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Evidence of a Dormant but Infective State of the Fish Pathogen *Pasteurella piscicida* in Seawater and Sediment

B. MAGARIÑOS, J. L. ROMALDE, J. L. BARJA, and A. E. TORANZO*

Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela 15706, Spain

Received 22 February 1993/Accepted 28 October 1993

The stability of *Pasteurella piscicida* strains in seawater and sediment microcosms at different temperatures (6 and 20°C) was investigated during a 1-month period. Three strains of *P. piscicida* showed similar survival kinetics. By a standard plate count method they survived in water and sediment for only 6 to 12 days, depending on the strain and type of microcosm. During this starvation period, the metabolic activity of the cells was reduced by more than 80%. Culturable cells of each *P. piscicida* strain persisted better in sediment than in water, as well as at 20°C compared to 6°C. However, in all the microcosms, the acridine orange direct counts remained at about 10^5 cells per ml during the experimental period, which demonstrated that *P. piscicida* possesses a capacity to enter a viable but not culturable state. Moreover, dormant cells were always resuscitated by the addition of fresh medium to the microcosms, since we recovered numbers of culturable cells similar to the acridine orange direct counts. These resuscitated cells exhibited the same respiration rate as that seen prior to the start of the experiments. Although the biochemical, physiological, and serological characteristics; lipopolysaccharides; membrane proteins; and plasmid content of *P. piscicida* strains were unaffected during the starvation conditions, the dormant cells were smaller (dwarf cells) and had increased surface hydrophobicity. The starved cells maintained their infectivity and pathogenic potential for fish, with 50% lethal doses similar to those of the original strains.

Application of the direct viable count methods has shown that when bacteria from humans and animals are exposed to oligotrophic aquatic environments, they may enter in a nonculturable starvation survival state in which the direct viable counts of the population are higher than the plate counts (47). Pathogens showing this characteristic include *Aeromonas salmonicida* (1, 35, 46), *Escherichia coli* (58), *Salmonella enteritidis* (48), *Vibrio cholerae* (7, 10, 58), *Vibrio vulnificus* (30, 39), *Legionella pneumophila* (21), and *Campylobacter jejuni* (44). This dormancy process or the loss of culturability was also observed in enteric bacteria physiologically injured by biotic environment stress (3, 4) or subjected to sublethal concentrations of antimicrobial agents (34, 49, 50).

The viable-but-nonculturable-phase phenomenon has serious repercussions for public and animal health, since some of these dormant cells maintain their infective and pathogenic potentials (10, 15, 49). However, the maintenance of infectivity is at present contentious, since in other studies conducted with *V. vulnificus* and *A. salmonicida*, the starved cells lost the capacity to infect their respective hosts (30, 46).

Pasteurella piscicida since 1969 has been one of the most significant agents of disease in aquaculture, causing significant losses of cultured fish in Japan (13, 29). Since 1990, this bacterium has also become a threatening problem in Europe (5, 8, 55). We have studied the phenotypic, antigenic, molecular, and virulence characteristics of this pathogen (32, 33), but reports concerning the viability of this microorganism in the aquatic environment are scarce (23, 53). Therefore, its mode of transmission has not yet been elucidated. In this study, we investigated the survival period of *P. piscicida* in different seawater and sediment microcosms, analyzing the capacity of this marine fish pathogen to enter a dormant state. The possible changes in the morphological, biochemical, and antigenic characteristics and plasmid content of this microorganism during the starvation period were studied. Moreover, the pathogenic capacity of the dormant cells for fish was analyzed.

* Corresponding author. Mailing address: Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela 15706, Spain. Phone: 34 (81) 563100, ext. 2548. Fax: 34 (81) 596904.

MATERIALS AND METHODS

Bacterial strains and preparation of the microcosms. Three strains of *P. piscicida* with different origins and sources of isolation were used in this study: strain DI 21, isolated from gilthead sea bream (*Sparus aurata*) in Spain; strain MP-7801, isolated from yellowtail (*Seriola quinqueradiata*) in Japan; and strain ATCC 17911, isolated in the United States from white perch (*Roccus americanus*) (32). All these strains proved to be pathogenic for fish, with 50% lethal doses (LD_{50}s) ranging from 10^4 to 10^5 CFU (33) (Table 1).

The survival assays were conducted in seawater and sediment (taken from Ría de Muros, Galicia, northwest Spain; pH 7.4; salinity, 15%) which were transported in cold storage containers and kept at 5°C until use (within 2 days). Water was filtered through 0.2-μm-pore-size membranes (Millipore, Madrid, Spain), and sediment was autoclaved at 120°C for 30 min. The experimental assays were conducted in Erlenmeyer flasks containing 100 ml of water or 80 g of sediment. Twenty milliliters of seawater was added to each sediment flask, and flasks were incubated at two different temperatures (6 and 20°C). To prepare the bacterial inocula, the strains were grown on brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl at 22°C for 24 h. Exponential-phase cultures of each strain were centrifuged, and the pellets were washed several times with phosphate-buffered saline (PBS; pH 7.4) and then resuspended and diluted in sterile seawater. A total of 12 micro-

TABLE 1. Origin, plasmid content, and virulence for turbot of the original and dormant *P. piscicida* strains

Strain	Origin	Plasmids (MDa)	LD_{50} (CFU) for turbot of:	
			Original strain (live cells[a])	Dormant strain
DI 21	Sea bream, Spain	50, 20, 7	3.2×10^5	4.0×10^5
MP-7801	Yellowtail, Japan	37, 6	6.0×10^4	5.4×10^4
ATCC 17911	White perch, United States	20, 7	4.0×10^5	4.2×10^5

[a] Killed cells were not virulent for turbot, with LD_{50} values of >10^8 CFU.

cosms prepared in duplicate were inoculated with the different strains to obtain an initial bacterial concentration of approximately 10^4 cells per ml. Flasks were incubated in the dark on a rotary shaker.

Direct plate counts and epifluorescence microscopy. Culturability was determined by the spread plate technique. Samples (0.5 ml) were taken at 0, 12, and 24 h and then daily for a period of 30 days. Samples were serially diluted in PBS and plated in duplicate on marine agar (MA; Difco) and MA 1/10 diluted with distilled water in order to obtain low-nutrient medium. This diluted MA was supplemented with NaCl to a 3% final concentration (which was the original percentage in the MA). After incubation for 48 h at 25°C, the number of CFU per milliliter was determined. When culturable bacteria could not be recovered, samples (1 ml) taken directly from the flasks were seeded onto MA plates to obtain a detection limit of 1 CFU/ml. The regression coeficients of the data were compared by using a standard analysis of variance (51). In addition, the total viable number of bacteria in each microcosm was counted by epifluorescence microscopy employing the acridine orange staining method. The samples were diluted, filtered onto 0.20-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.), prestained with irgalan black, and stained with 0.01% acridine orange (18). All solutions were sterilized by filtration. After 5 min of staining, filters were washed twice with distilled water. The prepared filters were observed during a 1-month period for acridine orange direct counts (AODCs) and changes in the morphology and size of the cells.

The metabolic activity of the cells in all the microcosms was determined by measuring the A_{600} with 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (40). The results were expressed as the percentage of the remaining respiratory activity, taking as 100% the value at zero time.

Resuscitation condition. When culturable bacteria were below the detection limit by the plate count method (1 CFU/ml), 10 ml of marine broth (Difco) was added to each microcosm. Flasks were incubated at 6 and 20°C as described above. After 24 and 48 h samples were withdrawn, spread onto MA plates, and incubated at 25°C to confirm absence or presence of *P. piscicida* cells. The resuscitation experiments were also conducted after a 1-month period.

Phenotypic and serological analysis. The possible morphological, biochemical, physiological, and serological changes of the three starved *P. piscicida* strains were analyzed. The dormant cells were recovered after 12- and 30-day periods by centrifugation of each water and sediment microcosm. The pellets were washed several times with sterile seawater and then used for the different assays. The taxonomic analysis was performed using conventional plate and tube tests as well as API ZYM systems (bioMerieux S.A., Madrid, Spain) (32, 33, 55). In addition, the biological activities of the extracellular products of the original and starved strains were also analyzed by the API ZYM system and standard plate methods as described previously (33).

The possible serological changes of the dormant cells were analyzed by a slide agglutination test using the respective O antigens and the antisera against strains DI 21 and ATCC 17911 raised in New Zealand rabbits (32, 56). Cross-quantitative tests were conducted on microtiter plates by following the procedure described by Roberson (43).

Analysis of cell surface components and hydrophobicity. The lipopolysaccharides (LPS) and the total and outer membrane protein profiles of the nonculturable *P. piscicida* strains were analyzed by the methods of Hitchcook and Brown (17) and Crosa and Hodges (11), respectively. Moreover, the plasmid profiles of the strains in the dormant state were compared with those of the original isolates by following the procedures of Birnboim and Doly (6).

The cell surface hydrophobicity of the strains was assayed by the salt aggregation test. The assay was performed basically as described Lindahl et al. (31). Bacterial cultures were resuspended to an A_{420} of 1.0 in 0.002 M sodium phosphate buffer (pH 6.8). These suspensions (30 μl) were mixed with an equal volume of various molarities of buffered ammonium sulfate (Panreac; Montplet & Esteban S.A., Barcelona, Spain) solutions that ranged from 0.05 to 4.0 M. Bacterial suspensions mixed only with PBS were used as negative controls.

Virulence for fish. The virulence of the dormant strains for fish was measured by determining their LD_{50}s, evaluated by following the method of Reed and Müench (41). The starved cells, obtained by centrifugation as described above, were injected intraperitoneally in turbot (5 g) at doses ranging from 10^6 to 10^3 CFU (eight fish being used per dose) and maintained at 18 to 20°C with aeration as previously described (33, 54). In parallel, the original strains and sterile seawater were also inoculated as positive and negative controls, respectively. Moreover, formalin- and heat-killed cells from *P. piscicida* were used also as negative controls. The dead fish were examined to confirm the reisolation of the inoculated strains from the internal organs (liver, kidney, and spleen) by streaking directly onto brain heart infusion agar plates (Difco) supplemented with 2% NaCl.

RESULTS

Survival and resuscitation of viable but not culturable cells in different microcosms. The stability in seawater and sediment microcosms of the three strains of *P. piscicida* utilized in this study is shown in Fig. 1 and 2, respectively. The kinetics of survival was very similar for all strains regardless of their geographic origin and source of isolation. In all the cases, by the standard plate count method, the number of culturable cells declined very rapidly. A loss of about 0.5 to 1 log unit was detected within the first 2-day incubation, and after 6 to 12 days (depending on the strain tested and the type of microcosm) we were unable to recover culturable cells of *P. piscicida*. In all the microcosms the stability of the *P. piscicida* strains was greater in sediments than in water, with differences in the order of 0.5 to 1 log unit. In addition, the survival in seawater and sediment was directly associated with the temperatures, since the number of culturable bacteria was slightly higher at 20°C than at 6°C for each strain and microcosm. The differences among the dynamics of survival were statistically significant in two of the strains

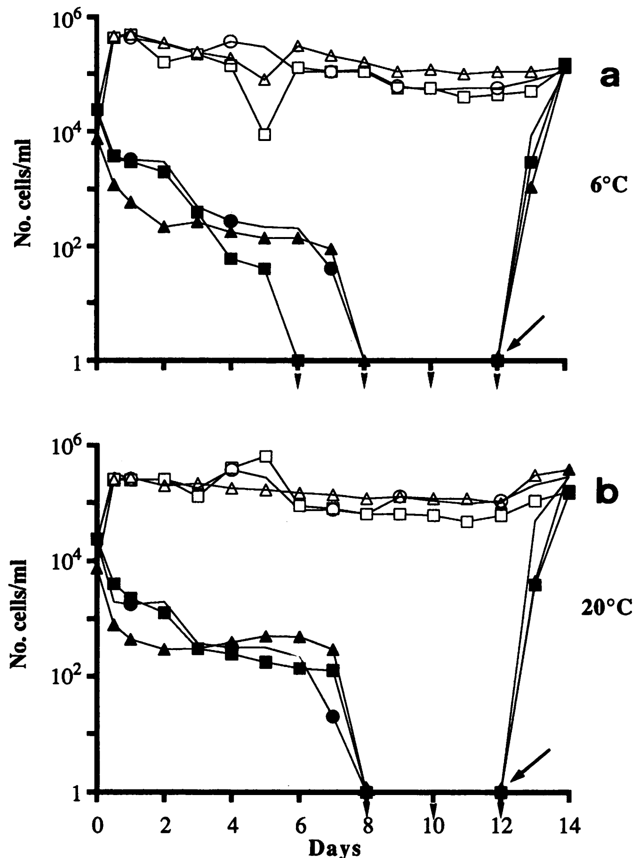

FIG. 1. Survival of different *P. piscicida* strains in seawater at 6°C (a) and 20°C (b). □ and ■, strain DI 21; △ and ▲, strain MP-7801; ○ and ●, strain ATCC 17911. Open symbols, AODCs; closed symbols, CFU. Arrows indicate the addition of fresh medium to the microcosms. Arrowheads indicate that CFU values are below the detection limit.

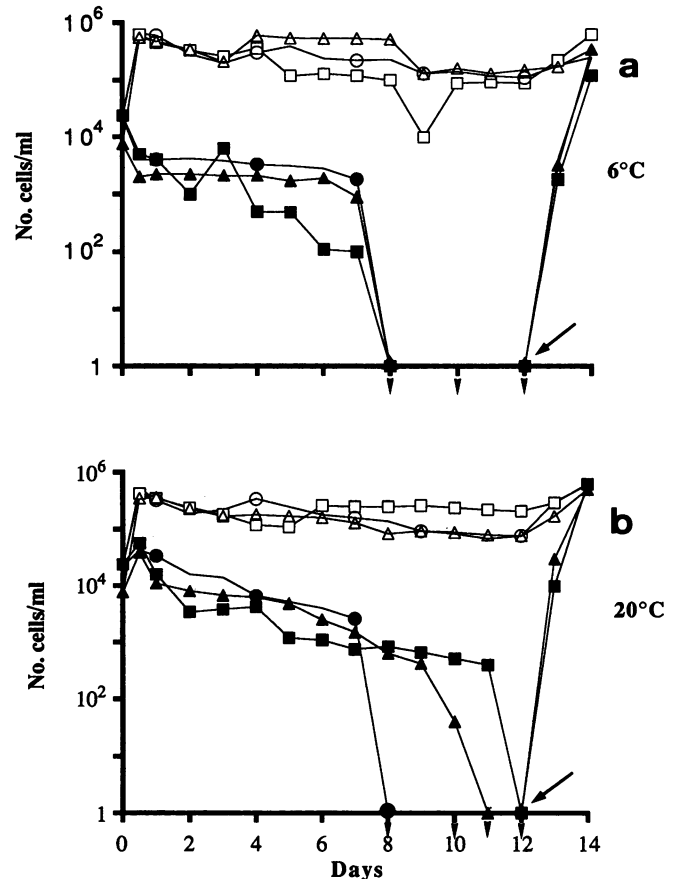

FIG. 2. Survival of different *P. piscicida* strains in sediment at 6°C (a) and 20°C (b). □ and ■, strain DI 21; △ and ▲, strain MP-7801; ○ and ●, strain ATCC 17911. Open symbols, AODCs; closed symbols, CFU. Arrows indicate the addition of fresh medium to the microcosms. Arrowheads indicate that CFU values are below the detection limit.

tested (MP-7801 and ATCC 17911) only when we compared the results found in sediments at 6 and 20°C ($P < 0.01$) and those obtained in water and sediment at 6°C ($P < 0.001$). It is interesting to note that from the start of survival experiments it was not possible to recover any culturable *P. piscicida* cells in diluted MA. Because the three strains followed similar inactivation kinetics, Fig. 3 demonstrates the stability of only the Spanish isolate DI 21 in seawater and sediment at different temperatures.

By epifluorescence microscopy it was found that after an initial increase of 1 to 1.5 log units, the AODCs in seawater and sediment remained near constant at approximately 10^5 cells per ml through the experimental period (Fig. 1 to 3). As it could be expected, after 6 to 8 days of starvation, the metabolic activity of the cells was reduced 80 to 90% (depending on the strain and microcosm), placing the cells in a state of near dormancy. Figure 4 shows the reduction of the respiratory activity of a representative strain (DI 21) in seawater and sediment microcosms at 20°C. The addition to the microcosms of fresh marine broth after 12 days (indicated by arrows) induced the resuscitation and possible growth of *P. piscicida* strains from the dormant state. In fact, this reactivation process allowed recovery of numbers of culturable cells similar to those obtained by acridine orange method. Moreover, these resuscitated cells showed the same level of metabolic activity as was seen prior to the initiation of the starvation. Identical results were obtained when the resuscitation experiments were repeated at 1 month of starvation.

Morphological and biochemical characteristics and cell surface hydrophobicity. The results of this study indicated that during the starvation period the three *P. piscicida* strains used changed morphologically. Whereas the cells inoculated into the microcosms were rods with a size of approximately 1.7 by 2.5 μm, nonculturable cells of *P. piscicida* became smaller with a coccoid appearance (1.3 by 1.5 μm). After resuscitation, cells recovered their original size and morphology.

Although no changes were detected in the biochemical or physiological characteristics of the dormant *P. piscicida* strains, the hydrophobicity was higher during the nonculturable phase than in the original strains. While the original *P. piscicida* strains agglutinated in a range of 1.5 to 2 M $(NH_4)_2SO_4$, the dormant cells of *P. piscicida* exhibited salt aggregation test values of 0.5 to 1 M.

The extracellular products obtained from the starved cells showed similar profiles in the API ZYM system and displayed the same enzymatic activities (caseinase, gelatinase,

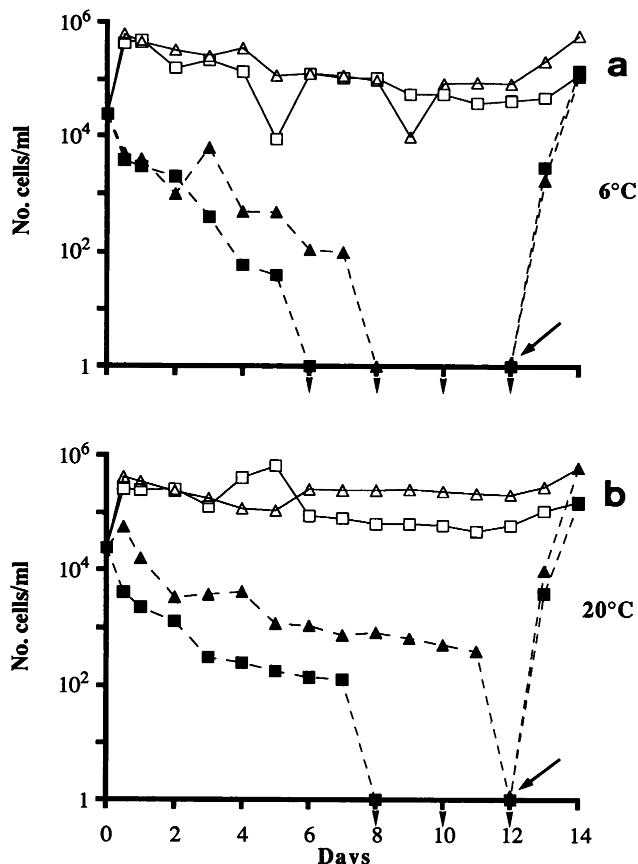

FIG. 3. Comparison of the survival in water (□ and ■) and sediment (△ and ▲) of *P. piscicida* DI 21 at 6°C (a) and at 20°C (b). Open symbols, AODCs; closed symbols, CFU. Arrows indicate the addition of fresh medium to the microcosms. Arrowheads indicate that CFU values are below the detection limit.

phospholipase, and chondroitinase) by standard plate methods as those of the original strains (33).

The serological assays demonstrated that the *P. piscicida* dormant cells produced agglutination titers similar to those of the original strains (values of 20,480 for strain DI 21 and of 40,960 for strains MP-7801 and ATCC 17911) with the antisera raised against the Spanish isolate DI 21 and the American strain ATCC 17911.

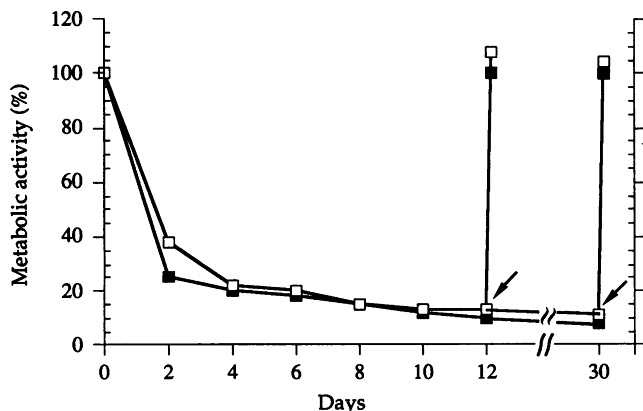

FIG. 4. Metabolic activity of *P. piscicida* DI 21 in seawater (■) and sediment (□) microcosms at 20°C. Arrows indicate the addition of fresh medium to the microcosms.

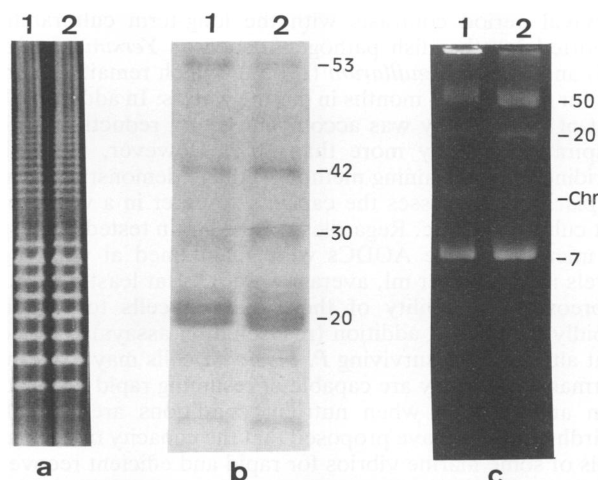

FIG. 5. Comparison of the LPS (a), outer membrane proteins (b), and plasmid contents (c) between the original (lanes 1) and dormant (lanes 2) *P. piscicida* DI 21 strain. Numbers to the right indicate the molecular sizes of the outer membrane proteins and plasmids in kilodaltons and megadaltons, respectively. Chr, chromosomal band.

Analysis of cellular surface constituents, plasmid content, and virulence assays. The viable but not culturable cells of all *P. piscicida* analyzed showed the same LPS and protein profiles as the original isolates. The LPS of the dormant strains exhibited the ladder pattern of smooth type characteristic of *P. piscicida* strains, with no changes being observed either in the O-specific polysaccharide chain or in the low-molecular-weight core oligosaccharide (Fig. 5a). Similarly, in both starved and original isolates four major outer membrane protein bands of 20, 30, 42, and 53 kDa were detected (Fig. 5b). In addition, the plasmids harbored by the strains in the dormant state were identical to those in the original isolates (Table 1). Figure 5c shows the plasmid profile of *P. piscicida* DI 21. Three plasmid bands of 50, 20, and 7 MDa were shared by the original and the nonculturable *P. piscicida* cells.

The challenge experiments demonstrated that the nonculturable cells of the three *P. piscicida* strains maintained their pathogenic capacity for turbot, with LD_{50}s ranging from 10^4 to 10^5 cells, which are similar to those exhibited by the original strains (Table 1). The inoculated strains were always reisolated in pure culture from the internal organs of all dead fish. As we expected, no mortalities were produced when fish were injected with killed cells from *P. piscicida*.

DISCUSSION

Although pasteurellosis caused by *P. piscicida* is a very old and threatening disease affecting important marine fish species such as seriola, sea bream, and sea bass (32, 55), the organism's behavior outside the host is unknown. In fact, the route of infection and possible reservoirs in the aquatic environment of this pathogen are not yet clarified.

In this study we have found that the *P. piscicida* strains are able to survive in a culturable state in seawater and sediment for only 6 to 12 days. Similar results were described by Rose et al. (46) for *A. salmonicida*. This short

survival period contrasts with the long-term culturability reported in other fish pathogens, such as *Yersinia ruckeri* (45) and *Vibrio anguillarum* (19, 53), which remain culturable for more than 3 months in marine waters. In addition, the lost of culturability was accompanied of a reduction of the respiration rate by more than 80%. However, using the acridine orange staining method we have demonstrated that *P. piscicida* possesses the capacity to enter in a viable but not culturable state. Regardless of the strain tested and type of microcosm, the AODCs were maintained at very high levels (10^5 cells per ml, average value) for at least a month. Moreover, the ability of these starved cells to respond rapidly to nutrient addition (resuscitation assays) indicated that although the surviving *P. piscicida* cells may exist in a dormant state, they are capable of resuming rapid resuscitation and division when nutrient conditions are suitable. Flärdh et al. (14) have proposed that the capacity of dormant cells of some marine vibrios for rapid and efficient recovery can be associated with the ribosomal counts. In fact, they demonstrated that the ribosomes existed in large excess over the apparent demand for protein synthesis, which in turn provides to the starved cells an excess of protein synthesis capacity to immediately initiate an upshift program when substrate or nutrient medium is added. It is important to point out that in the majority of the starvation experiments, it is difficult to ascertain whether the nonculturable cells were resuscitated, grew, or did both when the optimal environmental conditions were restored. However, in the present study, the overall data of AODCs, viable plate counts, and metabolic activity seem to indicate that although both processes may occur when fresh medium is added to the microcosms, the major proportion of the recovered culturable *P. piscicida* cells corresponded to resuscitated bacteria.

Together, these results indicate that although *P. piscicida* appears to be a very labile organism, the aquatic environment constitutes a reservoir and a vehicle of transmission of this pathogen. However, we must consider that in natural conditions other physicochemical factors (e.g., UV light, organic matter, and particles) as well as biological factors such as bacteriophages, protozoans, and bacterial antagonisms also play an important role in the survival of bacteria in marine waters (3, 4, 12, 24).

The three strains of *P. piscicida* presented similar survival patterns in the marine environment, with the number of culturable bacteria higher in sediment than in seawater and higher at 20°C than at 6°C. The effect of temperature in the dynamics of *P. piscicida* survival differs from that reported for pathogens like *Y. ruckeri*, *C. jejuni*, and *E. coli* (27, 45), in which the stability of the microorganisms was lower at high temperatures.

Throughout this study, we were unable to recover any culturable cells in diluted MA. This finding indicated that, in contrast to other fish pathogens such as *Y. ruckeri* (45), *P. piscicida* strains do not show an adaptation to low-nutrient-concentration conditions.

Although the variations in the protein, LPS, and plasmid profiles as well as in the RNA and DNA content have been described during the course of starvation of different marine bacteria (9, 20, 22, 28, 37, 38), the analysis of cell surface components and the phenotypic characteristics of the dormant cells of *P. piscicida* revealed that they maintain the same biochemical, physiological, and serological characteristics; LPS; and membrane proteins as the original strains. Moreover, the dormancy process did not cause loss of any plasmid harbored by the *P. piscicida* isolates. However, these dormant strains suffered reductions in their cell size (dwarf cells) which were accompanied by an increase of their degree of hydrophobicity. Similar drastic reduction in cell size and significant changes in the cell surface characteristics, which could be survival strategies, have been observed at various times during the starvation survival process of several bacteria in the aquatic environment (2, 16, 25, 26, 36, 42, 45). This cell miniaturization represents a response to the lack of utilizable nutrients because it increases the surface-volume ratio of the cells and therefore their ability to scavenge substrates from the environment.

At present, the maintenance of virulence capability is a subject of controversy. Whereas several authors have reported that the nonculturable cells of different pathogenic bacterial species such as *E. coli* (50, 58), *Y. enterocolitica* (49), *V. cholerae* (10), and *Y. ruckeri* (45) keep their infectivity capacity and pathogenic potential, other authors demonstrated that the dormant cells of fish and human pathogens such as *A. salmonicida* and *V. vulnificus* lost their infective ability (30, 46). In our study, we have clearly demonstrated that the *P. piscicida* starved cells showed an LD_{50} for fish similar to that of nonstarved strains.

All these results support the earlier findings of Janssen and Surgalla (23) and Toranzo et al. (53) that *P. piscicida* does not appear to persist for long in a culturable state away from fish. However, the existence in seawater and sediment of dormant but viable cells of *P. piscicida* which maintain their pathogenic potential for fish could explain why outbreaks of pasteurellosis can occur in fish populations which apparently have not come in contact with the pathogen. In addition, the phenomenon of dormancy in the aquatic environment of fish pathogens has special importance in aquaculture when different disinfectants such as UV radiation, ozone, and chlorine are used to inactivate bacterial pathogens present in farm effluents (52, 57). Although those authors have suggested that these treatments are effective to disinfect water in aquaculture facilities, they have not tested whether these injured populations, unable to form colonies by standard methods, can remain viable and infective in the environment.

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