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ORIGINAL ARTICLE

# Phenotypic variation and morphological changes in starved denitrifying *Aeromonas hydrophila*

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**Abstract** In this study, an *Aeromonas hydrophila* identified as a denitrifying bacterium by PCR detection of nitrate reductase (*nar*G) and nitrite reductase (*nir*K) genes was incubated in seawater microcosms for 8 months at room temperature and at 4 °C. A study of the phenotypic variation demonstrated that *A. hydrophila* becomes gelatinase-positive after the incubation in sea water. We noted that starved *A. hydrophila* becomes unable to produce leucine arylamidase, and that the starved strain appears to grow more slowly. Indeed, we also observed a severe decrease in cellular aggregation of *Aeromonas* after incubation. In addition, atomic force micrographs revealed a reduction in cell size.

**Keywords** *Aeromonas* · Seawater · Starvation · Phenotypic · Morphological

## Introduction

*Aeromonas hydrophila* is a Gram-negative enterobacterium distributed widely in aquatic environments (Hazen et al. 1978; Massa et al. 2001), and has long been known as a pathogen of amphibians, reptiles, and fish (Shotts et al. 1972; Austin and Adams 1996; Pathiratne and Jayasinghe 2001; Pearson et al. 2000). This bacterial species has also been reported to cause a wide variety of human infections (Janda and Duffey 1988; Thornley et al. 1997). Diseases caused by *A. hydrophila* (hemorrhagic septicemia, fin-tail rot, and epizootic ulcerative syndrome) have a major impact in aquacultures (Angka et al. 1995; Austin and Austin 1999).

During their life cycle, bacteria are exposed constantly to stressful environmental situations. Depending on the level of stress, they may develop adaptive responses that allow them to grow or at least survive. Mechanisms by which bacteria can cope with adverse situations involve regulation of gene expression that may lead to modified expression of physiological and phenotypical characteristics. Several studies have reported that stress factors, including salinity, nutrient depletion, temperature, pH, and atmosphere, affect bacterial cell shape, suggesting that morphological changes are correlated to adaptive mechanisms that allow bacteria to prolong survival under adverse conditions (Mattick et al. 2003a, b; Everis and Betts 2001; Alonso et al. 2002; Shi and Xia 2003; Alterman et al. 2004; Jydegaard-Axelsen et al. 2005; Piuri et al. 2005; McMahon et al. 2007). Bacterial populations under nutritional stress react in order to adapt cell metabolism and physiology to stressful conditions. As a consequence, a general increase in virulence and resistance against stress and chemotherapic as well as antibiotic agents is typically observed (Givskov et al. 1994). One of behaviors most frequently observed in the nutrient starvation response of Gram-negative bacteria is size reduction and cell morphology conversion from rod to coccoid shape (Kjelleberg et al. 1993). Despite extensive knowledge at the molecular level about the mechanism and regulation of the nutrient starvation response, the process by which the morphology transition from rod to coccoid shape occurs is not yet well understood.

The aim of this work was to study the adhesion ability, and phenotypical and morphological changes in denitrifying *Aeromonas hydrophila* incubated in natural seawater

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microcosms for 8 months. *nir*K and *nar*G genes were detected by PCR to confirm the denitrifying ability of this strain. Biochemical and enzymatic characterization was achieved using Api-20NE and Api–ZYM systems (bio-Merieux, Marcy l'Etoile, France). Adherence assays were performed on microtiter plates, and morphological changes were examined by atomic force microscopy (AFM).

## Materials and methods

### Bacterial strains and growth conditions

The experiments in this study were performed on *Aeromonas hydrophila* isolated from activated sludge of a Tunisian wastewater treatment plant maintained at −80 °C in Luria–Bertani broth (LB) supplemented with glycerol (15 %, v/v). For the experiments, cells were grown at 37 °C in tryptic soy broth [TSB, (Difco, Franklin Lakes, NJ)] for 24 h. Natural seawater (100 mL) from the Tunisian coast of Monastir (salinity 4 %, pH 8) was filtered through membranes (pore size, 0.22 μm; Millipore, Bedford, MA) and autoclaved three times (115 °C for 15 min) in 200 mL Erlenmeyer flasks. *A. hydrophila* cells were washed three times by centrifugation (13,000 rpm for 10 min at 20 °C) with autoclaved seawater and then suspended in 10 mL autoclaved seawater (Ben Abdallah et al. 2007). The microcosms (100 mL) were inoculated with these suspensions ($10^9$CFU/ml) and then incubated in a static state at room temperature (22–25 °C) and at 4 °C.

### PCR detection of *nir* and *nar* genes

The*nir*K gene was detected by PCR, according to the method previously described by Braker et al. (1998). *A. hydrophila* was grown overnight on Trypticase Soy Agar (TSA) at 37 °C. One colony was suspended in 1 mL LB broth (Sigma, Aldrich, France) and incubated for 24 h at 37 °C. DNA was extracted using a Wizard Genomic purification Kit (Promega, Madison, WI) according to the manufacturer's recommendations. DNA template was adjusted to 50 ng/μL using spectroscopy (Ultraspec 2100 pro, Amersham Biosciences Europe, Orsay, France).

A typical PCR (25 μL) contained 1 μM forward and reverse primers (*nir*S1F 5′-CCT A(C/T)T GGC CGC C(A/G)C A(A/G)A-3′; *nir*S6R 5′-CGT TGA ACT T(A/G)C CGG T-3′), a dNTP mix (100 μM each of dATP, dCTP, dGTP and dTTP), 1 U GO *Taq* DNA polymerase (Promega), 5 μL green Go *Taq* buffer (5×), and a DNA template (50 ng). The primers were designed to amplify a gene product of 514 bp for *nir*K and 500 bp for *nar*G encoding nitrite and nitrate reductase in denitrifying bacteria. Polymerase chain reaction conditions included initial denaturation (94 °C for 5 min), 30 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 40 s) and extension (72 °C for 40 min), followed by a final extension (72 °C for 10 min) at the end of cycling.

The presence of the *nar*G gene was detected by PCR using forward and reverse primers (*nar*GW9F 5′-(A/C)G(A/C/G/T) GG(A/C/G/T) TG(C/T) CC(A/C/G/T) (A/C)G (A/C/G/T)GG (A/C/G/T)GC-3′; *nar*GT38R 5′-AC(A/G) TC(A/C/G/T) GT(C/T) TG(C/T) TC(A/C/G/T) CCC CA-3′), as described previously by Nogales et al. (2002). PCR conditions included initial denaturation (95 °C for 5 min), 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 45 min), followed by a final extension (72 °C for 6 min) at the end of cycling. The PCR products (5 μL) were analyzed on 1 % (w/v) agarose gel stained with ethidium bromide (0.5 μg/mL), visualized under UV transillumination and photographed using a Gel Doc XR apparatus (Bio-Rad, Hercules, CA).

### MTT-assay for growth

Viability was analyzed using a cell proliferation kit MTT (Roche Diagnostics, Mannheim, Germany). The MTT-assay is based on cleavage of the yellowtetrazolium salt MTT (methylthiazolyldiphenyl-tetrazolium bromide) into purple formazan by metabolically active cells. In brief, *A. hydrophyla* and cells obtained after incubation for 8 months in seawater at room temperature were cultured in TSB for 1 week in a shaking incubator with humidified atmosphere (100 rpm, 37 °C, 5 % $CO_2$). Daily, 900 μL of the mixed suspension was removed and 100 μL MTT-solution (1 mg/ml MTT in PBS) was added. After an incubation period of 2 h at 37 °C, the cells were lyzed and the formazan crystals solubilized by adding 1 mL solubilization solution and incubation for 1 h at 37 °C. The solubilized formazan product was quantified photometrically at 570 nm.

### Phenotypical characterization of starved cells

The biochemical characters of *A. hydrophila* cells obtained after incubation in seawater at room temperature and at 4 °C were characterized using the Api 20NE system (bio-Merieux). The enzymatic profiles were achieved with Api–ZYM system (bio-Merieux) according to the manufacturer's instructions. Amylase, lecithinase (phospholipase), caseinase and lipase of starved cells were characterized according to method described previously (Ben Kahla-Nakbi et al. 2007) on TSA, with added 1 % (w/v) starch, 5 % (v/v) egg yolk, 5 % (v/v) skim milk, 1 % (v/v) Tween 80 (Merck, Darmstadt, Germany), respectively. After incubation for 24–48 h at 37 °C, a positive reaction is indicated by a clear halo around the colony. For amylase activity, addition of lugol solution is required to reveal a positive reaction. Biochemical

characteristics and enzymatic profiles were studied in triplicate.

### Microtiter plate adherence assay

The ability of *A. hydrophila* to form a biofilm on an abiotic surface was determined, in triplicate, using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously (Chaieb et al. 2007; Mahdhi et al. 2010) with some modifications. Following overnight incubation at 37 °C, the optical density of the bacteria at 595 nm ($OD_{595}$) was measured. An overnight culture grown in TSB at 37 °C was diluted to 1/100 in TSBg (2 %, w/v glucose). A total of 200 μL of these cell suspensions was transferred to a U-bottomed 96-well microtiter plate (Nunc). The cultures were removed and the microtiter wells were washed twice with phosphate-buffered saline (PBS) (7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$ and 130 mM NaCl at pH 7.4) to remove non-adherent cells and the plates were then dried in an inverted position. Adherent bacteria were fixed with 95 % ethanol and stained with 100 μL 1 % crystal violet (Merck) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 μL sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 595 nm ($OD_{595}$) using an automated Multiskan reader (Gio. de Vita, Rome, Italy). Adhesion ability was interpreted as strong ($OD_{595} \geq 1$), fair ($0.1 \leq OD_{595} < 1$) or slight ($OD_{595} < 0.1$).

### Determination of morphological changes by AFM

In order to visualize any morphological changes in the stressed cells, *A. hydrophila* cells were examined, in triplicate, by AFM (Nanoscope IIIA, Digital Instrument, VEECO; http://www.veeco.com/). For the experiments, cells were collected, washed three times with PBS, and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and simply dried in air according to the method previously described (Braga and Ricci 1998; Ben Abdallah et al. 2007).

## Results

### Amplification of denitrification genes

First, experiments with control bacteria were performed in order to develop and optimize the conditions for PCR amplification of transcripts for the denitrification genes *narG*, *napA*, *nirS*, and *nirK*. The presence of the nitrate reductase gene (*narG*) and nitrite reductase (*nirS*) gene in *A. hydrophyla* was confirmed by PCR amplification from genomic DNA prior to analysis of transcripts. As shown in Figs. 1 and 2, *A. hydrophila* is positive for *nar*G giving a 500-bp band, and is also positive for *nir*K giving a 514-bp band; this confirms the denitrifying ability of this strain.

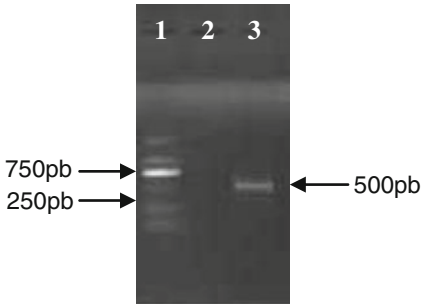


**Fig. 1** Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of the *nar*G gene. Lanes: *1* 250 bp DNA molecular size marker, *2* negative control, *3 Aeromonas hydrophila*

### MTT-assay for growth

A culture of *A. hydrophyla* grown for 7 days in TSB, and the same strain pre-incubated for 8 months in sea water, showed a difference in growth curve. The stressed strain appears to grow more slowly, as shown by the growth curve carried out with the MTT essay (Fig. 3).

### Enzymatic changes in starved *A. hydrophila*

The results of the API 20NE system showed some modifications in the biochemical profile of the *A. hydrophila* strain during their stays in seawater at room temperature and 4 °C. We noted that *A. hydrophila* becomes able to hydrolyze gelatin after 8 months of incubation and becomes β-galactosidase-negative at room temperature but not at 4 °C (Table 1).

Enzymatic characters studied in the Api-ZYM system showed that the investigated cells are able to assimilate the alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucosidase, α-mannosidase, and α-fucosidase before and

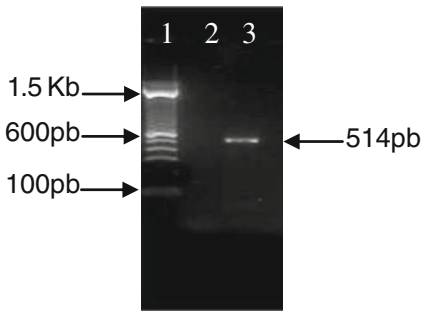


**Fig. 2** Agarose gel electrophoresis of PCR amplification of *nir*K gene. Lanes: *1* 100 bp DNA molecular size marker, *2* negative control, *3 A. hydrophila*

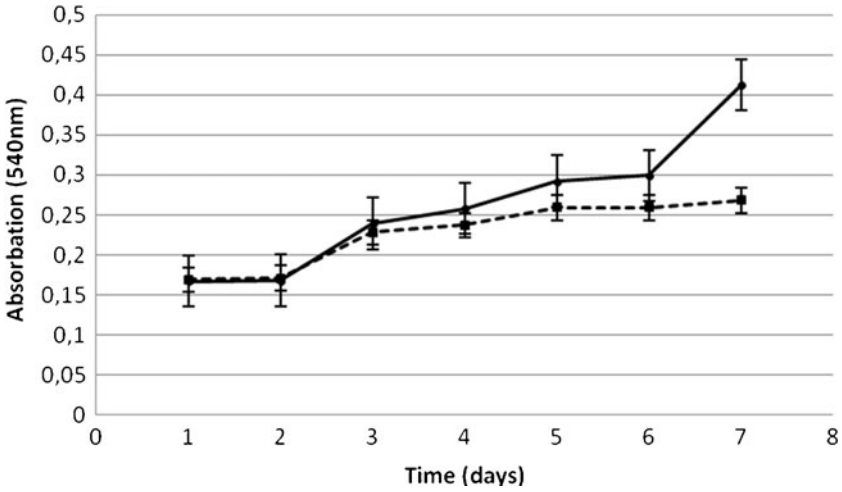


**Fig. 3** Growth curve of *A. hydrophila* with MTT assay. *Triangles* Fresh culture before incubation in seawater, *circles* after incubation for 8 months in seawater at room temperature

after incubation in seawater. After incubation for 8 months in seawater at room temperature, we noted that *A. hydrophila* becomes unable to produce leucine arylamidase (Table 2).

## Microtiter plate adherence assay

During cultivation studies, we observed a severe decrease in cellular aggregation of *Aeromonas* after 8 months of incubation in seawater microcosms. Thus, we investigated the effect that starvation stress in seawater had on biofilm formation in microtiter plate adherence assays (Table 3).

## Morphological variations of stressed strain

Alterations in cell morphology due to starvation stress in seawater were examined by AFM (Fig. 4). Control *A.*

**Table 1** Evolution of biochemical characters of *Aeromonas hydrophila* (Api 20NE system; bio-Merieux, Marcy l'Etoile, France)

| Enzymes | *A. hydrophila* | After 8 months of incubation in seawater | |
|---|---|---|---|
| | | Room temperature | +4 °C |
| $NO_3^-$ | + | + | + |
| TRP | − | − | − |
| Glu | + | + | + |
| ADH | + | + | + |
| URE | + | + | + |
| ESC | + | + | + |
| GEL | − | (+) | (+) |
| PNG | + | (−) | + |
| GLU | + | + | + |
| ARA | + | + | + |
| MNE | + | + | + |
| MAN | + | + | + |
| NAG | + | + | + |
| MAL | + | + | + |
| GNT | + | + | + |
| CAP | − | − | − |
| ADI | − | − | − |
| MLT | + | + | + |
| CIT | + | + | + |
| PAC | − | − | − |
| OX | + | + | + |

**Table 2** Modification of enzymatic profile of *A. hydrophila*

| Enzyme | *A. hydrophila* | After 8 months of incubation in seawater | |
|---|---|---|---|
| | | Room temperature | + 4 °C |
| Control | − | − | − |
| Alkaline phosphatase | + | + | + |
| Esterase (C4) | + | + | + |
| Esterase lipase (C8) | + | + | + |
| Lipase (C14) | + | (−) | + |
| Leucine arylamidase | + | (−) | (−) |
| Valine arylamidase | − | (+) | (+) |
| Cystine arylamidase | − | − | − |
| Trypsin | − | − | − |
| α-Chymotrypsin | − | − | − |
| Acid phosphatase | + | + | (−) |
| Naphthol-AS-BI - phosphohydrolase | + | + | + |
| α-Galactosidase | + | + | + |
| β-Galactosidase | + | + | + |
| β-Glucuronidase | − | − | − |
| α-Glucosidase | + | + | + |
| β-Glucosidase | + | + | + |
| N-Acetyl-β glucosaminidase | − | − | − |
| α-Mannosidase | + | + | + |
| α-Fucosidase | + | + | + |

**Table 3** Quantitative estimation of *A. hydrophila* biofilm formation on polystyrene microtiter plates

| | *A. hydrophila* | | After 8 months of incubation in seawater | |
|---|---|---|---|---|
| Mean $OD_{595}$ ± SD | 0.442±0.09 | (+)[a] | 2.710±0.28 | (++) |

[a] Strong (++); fair (+); slight (−)

*hydrophila* cells, whose length is 3–4 μm, have a rod shape, whereas, after 8 months of incubation in seawater microcosms, the cells adopt a coccoid shape form with a length of less than 2 μm.

## Discussion

The results in the present work showed that Gram-negative bacteria like *Aeromonas hydrophila* are able to adapt and survive under starvation conditions. Bacterial cells can sense and respond to changes in their external environment (Rosen et al. 2001). The ability of bacteria to sense and respond effectively to changes in the environment is crucial for their survival. In general, microorganisms do not respond to nutrient deprivation or starvation by simply arresting all metabolic activities and stopping growth. Many marine bacteria, especially *Aeromonas* spp., can survive for a long time under starvation conditions by sequential changes in cell physiology and gradual changes in morphology (Jiang and Chai 1996). Numerous studies have focused on the incidence of *Aeromonas* in the environment with regard to their role in public health (Hazen et al. 1978; Rippey and Cabelli 1989). Less is known concerning the overall physiology and ecology of the group. *Aeromonas* grow aerobically or anaerobically by fermentation (Farmer et al. 1992). Members of the genus reduce nitrate and nitrite. Nitrate respiration is supported by a typical membrane-bound NAR-type nitrate reductase and nitrite respiration is supported by a copper-containing nitrite reductases. The PCR products were the expected sizes (approximately 500 bp for *narG* and 514 bp for *nirK*) and, in most cases, sequencing or Southern hybridization confirmed that they were indeed the expected products (Nogales et al. 2002).

Our results showed that the stressed strain appears to grow more slowly, as shown by the growth curve carried out with the MTT assay as well as variations in the

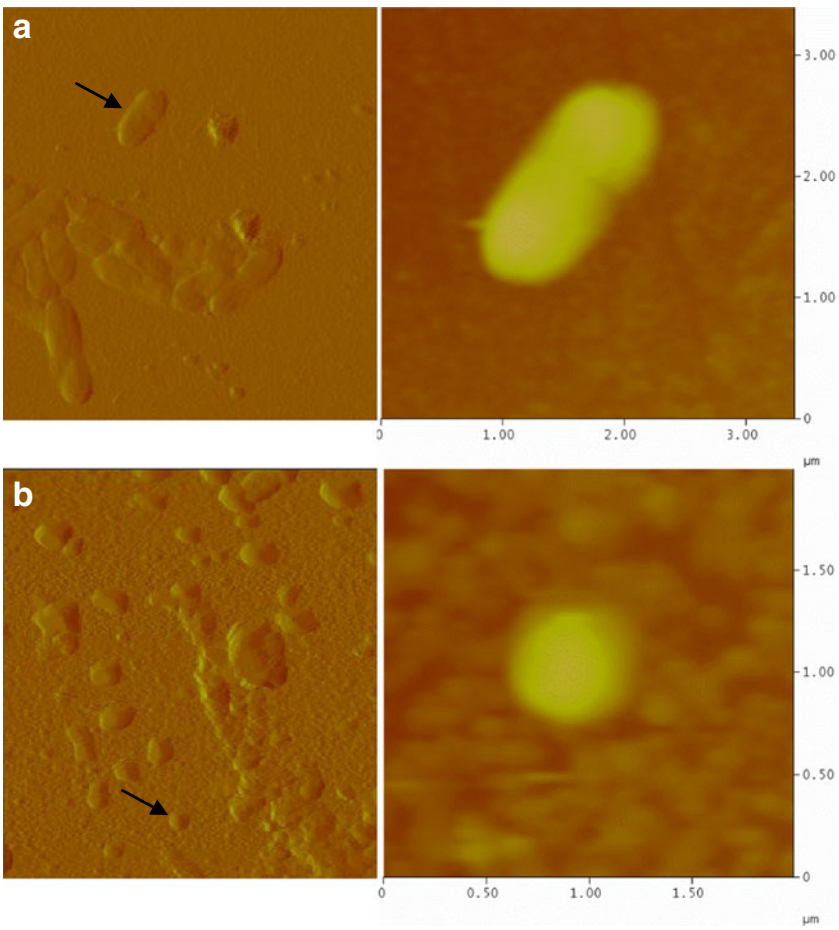


**Fig. 4a,b** Atomic force micrographs of *A. hydrophyla* cells exposed to starvation for 8 months in seawater. **a** Strain before incubation in seawater. **b** Strain after incubation for 8 months in seawater at room temperature

biochemical characteristics after 8 months in seawater. Indeed, this difference between the normal and starved cells is probably caused by the evolution of bacterial strains to the viable but non cultivable (VBNC) dormant state and the dramatic change in the physiology and morphology of strains after 8 months in seawater (Torella and Morita 1981; Jiang and Chai 1996). Various studies have indicated that growth conditions, such as the composition of the growth medium, the growth phase of the cells, incubation temperature, and the pH value (Russell et al. 1995; Wang et al. 2005) markedly affect the bacteria. The biochemical modifications observed in the starved strains are probably due to the osmolarity and/or oligotrophy of the medium. These modifications attest to the existence of physiological changes in *A. hydrophila* cells after this period of stress (Ben Abdallah et al. 2007). These new enzymatic activities are the result of the expression of certain genes involved in the survival of these germs under the stressing conditions of the marine environment (Gauthier et al. 1988). According to Costerton and Cheng (1975), the appearance of new enzymatic activity in stressed bacteria cells is due to starvation, which makes the bacteria able to change its nutrient pathways. So, the appearance of gelatinase activity in *Aeromonas hydrophila* is caused by starvation, which makes the bacteria able to change pathways of glucose use. The paucity of food in seawater can also lead to the loss of some features, either by repression of specific enzymes or following modifications at the bacterial-wall level.

Our findings show that starvation can influence significantly the capacity of *Aeromonas* cells to aggregate. Indeed, characteristics of the suspension medium such as pH, osmolarity and temperature are considered to be important factors in altering the physicochemical properties of a bacterial surface (Hamadi et al. 2004). Similar results have been found with *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* under different environmental conditions. Furthermore, the surface charges and hydrophobicity of bacteria were influenced by the environmental conditions (Briandet et al. 1999), in fact explaining the variation in bacterial capacity to aggregate. In addition, this change is probably caused by the change in the bacterial membrane fatty acid composition. Indeed, little is known about the influence on bacterial resistance to subsequent stresses of these modifications in membrane composition. One of the most important consequences of membrane fatty acid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukanaga 1990).

The atomic force micrographs revealed a reduction in the *Aeromonas* cell size caused by starvation in seawater. According to Morita (1993), several bacteria such as *Aeromonas*, can survive for long periods under stressing environmental conditions owing to the gradual changes in cellular physiology and morphology. The reduction in bacteria size, such as that seen in *Aeromonas*, during stress is a survival strategy (Jiang and Chai 1996). To confront nutrient limitation, bacteria may develop defense mechanisms to enhance their ability to survive periods of starvation. Some differentiating bacteria respond to starvation by marked alterations in their ultrastructure, producing spores or cysts.

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