Adhesion of *Enterococcus faecalis* in the Nonculturable State to Plankton Is the Main Mechanism Responsible for Persistence of This Bacterium in both Lake and Seawater

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The presence of enterococci in lake and seawater in an 18-month survey comparing molecular (PCR and quantitative PCR) and culture methods was evaluated, as well as the possibility that zooplankton could act as reservoirs for enterococci. Samples of both water and zooplankton were collected monthly from a Lake Garda site and an Adriatic Sea site. In lake water, the positive samples numbered 13 of 54 (24%) by culture and 32 of 54 (59%) when PCR was applied. In seawater, they numbered 0 of 51 by culture and 18 of 51 (35%) by PCR. Enterococci were found either totally bound to plankton or totally in water, depending on the presence or absence of plankton, respectively. These results clearly indicate that the PCR assay is a powerful tool for detecting fecal indicators and pathogens in the environment, thus providing a much more sensitive method than culture.

When bacteria of medical interest are released into the external environment, they find conditions that are often opposed to those encountered during infection, in that the environment (e.g., marine or freshwater) is often oligotrophic, cold, of high or very low osmolarity, and exposed to solar radiation (2, 6, 11). Starting in the early 1980s, Rita Colwell and her coworkers detected a survival strategy adopted by bacteria when exposed to environmental stresses to prevent rapid cell death. This strategy is known as the "viable but nonculturable" (VBNC) state (4–6, 14, 23, 27). This condition can be summarized as a dormant state into which bacteria enter and switch off most of the metabolic activities. In the VBNC state bacteria were shown to be unable to form colonies on conventional growth media, but cells maintained viability (6, 17, 18, 20, 32) and gene transcription, accompanied by specific mRNA production (9, 12, 20–22), and cell wall modifications (8, 17, 34, 35) took place. Furthermore, resuscitation, i.e., restoration of cell division, has been demonstrated in a part of the VBNC population on returning to suitable environmental conditions (7, 17, 18, 20, 29, 36). These bacterial forms are not detectable with the methods currently used to ascertain the microbiological quality of the environment. Thus, it could be concluded that the real bacterial-pathogen load in environments would be unidentified or underestimated (6, 14, 25, 28).

Another recently identified strategy which enables human pathogens to survive and persist when released in water is the ability to adhere to chitin-containing surfaces, such as those of zooplanktonic organisms (1, 3, 13, 15, 16, 26, 30, 37, 38). This strategy has been analyzed in detail for *Vibrio cholerae* and for other vibrios which are causative agents of human pathologies. It has been concluded that this may be the mode of persistence of vibrios in the environment in interepidemic periods (5, 14, 19, 31). In addition, vibrios adhering to zooplankton in both the culturable and nonculturable state were detected (19, 25). This strategy, however, could alter the evaluation of the microbiological load in waters, in that zooplankton, by binding bacteria, can withdraw and/or concentrate bacteria in a given volume of water.

Microbiological evaluation of surface waters usually involves the detection of specific pathogens and, most commonly, the detection of fecal indicators. Of all the indicators tested, enterococci are those which correlate best with the incidence of diarrheal diseases observed among swimmers (10). For this reason enterococci (mainly *Enterococcus faecalis* and *Enterococcus faecium*) are currently considered useful indicators of fecal contamination of waters. We have shown that enterococci can enter the VBNC state under exposure to oligotrophic environments (20, 22, 23). Enterococci in this state are no longer detectable in environmental samples if the culture method alone is applied (24).

In this study we describe the results of the monitoring of the microbiological quality of both freshwater and marine water by applying an approach consisting of detecting both culturable and nonculturable enterococci which are present in water and adherent to the plankton in order to evaluate to what extent the adhesion to plankton and the VBNC state may represent survival strategies and contribute to the formation of environmental reservoirs of these microorganisms.

Water sampling sites and conditions and microbiological

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TABLE 1. Occurrence of *E. faecalis* in lake water and plankton samples as determined by culture and molecular methods

Mo	No. of plankton >100 μm (organisms/m³)	Culture method			Quantitative PCR[a]		
		On plankton >100 μm (CFU/m³)	On plankton 64 to 100 μm (CFU/100 ml)	In water (CFU/100 ml)	On plankton >100 μm (no./m³)	On plankton 64 to 100 μm (no./100 ml)	In water (no./100 ml)
April 2001	250	0	0	0	0	0	$1.3 \times 10^6 \pm 4.9 \times 10^5$
May	300	0	3	300	0	0	$2.3 \times 10^7 \pm 5.3 \times 10^6$
June	850	0	0	25	550 ± 140	<50	$9.2 \times 10^6 \pm 2.1 \times 10^6$
July	3,300	600	0	16	$5.6 \times 10^5 \pm 1.4 \times 10^5$	$1,200 \pm 340$	$2.6 \times 10^3 \pm 1.2 \times 10^3$
August	10,000	750	0	0	$5.2 \times 10^8 \pm 7.8 \times 10^7$	800 ± 260	0
September	14,500	0	0	0	$7.3 \times 10^8 \pm 1.6 \times 10^8$	170 ± 120	0
October	1,150	0	0	0	$8.9 \times 10^4 \pm 2.4 \times 10^4$	70 ± 45	0
November	500	0	0	0	0	0	<50
December	200	0	0	0	0	0	$2.5 \times 10^6 \pm 7.9 \times 10^5$
January 2002	100	0	0	0	0	0	$1.8 \times 10^6 \pm 7.4 \times 10^5$
February	250	0	0	0	0	0	$1.1 \times 10^6 \pm 3.7 \times 10^5$
March	500	0	0	0	0	0	$3.7 \times 10^5 \pm 1.3 \times 10^5$
April	1,400	0	0	0	0	0	$5.3 \times 10^4 \pm 1.2 \times 10^4$
May	4,400	900	0	2	$1,100 \pm 270$	0	<50
June	9,000	500	0	5	$6.1 \times 10^6 \pm 1.9 \times 10^6$	240 ± 170	<50
July	7,500	3,000	0	$60 (28)^b$	$6.4 \times 10^4 \pm 4.5 \times 10^4$	90 ± 75	<50
August	6,000	0	1	0	$4.4 \times 10^6 \pm 7.8 \times 10^5$	120 ± 90	0
September	4,500	0	0	0	$8.6 \times 10^4 \pm 5.6 \times 10^4$	100 ± 80	0

[a] *E. faecalis*-equivalent cell number (no.) was calculated as described by Lleò et al. (24).

[b] Includes different enterococcal species: *E. faecalis*, *E. faecium*, and *Enterococcus hirae*. The *E. faecalis* count is in parentheses.

procedures. Samples of both freshwater and marine water were collected monthly between March 2001 and September 2002 from Lake Garda offshore from Lazise on the eastern side of the lake (Province of Verona, Italy) and from the Adriatic Sea offshore from Senigallia (Province of Ancona, Italy). Marine plankton organism sampling was carried out by dragging the water horizontally, at a depth of about 1 m, with a 200-μm-mesh plankton net, while for lake plankton organisms a 100-μm-mesh plankton net was used. Five to 10 m³ of water was dragged at each sampling. One-half of the dragged material was fixed with 2% (final concentration) formaldehyde for subsequent plankton characterization, while the other half was immediately transferred to the laboratory and processed within 6 h of collection for microbiological and molecular investigations. Ten liters of water was also collected and immediately filtered through a 100- or 200-μm net (lake and seawater, respectively) and then through a 64-μm net. This material was considered plankton ranging in size from 64 to 100 μm or 64 to 200 μm. At the same time as each plankton sampling, two additional liters of water was collected to evaluate the number of enterococci present in water.

To evaluate the load of culturable enterococci bound to zooplankton, 100 μl of the dragged material, after standing in a sonication bath for 30 s, was spread on *m-Enterococcus* agar (mEA; Becton Dickinson) and plates were incubated at 35°C for 3 days (39). To evaluate the enterococcal load present in water, the samples of fresh- or seawater were first filtered on a piece of the 64-μm net in order to remove the highly particulate matter (plankton included). One hundred milliliters of the resulting water was then filtered on a 0.22-μm-pore-size Millipore membrane (47 mm in diameter). Membranes were placed faceup on mEA plates, which were incubated at 35°C for 3 days (39).

To perform molecular detection of enterococci, DNA was extracted from either 1 ml of dragged material or 1 ml of 200× freshwater or seawater concentrated by filtration on 0.22-μm

Millipore membrane, as previously described (24). The target sequence for PCR amplification was a 444-bp fragment located on the *E. faecalis* chromosome within the *pbp5* gene, coding for penicillin binding protein 5 (PBP5), previously sequenced and demonstrated to be species specific (33). The fragment was amplified by PCR with two primers selected from within the gene: primer FWD (5′ CATGCGCAATTAATCGG 3′) and primer REV (5′ CATAGCCTGTCGCAAAAC 3′). To perform competitive PCR (cPCR), a deleted internal standard amplified by the same primers (24) was used. The sensitivity of the method was 50 cells per ml (24).

Zooplankton detection as a function of changes in physicochemical parameters in both freshwater and seawater. Lake Garda is the biggest Italian, lake with a surface of 368 km². The total volume is 49 km³, while the mean outflow rate of the Mincio river is 58 m³/s, so that total water replacement time is calculated to be 27 years. The resident population of the lakeside villages is 160,000 people, but this population is as much as 25 times greater in the warmest months (April to September) as a result of the booming tourist industry. The surface water temperature ranged from 7 to 26°C, and pH ranged from 7.9 to 9.1, while the only salt present was calcium carbonate at a concentration ranging from 150 to 180 mg/liter. The zooplankton (>100 μm) concentration varied according to temperature in that a peak was recorded during the summer (highest value in the summer of 2001, with 14,500 organisms/m³ of water, and 9,000 organisms/m³ of water in the summer of 2002), while during the winter (water temperature: 7°C) zooplankton practically disappeared (Table 1). The zooplankton comprised rotifers, cladocers, and copepods. Copepods, including *Calanoides* (*Copipodiaptomus steueri*) and *Cyclopoides* (*Mesocyclops leuckarti*) spp. were highly prevalent (>85%) among zooplankton families.

As far as the seawater was concerned, the water temperature changed seasonally, with a trend similar to that for Lake Garda, while zooplankton (>200 μm) counts per cubic meter

TABLE 2. Occurrence of *E. faecalis* in seawater and plankton samples as determined by culture and molecular methods

Mo	No. of plankton >200 μm (organisms/m³)	Level of *E. faecalis* by:					
		Culture method			Quantitative PCR[a]		
		On plankton >200 μm (CFU/m³)	On plankton 64 to 200 μm (CFU/100 ml)	In water (CFU/100 ml)	On plankton >200 μm (no./m³)	On plankton 64 to 200 μm (no./100 ml)	In water (no./100 ml)
April 2001	1,300	0	0	0	0	0	0
May	1,000	0	0	0	420 ± 260	480 ± 370	0
June	2,100	0	0	0	0	0	0
July	300	0	0	0	$8,400 \pm 3,750$	640 ± 290	$4.2 \times 10^4 \pm 2.7 \times 10^4$
August	2,600	0	0	0	$6.4 \times 10^4 \pm 2.9 \times 10^4$	360 ± 130	0
September	1,200	0	0	0	$1,900 \pm 640$	120 ± 85	0
October	1,500	0	0	0	0	220 ± 90	0
November	1,700	0	0	0	$1,450 \pm 730$	360 ± 125	0
December	2,000	0	0	0	0	0	0
January 2002	550	0	0	0	<50	0	0
February	60	0	0	0	0	0	0
March	500	0	0	0	0	0	0
April	900	0	0	0	<50	0	0
May	3,300	0	0	0	<50	0	0
June	300	0	0	0	0	0	0
July	3,700	0	0	0	<50	<50	<50
August	3,250	0	0	0	0	0	0

[a] *E. faecalis*-equivalent cell number (no.) was calculated as described by Lleò et al. (24).

of seawater were subject to substantial variability throughout the year, though this was not directly related to water temperature (Table 2). Copepods were highly dominant among the zooplankton organisms of the Adriatic Sea, and species such as *Paracalanus parvus*, *Clausocalanus clause*, *Centropages typicus*, *Oithona elgolandica* and *Acartia clause* were included. Cladocers such as *Penilia avirostris*, *Evadne* spp. and *Podon* spp. were detected, especially during the warm season.

Detection of enterococci adherent to zooplankton and in water by culture and molecular methods in both lake and seawater. Table 1 shows the results of the search for enterococci by the cultural method in Lake Garda. Enterococci adherent to zooplankton measuring more than 100 μm or in water were sporadically detected by the culture method in late spring and early summer in both 2001 and 2002. With the exception of one sample (August 2002), plankton ranging in size from 64 to 100 μm did not yield bound enterococci. When, however, detection of enterococci was performed in the same samples by the molecular method (PCR) a much higher number proved positive (32 of 54 samples), thus suggesting that enterococci were present in Lake Garda all year round. cPCR (quantitative PCR) yielded surprising results: in samples collected in summer and early fall (June to October), when the highest numbers of zooplankton elements were detected, only very large numbers of enterococci adhering to zooplankton were detected, while no enterococci were detected in water. In samples collected during the cold months (December to April), in which zooplankton counts were lower than 500 plankton organisms/m³, enterococci were detected only in water.

Table 2 shows the results of enterococcus detection in seawater. The culture method yielded negative results for all samples whether the samples contained zooplankton or not. When the enterococcal presence was evaluated by the cPCR method several samples proved positive (18 of 51). In particular, positive results were detected in samples containing zooplankton that were collected during the months May to November 2001. In comparison with results for samples collected from Lake Garda, the number of enterococci was much lower. During the next few months and in spring to summer 2002 the number of positive samples and the bacterial counts were less than in the previous year; once again, however, the positive samples were those containing zooplankton. Enterococci were detected in only 3 out of 16 water samples, but the equivalent plankton samples always proved positive.

To rule out the possibility that these positive samples may simply have been an artifact due to binding of enterococcal free DNA (originating from the lysis of dead microorganisms) to zooplankton, we performed an in vitro experiment in which zooplankton collected from Lake Garda previously shown to be negative for enterococci by PCR was placed in contact with *E. faecalis* purified DNA in the presence of Lake Garda water. After extensive washing with lake water, any DNA still bound to the copepods was detected by PCR. No positive results were detected in the three separate experiments performed.

To confirm the unexpected results of the field research, in which enterococci were prevalently bound to plankton, we tried to detect whole adherent enterococci using anti-D antibodies bound to microbeads. A sample (100 μl) of formalin-fixed plankton was mixed with a drop of Phadebact Strep D reagent (Boule Diagnostics) and allowed to stand for a few seconds at room temperature with gentle agitation. Figure 1 shows that zooplankton organisms collected from Lake Garda that proved negative for enterococci as detected by PCR did not present microbeads on their surfaces (Fig. 1B and D), while the same zooplankton organisms that were exposed in vitro to enterococci resuspended in lake water presented bound microbeads (Fig. 1A). Figure 1C shows zooplankton organisms from a sample that was negative for enterococci by the culture method but positive by PCR that presented microbeads adhering to their surfaces.

Finally, to rule out the possibility of detection by PCR of DNA of inactivated but structurally intact enterococci, we performed, on some PCR-positive samples, a search for *pbp5* mRNA, which was previously shown (22) to be a marker of the viability of nonculturable *E. faecalis* cells. All three water sam-
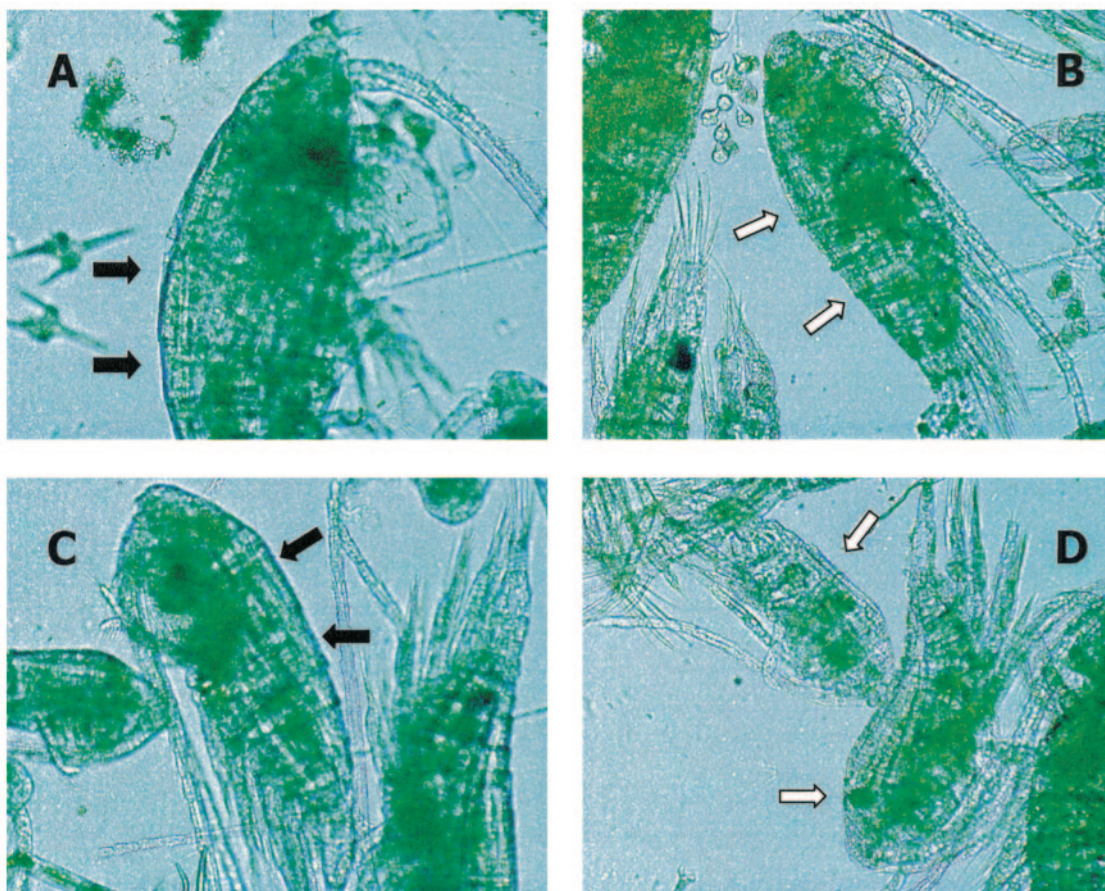
FIG. 1. Microscopic observation of lake zooplankton treated with anti-D antibody bound to latex microbeads (Phadebact Strep D reagent; Boule Diagnostic). Positive (A) and negative (B) controls consisted of zooplankton which was exposed, in vitro or not, to an *E. faecalis* culture. Black arrows (A and C) indicate the presence of latex microbeads as a black striation on the carapace; white arrows (B and D) indicate the carapace without adherent microbeads. (C) Sample collected from Lake Garda negative by culture but positive by PCR. (D) Sample collected from Lake Garda negative by both culture and PCR for enterococci.

ples and five plankton samples positive by PCR contained *pbp5* mRNA, as determined by reverse transcription-PCR (data not shown), thus indicating that at least part of the nonculturable enterococci detected by PCR were in the VBNC state.

The aim in this study was to compare culture and molecular (quantitative) methods of detecting *E. faecalis* in the water and/or adhering to zooplankton in order to define whether these survival strategies truly represent a matter for public concern. We show that molecular methods for the detection of enterococci resulted in a higher number of positive samples than the culture method. In particular, 13 of 54 (24%) samples of lake water were positive by the culture method, as against 32 of 54 (59%) when PCR was used. In seawater the respective figures were 0 of 51 (0%) by the culture method and 18 of 51 (35%) by PCR. Evaluation of *E. faecalis* cell numbers by cPCR yielded the highest values in lake water. This should not be surprising, in that lakes have a slow water replacement, which could result in serious environmental damage due to bacterial overload. Furthermore, the substantial discrepancy between the culture and molecular methods of detecting *E. faecalis* in both freshwater and seawater may be tentatively explained by the fact that this microorganism may persist in waters prevalently in the nonculturable state, like *V. cholerae* (4–6, 19, 25).

The most interesting result of this study was the observation that in Lake Garda *E. faecalis* is almost exclusively found either adhering to plankton or in water, and not both. This result was also confirmed by the results in seawater, although not to such an evident extent. One reason that can be adduced to explain this is the fact that plankton is always observed in coastal seawater all year round because of the continuous supply of warmer water and plankton from the open sea. Thus, in general, because plankton is observed all year round in the sea, positive plankton samples are much more prevalent than positive water samples.

To the best of our knowledge, this is the first description of a gram-positive bacterium that persists in the environment by adherence to plankton. Very recently, Whitman et al. (40) described the occurrence of *Escherichia coli* and enterococci in *Cladophora* (an alga) in nearshore water and beach sand of Lake Michigan.

REFERENCES

1. **Amako, K., S. Shimodori, T. Imoto, S. Miake, and A. Umeda.** 1987. Effects of chitin and its soluble derivatives on survival of *Vibrio cholerae* O1 at low temperatures. Appl. Environ. Microbiol. **53:**603–605.
2. **Barcina, I., P. Lebaron, and J. Vives-Rego.** 1997. Survival of allochthonous bacteria in aquatic systems: a biological approach. FEMS Microbiol. Ecol. **23:**1–9.
3. **Carli, A., L. Pane, L. Casareto, S. Bertone, and C. Pruzzo.** 1993. Occurrence of *Vibrio algynolyticus* in Ligurian coast rock pools (Tyrrhenian Sea, Italy) and its association with the copepod *Tigriopus fulvus* (Fisher 1860). Appl. Environ. Microbiol. **59:**1960–1962.
4. **Carroll, J. W., M. C. Mateescu, K. Chava, R. R. Colwell, and A. K. Bej.** 2001. Response and tolerance of toxigenic *Vibrio cholerae* O1 to cold temperatures. Antonie Leeuwenhoek **79:**377–384.
5. **Colwell, R. R.** 1996. Global climate and infectious disease: the cholera paradigm. Science **274:**2025–2031.
6. **Colwell, R. R.** 2000. Bacterial death revisited, p. 325–342. *In* R. R. Colwell, and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. ASM Press, Washington, D.C.
7. **Colwell, R. R., P. R. Brayton, A. Huq, B. Tall, P. Harrington, and M. Levine.** 1996. Viable but non-culturable *Vibrio cholerae* O1 revert to a culturable state in the human intestine. World J. Microbiol. Biotechnol. **12:**28–31.
8. **Costa, K., G. Bacher, G. Allmaier, M. G. Dominguez-Bello, L. Engstrand, P. Falk, M. A. De Pedro, and F. Garcia-del Portillo.** 1999. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. J. Bacteriol. **181:**3710–3715.
9. **Fisher-Le Saux, M., D. Hervio-Heath, S. Loaec, R. R. Colwell, and M. Pommepuy.** 2002. Detection of cytotoxin-hemolysin in nonculturable populations of environmental and clinical *Vibrio vulnificus* strains in artificial seawater. Appl. Environ. Microbiol. **68:**5641–5646.
10. **Fujioka, R. S.** 1997. Indicators in marine recreational water quality, p. 176–183. *In* C. J. Hurst (ed.), Manual of environmental microbiology. ASM Press, Washington, D.C.
11. **Gauthier, M. J.** 2000. Environmental parameters associated with the viable but nonculturable state, p. 87–112. *In* R. R. Colwell, and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. ASM Press, Washington, D.C.
12. **Heim, S., M. M. Lleò, B. Bonato, C. A. Guzman, and P. Canepari.** 2002. The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. J. Bacteriol. **184:**6739–6745.
13. **Hood, M. A., and P. A. Winter.** 1997. Attachment of *Vibrio cholerae* under various environmental conditions and to selected substrates. FEMS Microbiol. Ecol. **22:**215–223.
14. **Huq, A., I. N. G. Rivera, and R. R. Colwell.** 2000. Epidemiological significance of viable but nonculturable microorganisms, p. 301–323. *In* R. R. Colwell, and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. ASM Press, Washington, D.C.
15. **Huq, A., P. A. West, E. B. Small, M. I. Huq, and R. R. Colwell.** 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl. Environ. Microbiol. **45:**275–283.
16. **Huq, A., P. A. West, E. B. Small, M. I. Huq, and R. R. Colwell.** 1984. Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. Appl. Environ. Microbiol. **48:**420–424.
17. **Jians, X., and T. Chai.** 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable nonculturable cells. Appl. Environ. Microbiol. **62:**1300–1305.
18. **Kaprelyants, A. S., and D. B. Kell.** 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometry analysis of starvation and resuscitation. Appl. Environ. Microbiol. **59:**3187–3196.
19. **Lipp, E. K., I. N. G. Rivera, A. I. Gil, E. M. Espeland, N. Choopun, V. R. Louis, E. Russek-Cohen, A. Huq, and R. R. Colwell.** 2003. Direct detection of *Vibrio cholerae* in Peruvian coastal water and plankton by PCR. Appl. Environ. Microbiol. **69:**3676–3680.
20. **Lleò, M. M., B. Bonato, M. C. Tafi, C. Signoretto, M. Boaretti, and P. Canepari.** 2001. Resuscitation rate in different enterococcal species in the viable but non-culturable state. J. Appl. Microbiol. **91:**1095–1102.
21. **Lleò, M. M., B. Bonato, C. Signoretto, and P. Canepari.** 2003. Vancomycin resistance is maintained in enterococci during their permanence in the viable but nonculturable state and after division is resumed. Antimicrob. Agents Chemother. **47:**1154–1156.
22. **Lleò, M. M., S. Pierobon, M. C. Tafi, C. Signoretto, and P. Canepari.** 2000. mRNA detection by RT-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. Appl. Environ. Microbiol. **66:**4564–4567.
23. **Lleò, M. M., M. C. Tafi, and P. Canepari.** 1998. Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. Syst. Appl. Microbiol. **21:**333–339.
24. **Lleò, M. M., M. C. Tafi, C. Signoretto, C. Dal Cero, and P. Canepari.** 1999. Competitive polymerase chain reaction for quantification of nonculturable *Enterococcus faecalis* cells in lake water. FEMS Microbiol. Ecol. **30:**345–353.
25. **Louis, V. R., E. Russek-Cohen, N. Choopun, I. N. G. Rivera, B. Gangle, S. C. Jiang, A. Rubin, J. A. Patz, A. Huq, and R. R. Colwell.** 2003. Predictability of *Vibrio cholerae* in Chesapeake Bay. Appl. Env. Microbiol. **69:**2773–2785.
26. **Montanari, M. P., C. Pruzzo, L. Pane, and R. R. Colwell.** 1999. Vibrios associated with plankton in a coastal zone of the Adriatic Sea (Italy). FEMS Microbiol. Ecol. **29:**241–247.
27. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York, N.Y.
28. **Oliver, J. D.** 2000. The public health significance of viable but nonculturable bacteria, p. 277–300. *In* R. R. Colwell, and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. ASM Press, Washington, D.C.
29. **Oliver, J. D., and R. Bockian.** 1995. In vivo resuscitation and virulence towards mice of viable but nonculturable cells of *Vibrio vulnificus*. Appl. Environ. Microbiol. **61:**2620–2623.
30. **Pruzzo, C., A. Crippa, S. Bertone, L. Pane, and A. Carli.** 1996. Attachment of *Vibrio alginolyticus* to chitin mediated by chitin binding proteins. Microbiology **142:**2181–2186.
31. **Pruzzo, C., R. Tarsi, M. M. Lleò, C. Signoretto, M. Zampini, L. Pane, R. R. Colwell, and P. Canepari.** 2003. Persistence of adhesive properties of *Vibrio cholerae* after long term exposure to sea water. Environ. Microbiol. **5:**650–658.
32. **Rahman, I., M. Shahamat, P. A. Kirchman, E. Rissek-Cohen, and R. R. Colwell.** 1994. Methionine uptake and cythopathogenicity of viable but nonculturable *Shigella dysenteriae* type 1. Appl. Environ. Microbiol. **60:**3573–3578.
33. **Signoretto, C., M. Boaretti, and P. Canepari.** 1994. Cloning, sequencing and expression in *Escherichia coli* of the low-affinity penicillin-binding protein of *Enterococcus faecalis*. FEMS Microbiol. Lett. **123:**99–106.
34. **Signoretto, C., M. M. Lleò, and P. Canepari.** 2002. Modification of the peptidoglycan of *Escherichia coli* in viable but nonculturable state. Curr. Microbiol. **44:**125–131.
35. **Signoretto, C., M. M. Lleò, M. C. Tafi, and P. Canepari.** 2000. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl. Environ. Microbiol. **66:**1953–1959.
36. **Steinert, M., L. Emody, R. Amann, and J. Hacker.** 1997. Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR 32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **63:**2047–2053.
37. **Tamplin, M. L., A. L. Gauzens, A. Huq, D. A. Sack, and R. R. Colwell.** 1990. Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. Appl. Environ. Microbiol. **56:**1977–1980.
38. **Tarsi, R., and C. Pruzzo.** 1999. Role of surface proteins in *Vibrio cholerae* attachment to chitin. Appl. Environ. Microbiol. **65:**1348–1351.
39. **Toranzos, G. A., and G. A. McFeters.** 1997. Detection of indicator microorganisms in environmental freshwaters and drinking waters, p. 184–194. *In* C. J. Hurst (ed.), Manual of environmental microbiology. ASM Press, Washington, D.C.
40. **Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli.** 2003. Occurrence of *Escherichia coli* and enterococci in *Cladophora* (*Chlorophyta*) in nearshore water and beach of Lake Michigan. Appl. Environ. Microbiol. **69:**4714–4719.