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Survival of Nonculturable *Aeromonas salmonicida* in Lake Water

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The survival of *Aeromonas salmonicida* subsp. *salmonicida* was investigated in sterile and untreated lake water. In sterile lake water (filtered and autoclaved), it was found that cells of *A. salmonicida* entered a nonculturable but viable condition. Viability was determined by flow cytometry with the dye rhodamine 123, which is taken up and maintained within cells with a membrane potential. For survival studies in untreated lake water, *A. salmonicida* was marked with the *xylE* gene by using the plasmid pLV1013. Marked cells were detected by growth on tryptone soy agar and tryptone soy agar supplemented with kanamycin. Cells were also detected by polymerase chain reaction DNA amplification of the *xylE* gene and a chromosomal DNA fragment specific for *A. salmonicida* (pLV1013). The results indicated that *A. salmonicida* entered a nonculturable condition in untreated lake water over a 21-day study. The viability of nonculturable cells could not be determined in mixed samples; however, the presence of nonculturable cells containing both chromosomal and plasmid DNA was confirmed.

Aeromonas salmonicida subsp. *salmonicida* is the etiological agent of furunculosis in salmonids (19), is considered to be an obligate pathogen, and has a widespread distribution and a diverse host range. The survival of *A. salmonicida* in water has been well documented, and most studies using cell culture have shown that it cannot persist for long periods without a new host (1, 6, 13, 14, 16, 21, 24). A number of species of gram-negative bacteria have been shown to enter a physiological state under environmentally relevant conditions in which they become nonculturable but viable (NCBV) (2–4, 7, 18, 22, 23, 28). The ability of *A. salmonicida* to enter such a state has remained unclear and controversial (1, 16, 20). The existence of the NCBV state for *A. salmonicida* may explain how outbreaks of furunculosis can occur in fish populations which apparently have not come into contact with the pathogen. Cells of *A. salmonicida* shed by an infected fish into the surrounding water could become nonculturable and therefore would not be detected by growth on standard media. Nonculturable cells that remain viable could then infect a new fish and result in the outbreak of furunculosis. From these fish, culturable cells would then be detected by standard methods. Thus, the cycle from infected fish through a nonculturable condition and back to a culturable condition in a new host would be completed.

The basis for the nonculturable state for many bacteria has been the discovery that cells placed in conditions present in the environment cannot be recultured on the medium from which they were initially isolated or any other laboratory media. In some cases, viability has been proven through the use of the direct viable count, respiration studies, and infection studies. If viability has been proven, then the term NCBV can be used. If viability has not been shown, then the term NCBV cannot be used; therefore, we have adopted the term nonculturable to describe these cells (16).

A. salmonicida was first shown to enter an NCBV state by Allen-Austin et al. (1). Since then, evidence indicating that the assay they used for detecting viable cells is compromised by the presence of a small number of culturable cells remaining undetected within the sample has been provided (16, 20). This does not mean that the nonculturable cells they found were not viable but that the test they used did not prove their viability. Cells of *A. salmonicida* in a nonculturable state have been shown to be morphologically intact and contain DNA, RNA, plasmids, and bound fatty acids (15, 16). The nonculturable cells also maintain a rough cell appearance, and an external protein matrix remains attached to the cell surface (15). Viability assays using the direct viable count (11) and respiration measurements (29) have been employed to try and determine whether these nonculturable cells remain viable (16). *A. salmonicida* MT432 was found to be resistant to nalidixic acid, which prevents the use of the direct viable count. The detection of respiration in these cells would also prove their viability; however, such tests were negative (16) and could indicate a low respiration rate rather than the presence of nonviable cells. The revival of nonculturable cells to a culturable condition would also prove their viability. To date, it has not been possible to revive these cells in various media, whole dead fish, fish tissue, or live fish (15, 16). This may indicate equally our inability to identify the appropriate revival conditions as well as the presence of nonviable cells.

This article describes the detection of viable cells of *A. salmonicida* that have entered a nonculturable state by the use of a new viability assay to detect cells with a membrane potential. In addition, the direct detection of *A. salmonicida* in untreated lake water by culture and direct detection of plasmid and chromosomal DNA by polymerase chain reaction (PCR) is presented and used to monitor the survival of cells in a mixed population.

MATERIALS AND METHODS

Bacterial strains and culture. *A. salmonicida* subsp. *salmonicida* MT432 (20) was provided by A. L. S. Munro (Marine Laboratory, Aberdeen, United Kingdom) and maintained on

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tryptone soy agar (TSA; Oxoid, Basingstoke, United Kingdom). In this study, *A. salmonicida* MT432 was marked genetically with the plasmid pLV1013 (26) which harbors the *xylE* gene. The broad-host-range plasmid construct pLV1013 was mobilized from *Escherichia coli* ED8654 into *A. salmonicida* by means of a nonquantitative triparental mating procedure which involved the mobilization functions of pNJ5000 (26). Samples consisting of 10 μl of cells from overnight nutrient broth (NB) cultures (37°C) of *E. coli* CA60(pNJ5000) and ED8654(pLV1013) and an overnight tryptone soy broth (TSB) culture (20°C) of *A. salmonicida* were overlaid successively on a nutrient agar plate and allowed to dry. After overnight incubation at 20°C, cells were resuspended in 0.5 ml of phosphate-buffered saline (0.1 M, 0.85% [wt/vol] NaCl [pH 7.5]) and plated onto TSA containing 25 μg of nalidixic acid ml^{-1} and 50 μg of kanamycin ml^{-1}. The plates were then incubated at 20°C for 2 days, and resistant colonies were checked for the presence of the *xylE* gene.

For release studies, cultures were grown at 20°C for 18 h in 50 ml of TSB or in 50 ml of TSB containing 50 μg of kanamycin ml^{-1} for strains harboring the plasmid pLV1013. The cells were harvested by centrifugation at 3,400 × *g* for 10 min and washed three times in sterile distilled water. The final pellet was resuspended in 50 ml of sterile distilled water. An acridine orange direct count was used to determine cell density (9).

Freshwater samples and release systems. Water samples were collected from the Northern Basin of Windermere (Cumbria, United Kingdom). Sterile lake water was prepared by filtering samples through a 0.22-μm-pore-size membrane (Millipore, Watford, Cumbria, United Kingdom) and autoclaving the filtrate at 120°C for 30 min. Sterile lake water release systems were prepared by placing 500 ml of sterile lake water in a 1-liter conical flask capped with a cotton wool bung. Lake water systems were prepared by placing 12 liters of untreated lake water in a 15-liter glass tank (25 cm^3) covered with a perspex lid. All release systems were performed in triplicate and incubated in the dark at 10°C.

Cell counts. Acridine orange direct counts were performed by the method of Jones and Simon (9). Colony-forming ability was determined in triplicate on samples plated on TSA. For pLV1013-containing strains, colony-forming ability was determined on TSA and TSA containing kanamycin (50 μg ml^{-1}). Sample sizes ranged from 0.1 ml for spread plate counts to 10 to 50 ml for filtered samples. Sample filtration was performed on a 0.22-μm-pore-size filter (4.5 cm in diameter); after filtration, the filter was removed from the apparatus and placed face up on the growth medium. All plates were incubated at 20°C for 3 days. Colonies containing the *xylE* gene were identified by spraying plates with a 1% (wt/vol) solution of catechol and observing the appearance of a yellow coloration (26).

Rhodamine 123 viability assay. The rhodamine 123 assay used was a modification of the method developed by Daiper et al. (5). At various intervals after the initial inoculation of sterile lake water release systems, 50 ml was removed and centrifuged at 8,000 × *g* for 15 min. The cell pellet was resuspended in 1 ml of sterile lake water. To the cell suspension, rhodamine 123 (2 μg ml^{-1}; Sigma Biochemicals, Ltd., Poole, United Kingdom) and EDTA (1 mM, pH 8.0) were added, and the suspension was incubated at room temperature for 30 min. To repeat samples, rhodamine (2 μg ml^{-1}) and gramicidin S (10 μg ml^{-1}; Sigma Biochemicals) were added, and the samples were incubated at room temperature for 30 min. Each cell suspension was analyzed in a FACStar Plus flow cytometer (Becton Dickinson). The three parameters measured were forward light scatter, 90°C side scatter, and fluorescence at 520 nm. The laser power at 488 nm was set at 0.2 W, and all recordings were taken on a log scale. The photomultiplier voltages were set at 450 V for the side-scatter detector and 550 V for the fluorescence detector. For each sample, 5,000 events were recorded. In addition, to calculate the cell concentration of the sample, the sweep trigger rate was recorded 10 times and the flow rate of sample through the cytometer was determined. The FACStar Plus computing package was used to determine the proportion of cells that were fluorescently labelled and to display the data as single dot plots and fluorescence histograms.

DNA amplification. Samples of lake water release systems (50 ml) were taken and centrifuged at 58,000 × *g* for 30 min. Cell pellets were resuspended in 1 ml of ultrapure (UP) water and recentrifuged. The cell pellet was then resuspended in 20 μl of UP water and placed in liquid nitrogen for 5 min. Frozen samples were transferred to −20°C for storage.

The amplification of DNA by PCR was performed by using the DNA AmpliTaq kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the Omnigene thermal cycler (Hybaid, Teddington, United Kingdom). For DNA amplification of cultured cells, one colony was suspended in 1 ml of UP water and diluted 1:100 in UP water. For the amplification of DNA from release systems, frozen cell samples were defrosted quickly and used undiluted. All amplifications were carried out in a final volume of 50 μl and covered with 50 μl of mineral oil. To each sample, 2 μl of cell suspension was added. The composition of the reaction mix was 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and 100 pmol of each primer. When the reaction mix was prepared, it was heated to 98°C for 10 min and cooled to 80°C, and 2.5 U of AmpliTaq was added. The sample was heated to 95°C for 5 min, and the following cycle was repeated 30 times: 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min. After cycling, the tube was held at 72°C for 7 min and cooled to 30°C. Samples were stored frozen at −20°C.

The primers for amplification of the *xylE* gene were 5′-CTGAAGGCTTGGACCGAAGT-3′ and 5′-GGTCCGGG TAGTTGTAATCT-3′. These primers bind to 20-bp regions of the *xylE* gene and amplify the region between bases 130 and 829. The *A. salmonicida* chromosomal primers hybridize to a region of specific DNA cloned and sequenced by Hiney et al. (8). The chromosomal primers 5′-CGTTGGATA GGCTCTTCCT-3′ and 5′-CTCAAAACGGCTGCGTACCA-3′ amplify a region of DNA between bases 31 and 453 on this fragment.

PCR products were detected by agarose gel electrophoresis carried out in 0.7% (wt/vol) agarose by the method of Maniatis et al. (12). In addition, radiolabelled probes were used to detect amplified DNA by filter hybridization. The probes were end-labelled with polynucleotide kinase (Boehringer, Lewes, United Kingdom) and gamma-labelled ^{32}P (Amersham International, Amersham, United Kingdom) by the method outlined in Maniatis et al. (12). The *xylE* gene probe hybridized to a 20-bp region of the gene between bases 561 and 580, 5′-CGTCGCCCAGTTTCTCAGTC-3′. The *A. salmonicida* chromosomal probe hybridized to a region between bases 218 and 237 on the chromosomal DNA fragment, 5′-AATCAAAAGTTGCAATTCTT-3′. PCR products were heated to 100°C for 5 min, and 2 μl was transferred immediately to a nylon membrane (Amersham) and allowed

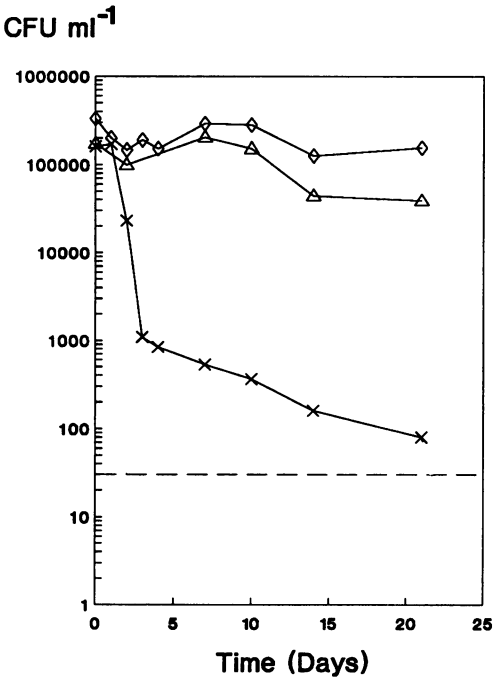

FIG. 1. Survival of *A. salmonicida* in sterile water. CFU were measured on TSA (×). Cells were measured by acridine orange direct count (◇) and by rhodamine 123 viable cell count (△). The broken line indicates the detection limit for CFU on TSA.

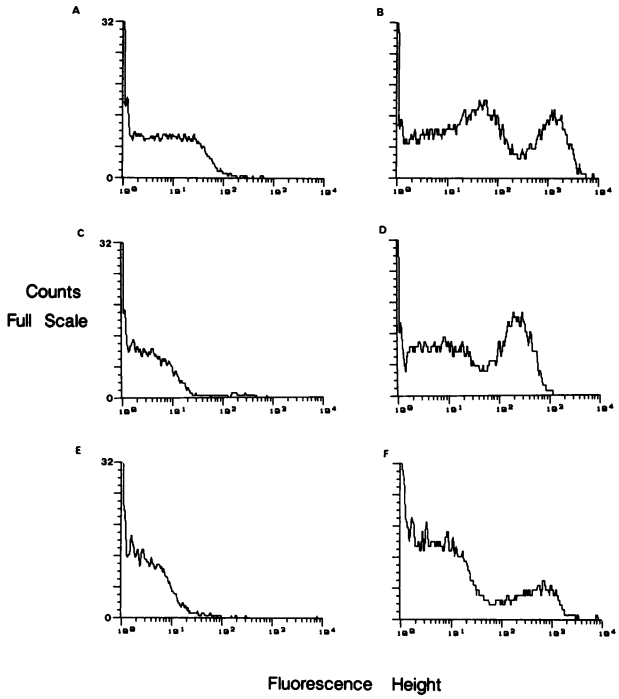

FIG. 2. Detection of viable *A. salmonicida* in sterile lake water release systems by using rhodamine and flow cytometry. (A) Day 0 cells detected with rhodamine and gramicidin S; (B) day 0 cells detected with rhodamine; (C) day 7 cells detected with rhodamine and gramicidin S; (D) day 7 cells detected with rhodamine; (E) day 21 cells detected with rhodamine and gramicidin S; (F) day 21 cells detected with rhodamine. Horizontal axes show arbitrary logarithmic values of fluorescence intensity (520 nm) which are reproducible under the conditions described in Materials and Methods.

to dry. The membrane was UV treated, and DNA hybridization was performed at 50°C as described previously (17).

RESULTS

Survival of *A. salmonicida* in sterile lake water. The survival of *A. salmonicida* in sterile lake water is illustrated in Fig. 1. Over the 21-day study, the detection of fluorescent cells stained with acridine orange did not decline significantly from the initial inoculation level. The numbers of cells able to form colonies on TSA declined rapidly after inoculation from 1×10^5 CFU ml^{-1} on day 0 to 5×10^2 CFU ml^{-1} on day 7; after this, there was a slow decline to 9×10^1 CFU ml^{-1} on day 21. The detection of viable cells over the 21-day period by the rhodamine assay is illustrated in Fig. 1 and 2. The fluorescence histograms obtained by flow cytometry (Fig. 2) indicate that on day 0 a large proportion of the cells detected were able to take up the rhodamine dye; this uptake was abolished in the presence of gramacidin S. On days 7 and 21, cells that were able to take up rhodamine remained, although the percentage of fluorescent cells (viable) relative to nonfluorescent cells (nonviable) had decreased by day 21. Therefore, on day 21, a population of 1×10^5 cells ml^{-1} was present, of which 3×10^4 cells ml^{-1} (30%) were viable and 9×10^1 cells ml^{-1} were able to form colonies. The results from repeat microcosms varied with respect to the survival of CFU. The reasons for this variability are not fully understood; however, differences in the nutrient level of the water are probably an important factor. Figure 1 indicates an intermediate response in which the number of CFU declined at a moderate rate. At one extreme, the number of CFU declined rapidly and within 7 days were found to fluctuate around the detection limit of 3 cells 100 ml^{-1} (filter plate counts). Therefore, on day 21, the number of cells in the water sample was 1.5×10^5 cells ml^{-1}, the number of viable cells was 5.6×10^4 cells ml^{-1} (37%), and the number of cells able to form colonies was 4.0 CFU 100 ml^{-1} (0.00002%). At the other extreme, the survival of CFU over the 21-day period showed very little decline. On day 21, this population was found to contain 1.2×10^5 cells ml^{-1}, of which 6.9×10^4 cells ml^{-1} (57%) were viable and 5.2×10^4 cells ml^{-1} (43%) were able to form colonies.

Survival of *A. salmonicida*(pLV1013) in untreated lake water. The survival of CFU of *A. salmonicida*(pLV1013) in lake water is illustrated in Fig. 3. After inoculation at 9.2×10^4 CFU ml^{-1}, the recovery of *xylE* colonies declined rapidly over the 21-day study. Over the first 10 days, there was no difference in the recovery of colonies of *A. salmonicida*(pLV1013) on TSA and TSA with kanamycin. The detection limit of marked cells was improved from 3×10^2 CFU ml^{-1} on TSA to 1 CFU 100 ml^{-1} on TSA with kanamycin by using the filter counting method. Filter counts were not possible on TSA alone because of interference from indigenous bacteria growing on the filter surface. It was possible to detect colonies of *A. salmonicida*(pLV1013) from days 10 to 14 on TSA containing kanamycin. After 14 days, *A. salmonicida*(pLV1013) had declined to below the detection limit for filter plate counts.

The detection of *A. salmonicida*(pLV1013) in the lake water release systems described above was possible by PCR amplification of the *xylE* gene and the chromosomal DNA marker. The amplification of the *xylE* gene from lake water

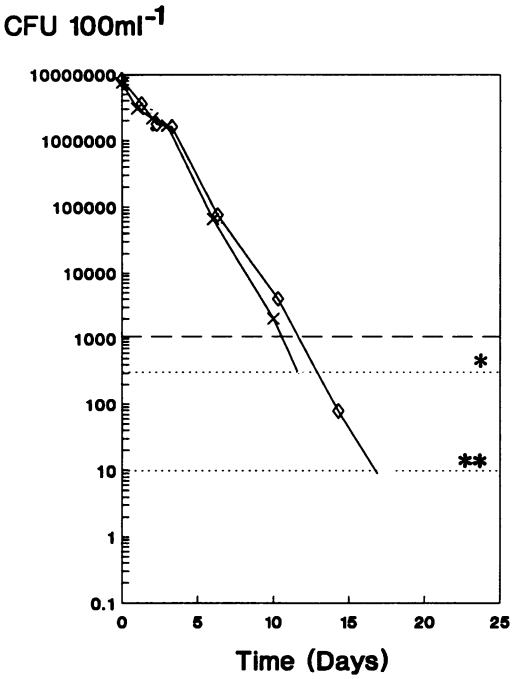

FIG. 3. Survival of *A. salmonicida*(pLV1013) in nonsterile lake water. CFU were detected on TSA (×) and on TSA containing kanamycin (◇). Detection limits for CFU on TSA (. . .*), CFU on TSA containing kanamycin (. . .**), and PCR amplification of the *xylE* gene and chromosomal marker (– – –) are shown.

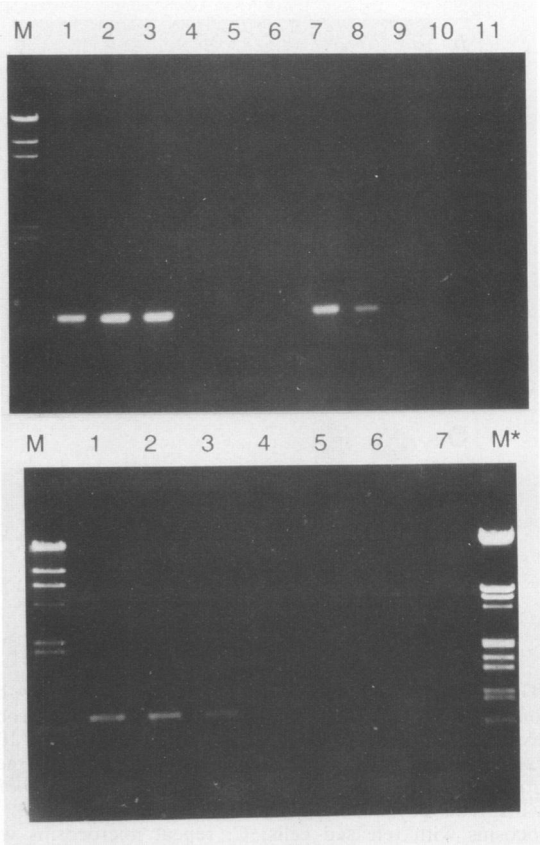

FIG. 4. Agarose gel illustrating PCR products by using *xylE* primers and cells extracted from lake water release systems on days 0 (top) and 21 (bottom). (Top) Lanes: 1 to 3, repeat microcosms inoculated with *A. salmonicida*(pLV1013); 4 to 6, repeat microcosms with no released cells; 7 to 9, cells released into lake water at 10^3, 10^2, and 10^1 cells ml^{-1}, respectively; 10, no released cells; 11, PCR control containing no target DNA; M, lambda DNA cut with *Hin*dIII. (Bottom) Lanes: 1 to 3, repeat microcosms inoculated with *A. salmonicida*(pLV1013); 4 to 6, repeat microcosms with no released cells; 7, PCR control containing no target DNA; M, lambda DNA cut with *Hin*dIII; M*, lambda DNA cut with *Hin*dIII and *Eco*RI.

containing released *A. salmonicida*(pLV1013) is shown in Fig. 4. The detection limit for this method on day 0 was approximately 10 cells per ml of lake water. The *xylE* DNA fragment was amplified from lake water samples on day 0 and from samples incubated for 7, 14, and 21 days. The amplified fragment also reacted with the oligonucleotide probe for the central region of the *xylE* gene (Fig. 5). The amplification of the chromosomal fragment from lake water with released cells is shown in Fig. 6. Amplification of the marker was possible on days 0 and 21. The oligonucleotide probe to the center of this fragment also hybridized with the amplified DNA (Fig. 7). In all cases, no amplification of DNA occurred on any day in lake water samples to which *A. salmonicida*(pLV1013) had not been added.

Selection of cells resistant to entering a nonculturable condition. Sterile lake water release systems were inoculated with 2×10^5 cells of *A. salmonicida* MT432 ml^{-1}. Within 6 days, the number of CFU declined to approximately 2 CFU 100 ml^{-1}. After incubation for a further 6 months at 10°C, subsamples (50 ml) were filtered, and the filters were removed and incubated on TSA for 3 days. A total of 10 colonies were obtained from a total of 500 ml of lake water. A direct acridine orange count indicated the presence of a nonculturable population of 1.2×10^5 cells ml^{-1}. All 10 colonies produced a brown diffusible pigment on TSA during subculture and had an API 20NE profile identical to that of *A. salmonicida*; these were termed environmental isolates. The plasmid content of the isolates was investigated by the sucrose gradient method (25), and the profile is shown in Fig. 8. The presence of a number of plasmids with the same mobility as that of plasmids found in *A. salmonicida* MT432 can be seen. When the environmental isolates were cultured in TSB and inoculated into sterile lake water, the survival of CFU declined rapidly over the 7-day study (Table 1). The acridine orange direct count indicated that the number of cells within samples was maintained at approximately the same level as the inoculation density. The percentage of nonculturable cells within each sample on day 7 was similar for all environmental isolates and *A. salmonicida* MT432. This indicates that none of the environmental isolates recovered from the original release system resisted reentering a nonculturable condition.

DISCUSSION

The survival of *A. salmonicida* in sterile and untreated lake water was investigated. It was discovered that, in sterile lake water, *A. salmonicida* could enter a NCBV state. In previous studies, this has not been possible because of problems associated with determining the viability of cells in sterile lake water (15, 16). The use of membrane potential to detect viable cells with rhodamine was both rapid and reliable. Flow cytometry proved to be an ideal method to determine the number of cells that were viable (fluorescent)

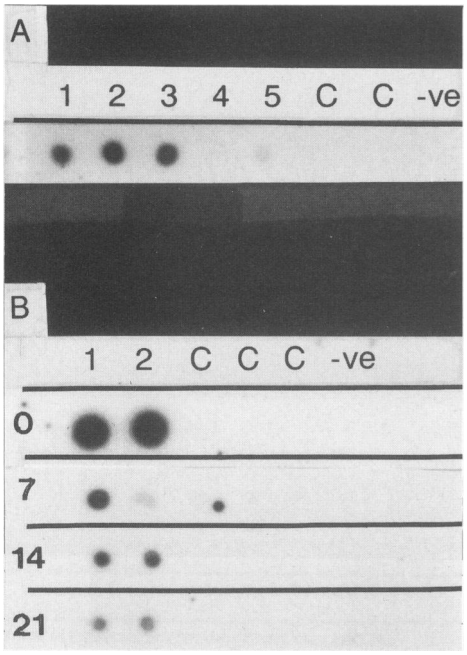

FIG. 5. Detection of *A. salmonicida*(pLV1013) by DNA hybridization to the PCR products by using *xylE* primers and probe. (A) Lanes: 1 to 5, dilutions of cells released into lake water at 10^3, 10^2, 10^1, and 10^0 cells ml^{-1}; C, no released cells; −ve, PCR-negative control containing no target DNA. (B) Detection of *A. salmonicida*-(pLV1013) on days 0, 7, 14, and 21. Lanes: 1 and 2, repeat microcosms with released cells; C, repeat microcosms with no released cells; −ve, PCR-negative control containing no target DNA.

and to monitor the effect of the ionophore gramicidin S, which was used to abolish the membrane potential of the cells. This method has been used to determine the viability of *E. coli*, *Staphylococcus aureus*, and *Pseudomonas putida* (5, 10). The main limitation to the use of rhodamine 123 with gram-negative bacteria is the permeability of the cell wall,

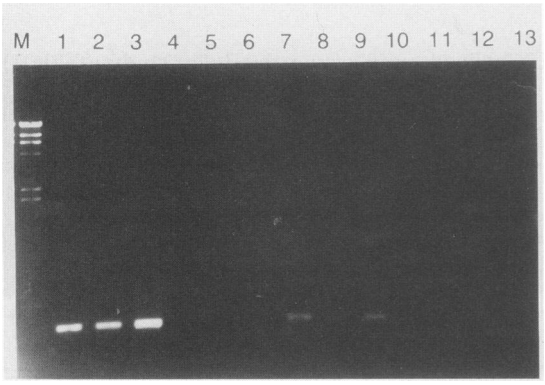

FIG. 6. Agarose gel illustrating PCR products by using *A. salmonicida* chromosomal primers and cells extracted from lake water release systems on days 0 and 21. Lanes: 1 to 3, repeat microcosms inoculated with *A. salmonicida*(pLV1013) on day 0; 4 to 6, repeat microcosms with no released cells on day 0; 7 to 9, repeat microcosms inoculated with *A. salmonicida*(pLV1013) and incubated for 21 days; 10 to 12, repeat microcosms with no released cells after 21 days; 13, PCR-negative control containing no target DNA; M, lambda DNA cut with *Hin*dIII.

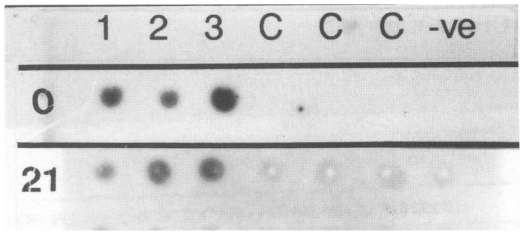

FIG. 7. Detection of *A. salmonicida*(pLV1013) by DNA hybridization to PCR products with chromosomal primers and probe on day 0 and 21. Lanes: 1 to 3, repeat microcosms with released cells; C, repeat microcosms with no released cells; −ve, PCR-negative control containing no target DNA.

which must allow access of the dye for uptake. This may be the reason that a large proportion of freshly cultured. *A. salmonicida* cells remain unstained with rhodamine at the start of this study. Daiper et al. (5) also had difficulty using the rhodamine assay with a number of bacteria, including *A. salmonicida*. To overcome this, pretreatment of cells with EDTA is required, and different species require treatment with different concentrations of EDTA for various times. In some cases, the presence of thick coatings on the surface of cells like capsular material may prevent the use of this technique. Bacteria under starvation conditions may also require treatment different from that of freshly cultured cells. In this experiment, various concentrations of EDTA, rhodamine, and gramicidin S were used at each time point; the best treatment for all days is presented in the results. The rhodamine assay may provide an ideal viability assay for many bacteria that do not respond well to other viability assays such as those strains resistant to nalidixic acid.

The presence of NCBV *A. salmonicida* in sterile lake water agrees with our previous findings that nonculturable cells remained intact, contained DNA and RNA, bound fatty acids, and showed no signs of cell decay that would be expected with nonviable cells (15, 16). However, these methods can be used only on populations released in sterile lake water systems since the cannot discriminate between cells of *A. salmonicida* and other bacteria.

In previous studies on the survival of *A. salmonicida* in mixed populations, the presence of colonies with a brown diffusible pigment was used to indicate the presence of released cells. This indicator is limited since natural popu-

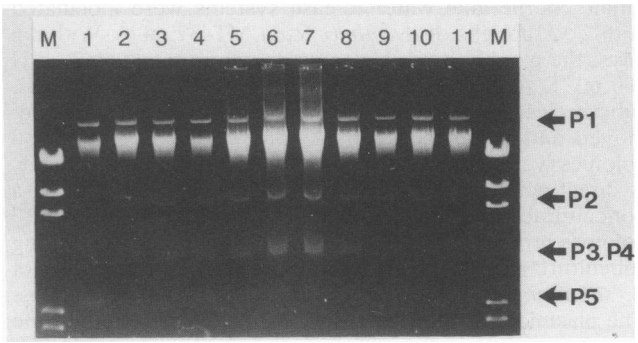

FIG. 8. Plasmid profiles of *A. salmonicida* MT432 and environmental isolates recovered from sterile lake water release systems. Lanes: 1, *A. salmonicida* MT432; 2 to 11, 10 environmental isolates; M, lambda DNA cut with *Hin*dIII. Plasmid bands P1 to P5 are indicated.

TABLE 1. Survival of *A. salmonicida* environmental isolates in sterile lake water from Windermere.

Isolate	CFU ml^{-1}					Direct acridine orange count on day 6[a] (cells ml^{-1})
	Day 0	Day 1	Day 2	Day 3	Day 6	
Environmental isolate						
1	8.9×10^4 ($\pm 2.0 \times 10^4$)	8.1×10^3 ($\pm 4.1 \times 10^3$)	1.8×10^3 ($\pm 1.6 \times 10^3$)	262 ($\pm$285)	0.0 ($\pm$0.0)	1.1×10^5 ($\pm 6.6 \times 10^3$)
2	1.0×10^5 ($\pm 1.6 \times 10^4$)	1.1×10^4 ($\pm 6.0 \times 10^3$)	455 ($\pm$365)	12 ($\pm$24)	1.1 ($\pm$3.1)	8.3×10^4 ($\pm 1.2 \times 10^4$)
3	1.4×10^5 ($\pm 5.0 \times 10^4$)	1.1×10^4 ($\pm 5.7 \times 10^3$)	292 ($\pm$207)	11 ($\pm$9)	0.0 ($\pm$0.0)	9.1×10^4 ($\pm 3.7 \times 10^3$)
4	1.1×10^5 ($\pm 1.6 \times 10^4$)	3.3×10^3 ($\pm 1.1 \times 10^3$)	119 ($\pm$70)	10 ($\pm$6.7)	0.0 ($\pm$0.0)	9.4×10^4 ($\pm 1.2 \times 10^4$)
5	1.2×10^5 ($\pm 2.0 \times 10^4$)	4.1×10^3 ($\pm 4.3 \times 10^3$)	136 ($\pm$188)	2.2 ($\pm$4.2)	3.3 ($\pm$9.4)	7.4×10^4 ($\pm 7.0 \times 10^3$)
6	1.7×10^5 ($\pm 5.3 \times 10^4$)	1.2×10^4 ($\pm 5.4 \times 10^3$)	256 ($\pm$117)	16.7 $\pm$8.2)	3.3 ($\pm$9.4)	3.9×10^5 ($\pm 4.3 \times 10^4$)
A. salmonicida MT432	1.4×10^5 ($\pm 3.1 \times 10^4$)	6.2×10^3 ($\pm 2.4 \times 10^3$)	1.9×10^3 ($\pm 1.1 \times 10^3$)	219 ($\pm$197)	0.0 ($\pm$0.0)	8.5×10^4 ($\pm 2.3 \times 10^4$)

[a] The direct acridine orange counts on day zero were 10^5 cells ml^{-1} for every isolate.

lations may harbor brown-pigmented bacteria and its production may not be stable upon reisolation of cells from water. In our experiments, we found that after a few days in lake water only colonies that had been incubated for more than a week on the initial isolation media showed a detectable brown pigment. However, younger colonies could be subcultured and rapidly produced a brown pigment. The use of the *xylE* marker system overcomes this problem since the presence of indigenous bacteria with a *xylE* gene in lake water has not been detected (17, 27). Colonies of *A. salmonicida*(pLV1013) could be detected in lake water on TSA at a detection level of 3×10^1 cells ml^{-1}. Initial results indicated that *A. salmonicida* quickly declined below this level. To improve the detection limit, filtration was used in combination with TSA containing kanamycin. The initial isolation of cells that had been in lake water for up to 10 days indicated that kanamycin had no inhibitory effect on the growth of cells to form colonies. To determine the presence of nonculturable cells, it was important to determine if any culturable cells of *A. salmonicida* were maintained at or around the PCR detection level (10^1 cells ml^{-1}). Only when the detection of marker DNA was possible in a population in which the level of culturable cells had declined below this detection limit could the presence of a nonculturable population be determined. The detection of both *xylE* DNA and *A. salmonicida* chromosomal DNA in lake water in which the culturable population had declined to below 10^1 CFU ml^{-1} was possible from day 14 on. This indicates that a nonculturable population of *A. salmonicida* cells was present. The nonculturable population may still be transient and not maintained for a long period in lake water. However, we have shown that this population can be formed and survives longer than culturable cells. The detection of DNA specific to a released population does not prove that viable cells are present; hence, we have used the term nonculturable for these cells. PCR detection of the chromosomal fragment isolated by Hiney et al. (8) proved ideal for the detection of *A. salmonicida* in mixed lake water populations and may provide a rapid method for detecting nonculturable cells of *A. salmonicida* in other environments. The development of other detection techniques to determine the presence of cells and their viability is important to the future study of the presence of a NCBV population of *A. salmonicida* in mixed populations. Flow cytometry could provide the ideal method for this since the dual labelling of cells with rhodamine and a fluorescently labelled antibody should be possible.

In all of our sterile lake water release systems, a small number of culturable *A. salmonicida* cells remained after 21 days at 10°C. These cells have the potential to infect fish either directly from the small number of cells remaining or by enrichment through the input of nutrients into the water prior to infection. Why these few cells survive in a culturable condition when the majority of the population becomes nonculturable is not understood. That they then resist becoming nonculturable could be attributed to either chance or a change within the cells. Our results further indicate that the resistance to entering a nonculturable condition after the regrowth of surviving cells in laboratory media is not maintained. Therefore, selection of a population resistant to becoming nonculturable is not inherited. In addition, the plasmids associated with the original *A. salmonicida* MT432 strain are maintained in culturable cells for more than 6 months in sterile lake water systems. This indicates that they are very stable under these conditions. In sterile lake water release systems, a nonculturable population develops with the retention of a subpopulation of culturable cells at a density as low as 1 CFU 100 ml^{-1}. If this was also the case in untreated lake water containing a mixed microbial population, then these cells would remain undetected without an appropriate selective media to reduce the growth of the indigenous population. This could also explain how furunculosis can occur in populations that had apparently not come into contact with the pathogen, since standard media would not permit the detection of cells at such a low level. By using the *xylE* marker system and kanamycin resistance, it was possible to filter 100 ml of untreated lake water and easily detect marked colonies. By using this method, it was found that culturable cells of *A. salmonicida* did not survive at a population density above 0.3 CFU 100 ml^{-1} after a period of 8 days. This would indicate that in mixed microbial populations, *A. salmonicida* would not be expected to survive in a culturable condition for very long periods. It is possible that culturable cells still remain within the system at even lower levels and provide the route for reinfection of fish.

The significance of an NCBV population of *A. salmonicida* in the spread of furunculosis in lake water needs further investigation. Cells in this condition do not appear to infect fish (21) and cannot be revived in dead fish, fish tissue, or various nutrients (15, 16). However, the identification of an appropriate revival system is difficult because of the multitude of culture conditions that are possible. These could include ingestion by protozoa and recovery within the cell, attachment to other microorganisms including algae, accumulation within invertebrate filter feeders, or attachment to

other particles and animals. A study of the distribution of *A. salmonicida* cells in the environment using methods that do not require their culture such as PCR and immunofluorescence may provide a rapid insight into the most favorable conditions for the revival of *A. salmonicida*.

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