bacteria to the same level as in stationary cells. These differences were statistically significant ($P < 0.05$). This may be due to the fact that the surfaces of exponentially growing cells bear a higher negative charge than those of old cells (i.e., stationary cells and cells in the VBNC state), which exerts a repulsive force when bacteria approach copepods. By masking the negative charges of exponentially growing cells, the bivalent cations may encourage the two surfaces to approach one another, with a consequent net increase in enterococcal adhesion.

As far as the effects of sugars are concerned (Table 2), the only marked effect on the inhibition of interaction with both copepods and chitin particles was observed for GlcNAc, with reduction rates ranging from 40 to 82%, as opposed to the nonefficacy of D-glucose and D-fructose. D-Mannose exerted significant inhibitory activity ($P < 0.05$) only on the adhesion of enterococci to copepods, irrespective of the growth phase and state of the cells, while no effect on cell adhesion to purified chitin particles was detected. This suggests a role for bacterial surface lectins in adhesion to copepods. That chitin may be involved in the adhesion of enterococci is documented by the inhibition exerted by GlcNAc on adhesion to either copepods or purified chitin, while D-mannose probably acts on a receptor located in the mucilaginous materials present on the carapace surface (7).

Surface hydrophobicities of *E. faecalis* cells under different growth conditions. In a further experiment, we evaluated whether the differences in adhesion capabilities of *E. faecalis* cells were due to differences in surface hydrophobicity. Cell surface hydrophobicity was measured as described by Rosenberg et al. (30). Briefly, 300 μl of *n*-hexadecane was added to 3 ml of exponentially growing, stationary, and VBNC cells at an OD_{470} of 1.0. After a 10-min incubation at 37°C and a 30-s shaking, the OD_{470} was evaluated in the aqueous phase. The percentage of bacterial adhesion to hydrocarbon (BATH) was

calculated by applying the following equation: $[1 - (OD_F/OD_I)] \times 100$, where OD_I and OD_F were the ODs of cells at the beginning and at the end of the experiment, respectively. The percentages of enterococcal adhesion to hydrocarbon slightly increased from exponentially growing (BATH = 1.48% $\pm$ 0.21%), to stationary (BATH = 2.29% $\pm$ 0.31%), and to VBNC cells (BATH = 3.0% $\pm$ 0.29%). These differences, however, were not statistically significant ($P > 0.05$).

***E. faecalis* cell wall molecules that interact with chitin.** Because the copepod carapace is essentially made up of chitin, in a second phase of the research, we explored the possibility that *E. faecalis* surface molecules could be involved in chitin binding. Protein extraction from the *E. faecalis* cell wall by lithium chloride was performed as described previously (19). Briefly, 1 liter each of a stationary-phase and a VBNC culture of *E. faecalis* 56R was collected by centrifugation at 4°C, and the resulting pellet was washed twice with cold Na-phosphate buffer (0.01 M, pH 7.2). The pellet was resuspended in 2 M LiCl and incubated at RT for 60 min under gentle agitation. Particulate material was sedimented out by centrifugation at 8,000 $\times$ *g* for 15 min at 4°C. Supernatants were thoroughly dialyzed against sterilized lake water with four changes. The extracts were concentrated by ultrafiltration using a Vivascience VIVASPIN concentrator equipped with a 5,000-molecular-weight-cutoff polyethersulfone membrane. Two hundred microliters of a suspension containing 40 μg/ml of proteins was placed in contact with 25 mg of chitin and allowed to stand at RT for 1 h under gentle agitation. Chitin was sedimented by centrifugation and the supernatant removed with a pipette. Chitin was washed four times with lake water. CBPs were solubilized in boiling sodium dodecyl sulfate (SDS) in loading buffer and separated by SDS-polyacrylamide gel electrophoresis (29). After migration, proteins were detected by silver staining (Bio-Rad Laboratories). Figure 2 shows the electropherogram of cell wall proteins and the corresponding CBPs of stationary and VBNC cells. Few proteins were present in the stationary-growth-phase *E. faecalis* 56R wall, and the following molecular masses were calculated: 91, 84, 80, 74, 71, 55, 49, 43, 39, and 35 kDa. Six of 10 proteins present in the stationary cells were displayed by the *E. faecalis* VBNC cells. The molecular masses were 91, 80, 71, 49, 39, and 35 kDa. Of these wall proteins, only four displayed chitin-binding capabil-
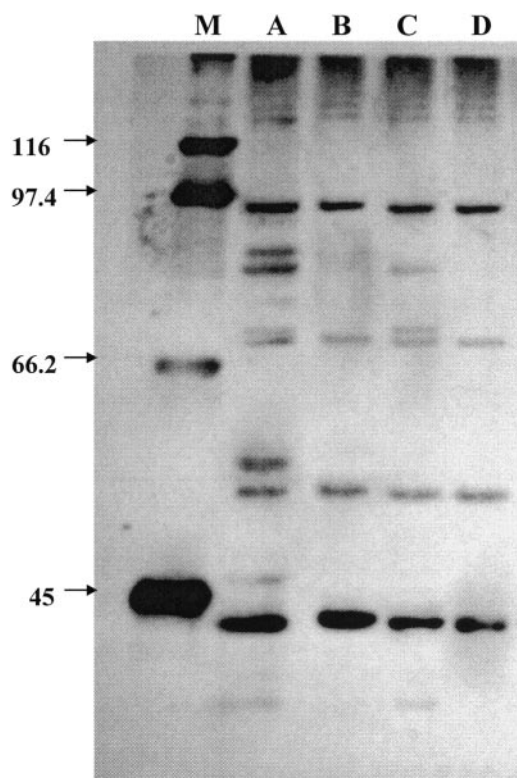
FIG. 2. SDS-polyacrylamide gel electrophoresis of cell wall proteins and the corresponding CBPs of *E. faecalis* 56R grown to the stationary phase and in the VBNC state. Lane A, cell wall proteins of stationary-phase cells; lane B, CBPs corresponding to those in lane A; lane C, cell wall proteins of VBNC cells; lane D, CBPs corresponding to those in lane C; lane M, molecular mass markers (in kilodaltons) as indicated on the left.

ity, whether they were in stationary or VBNC cells. Their molecular masses were 90, 71, 49, and 39 kDa.

Extraction of lipoteichoic acid (LTA) was done with 45% aqueous phenol at 68°C, as described by Kessler and Shockman (18), with modifications introduced by Signoretto et al. (33). Purified LTA was placed in contact with 25 mg of chitin and allowed to stand at RT for 60 min under gentle agitation. The supernatant was removed as stated above for CBPs, chitin was washed four times with lake water, and total bound phosphorus was evaluated (6). Table 3 shows the results of these experiments. In all cases, i.e., with exponentially growing, stationary, and VBNC cells, roughly 25 to 30% of LTA was tightly bound to purified chitin.

TABLE 3. Determination of *E. faecalis* 56R LTA bound by chitin particles

Type of cells	Phosphorous in LTA (mg/dl)		
	Total	Bound to chitin	Surnatant[a]
Exponential	7.92 ± 0.13	2.44 ± 0.06	5.72 ± 0.11
Stationary	6.18 ± 0.09	2.01 ± 0.09	4.45 ± 0.06
VBNC	5.73 ± 0.11	1.66 ± 0.05	3.83 ± 0.05

[a] Liquid fraction after chitin removal. Values correspond to the unbound LTA fractions.

The finding that either some cell surface proteins or LTA is capable of in vitro chitin-binding activity supports the direct involvement of chitin as a target for enterococcal adhesion to copepods. In particular, we have found four proteins located in the cell wall of *E. faecalis* which are present in both stationary and VBNC cells and act as CBPs. This situation may be reminiscent of that of *V. cholerae* (29, 35). The 90-kDa cell wall protein may correspond to *E. faecalis* autolysin (4), a peptidoglycan lytic enzyme involved in remodeling peptidoglycan for cell surface extension during cell growth and division (12). The proteins with molecular masses of 39 and 49 kDa may be the result of a proteolytic cleavage of autolysin. That a bacterial surface muramidase may be involved in chitin binding is not surprising due to a number of structural similarities between peptidoglycan and chitin. We evaluated the adhesion capability of a Lyt mutant of *E. faecalis* (3), but no impairment of cell adhesion was observed (data not shown). This suggests that multiple factors may be involved in copepod and chitin binding and is in accordance with the identification of four cell wall proteins and with LTA as being involved. Finally, it is reasonable to assume that LTA may be involved in binding to both biotic and abiotic surfaces, inasmuch as involvement of this wall polymer has been previously demonstrated in adhesion to mammalian cells (25).

Our results as a whole indicate that copepods may constitute an additional environmental reservoir of enterococci, thus suggesting the advisability of redesigning the protocols currently used for microbial detection during the evaluation of the microbiological quality of environmental samples.

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