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Effect of low-nutrient seawater on morphology, chemical composition, and virulence of *Salmonella typhimurium*

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Abstract The response of *Salmonella typhimurium* to low nutrient levels was determined by measuring the concentrations of lipids, carbohydrates, DNA, RNA, and proteins over a 32-day starvation period. Ultrastructural integrity was observed by transmission electron microscopy. Lipid and carbohydrate content of bacterial cells rapidly declined within the first 16 days, while DNA and proteins exhibited a more gradual decline over the 32 days of starvation. In contrast, RNA content did not decrease appreciably upon nutrient starvation. Structural damage occurred especially after 16 days of starvation. After 32 days of nutrient deprivation, we recorded degenerative cellular forms, a coccoidal cell shape, a decrease in cellular volume, and the loss of the three-layered outer membrane. The morphological and structural alterations correlated with virulence in infected animals. We observed a decrease in virulence of *S. typhimurium* after 9, 16, and 32 days of starvation, reaching a maximal decrease after 32 days of nutrient deprivation. The decrease in virulence correlated to surface hydrophobicity alterations, adherence to eukaryotic cells, and phagocytosis.

Key words Bacterial starvation · Seawater · *Salmonella typhimurium* morphology · Surface hydrophobicity Virulence

Abbreviations BHI brain heart infusion · *PBS* phosphate-buffered saline · *TE* Tris-EDTA buffer · F_t phagocytic index · K_t Killing index

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Introduction

While much is known about the survival of marine bacteria under poor growth conditions (Hood and Ness 1982; Amy and Morita 1983a, b; Baker et al. 1983; Harder and Dijkhelzen 1983, Oliver and Stringer 1984; Nystrom et al. 1988; Oliver et al. 1991), information concerning survival under poor growth conditions of bacteria from other habitats, such as the mucosal surface of higher organisms, is very scarce. Gastrointestinal bacteria from both humans and domestic animals are frequently found in sea water; contamination with particular enteric bacilli varies in the geographic region in the world.

It is well-known that the survival of a marine bacterium would be expected to depend on its ability to express specific sets of genes resulting in a phenotype appropriate for a particular circumstance. For example, the response of the marine *Vibrio* strain S14 during transition from growth to multiple-nutrient starvation is complex and includes a time-dependent series of molecular and physiological rearrangements accompanied by a sequential synthesis of starvation-inducible proteins (Oliver and Stringer 1984; Marden et al. 1985; Nystrom et al. 1988, 1990, 1992).

Further data has recently been gathered for bacteria connected with higher organisms; *Vibrio cholerae,* for example, is able to survive long periods of low-nutrient stress upon nutrient deprivation in a closed system, showing an initial increase in viable cells, followed by a decrease in the viable population until a constant level is reached (Amy and Morita 1983b). The data suggest that certain lipids and carbohydrates may provide the endogenous energy sources needed for domancy preparation and cell maintenance under nutrient starvation. It has been shown that *Escherichia coli* is able to synthesize new proteins that enhance its survival (Reeve et al. 1984); these proteins enable the cells to shift into a starvation-induced maintenance physiology. In *V. cholerae,* these synthesized proteins also enhance survival (Hood et al. 1986). Studies of the survival process in carbol-starved *E. coli* (Groat et al. 1986) and phosphate-starved *S. typhimurium* (Foster

and Spector 1986) have also revealed a short temporal expression of starvation-specific proteins.

Biological environmental pollution poses problems for all advanced societies; many of these problems have been well defined and are under investigation, but most of them are still undefined and require further study. The waters that surround highly populated areas present a particularly dramatic situation. Contamination of the waters with physical, chemical, and especially biological pollutants often raises a serious health hazard. One identifiable problem, within the large scope of water pollution, is that immediately surrounding the coasts of densely inhabitated centers, sewage contains a large quantity of biological pollutants.

The increasing number of higher organisms necessarily involves an increase in the microbic load on the environment, particularly in seawater. This raises the need, therefore, for more detailed studies on the possible risks of infection from pathogenic microorganisms that can be transmitted through these waters.

Little is known about changes in the interactions between human host cells and microorganisms under starvation (Galdiero et al. 1993). In this work, we examined the survival rate, morphological modifications, and chemical composition of the pathogenic bacterium *S. Typhimurium* and the successive interaction with the host organism after starvation.

Materials and methods

Microorganisms and culture conditions

S. typhimurium strain 74 NCTC was grown in brain-heart infusion (BHI) broth at 37° C with constant agitation. The cells were harvested in the logarithmic growth phase, centrifuged, washed three times in distilled water, and resuspended in 10 ml sterilized seawater. Cells were inoculated (10^7 cells/ml) into sterile 2-l flasks containing 1 l seawater and incubated at 22° C in the dark. At 0, 3, 6, 9, 16, and 32 days after inoculation, cells were counted by standard plate counting, acridine orange direct counting (Hobbie et al. 1977), and direct viable counting (Kogure et al. 1979). *S. typhimurium* cells were harvested by centrifugation for chemical and biological analyses.

Electron microscopy

Cells in suspension were fixed in 2.5% glutaraldehyde in 0.002 *M* phosphate-buffered saline (PBS) with 0.01% $CaCl_2$ to prevent cell rupture, for 1 h at room temperature. They were then post-fixed in an aqueous solution of OsO_4 (1%) for 90 min, dehydrated in a graded series of ethanol and embedded in TAAB 812 resin. Ultrathin sections cut on an ultramicrotome (LKB ultrotome NOVA) were collected on uncoated copper grids, stained with aqueous uranyl acetate and lead citrate according to standard procedures, and observed in a Zeiss EM 109 electron microscope at an accelerating voltage of 80 kV.

Analytical methods

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Quantitative analysis of neutral sugar was determined by the phenol-sulfuric acid method of Dubois et al. (1956), which utilizes phenol as the specific organic color developing agent. Total lipids were determined by the sulfophosphovanillin reaction (Boehringer Mannheim GmbH).

RNA was extracted by a procedure based on the methods of Bolton (1966), Kirby (1968), and Johnson (1981). Briefly, cells were washed in Tris-EDTA buffer (TE; Tris-HCl 0.01 M, EDTA 0.001 M, pH 7.5) and were resuspended after centrifugation in 0.05 *M* Tris-HCl plus 25% sucrose, pH 7.5. They were then lysed by adding lysozyme (10 mg/ml) and an EDTA-free detergent [TritonX 100 (10%) in Tris-HCl 1 *M*, pH 8]. To eliminate contaminating RNases, ribonucleoside vanadyl complexes (Sigma) were added to a final concentration of 10 mm. After centrifugation, the supernatant was recovered and 50 µg DNaseI was added. After incubation at 37° C for 20 min, sodium acetate (3.3 *M* and an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2, v:v:v) saturated with 50 m*M* Tris-HCl (pH 7.5) was added and mixed and the samples were centrifuged at 8 000 × *g*.

RNA was precipitated from the aqueous phase by addition of absolute ethanol and incubation at –20° C overnight. Purity of the RNA was determined by the ratio of A_{260} to A_{280}. Absorbance was measured with a Pharmacia LKB Ultra Spec 3 spectophotometer, and the amount of RNA was calculated by comparison with a standard curve.

DNA was extracted according to Marmur (1961). The DNA pellet was washed twice in PBS resuspended in TE containing lysozyme (10 mg/ml) and sucrose (1 *M* final concentration) and was incubated in a 37° C water bath for 1 h. After centrifugation, the pellet was resuspended in TE containing pronase (20 mg/ml) and incubated for 30 min in a 37° C water bath. After incubation, 0.5 *M N*-lauroylsarcosine was added and the sample was incubated for 30 min at 37° C. The same incubation was carried out after adding RNase (10 mg/ml). DNA was extracted by washing with phenol-chloroform (1:1, v:v) and precipitated by incubating overnight at –20° C. DNA purity was determined by comparison of the absorbance at 280 and 260 nm.

Adherence to eukaryotic cells

Monolayers of Hep2 cells were grown on 6 × 10 mm slides in a 24-well microplate (Falcon). Each well contained 1 ml cell suspension (6 × 10^5 cells/ml) in Eagle's medium. The slides were incubated for 48 h at 37° C in 5% CO_2. Before inoculation, the cells were washed three times with 0.002 *M* PBS. Cell cultures were inoculated with 0.2 ml bacterial suspension (3 × 10^8 bacteria/ml as measured by a standard plate count) to obtain a final concentration of about 5 × 10^7 bacteria/well. Slides were incubated for 3 h at 37° C in 5% CO_2, and were then washed three times with PBS to remove non-adherent bacteria. The cells were fixed by incubating overnight with 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) containing 0.1 *M* sucrose, and were then washed three times with the same buffer. They were stained for 5 min in acridine orange (50 mg/l) in cacodylate buffer (0.1 *M*), and were observed by fluorescence microscopy (Leitz Dialux 22/22 EB with a 120 Z mercury lamp, a BG 30 excitation filter and a KP 500 suppression filter).

An adhesion index was calculated on the percentage of Hep2 cells with more than 50 adherent bacteria.

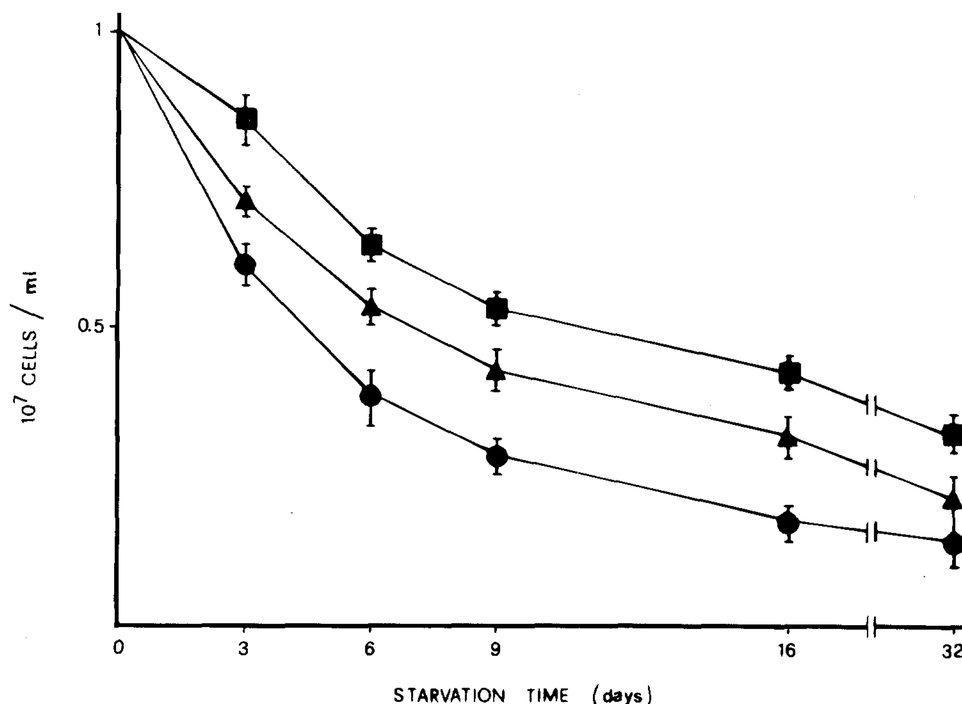
Aggregation test

To detect aggregates, Lijungh's technique was used (Lijungh et al. 1985). Briefly, bacteria were washed three times in PBS and resuspended in the same buffer to obtain a final concentration of 10^8 cells/ml as measured by a standard plate count. This suspension (50 µl) was mixed with an equal volume of a solution of ammonium sulfate at various molarities (0.2 *M*, 1.8, and 3.2 *M*). The presence or absence of aggregates was checked after agitating the sample for 1 min at room temperature.

Chromatographic-hydrophobic interaction

To detect hydrophobic interactions, the technique of Tylewska et al. (1979), was used. Briefly, Pasteur pipettes were filled with

Fig. 1 Effect of nutrient deprivation on *Salmonella typhimurium* cell number. ● Standard plate counts; ▲ direct viable counts; ■ acridine orange direct counts



phenyl-sepharose CL-4B (Pharmacia AB Laboratory Separation Division, Uppsala) up to 30 mm (0.6 ml gel). The columns were then balanced with 8 ml double-distilled water or with 1 *M* ammonium sulfate (pH 7); 100 µl of the bacterial suspension (2×10^{12} cells/ml as measured by a standard plate count) was washed three times with water or with ammonium sulfate solution, placed onto the gel, and eluted with the same solution.

Phagocytosis

Peritoneal macrophages from the Morini mice strain were used. Peritoneal cells were collected with sterile PBS, concentrated by centrifugation, and washed three times in RPMI 1640 medium (Labtek Laboratories, Eurobio, Paris).

Cell viability was evaluated with the trypan blue exclusion test. Cells were suspended at a concentration of 2.5×10^6 cell/ml in RPMI 1640.

Since opsonic serum was used, inactivated pig serum (heated at 56°C for 30 min) was added to bacterial suspensions and incubated at 37°C for 30 min. For phagocytosis measurements, 2.5×10^6 peritoneal macrophages and 1×10^6 bacteria (as measured by standard plate count) were mixed. The bacteria-cell suspension was incubated for up to 60 min at 37°C with constant shaking.

The phagocytic index (F_t), according to Van Zwet et al. (1975) is defined as the decrease in the number of viable bacteria in the supernatant during a given interval and calculated according to the equation $F_t = \log N_{t=0} - \log N_t$, where *N* is the number of viable bacteria in the supernatant and *t* is time.

For the determination of the intracellular killing of microorganisms by peritoneal macrophages, the cells, obtained after centrifugation at $110 \times g$, were distrupted by freezing in ice-cold distilled sterile water. The killing index (K_t) is defined as the decrease in the number of viable intracellular microorganisms during a given interval and calculated according to the equation $K_t = \log N_t = 0 \log N_t$, where *N* is the number of viable mircroorganisms in the peritoneal macrophages and *t* is time.

Virulence

Experimental infection in mice was induced by intraperitoneal inoculation of 8×10^5 cells of *S. typhimurium*/mouse as measured by standard plate count. Mortality in infected mice was monitored over the next 7 days and compared to control mice kept under the same environmental conditions.

Statistics

All experiments were carried out in triplicate; results were expressed as mean ± SD. Comparisons between tests were done by Student's *t*-test with statistical significance considered to be $P < 0.05$.

Results

Cell viability

We observed a progressive decrease in viable cell numbers beginning 3 days after nutrient deprivation, and progressively reaching the lowest point after 32 days (Fig. 1). At this time there were nearly four times fewer viable cells (direct viable count).

Morphology

Transmission electron micrographs of ultrathin sections of cells suspended in seawater showed remarkable differences in cell morphology compared to cells cultured in BHI broth (Fig. 2A). After 6 days of starvation, the cells were reduced in size, the cell wall was little distorted, and the cytoplasm was not as dense as that of nourished cells. Variation in the appearance of nuclear material was also observed. The nuclear area contained fine and coarse, often branched, filaments with amorphous electron-dense material (Fig. 2B). The outer membrane appeared to be more wrinkled after 16 days of starvation than after 6 days

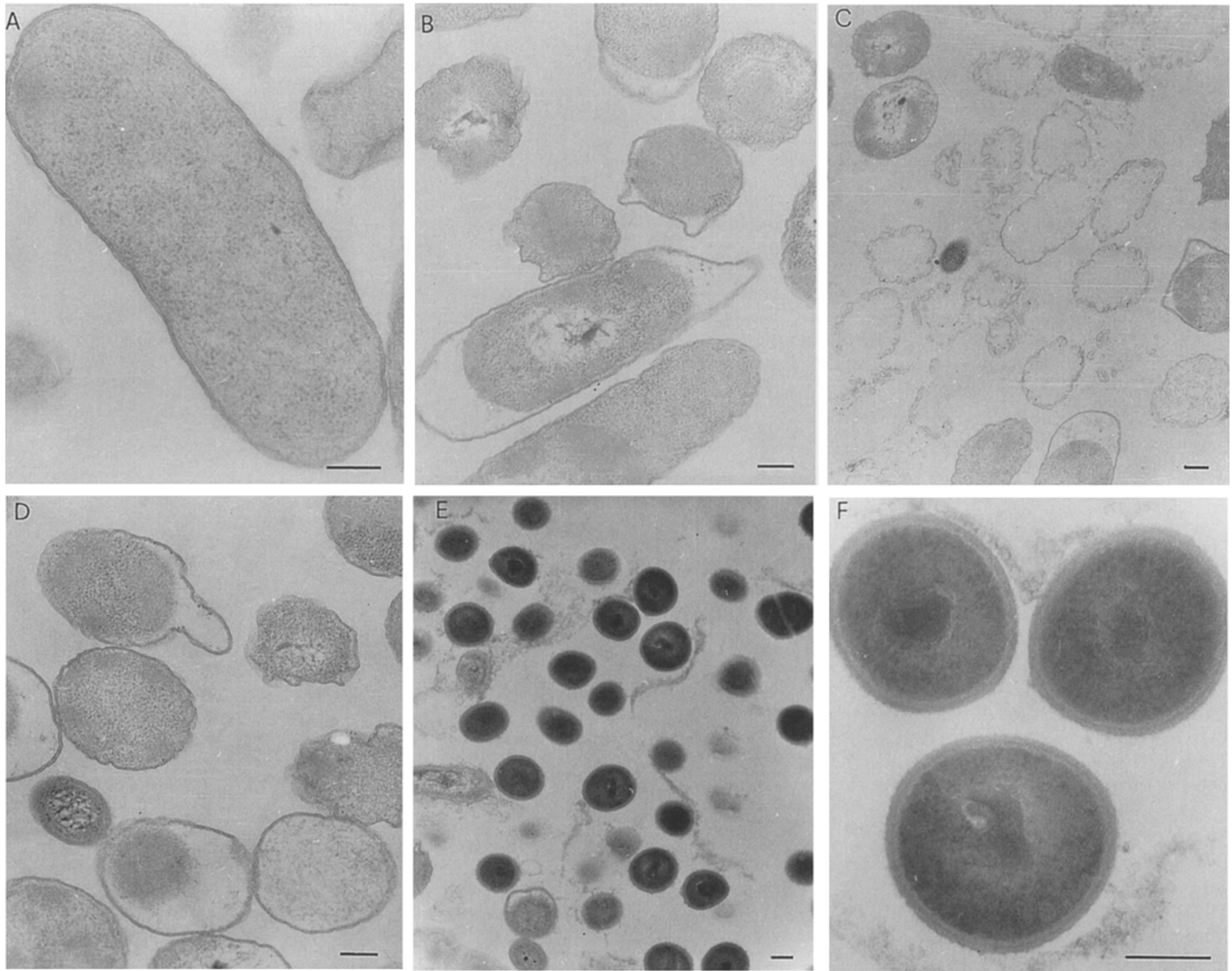


Fig. 2 Transmission electron micrographs of ultrathin sections of *Salmonella typhimurium*. **A** Non-starved *S. typhimurium* cell, *bar* 0.2 μm. **B** *S. typhimurium* cells after 6 days of starvation, *bar* 0.2 μm. **C–D** *S. typhimurium* cells after 16 days of starvation, *bar* 0.2 μm. **E–F** *S. typhimurium* cells after 32 days of starvation, *bar* 0.2 μm

(Fig. 2C). Electron microscopy of the cells after 6 and 16 days of starvation conditions also revealed an enlarged periplasmic space, sometimes with a low electron density. After 16 days of starvation, *S. typhimurium* cells exhibited major structural damage; spheroplastlike bodies that appeared to have lost most of the cytoplasmic constituents were present. Free vesicles, presumably *S. typhimurium* membrane debris, were also frequently observed (Fig. 2D). After 32 days of starvation, ultrathin sections revealed vescicles as well as other degenerative cellular forms (Fig. 2E). Many cells became coccoid and decreased in volume, all granules and inclusion bodies were lost and the nuclear region (electronuclear area) was compressed into the center of the cell surrounded by a denser cytoplasm (Fig. 2F).

Biochemical analysis

The response of the cells with regard to total carbohydrate, lipid, and protein content followed a similar pattern (Table 1). After 6 days of starvation 68.2% of the protein, 83% of the carbohydrate, and 86% of the total lipid had disappeared. These parameters continued to decrease until day 16, reaching their lowest point on day 32.

DNA concentration exhibited a gradual but constant decline over the 32-day starvation period. By day 32, approximately 75% of the total DNA had disappeared.

In contrast to DNA, RNA levels changed very little upon starvation. For example, after 6 days there was only a 2% decrease in concentration, after 16 days only a 4% decrease, and after 32 days, a 20% decline was observed.

Hydrophobicity

Surface hydrophobicity of the bacterial suspension was evaluated by absorption on phenyl-sepharose CL-4B columns (Table 2). A high ionic strength solution was

Table 1 Changes in quantities of *Salmonella typhimurium* components during starvation in seawater

Length of starvation	Carbohydrates (fg/cell)	Lipids (fg/cell)	Proteins (fg/cell)	DNA (fg/cell)	RNA (fg/cell)
0	4150 ± 200	2700 ± 160	1.36×10^5 ± 360	10 ± 0.316	27.5 ± 5.2
3 days	1170 ± 100	1800 ± 130	4320 ± 200	10.5 ± 0.324	27.1 ± 5.1
6 days	710 ± 80	400 ± 60	1170 ± 100	6.7 ± 0.258	26.9 ± 5.1
9 days	520 ± 70	400 ± 60	1030 ± 100	5.2 ± 0.228	26.8 ± 4.9
16 days	350 ± 50	400 ± 58	980 ± 90	2.63 ± 0.162	26.3 ± 4.8
32 days	80 ± 20	350 ± 50	750 ± 60	2.48 ± 0.157	21.9 ± 4.6

Table 2 Surface hydrophobicity of *Salmonella typhimurium* cells after nutrient deprivation evaluated by adsorption test on phenyl-sepharose and by aggregation test in ammonium sulfate. – Absence of aggregation; ± presence of some small aggregates; + presence of visible aggregation; ++ aggregation with formation of large aggregates

Length of starvation	Adsorption on phenyl-sepharose[a] (%)	$(NH_4)_2SO_4$		
		0.2 *M*	1.8 *M*	3.2 *M*
0	60 ± 3	+	++	++
3 days	45 ± 2	+	+	+
6 days	43 ± 1	+/–	+/–	+
9 days	40 ± 3	+/–	+/–	+/–
16 days	38 ± 2	+/–	+/–	+/–
32 days	35 ± 1	–	–	+/–

[a] Bacterial suspensions eluted with distilled water showed no adhesion to phenyl-sepharose

used to promote the hydrophobic interaction and adherence between the bacteria and the gel. The pH was kept near neutrality to neutralize the effect of the hydrogenionic concentration on the hydrophobic interaction. The suspensions sampled at days 3, 6, 9, 16, and 32, by elution with distilled water, did not interact with phenyl-sepharose, while those eluted with 1 *M* ammonium sulfate showed a gradual decrease in adsorption of bacteria to the gel, compared to the values obtained with suspensions at time 0. After 3 days of starvation, the bacteria showed a 25% decrease in adsorption which continued progressively over time to reach a value of 41% after 32 days.

Surface hydrophobicity was also measured using the aggregation test in solutions of $(NH_4)_2SO_4$ (Table 2). Bacteria that aggregate in 0.2 to 1.8 *M* ammonium sulfate buffer are considered to have a highly hydrophobic surface, while cells with lower hydrophobicity aggregate in higher concentrations of ammonium sulfate. The results indicate a decrease in surface hydrophobicity. Table 2 shows that the longer *S. typhimurium* is in seawater, he higher the $(NH_4)_2SO_4$ concentrations necessary for aggregation.

Adherence to eukaryotic cells

Adhesion tests of *S. typhimurium* suspensions at time 0 and after 3, 6, 9, 16, and 32 days of nutrient deprivation indicated that after starvation, *S. typhimurium* cells were significantly less adhesive than control *S. typhimurium* cells. In fact, the percentage of adhesive cells progressively decreased, reaching a maximal decrease of 40% after 32 days of starvation (Fig. 3).

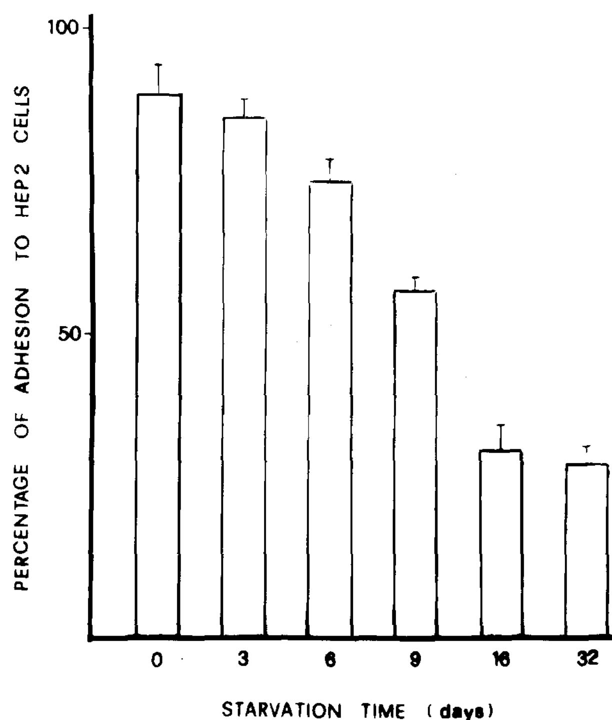


Fig. 3 Percent adhesion of *Salmonella typhimurium* cells after starvation to Hep2 cells. Results are the mean of three individual experiments ± SD

Phagocytosis and intracellular killing

Table 3 reports the susceptibility to phagocytosis by peritoneal macrophages of bacteria suspended in seawater for 3, 6, 9, 16, or 32 days, compared with that of *S. typhimurium* grown in BHI broth. The phagocytic index (F_t) of bacteria suspended in seawater progressively increased, as compared to *S. typhimurium* grown in BHI broth, reaching a maximum value (1.024) after 32 days of contact with seawater, indicating that bacteria left in contact with seawater are more susceptible to phagocytosis by peritoneal macrophages.

Table 3 also summarizes the results of intracellular killing by peritoneal macrophages of bacteria left in seawater for 3, 6, 9, 16, and 32 days, as compared to *S. typhimurium* grown in BHI broth. The killing index (K_t) values also progressively increased when the microorgan-

Table 3 Phagocytosis and intracellular killing by peritoneal macrophages of *Salmonella typhimurium* after nutrient deprivation in seawater. The values are the mean of three experiments

Length of starvation	Phagocytic index	Killing index
0	0.403	0.137
3 days	0.489	0.227
6 days	0.545	0.361
9 days	0.766	0.395
16 days	1.002	0.524
32 days	1.024	0.534

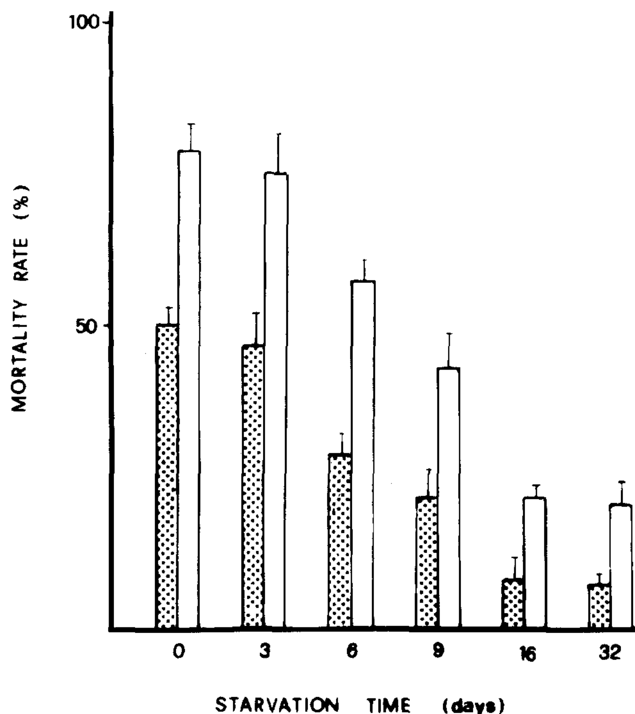


Fig. 4 Mortality of mice 2 (▒) and 7 days (□) after the injection of *Salmonella typhimurium* cells subjected to various starvation periods. Results are the mean of three individual experiments ± SD

isms were left in contact with seawater, reaching a maximum value (0.634) after 32 days.

Virulence

Animals were infected by intraperitoneal inoculation of 8×10^5 *S. typhimurium* cells. Animals infected with *S. typhimurium* at time 0 showed a 50% mortality within 48 h, reaching 80% after 7 days of inoculation. The contact of the bacteria with seawater reduced the mortality of infected animals (Fig. 4). Animals infected with *S. typhimurium* that had been deprived of nutrients for 6 days, had a mortality of 30% after 48 h, and 50% after 7 days. This reduced pathogenicity was observed with *S. typhimurium* also after 9, 16, and 32 days of starvation, reaching maximal reduction after 16 days of nutrient deprivation. The infected animals had a mortality of 10% after 48 h, and 30% after 7 days.

Discussion

In this study, we approached the problem of the persistence of virulence in *S. typhimurium* cells suspended in seawater for up to 32 days. The results showed that already after 6 days, the bacterial cells lose their surface hydrophobicity and become more susceptible to phagocytosis by peritoneal macrophages. A decrease in hydrophobicity and, therefore, in adhesiveness, generally leads to a greater resistance to phagocytosis (Van Oss et al. 1975). We can hypothesize that the structural alterations observed reveal hydrophilic components specific for the appropriate receptors on the surface of the macrophages.

After about 16 days, the capacity to induce experimental infection in the mouse was dramatically reduced. Even an extremely high bacterial load did not lead to experimental infection. It is highly improbable that the elevated bacterial loads used in our study could be found in even the most polluted waters. The decrease in virulence of *S. typhimurium* cells suspended in seawater was accompanied by notable alterations, both in chemical composition and in cell structure.

The cellular content of proteins, lipids, and carbohydrates underwent a drastic reduction within even a few days of contact with seawater. After 6 days of starvation, 68.2% of the protein, 83% of the carbohydrate, and 86% of the total lipid had disappeared. These parameters continued to decrease until day 16, reaching their lowest point on day 32.

It has been proposed that cells do not merely passively, go into dormancy, but rearrange their cellular constituents into different compounds so that survival is enhanced (Amy and Morita 1983b), and there have been a number of studies that have examined compositional changes in aquatic bacteria exposed to low nutrients. For example, when the marine Vibrio ANT-300 was starved for 21 days at 5° C, phospholipids decreased by 65% (Oliver and Stringer 1984). In *Vibrio cholerae,* phospholipids declined 99.8% during the first week of starvation at 22° C and total carbohydrates also exhibited a rapid and immediate decline (Hood et al. 1986). The rapid disappearance of lipids and carbohydrates in *V. cholerae* suggest that they are used as an endogenous energy source to prepare the cell for dormancy, a strategy for a cell to use more energy-efficient available compounds to carry out these activities.

During the first 16 days, DNA degradation was relatively rapid, followed by a slower progressive degradation. This decrease may reflect a decreased rate of cell division and, therefore, a reduction in DNA synthesis since the values obtained are all normalized to the same number of total cells.

RNA levels, on the other hand, are not substantially modified during starvation and only show a significant decrease after 32 days. This finding correlates with other reports on *E. coli, S. typhimurium* and marine *Vibrio* species, showing the need for continued transcription and protein synthesis for bacterial survival (Groat et al. 1986; Galan and Curtiss 1990; Nystrom et al. 1990).

Substantial morphological differences were observed between the cultures of *S. typhimurium* grown in BHI broth and cells suspended in seawater. During starvation the cell envelope structure became less rigid, leading to changes in cell shape and size that might account for the pleomorphism. Electron microscopy of the cells after 6 and 16 days of starvation conditions also revealed an enlarged periplasmic space. The cytoplasmic membrane, partially pulled away from the rigid cell wall, maintained several points of contact with the murein layer and outer wall. The morphology of the nuclear region was grossly altered. The chromosomal material, found in both normal and starved cells predominantly concentrated in the central portion of the cell, contained large envelope fragments in the starved cell and was surrounded by a denser cytoplasm.

In addition, we observed the presence of some coccoidal cells after 32 days of starvation. This morphological rearrangement of *S. typhimurium* is an interesting consequence of this starvation period and was also observed in *V. cholerae* cells after nutrient deprivation (Hood et al. 1986). The results obtained bring to light an interesting correlation among morphological alterations, chemical composition modifications, and virulence of *S. typhimurium* cells.

Since salmonellosis is a gastrointestinal disease, virulence depends on the survival of bacterial cells in the transit through the mouth and the highly acidic stomach. The morphological and structural modifications observed, and the resulting alterations in virulence, lead us to hypothesize that cells staying in seawater for a considerable length of time no longer have the capability to withstand the gastric "barrier."

If extended and completed for other pathogenic microbial species, these results could further amplify the discussion on acceptable limits of bacterial loads in the waters of our environment.

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