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Behavior of an *Aeromonas hydrophila aroA* Live Vaccine in Water Microcosms

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Genetically modified auxotrophic mutants of different fish pathogens have been used as live vaccines in laboratory experiments, but the behavior of the strains after release into aquatic ecosystems has not been characterized. We previously constructed and characterized an *aroA* mutant of *Aeromonas hydrophila* and studied the protection afforded by this mutant as a live vaccine in rainbow trout. In this work, we describe the survival of this strain in aquatic microcosms prepared from fish water tanks. The *aroA* mutant disappeared rapidly in nonfiltered, nonautoclaved fish tank water, declining below detection levels after 15 days, suggesting an inhibitory effect of the autochthonous microflora of the water. When the *aroA* strain was used to inoculate sterilized water, its culturability was lower than that of wild-type strain *A. hydrophila* AG2; after long periods of incubation, *aroA* cells were able to enter a viable but nonculturable state. Entry into this nonculturable state was accompanied by changes in the cell morphology from rods to spheres, but the cells appeared to remain potentially viable, as assessed by the preservation of cell membrane integrity. Supplementation of the culture medium with sodium pyruvate favored the culturability and resuscitation of the two *A. hydrophila* strains at low temperatures (6 and 16°C). These results contribute to a better understanding of the behavior of the *aroA* strain in natural environments and suggest that the inactivation of the *aroA* gene may be beneficial for the safety of this live vaccine for aquacultures.

Aeromonas hydrophila is a gram-negative enterobacterium widely distributed in aquatic environments (22, 24, 26, 45), and it has long been known as a pathogen of amphibians, reptiles, and fish (4, 19, 52, 55–57, 66). Also, this bacterial species has been reported to cause a wide variety of human infections (2, 28, 29, 42, 70). Diseases caused by *A. hydrophila* (hemorrhagic septicemia, fin-tail rot, and epizootic ulcerative syndrome) have a major impact in aquacultures (3, 5). At present, no vaccines for the protection of farmed fish against *A. hydrophila* infections are commercially available, although several studies have proved that various vaccine formulations may provide protection (15, 33, 37, 51, 59).

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19) is present in bacteria, where it has a function in the biosynthesis of aromatic amino acids (58). Dysfunction of the *aroA* gene, encoding this enzyme, leads to auxotrophy of the organism for 4-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). Making use of this dysfunction is finding widespread applications for the development of vaccines for human diseases, such a cholera, tetanus, leishmaniasis, and typhoid (27, 36, 63, 68, 76), and has also been applied to protection against various fish diseases (71, 73).

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Hernanz Moral et al. previously constructed and characterized a nutritional derivative of a virulent *A. hydrophila* strain (AG2) for use as a live vaccine in rainbow trout (*Oncorhynchus mykiss*) (25). This method blocks the aromatic amino acid pathway, such that the strain cannot grow unless provided with a metabolite not available in fish tissues. Blocking of aromatic amino acid biosynthesis by mutation of the *aroA* gene causes a loss of virulence, so that the intraperitoneal injection of 10^8 CFU ml^{-1} in trout produces no ill effects, whereas the 50% lethal dose of the virulent wild-type strain is 10^6 CFU ml^{-1} (25).

The use of *aroA* mutant strains in fish vaccination by different routes of administration may facilitate the spread of these bacteria into aquatic environments. Because licensing regulations require assessing the risks associated with the release of genetically modified microorganisms, the persistence and behavior of mutant strains in natural environments need to be carefully studied. Several studies concerning the survival of *A. hydrophila* strains in aquatic environments, such as distilled water (44), mineral water (18, 45, 47), tap water (43), and seawater (40), have been published. Microcosms are often used in these studies, most of them applied to assess the health impact of the presence of motile *Aeromonas* species in drinking water, because the production of a wide range of virulence factors is a common feature of virulent strains (10, 13, 53, 54, 64).

Under adverse conditions, such as those present in some natural aquatic environments, *A. hydrophila* strains are able to

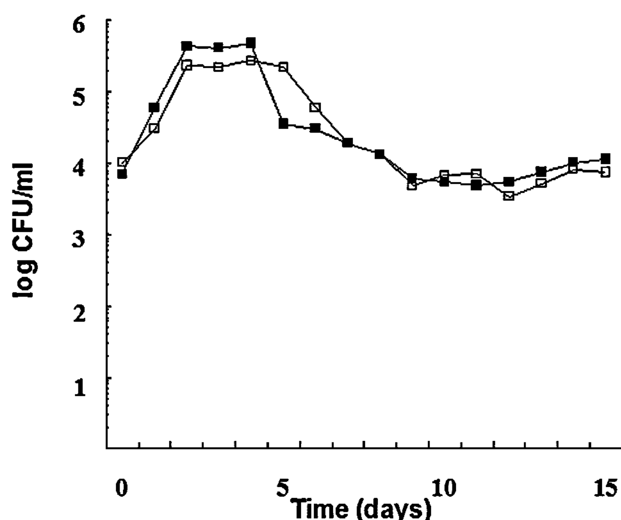

FIG. 1. Growth of autochthonous bacteria in nonsterilized (nonautoclaved, nonfiltered) fish tank water at 16°C (open squares) and 20°C (filled squares). Each point in the curve represents the mean of two independent experiments.

enter a viable but nonculturable (VBNC) or an active nonculturable (ANC) state (40, 44, 75), from which they may be resuscitated under permissive conditions (40, 75). A VBNC or an ANC state allows microorganisms long-term permanence in the environment and makes their isolation for diagnostic procedures difficult (9, 31).

Interestingly, it was reported that *A. hydrophila* can remain in aquatic environments for prolonged periods in the VBNC state when cultured in a 0.35% NaCl solution at pH 7.5 and at 25°C for 50 days, although cells in this state were not pathogenic to goldfish, *Carassius auratus* (60). Because of their importance for the safety of an *aroA* live vaccine, the behavior and culturability of *aroA* cells in different water microcosms were compared with those of wild-type strain AG2 in this work. The morphological modifications and cell viability shown by the nonculturable bacteria and resuscitation from the VBNC or ANC state in culture medium supplemented with sodium pyruvate were studied. The effect on culturability of the growth phase of the vaccine and the effect of fish-associated autochthonous microflora present in water were also investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. This study was performed with a previously described (25) *A. hydrophila aroA* mutant and wild-type strain *A. hydrophila* AG2, which were identified by PCR amplification of the *aroA* gene of *A. hydrophila* (14). Stock cultures were frozen at −80°C with 20% (vol/vol) glycerol, and the strains were routinely cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB) (Cultimed, Barcelona, Spain) at 22°C.

Survival assays in fish tank water microcosms and bacterial counts. Thirty rainbow trout, *O. mykiss* (20 to 35 g), were placed in a 100-liter plastic tank supplied with running well water at 16°C, maintained under constant photoperiod conditions (12 h of light and 12 h of darkness), and fed every other day with commercial trout pellets (Trouw, Burgos, Spain). After 14 days, when the autochthonous aquatic microflora was well developed (reaching about 10^4 CFU ml of water^{-1}; see Fig. 1), water samples from the fish tank were collected in 1-liter sterile glass bottles and split into subsamples of 200 ml that were used for different purposes in each of the following experiments: (i) nonautoclaved, nonfiltered (i.e., nonsterilized) water without any inoculation, to observe the behavior of the autochthonous microflora; (ii) nonsterilized water inoculated (see below) with the *aroA* strain; and (iii) autoclaved (121°C for 15 min), filtered (bottle-top 0.2-μm-pore-size cellulose acetate filter [Corning]) (i.e., sterilized) water inoculated with strain AG2 or with the *aroA* mutant. The bottles were incubated at 6, 16, or 20°C without shaking and in the dark.

For survival assays, the *A. hydrophila* strains were grown in TSB with orbital shaking (150 rpm) at 25°C. This culture medium was selected for the survival studies because it allows the easy growth of *Aeromonas* species (5) and, in particular, *aroA* cells grown in TSB provided significant protection in vaccination-challenge studies with trout (25, 74). Inocula were prepared from cells harvested from earlier (14-h) and later (24-h) stationary-growth-phase cultures. The cells were harvested by centrifugation (2,000 × *g*, 30 min, 4°C) and washed three times in phosphate-buffered saline (PBS) to eliminate the possibility of carryover of nutrients; the pellets were resuspended in the same type of water to be inoculated. The fish water tank microcosms were inoculated to give a final concentration of approximately 10^4 CFU ml^{-1} in 200 ml of water.

Culturable bacterial counts were determined from an undiluted sample and from four samples serially diluted in PBS by the standard plate count technique immediately after inoculation of water and at regular intervals postinoculation. As the *A. hydrophila aroA* strain contains a stable intrachromosomical kanamycin resistance cassette and natural ampicillin resistance, samples containing autochthonous microflora and the *aroA* strain were plated on TSA containing ampicillin (200 μg/ml; A2804 σ) and kanamycin (50 μg/ml; K0879 σ) to inhibit the growth of autochthonous microorganisms; CFU were enumerated after 36 h of incubation at 22°C. Samples from sterilized water inoculated only with strain AG2 or the *aroA* strain were plated on TSA without antibiotics; CFU were enumerated as described above. Water samples from bottles containing the autochthonous microflora of the fish tank were plated on TSA; populations were determined after 7 days of incubation at 22°C. All platings were done in triplicate, the experiments were independently repeated two times, and the mean log CFU were calculated.

Solid medium resuscitation experiments and effects of sodium pyruvate on recovery of strains. During the experiments, samples from the various microcosms were serially diluted and used to inoculate TSA amended with sodium pyruvate (0.1% [wt/vol]) as described by Mizunoe et al. (48) and Wai et al. (75). Sodium pyruvate was added directly to TSA prior to autoclaving.

SEM and detection of viable cells with a *Bac*Light viability kit. For scanning electron microscopy (SEM) studies, samples of bacteria were obtained from the inocula (time zero) and from the various microcosms at 90 days postinoculation. Cells at earlier (14-h) and later (24-h) stationary growth phases in TSB were harvested by centrifugation (2,000 × *g*, 30 min, 4°C), washed three times in PBS, and collected by filtration of the cell suspensions through 0.2-μm-pore-size sterile filters (Corning). Bacteria from the various microcosms were harvested by filtration of water through the same type of filters. The filters were fixed with 2% (vol/vol) glutaraldehyde (Polysciences, Warrington, Pa.) in PBS at 4°C for 1 h, dehydrated in a graded ethanol series, and then coated with gold in a sputter coater (Balzers SCD004). The samples were examined in a JEOL JSM 6100 scanning electron microscope at 20 kV.

For detection of bacteria with intact or damaged cell membranes, cells were stained with a LIVE/DEAD *Bac*Light viability kit (L-7007; Molecular Probes). Samples of *aroA* cells incubated in sterilized water at 6°C for 70 days were prepared by filtration of 30 ml of such water through Isopore 0.2-μm-pore-size black polycarbonate filters (Millipore). The filters were stained with the kit dyes according to the manufacturer's instructions, placed over a slide, immersed in mounting oil, and covered with a coverslip. The samples were observed in an inverted microscope (Eclipse TE-300; Nikon) equipped with an epifluorescence attachment, and separate band-pass filter sets were used to observe fluorescence from SYTO 9 dye (B-2 filter; Nikon) and from propidium iodide (G-2A filter; Nikon). The images were captured with a cooled charge-coupled device camera (DX 30; Kappa) and KAPPA ImageBase software at a ×40 objective.

RESULTS

Figures 1 to 4 show the growth of the autochthonous microflora in the fish tank water, the survival of the *A. hydrophila aroA* strain in nonsterilized water, and the survival of strain AG2 and the *aroA* strain in sterilized water at various temperatures. In nonsterilized water, five to seven different bacterial phenotypes were observed, and these microflora grew actively

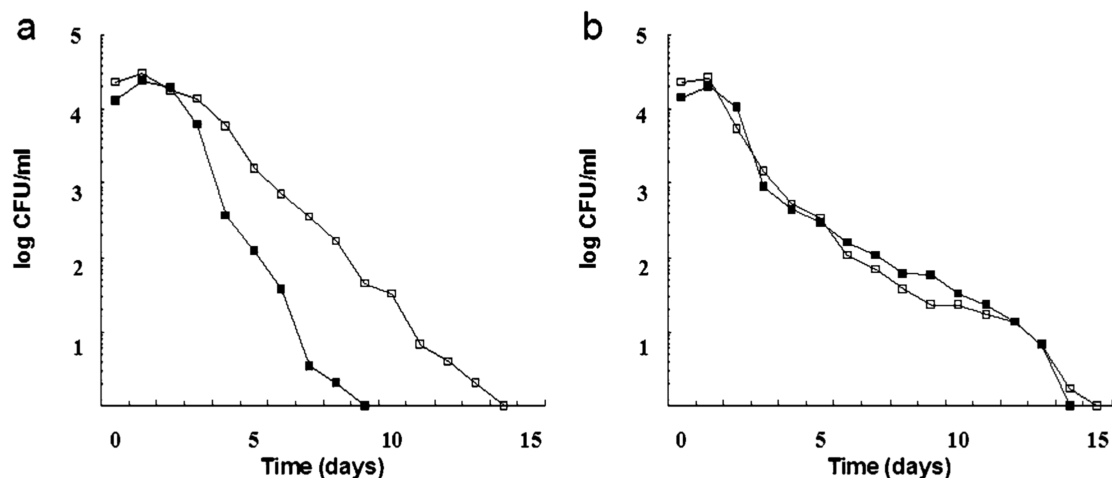

FIG. 2. Culturability of *A. hydrophila aroA* in the presence of autochthonous microflora in nonsterilized fish tank water at 16°C (a) and 20°C (b). Open squares represent CFU for inocula prepared from 24-h-old cultures, and filled squares represent CFU for inocula prepared from 14-h-old cultures. Each point in the curve represents the mean of two independent experiments.

after bottling to reach approximately 10^6 CFU ml^{-1} in the first week at 16 and 20°C (Fig. 1).

The behavior of the *aroA* strain at 16 and 20°C in nonsterilized water was initially characterized by a remarkable increase in the numbers of CFU, independently of the growth phase at which the cells were collected for the inocula (Fig. 2). In sterilized water, only strain AG2 showed such an initial increase (Fig. 3), and the culturability of the *aroA* strain declined from the time of inoculation, a phenomenon that was more prominent at 6°C (Fig. 4).

After the initial growth, the *aroA* strain disappeared rapidly in nonsterilized fish tank water (Fig. 2) and declined to below the detection level after 15 days. The culturability of the *aroA* strain in nonsterilized water was higher for inocula prepared from late-stationary-growth-phase cultures in TSB for experiments carried out at 16°C (Fig. 2a), but there were no differences at 20°C (Fig. 2b).

In sterilized water at 16 and 20°C, both strains showed prolonged culturability periods, but strain AG2 was able to survive much longer than the mutant strain (Fig. 3). The *aroA* strain became nonculturable more rapidly in sterilized water at 6°C, declining to below the detection level on TSA after 42 days of incubation (Fig. 4). When TSA was supplemented with sodium pyruvate, a positive effect on culturability was clearly observed at 6°C (*aroA* strain) and 16°C (*aroA* strain and strain AG2) but not at 20°C (Fig. 3). After incubation times at which the *aroA* strain was nonculturable on TSA (i.e., after 42 to 45 days at 6°C, 95 days at 16°C, and 115 days at 20°C in sterilized water), resuscitation of the mutant strain on sodium pyruvate-amended TSA was observed (Fig. 3 and 4).

SEM studies demonstrated remarkable changes in the morphology of the *aroA* cells after long incubation periods in sterilized water. *aroA* cells collected at time zero from either early- or late-stationary-growth-phase cultures in TSB showed typical rod shapes, with average lengths of 1.5 to 2.5 μm and average diameters of 0.6 to 0.8 μm (Fig. 5a and b). AG2 cells

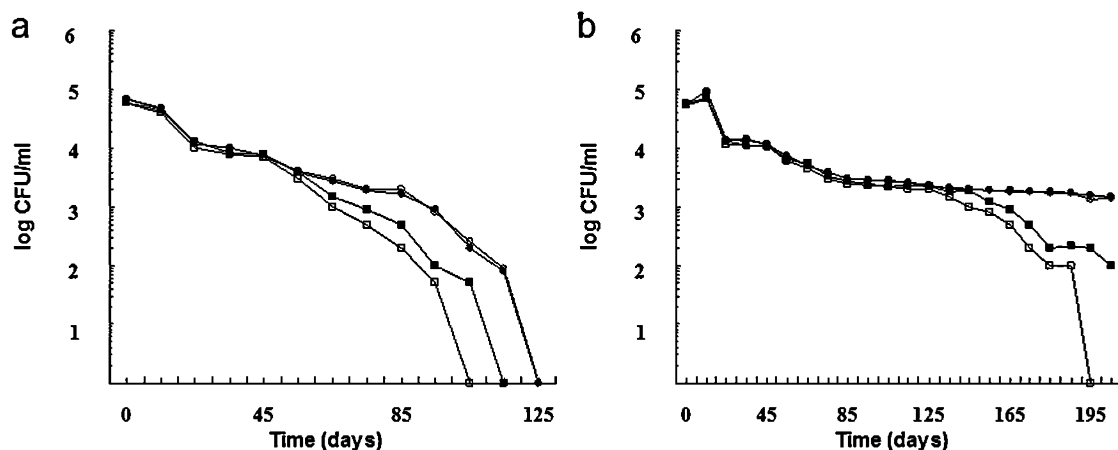

FIG. 3. Culturability of the *A. hydrophila aroA* strain (a) and of wild-type strain AG2 (b) in sterilized (filtered and autoclaved) fish tank water. CFU were determined on TSA plates (open symbols) or on TSA supplemented with sodium pyruvate (filled symbols) at 16°C (squares) and 20°C (circles). Each point in the curve represents the mean of two independent experiments.

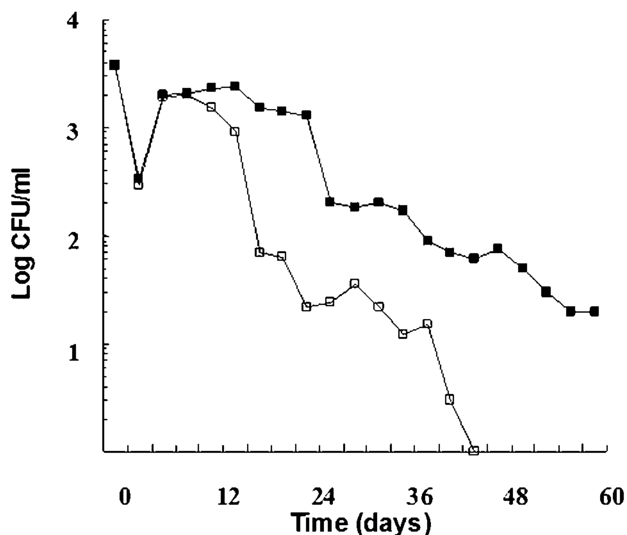

FIG. 4. Culturability of the *A. hydrophila aroA* strain in sterilized fish tank water at 6°C. CFU were determined on TSA plates (open squares) or on TSA supplemented with sodium pyruvate (filled squares). Each point in the curve represents the mean of two independent experiments.

collected from TSB cultures at the same growth phases showed a similar morphology (data not shown). After incubation in sterilized water for 90 days, *aroA* cells became coccoid or spheroid in shape, with a remarkable reduction in length and with diameters of ca. 0.5 μm (Fig. 5c and d). Most AG2 cells maintained coccobacillary shapes, and some dividing AG2 cells were observed (Fig. 6).

Staining of *aroA* cells sampled from sterilized water at 70 days of incubation at 6°C demonstrated the presence of cells with damaged (Fig. 7a) and intact (Fig. 7b) cell membranes. The use of separate filter sets to observe fluorescence for the two dyes in the bacterial viability kit on the same microscopic field allowed us to clearly distinguish cells stained with propidium iodide, which penetrates only cells with damaged membranes, from those stained with SYTO 9 dye, which labels all bacteria.

DISCUSSION

The safe use of live vaccines and/or genetically modified microorganisms requires evaluating important questions about potential hazards related to release, spread, and survival in natural environments. Our study reveals the effect of the *aroA* mutation on the behavior of the *A. hydrophila* live vaccine in freshwater at temperatures (16 to 20°C) at which the efficacy of this vaccine has been tested (74) and that are similar to those at which vaccination in fish farms is usually carried out (38) or at a temperature that can be found in salmonid rivers (6°C).

In the present study, the *aroA* strain was able to start to grow at 16 and 20°C, reaching a maximum cell density at 24 to 36 h; afterward, however, it rapidly lost its culturability in a microcosm containing water and fish-associated autochthonous microflora. The initial rapid growth seen with the *A. hydrophila*

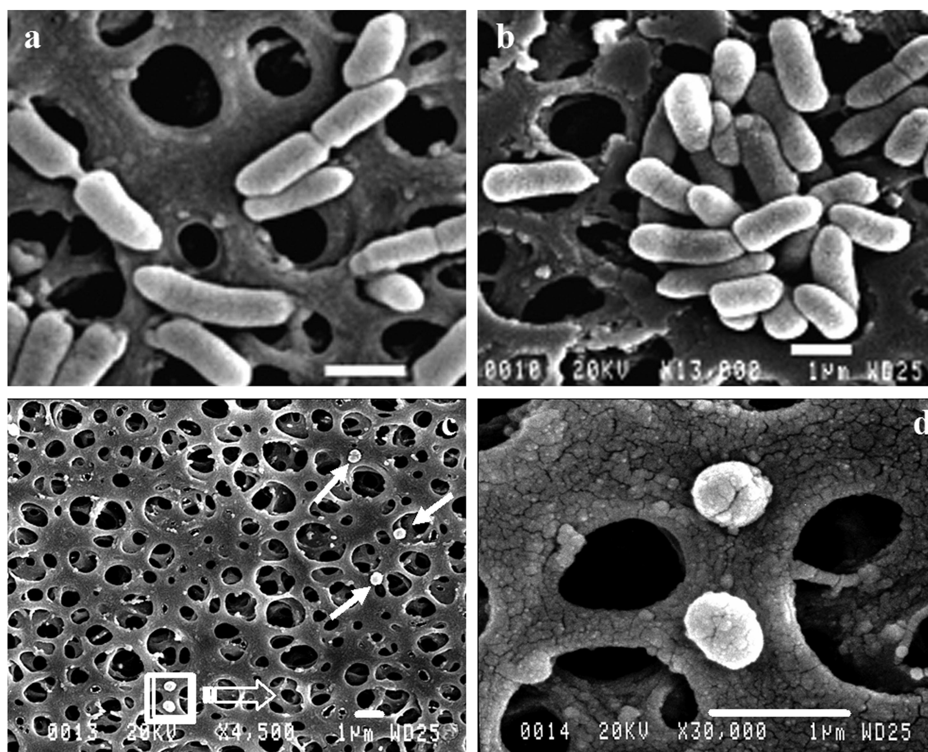

FIG. 5. SEM images of *aroA* cells collected from earlier (14-h) (a) and later (24-h) (b) stationary-growth-phase cultures in TSB and after 90 days of incubation in sterilized fish tank water at 20°C (c and d). Arrows in panel c indicate coccoid cells, and the boxed field is magnified in panel d. Bars, 1 μm.

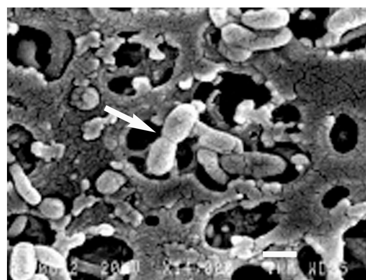

FIG. 6. SEM image of AG2 cells after 90 days of permanence in sterilized fish tank water at 20°C. The arrow shows a dividing cell. Bar, 1 μm.

aroA strain after inoculation of nonsterilized water microcosms has also been reported for other *A. hydrophila* strains (10, 18) as well as for other species, such as *Vibrio vulnificus* (11), *Pseudomonas aeruginosa* (35, 69), and *Photobacterium damselae* subsp. *damselae* (21), and appears to be correlated with the water volume and the recipient surface (34).

The decline in the culturability of the *aroA* strain in nonsterilized fish tank water suggests an inhibitory effect of the natural aquatic microflora on the growth and survival of the mutant. A similar decline in culturability has been reported for other *A. hydrophila* strains in tap water (32, 43) and seawater (40) microcosms. In fish farms and natural aquatic environments, introduced or resident bacteria may be regulated by biotic or abiotic factors, such as protozoan predation (16, 23, 65, 67). When considering the effect of the growth phase at which the *aroA* cells were harvested to prepare the inocula, we observed differences in survival rates in nonfiltered, nonautoclaved water (higher for the cells collected from the late stationary growth phase) only at 16°C and not at 20°C; this result might have been due to the rapid growth of the autochthonous microflora at 20°C. Similar results have been reported for *A. hydrophila* incubated in filter-sterilized seawater (40), where differences in resistance to seawater stress between starved cells and those from the exponential growth phase were evident at 5°C but not at 20°C.

In sterilized water, wild-type strain AG2 showed a clear fitness advantage over the *aroA* mutant strain, probably due to the effect of the inactivation of the *aroA* gene. Thus, at 16 and 20°C, strain AG2 survived in sterilized water for a longer period than did the mutant strain; after 45 days of incubation, CFU counts for the wild-type strain were higher than those for the *aroA* strain. Maximum cell densities found in this study for wild-type strain AG2 in sterilized water were of the same order of magnitude as those previously reported for other *A. hydrophila* strains in various types of water (43, 61, 72).

A decline in culturability after long periods of incubation in sterilized water may be due either to cell death or to entry into a VBNC or an ANC state (6, 30, 41, 77). Rahman et al. (60) reported that *A. hydrophila* cultured in a 0.35% NaCl solution at pH 7.5 and 25°C for 50 days showed a VBNC state characterized by the presence of viable but nonculturable bacteria. In our study, the culturability of the *aroA* strain declined more rapidly at 6°C than at the higher temperatures, and it has been reported that low temperatures seem to favor entry into the VBNC or ANC state (21, 75). To clarify this point, we tested a method for in vitro resuscitation, studied the morphological changes, and assessed the viability of the nonculturable *A. hydrophila* cells by using an indirect labeling method to distinguish cells with damaged and intact cell membranes.

After 90 days of incubation in sterilized water, when the culturability of the *aroA* cells on TSA was at its lowest level, we found significant differences in cell morphology between the mutant strain and the wild-type strain. Similarly, in oligotrophic and starvation microcosms, similar changes in cell size and morphology have been described for various bacteria (20, 30). It has been proposed that reductions in cell size and in metabolic activity confer better resistance to environmental stress (8, 50).

Although indirect labeling methods to assess the viability of nonculturable bacteria are not always reliable (7), nonculturable *aroA* cells appearing as coccoid or round forms in our study appeared to be in a true VBNC or ANC state, as some of them were not stained for propidium iodide; this result indicates that they retained intact cell membranes. A similar pattern of staining for fluorescein diacetate and ethidium bromide was used to assess the presence of viable *A. hydrophila* cells in the VBNC state after prolonged incubation in a 0.35% NaCl solution (60). Moreover, *aroA* cells could be resuscitated on TSA supplemented with sodium pyruvate. Sodium pyruvate and H_2O_2-degrading agents seem to be effective compounds for recovering the physiological state after cell damage or a loss of culturability (1, 12, 46, 48, 49). In our study, amendment

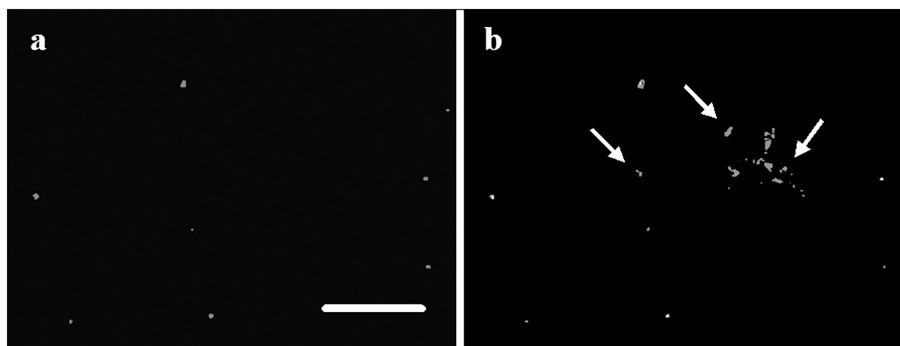

FIG. 7. Fluorescence microscopy of a sample of *aroA* cells collected from a sterilized fish tank water microcosm after 70 days of incubation at 6°C. The cells were stained with the LIVE/DEAD *Bac*Light viability kit. The images show the same field of a sample viewed for propidium iodide fluorescence (a) and for SYTO 9 fluorescence (b). The arrows in panel b indicate cells that were not stained for propidium iodide. Bar, 5 μm.

of TSA with sodium pyruvate notably improved the culturability of the *aroA* and AG2 cells. This effect was greater at 6 and 16°C than at 20°C. A similar effect of temperature on the resuscitation of *A. hydrophila* strain HR7 was reported by Wai et al. (75). This effect may be explained by the fact that 20°C is closer to the optimal growth temperature of the bacteria.

Therefore, attenuation of the live vaccine, which is related to the decreased ability of the bacteria to persist in host tissues (25, 74), may also modulate survival in water. Other authors have reported that mutations in strains used as live vaccines may have consequences for resistance to various environmental stresses (17, 62). The mutation in the *A. hydrophila aroA* gene which blocks the aromatic amino acid pathway may hinder the ability of the mutant to utilize a wide range of nutrients found in aquatic environments; this scenario would favor the disappearance of *A. hydrophila aroA* cells by nutrient competition in the presence of other species. This phenomenon has been demonstrated in fresh and salty waters for *A. hydrophila* strain NCTC 8049 transformed with plasmid pVL1013 (39).

In conclusion, this study suggests that the *aroA* live vaccine has a lower survival potential in water than wild-type strain AG2, and it is of particular interest that the mutant disappeared rapidly in a microcosm similar to the natural aquatic environment of fish farms. Further studies, such as those considering the effects of other culture media that would be more appropriate for the commercial production of the vaccine, are needed. However, our results contribute to a better understanding of this type of genetically modified vaccine and suggest that the inactivation of the aromatic amino acid pathway, which allows the development of attenuated live vaccines that retain a high level of antigenicity, may also affect the survival of the strain in water and contribute to the safety of these vaccines when released into natural environments.

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