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# Survival of *Aeromonas salmonicida* in Lake Water

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**The survival of *Aeromonas salmonicida* subsp. *salmonicida* in lake water was investigated by using a variety of techniques. They included acridine orange epifluorescence, respiration, cell culture, cell revival, flow cytometry, plasmid maintenance, and membrane fatty acid analysis. During a 21-day study, *A. salmonicida* became nonculturable in sterile lake water samples. Flow cytometry and direct microscopy indicated that cells were present. Although the nonculturable cells could not be revived, the recovery method did indicate that the presence of low numbers of culturable cells within samples could produce misleading results. Plasmid DNA, genomic DNA, and RNA were maintained in the nonculturable cells; in addition, changes in the fatty acid profiles were also detected. Although viability could not be proven, it was shown that the morphological integrity of nonculturable cells was maintained.**

*Aeromonas salmonicida* is the causative agent of furunculosis in fish (18). It has been established that infected fish shed large numbers of virulent bacteria into the surrounding water (15). The fate of released cells is poorly understood. Most studies have shown that *A. salmonicida* does not survive for long periods in water samples (2, 15, 19, 20); longer periods of persistence have been recorded in river sediments and on sand particles (16, 23). The factors that affect the survival of bacteria in the environment are wide-ranging, and a need for an understanding of how heterotrophic bacteria survive in low-nutrient environments is required (21).

Certain species of gram-negative bacteria have been shown to enter a physiological state under low-nutrient conditions, in which they become nonculturable but viable (NCBV) (7). Bacteria capable of this change include *Escherichia coli* (5, 7, 28), *Vibrio cholerae* (4, 7, 28), *Vibrio vulnificus* (14), *Shigella sonnei* (7), *Shigella flexneri* (7), *Salmonella enteriditis* (22), and *Aeromonas salmonicida* (2). The scientific basis for this state has been the discovery that cells placed in low-nutrient conditions cannot be recultured on the medium from which they were initially isolated or on other laboratory media. Cells in this state can be detected by acridine orange epifluorescence microscopy, which does not measure viability. In a number of cases, nonculturable cells have been shown to respond to yeast extract and nalidixic acid treatment (1, 12), to respire, and also to retain their pathogenicity. Cells showing these characteristics are considered to be viable, and the term NCBV can be used. The existence of NCBV *A. salmonicida* in water may explain how outbreaks of furunculosis can occur in fish populations which apparently have not come in contact with the pathogen. It is difficult to distinguish cells which have entered this physiological state (NCBV) from those that are nonviable. Recently, evidence has been presented against the existence of an NCBV state in *A. salmonicida* (19, 20). This evidence is based on differences in the methodology used to determine whether nonculturable cells are viable. In addition, nonculturable cells of *A. salmonicida* do not appear to be able to infect fish (20). This disrupts the cycle (from a host to a nonculturable state and then back into a new host). As a consequence, free NCBV *A. salmonicida* cells transported in water may not be important in the spread of the disease over long distances.

The NCBV state also has important implications with respect to monitoring the spread of other pathogens and genetically engineered microorganisms in the environment. If isolation methods which rely on colony-forming ability are used as the sole measure of bacterial survival, they may greatly underestimate the number of cells actually present. It is possible that the NCBV state is a survival strategy associated with pathogenic bacteria for maintaining themselves in the environment between hosts. Nonpathogenic bacteria may also enter a NCBV state. However, the NCBV issue is confused in some cases because the term NCBV has been used without confirmation of viability.

Plasmid maintenance is also an important consideration in monitoring pathogens and genetically engineered microorganisms in the environment, since pathogenicity and resistance functions can be plasmid encoded and since recombinant DNA may be inserted into plasmids for environmental release (27). Certain plasmids in bacteria are stably maintained when released into microcosms; others undergo a variety of rearrangements or are lost (9, 17, 24). *E. coli* can maintain plasmids after entering a nonculturable state (6). In this study we assessed the maintenance of plasmid DNA in *A. salmonicida* to provide information on its ability to infect fish and on plasmid survival in nonculturable cells.

## MATERIALS AND METHODS

**Bacterial culture.** *A. salmonicida* subsp. *salmonicida* MT432 (19) was provided by A. L. S. Munro, Marine Laboratory, Aberdeen, United Kingdom. This strain was initially isolated from Atlantic salmon with clinical furunculosis, and cultures were maintained on tryptone soya agar (TSA; Oxoid, Basingstoke, United Kingdom). For release studies, cultures were grown at 20°C for 18 h in 50 ml of tryptone soya broth (Oxoid). The cells were collected by centrifugation at 10,000 × *g* for 10 min and washed three times in sterile distilled water. The final pellet was resuspended in 50 ml of distilled water, and an acridine orange direct count was used to determine cell density.

**Freshwater samples and release systems.** Water samples were collected from Lake Windermere (Cumbria, United

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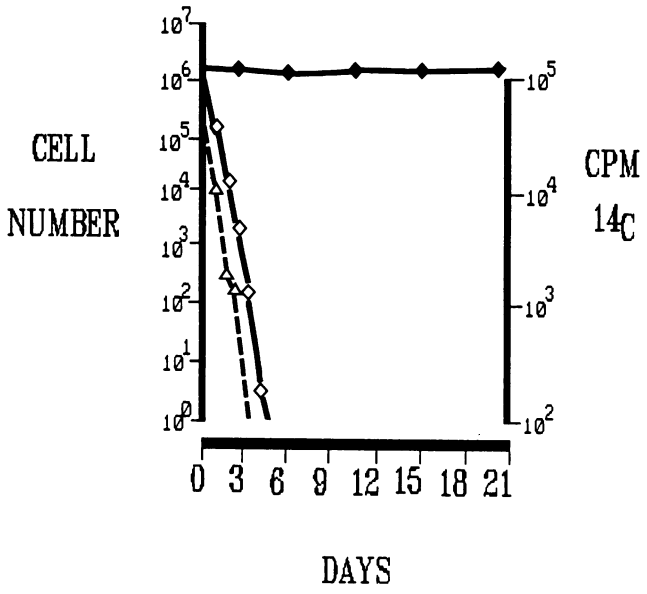


FIG. 1. Survival of *A. salmonicida* in lake water over 21 days. Symbols: ◆, detection of acridine orange-stained cells; ◇, CFU; and △, cell respiration.

Kingdom), filtered through a 0.22-μm-pore-size membrane (Millipore, Waterford, United Kingdom), and autoclaved at 120°C for 30 min. Release systems were constructed by placing 500 ml of sterile lake water in 1-liter conical flasks capped with cotton wool bungs. In addition, 20-liter sterile lake water systems were prepared in 21-liter glass containers by three cycles of autoclaving at 121°C for 30 min. All release systems were inoculated with *A. salmonicida* to a final cell concentration of approximately $10^6$ cells per ml and were incubated at 10°C.

**Cell counts.** Acridine orange direct counts were performed by the method of Jones and Simon (11). Colony forming ability was determined in triplicate on samples plated on TSA. Sample sizes ranged from 0.1 ml for spread plate counts to 10 and 100 ml for filtered samples (filters were removed from the apparatus and placed face up on TSA). The sample size was adjusted during the study to follow the decline of the host.

**Flow cytometry.** At various intervals following initial inoculation of release systems, 5 ml was removed and 100 μl of 4% formaldehyde was added. The samples were stored at 4°C for up to 30 days. In addition, duplicate samples were taken on days 0, 7, 14, and 28 and analyzed immediately with no formaldehyde addition. Each cell suspension was analyzed in a FACStar Plus flow cytometer (Becton Dickinson). The two parameters measured were forward light scatter and 90° side scatter. The laser power at 488 nm was set at 0.2 W,

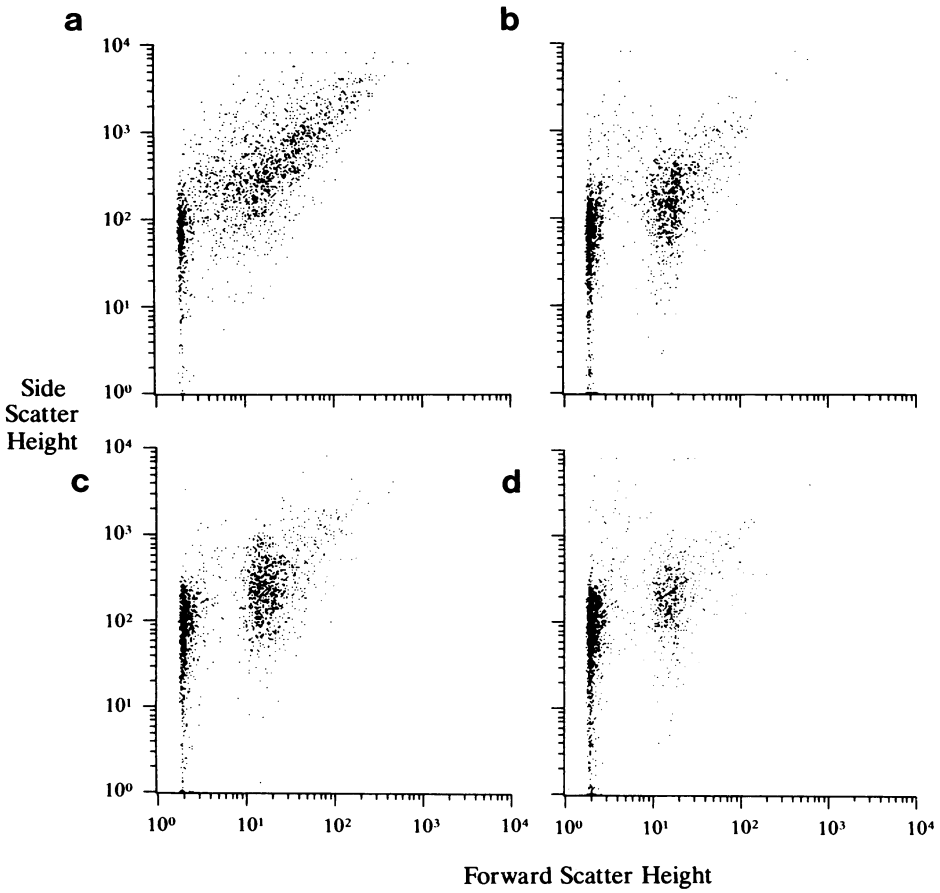


FIG. 2. Plots of side scatter versus forward scatter of cells recovered from release systems on days 0 (a), 7 (b), 14 (c), and 21 (d). Axes show arbitrary logarithmic values which are reproducible under the conditions described in Materials and Methods.

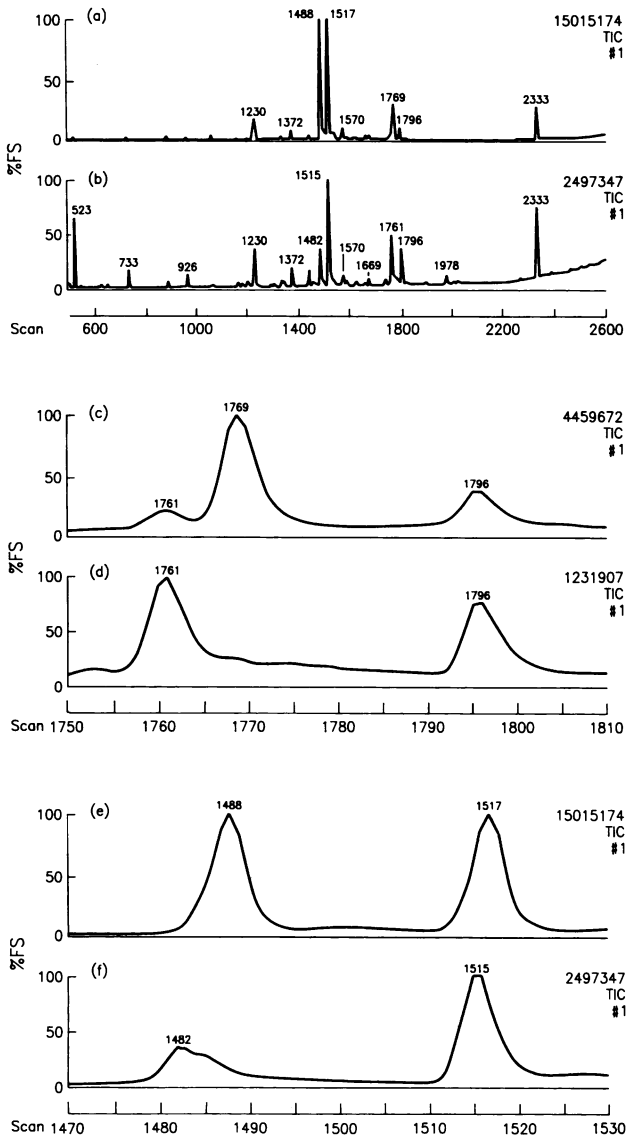


FIG. 3. Distribution of total FAME recovered from phospholipids of fresh cells (a) and nonculturable cells (day 21) (b) of *A. salmonicida*. The positions at which specific FAME elute in panel a are as follows: $C_{10:0}$, 733; $C_{12:0}$, 962; $C_{14:0}$, 1,230; $C_{15:0}$, 1,372; $C_{16:1}$, 1,488; $C_{16:0}$, 1,517; $C_{18:1}$, 1,769; $C_{18:0}$, 1,796. Panels c and e show $C_{18}$ and $C_{16}$ FAME, respectively, isolated from cells on day 0. Panels d and f show $C_{18}$ and $C_{16}$ FAME, respectively, obtained from 21-day-old cells. %FS, percentage of the full scale of the total ionization current (TIC).

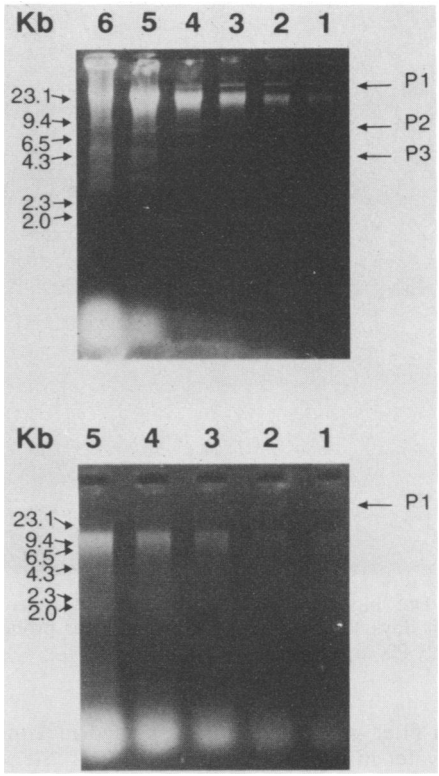


FIG. 4. Agarose gel electrophoresis plate illustrating DNA from the fractions obtained by the sucrose gradient method for DNA preparation. Fractions taken from the bottom of the ultracentrifuge tube (lanes 1) contain DNA of a larger size than fractions from higher up the gradient (lanes 2 to 6). *A. salmonicida* DNA extracted from fresh cultures (top) and after 21 days in lake water systems (bottom) are shown. Molecular sizes are shown to the left in kilobases.

and all recordings were taken on a log scale. For each sample, 2,000 events were recorded. The data were displayed as a single dot plot with forward light scatter versus side scatter.

**Revival studies.** At intervals over the release study, 1-ml samples were taken and diluted 10-fold in distilled water to $10^{-6}$. Larger samples of 10 and 100 ml were also taken which were not diluted. To each cell suspension, tryptone soya broth was added to 0.1% (vol/vol) and incubated at 20°C for 5 days. After incubation, a 0.1-ml subsample was tested for growth on TSA.

**Respiration studies.** At intervals over the release study, 20-ml samples were taken and placed in 50-ml hypovials. To this, 0.25 μCi of uniformly labeled [$^{14}$C]glucose (245 mCi/mmol; Amersham Corp., Amersham, United Kingdom) was added, and the samples were incubated at 10°C. Formaldehyde (1 ml) was added to stop the reaction after 0, 6, and 10 h. For experiments measuring the uptake of glucose, the samples were filtered through 0.22-μm-pore-size durapore filters (Millipore) and washed with 50 ml of sterile lake water. The filters were then placed in 10 ml of scintillation fluid (Uni-solve; Koch-lite, London, United Kingdom) and vortexed for 15 s. $^{14}$C was counted under standard conditions in a Tracer Analytic Mark II scintillation counter. For measuring $CO_2$ production, 0.25 ml of 10% (vol/vol) sulfuric acid was added to the fixed cells. Phenyldiamine (0.25 ml) was injected into a chamber above the surface of the sample. The vials were incubated at 30°C for 18 h, and the phenyldiamine was then removed, added to scintillation fluid, and counted as described above.

In a separate series of experiments, respiration was assayed by the 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT)-formazan method of Zimmermann et al. (30).

**Cell collection.** For DNA hybridization and fatty acid analysis, cells were collected from 200-ml samples by filtration through a 0.22-μm-pore-size filter (Millipore). Samples were filtered until approximately 1 ml was remaining, and

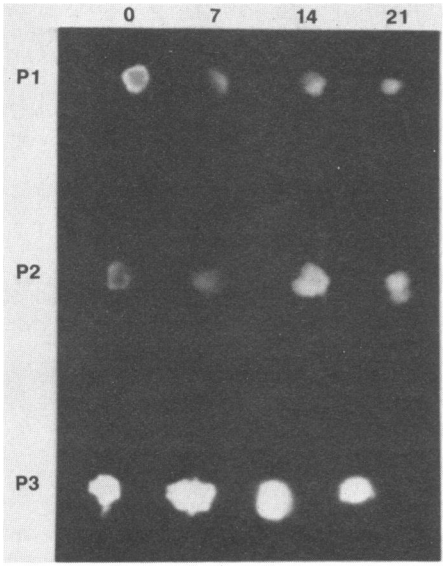


FIG. 5. Dot blot of whole cells recovered from lake water systems on days 0, 7, 14, and 21. Cells were probed with the plasmids P1, P2, and P3.

then each filter was removed and placed in 1 ml of sterile distilled water in an Eppendorf tube (2 ml). Sterile distilled water (1 ml) was added, and the suspension was vortexed for 1 min. The filter was removed, and the cell suspension was centrifuged at 15,000 × *g* for 15 min. Cell pellets were resuspended in 20 μl of distilled water and frozen at −20°C until required.

**Fatty acid analysis.** Lipids were extracted with chloroform-methanol (1:2 [vol/vol], 180 μl). The cells were warmed at 65°C for 10 min in a sealed vial, cooled, and centrifuged. The combined supernatant from three extractions was diluted with chloroform-water to give a final volumetric ratio of 2:2:1.8 (chloroform-methanol-water), and then the organic layer was separated and the aqueous layer was reextracted. The combined chloroform extracts were dried over anhydrous $Na_2SO_4$ and concentrated to 200 μl in a stream of nitrogen. The lipid extract was separated into three general classes by sequential elution from a silica gel (500 mg) with 5 ml of chloroform, acetone, and methanol (10). The methanol eluate, which contained phospholipids, was concentrated to 50 μl, diluted with an equal volume of toluene, and subjected to mild alkaline methanolysis to convert only esterified fatty acids into methyl esters as described by White et al. (26). The reaction product was dissolved in 10 μl of chloroform and stored under $N_2$ at −20°C.

To separate the constituent fatty acid methyl esters (FAME), a fused capillary column (30 m by 0.25 mm) coated with DB-5 (0.25 μm) was used (J & W Scientific, Folsom, Calif.). The system was programmed over the temperature range of 150 to 270°C at 4°C per min, using helium at 50 kPa as a carrier gas and an on-column injector for sample introduction. The gas chromatography column had a direct capillary interface into the ion source of a VG Trio 1 quadrupole filter mass spectrometer (VG Masslab Ltd., Manchester, United Kingdom). This was operated in the electron impact (EI+) mode at 70 eV with a source temperature of 220°C and scanning from 40 to 500 atomic mass units in 1 s. Mass spectral data were acquired and edited using an Intel data system (Intel Corp., Santa Clara, Calif.). Saturated FAME were identified by a combination of cochromatography with authentic compounds and by mass spectral comparison with the NIST library data base. Monoenoic FAME were recognized from their molecular ion ($M^+$) and characteristic fragmentation. The site and stereochemistry of the double bond were determined by epoxide formation and conversion to diols (29). Double bonds were identified from the mass spectrometry features of either derivative type. The olefin geometry was obtained from comparison of retention times with published values.

**DNA analysis.** Plasmid DNA was isolated and identified by the sucrose gradient method of Wheatcroft and Williams (25). Samples were run on 0.8% (wt/vol) agarose gels and were stained with ethidium bromide. From the plasmid profile of *A. salmonicida*, three plasmids (P1, P2, and P3) were selected and purified by electrophoresis in low-melting-point agarose. Plasmids were labeled with [$^{32}$P]dATP by using a random hexanucleotide priming kit (Boehringer, United Kingdom).

Cell suspensions recovered from water in 20-μl volumes were placed on nylon filters. The cells were lysed and hybridized as described previously (17). For direct plasmid analysis, cells were collected from 20-liter water samples by filtration through a 0.22-μm-pore-size filter (Sartorius, United Kingdom). The filter membrane was removed and covered with 10 ml of sterile distilled water. The filter was shaken at 20 rpm for 1 h and removed. The cells were collected from the supernatant by centrifugation at 10,000 × *g* for 15 min. DNA was extracted by using the sucrose gradient method (25).

## RESULTS

**Cell counts.** After release at $10^6$ cells $ml^{-1}$, the acridine orange direct count did not alter significantly over the 21-day period (Fig. 1). Microscopic examination indicated that during the release period the cells had decreased in length (by approximately 20%) and had become rounded in comparison with cells in the initial inoculum. CFU counts on TSA declined rapidly from the initial inoculation level to very low or undetectable levels after day 8. Small numbers of colonies (<3) could be detected in some 100-ml filtered samples; these are not illustrated in Fig. 1 because such low levels give unreliable results. However, their presence is important when revival results are considered.

**Respiration.** The ability of cells in sterile lake water to take up [$^{14}$C]glucose and to produce $^{14}CO_2$ is illustrated in Fig. 1. Both uptake and respiration showed a rapid decline over the release period; undetectable levels (below $10^2$ cpm) were reached after only 3 days.

Microscopic examination of cells treated with INT-formazan showed positive respiration on days 0 and 2 but showed no respiration on days sampled after this.

**Flow cytometry.** Figure 2 illustrates the forward light scatter-side scatter plots of cell samples taken from sterile lake water systems at different times. The population appears to become less varied with time and forms a tighter distribution. This suggests that the cells have become more uniform. No differences in results were found between cells treated with formalin and cells sampled directly.

**Revival studies.** The revival of cells from water samples on days 0, 9, and 28 can be seen in Table 1. As illustrated, revival was only observed when viable colonies were also recovered directly on TSA plates. Samples containing cells that could be stained with acridine orange but could not be cultured on TSA failed to be revived.

TABLE 1. Revival of cells from lake water systems

| Day | Lake water system | Direct AO count ($10^8$)/100 ml[a] | CFU/100 ml | Growth in revival studies[b] | | |
|---|---|---|---|---|---|---|
| | | | | 100 ml | 10 ml | 1 ml |
| 0 | 1 | 2.5 | $3 \times 10^8$ | + | + | + |
| | 2 | 2.8 | $1 \times 10^8$ | + | + | + |
| | 3 | 2.5 | $3 \times 10^8$ | + | + | + |
| 7 | 1 | 2.3 | 0 | − | − | − |
| | 2 | 2.0 | 1 | + | − | − |
| | 3 | 2.1 | 0.6 | + | − | − |
| 21 | 1 | 2.8 | 0.6 | + | − | − |
| | 2 | 2.4 | 0 | − | − | − |
| | 3 | 2.5 | 0.3 | + | − | − |

[a] AO, acridine orange.
[b] +, growth was recorded in tubes; −, no growth was recorded in tubes.

**Fatty acid analysis.** Figure 3 shows the results of fatty acid analyses of *A. salmonicida*. In fresh cultures, *cis*-16:1Δ9 was the most dominant fatty acid species and *cis*-18:1Δ11 was next in abundance. Cells held in water samples over 21 days displayed a lower abundance, relative to saturated analogs, of $C_{16:1}$ (scan 1,488) and $C_{18:1}$ (scan 1,769) unsaturated fatty acids (Fig. 3c through f). The appearance of peaks (scan 1,482 and 1,761 for $C_{16:1}$ and $C_{18:1}$ fatty acids, respectively) with shorter retention times suggested that the site of the double bond had changed. However, insufficient material was harvested from release systems to determine the position of the double bond in 21-day-old cells. Long-chain fatty acid species which were not detected on day 0 were also found on day 21, along with an increase in short-chain fatty acids.

**DNA analysis.** By using the three plasmid probes P1, P2, and P3 (Fig. 4), a positive hybridization result was recorded throughout the time course (Fig. 5). Plasmid extraction was performed directly on cells recovered from 21-day-old water systems to confirm the maintenance of plasmids at the end of the study. The results are presented in Fig. 4. Plasmid P1 can be clearly seen in these cells. At the positions where plasmids P2 and P3 normally appear, an area stained heavily with ethidium bromide can be seen. However, individual plasmids could not be resolved in this region. Therefore, it is difficult to determine whether plasmids P2 and P3 were retained or lost. Since a positive hybridization signal was recorded, these plasmids are probably present. Some degradation of chromosomal DNA under release conditions was the probable cause of smearing in this region. The presence of chromosomal DNA and cellular RNA was also observed in 21-day-old cells.

## DISCUSSION

In this study it was shown that *A. salmonicida* entered a nonculturable state. This term is used to describe cells that can be seen under the microscope with acridine orange staining but that cannot be cultured on our standard media. A reduction in the size of nonculturable cells was observed; this effect has been well documented for many cells that enter starvation conditions, and it has been proposed that it is a response used to minimize cell maintenance requirements. Flow cytometry also proved useful in detecting cells and illustrated that a change in the shape of the cells had occurred within the population.

It is difficult to assess whether nonculturable cells of *A. salmonicida* remained viable. Cells in a nonculturable state were found to contain DNA, RNA, and bound fatty acids. This indicates that they were present as more than empty cells that could be stained with acridine orange. However, nonculturable cells lost their ability to take up glucose and release $^{14}CO_2$. Respiration was also not detected by INT-formazan treatment combined with microscopic examination (3, 30). This indicates that the cells had a very low or nonexistent metabolic rate for these compounds. This could mean that the cells either have shut down their metabolic rate and maintained viability or have become nonviable. One method commonly used to detect viable cells in a nonculturable state is the yeast extract and nalidixic acid technique (12). This method did not work with *A. salmonicida*, probably because *A. salmonicida* exhibits nalidixic acid resistance.

Recovery of cells in low concentrations of tryptone soya broth would also have confirmed the presence of viable cells. This method was used by Allen-Austin et al. (2) for the revival of *A. salmonicida* from a nonculturable state. However, in this study only when culturable cells were detected in the initial sample could cells be apparently revived. This would indicate that it is the growth of a few culturable cells which remain in the release system that results in the false recovery of nonculturable cells. This agrees with the findings of Rose et al. (19) and emphasizes the point that differences in sample volume between revival and culture studies should not exist. It is likely that the presence of culturable cells reported by Allen-Austin et al. (2) led to the apparent recovery of nonculturable *A. salmonicida*. It would therefore seem essential to determine the absence of all culturable cells from such samples. The simplest method for this would probably be filter plate counts. This is an important criterion both for medium recovery procedures as well as for infection studies, since even small numbers of culturable cells in water samples may be able to cause infection.

Interesting changes in the fatty acid composition of phospholipids were observed. Linder and Oliver (14) reported a reduction in $C_{16}$ and $C_{16:1}$ fatty acids when *V. vulnificus* entered a nonculturable state and *E. coli* was starved. In this study the changes that have taken place have been identified as a double-bond shift in $C_{16:1}$ and $C_{18:1}$ fatty acids. However, the position of the double-bond shift was not resolved. The increases in both long- and short-chain fatty acids have also been found in nonculturable cells (14). The changes in fatty acid composition when *A. salmonicida* enters a nonculturable state may indicate membrane alterations for survival purposes. This may be linked to changes in the fatty acid composition of a cell for the efficient uptake of nutrients or for strengthening the membrane (8).

This work also has implications for the release of genetically engineered microorganisms into the environment, since it confirms the findings that plasmid DNA can be maintained in nonculturable cells. Whether these cells are of any ecological significance and whether the DNA can be transferred out of these cells into a new host have yet to be determined. If plasmids in *A. salmonicida* persist in the environment after detection of the host is no longer possible, it may be necessary to develop new DNA detection methods to assess their presence.

Reinfection of fish with nonculturable *A. salmonicida* has not been demonstrated, and there is no proof that NCBV cells cause the spread of furunculosis in water. If future work confirms their inability to infect fish, then DNA detection methods would prove too sensitive, because nonculturable cells would contain DNA and give positive results but would have no significance to outbreaks of furunculosis in

fish. This may be an important problem in the development of sensitive DNA detection methods for other pathogenic bacteria.

Other bacteria do not appear to enter a nonculturable state in the same way when released into water samples and are detectable by cell growth (9, 13, 17). Entry into this state may be influenced by many parameters, such as temperature and cell density. It seems that bacteria that enter this particular state are commonly those associated with infections. Certainly there is the requirement for an appropriate revival system for their initial isolation from the environment. For *V. cholerae*, *E. coli*, and *A. salmonicida*, this has been an animal model. Survival studies have shown that after revival these cells enter an NCBV state. There are several lines of evidence that support the existence of NCBV cells. However, many nonculturable bacteria in environmental samples may simply be nonculturable because the correct conditions for their isolation have not yet been identified and not because the cells have entered into this specialized survival state. Further work on these bacteria may clarify this situation.

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## REFERENCES

1. **Al-Hadithi, S. A., and R. Goulder.** 1989. An alternative approach to the yeast extract-naldixic acid method for determining the proportion of metabolically active aquatic bacteria. Lett. Appl. Microbiol. **8**:87–90.
2. **Allen-Austin, D., B. Austin, and R. R. Colwell.** 1984. Survival of *Aeromonas salmonicida* in river water. FEMS Microbiol. Lett. **21**:143–146.
3. **Baker, K. H., and A. L. Mills.** 1982. Determination of the number of respiring *Thiobacillus ferrooxidans* cells in water samples by using combined fluorescent antibody–2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride staining. Appl. Environ. Microbiol. **43**:338–344.
4. **Baker, R. M., F. L. Singleton, and M. A. Hood.** 1983. Effects of nutrient deprivation on *Vibrio cholerae*. Appl. Environ. Microbiol. **46**:930–940.
5. **Barcina, I., J. M. González, J. Iriberri, and L. Egea.** 1989. Effect of visible light on progressive dormancy of *Escherichia coli* cells during the survival process in natural fresh water. Appl. Environ. Microbiol. **55**:246–251.
6. **Byrd, J. J., and R. R. Colwell.** 1990. Maintenance of plasmids pBR322 and pUC8 in nonculturable *Escherichia coli* in the marine environment. Appl. Environ. Microbiol. **56**:2104–2107.
7. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3**:817–820.
8. **Fukunaga, N., and N. J. Russel.** 1990. Membrane lipid composition and glucose uptake in two psychotolerant bacteria from Antarctica. J. Gen. Microbiol. **136**:1669–1673.
9. **Griffiths, R. P., C. L. Moyer, B. A. Caldwell, C. Ye, and R. Y. Morita.** 1990. Long-term starvation-induced loss of antibiotic resistance in bacteria. Microb. Ecol. **19**:251–257.
10. **Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White.** 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol. Ecol. **31**:147–158.
11. **Jones, J. G., and B. M. Simon.** 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with respect to a new method for dyeing membrane filters. J. Appl. Bacteriol. **39**:317–329.
12. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
13. **Liang, L. N., J. L. Sinclair, L. M. Mallory, and M. Alexander.** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. **44**:708–714.
14. **Linder, K., and J. D. Oliver.** 1989. Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl. Environ. Microbiol. **55**:2837–2842.
15. **McCarthy, D. H.** 1977. Some ecological aspects of the bacterial fish pathogen-*Aeromonas salmonicida*. Soc. Appl. Bacteriol. Symp. Ser. **6**:299–324.
16. **Michel, C., and A. Dubois-Darnaudpeys.** 1980. Persistence of the virulence of *Aeromonas salmonicida* strains kept in river sediments. Ann. Rech. Vet. **11**:375–380.
17. **Morgan, J. A. W., C. Winstanley, R. W. Pickup, J. G. Jones, and J. R. Saunders.** 1989. Direct phenotypic and genotypic detection of a recombinant pseudomonad population released into lake water. Appl. Environ. Microbiol. **55**:2537–2544.
18. **Popoff, M.** 1984. Genus III. *Aeromonas* Kluyver and Van Niel 1936, 398$^{AL}$, p. 545–548. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
19. **Rose, A. S., A. E. Ellis, and A. L. S. Munro.** 1990. Evidence against dormancy in the bacterial fish pathogen *Aeromonas salmonicida* subsp. salmonicida. FEMS Microbiol. Lett. **68**: 105–108.
20. **Rose, A. S., A. E. Ellis, and A. L. S. Munro.** 1990. The survival of *Aeromonas salmonicida* subsp. *salmonicida* in sea water. J. Fish Dis. **13**:205–214.
21. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51**:365–379.
22. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but not recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30**:334–337.
23. **Sakai, D. K.** 1986. Electrostatic mechanism of survival of virulent *Aeromonas salmonicida* strains in river water. Appl. Environ. Microbiol. **51**:1343–1349.
24. **van Overbeek, L. S., J. D. van Elsas, J. T. Trevors, and M. E. Starodub.** 1990. Long-term survival of and plasmid stability in *Pseudomonas* and *Klebsiella* species and appearance of nonculturable cells in agricultural drainage water. Microb. Ecol. **19**: 239–249.
25. **Wheatcroft, R., and P. A. Williams.** 1981. Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. J. Gen. Microbiol. **124**:433–437.
26. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40**:51–62.
27. **Winstanley, C., J. A. W. Morgan, R. W. Pickup, J. G. Jones, and J. R. Saunders.** 1989. Differential regulation of lambda $p_L$ and $p_R$ promoters by a cI repressor in a broad-host-range thermoregulated plasmid marker system. Appl. Environ. Microbiol. **55**:771–777.
28. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Atwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8**:313–323.
29. **Yruela, I., A. Barbe, and J. O. Grimalt.** 1990. Determination of double bond position and geometry in linear and highly branched hydrocarbons and fatty acids from gas chromatography-mass spectrometry of epoxides and diols generated by specific resin hydration. J. Chromatogr. Sci. **28**:421–427.
30. **Zimmermann, R., R. Iturriaga, and J. Becker-Birk.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.