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Structural Changes During Lysis of a Psychrophilic Marine Bacterium[1]

J. Y. D'AOUST AND D. J. KUSHNER
Department of Biology, University of Ottawa, Ottawa, KIN 6N5, Ontario, Canada

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The marine psychrophile, a red, gram-negative motile rod with a single polar flagellum, is stable when suspended in 0.1 M Mg^{2+} plus 0.5 M NaCl at 0 C and neutral *p*H but lyses if the salt composition of the medium is changed, the temperature raised above 20 C, or the *p*H lowered. Lysis is accompanied by a fall in turbidity, a release of ultraviolet-absorbing substances, and a loss of deoxyribonucleic acid and ribonucleic acid. Ultrastructural changes accompanying lysis were studied. Thin sections of cells fixed while intact showed a triple-layered cell wall and cytoplasmic membrane, each 6.0 to 7.5 nm thick. Mesosomes were also observed. Either Na^+ or Mg^{2+} could maintain wall integrity, whereas Mg^{2+} was needed for membrane integrity. In distilled water, lysis was very extensive, and much material was released as wall fragments and as vesicles which probably came from the wall and cytoplasmic membrane. Lysis at 37 C resulted in degradation of the wall and liberation of wall fragments. The cell membrane was rarely observed as a triple-layered structure in such temperature-lysed cells. After lysis at *p*H 5.0, the cell wall was distorted, and only a suggestion of the cell membrane remained. Replicas showed that this organism had a matted surface which was distorted under different conditions of lysis.

Previous work from our group was concerned with the physiology of a red-pigmented psychrophilic marine bacterium, NRC 1004 (11, 14, 18). This short, gram-negative motile rod grows in a temperature range of 0 to 19 C. At higher temperatures, death and lysis occur rapidly (11), accompanied by a release of hexosamines and a degradation of lipid phosphorus, presumably through the action of a cell-bound phosphatidase (11, 14). Cells are also lysed by low ionic strength or acid *p*H values. The surface effects of different kinds of lysis were studied by microelectrophoresis (18).

This organism differs from certain other marine bacteria in its ionic requirements for stability. Monovalent salts or sucrose can maintain the stability of other marine bacteria, whereas the red psychrophile also needs divalent cations. Though certain divalent cations can maintain the stability of this organism, it needs both NaCl (0.5 M) and Mg^{2+} ions (0.1 M) for growth (14, 17).

We present here the results of an electron microscope study of the relationships between ionic requirements for stability, chemical changes occurring during lysis, and alterations in cellular fine structure.

MATERIALS AND METHODS

Cells were grown in shaken cultures at 10 C in a medium containing 0.5 M NaCl, 0.04 M $MgCl_2$, 0.04 M KH_2PO_4, 10^{-8} M $FeCl_2$, 1.5×10^{-4} M $CaCl_2$, and 0.8% (w/v) tryptone; *p*H 7.0. After 48 to 60 hr of growth, the bacteria were harvested by centrifugation, washed once, and suspended in an "all salts" solution (growth medium without the nutrients).

Fixation in 2% glutaraldehyde (made up in "all salts" solution) and postfixation in buffered 1% OsO_4 (Michaelis acetate-Veronal buffer, *p*H 6.3) followed by staining in 1% aqueous uranyl acetate did not satisfactorily show cell wall or cytoplasmic membrane morphology. Several combinations of fixatives, stains, and buffering systems were tested; best results were obtained when washed cells were fixed with 4% formalin, (made up in "all salts" solution) for 5 hr at 10 C. Cells were then stabilized with 1% buffered uranyl acetate (Michaelis acetate-Veronal buffer, *p*H 6.1) for 1 hr at 10 C. Dehydration was carried out in a graded series of alcohols. The specimen was then infiltrated in a 1:1 (w/v) mixture of Epon 812 epoxy resin and 100% acetone for 1 hr and subsequently polymerized at 55 C for 2 to 3 days in freshly prepared resin.

Preparation of samples for surface replication was carried out according to a technique elaborated by

[1] A preliminary report of some of these findings was presented at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

Kushner and Bayley (15), with the following modifications. The cell sample was smeared on a precooled glass slide and dried in vacuo at 10 C. The slide mount was immediately shadowed with platinum-carbon and strengthened by a film of evaporated carbon. The replica was floated onto a water surface, placed on a Formvar-carbon-coated grid and permitted to air dry. Addition of hydrofluoric acid was not needed to float replicas off slides (15); occasionally the replicas were freed of organic matter before electron microscopic observation by treatment with H_2O_2.

For negative staining, a loopful of young cells (36-hr culture) was spread on a precooled Formvar-carbon-coated grid. After removal of excess liquid with filter paper, a loopful of cold aqueous 2% ammonium molybdate, *p*H 7.1, was applied to the grid and dried in air.

To test the effects of ions on lysis and cell ultrastructure, 0.1-ml samples of a thick cell suspension were suspended in 9.9 ml of water or aqueous solutions of salts at specified concentrations. The cells were permitted to equilibrate to a constant turbidity (absorbance at 660 nm); 2 hr was usually required. The cells were then sedimented at 15,000 $\times$ *g* for 15 min, and the absorbance of the supernatant fluid was read at 260 nm. Cell pellets were fixed and embedded as described above. Lysis was also induced by exposing washed cells to acidic *p*H ("all salts" solution, *p*H 5.0, 7 C) and high temperature ("all salts" solution, *p*H 7.0, 37 C). After suspensions had reached constant turbidity (usually 2 hr), suspensions were centrifuged as above, and pellets were fixed and embedded.

Studies were also made of the effects of distilled water, high temperature, and low *p*H on surface structure. After the turbidity became constant, samples for surface replication were taken directly from the treated suspension without centrifuging.

Analytical methods. Dry weight of cells was measured turbidimetrically by using a standard curve relating salt-free dry weight to absorbance at 660 nm. Salt-free dry weight was determined by drying a thick suspension in "all salts" solution to constant weight at 105 C and subtracting the weight of a separately dried sample of "all salts" solution.

For nucleic acid determinations, cells or pellets remaining after lysis were extracted with cold and then with hot trichloroacetic acid as described by Schneider (22). The intermediate lipid extractions could be omitted without affecting nucleic acid measurements. Deoxyribonucleic acid (DNA) was measured in hot trichloroacetic acid extracts with the diphenylamine reagent (5), by using a highly polymerized calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) as standard. Ribonucleic acid (RNA) was measured in hot trichloroacetic acid extract by the orcinol reagent (3), with a yeast RNA (Worthington Biochemical Corp.) used as standard.

RESULTS

Motility of the marine psychrophile depends on a single long polar flagellum, which can be seen in negatively stained preparations (Fig. 1). About 30% of the cells investigated by this method had such a flagellum, which could also be demonstrated in a much lower proportion of cell replicas. Possibly, flagella were lost when cells were dried in vacuo.

Ultrastructure of intact cells. Examination of thin sections of cells fixed with formalin in "all salts" solution showed that these were surrounded by two triple-layered structures, each 6.0 to 7.5 nm wide (Fig. 2), which we consider to be the cell wall and cytoplasmic membrane. Mesosomes, presumably originating as invaginations of the cytoplasmic membrane (7, 21, 22), were seen in several preparations (Fig. 2B). Thin sections of cells suspended in 0.5 M NaCl plus 0.1 M $MgCl_2$, which preserves cells stability (reference 14 and Table 1), were similar to sections of cells in "all salts" solution.

Chemical and structural changes accompanying lysis. Effects of environmental changes on cell stability and chemical composition are shown in Table 1. As observed earlier (11, 14), the bacterium was stable at low temperatures in "all salts" solution or in 0.5 M NaCl plus 0.1 M $MgCl_2$. The turbidity remained constant, and only small amounts of ultraviolet (UV)-absorbing material were released from the cells. When the NaCl plus $MgCl_2$ was diluted, turbidity fell, and more UV-absorbing materials appeared in the supernatant fluid. In 0.5 M NaCl alone, turbidity fell slowly, and the final release of UV-absorbing substances approached that found in distilled water. At lower NaCl concentrations, turbidity fell more rapidly, and the release of UV-absorbing substances increased. Cells were not stable in $MgCl_2$ solutions of 0.1 M or lower. However, $MgCl_2$ solutions were better able to prevent release of UV-absorbing substances than fivefold greater concentrations of NaCl.

Even after extensive lysis, substantial amounts of DNA and RNA remained associated with the residual cellular material. Solutions of 0.5 M NaCl or of 0.1 M $MgCl_2$ were better able to prevent loss of cellular DNA than loss of RNA. Extensive loss of nucleic acids took place after lysis at 37 C.

Almost all cells in "all salts" solution and in 0.5 M NaCl plus 0.1 M $MgCl_2$ were rod-shaped and dense under phase contrast. In 0.3 M NaCl plus 0.06 M $MgCl_2$, about 75% were rod-shaped and the rest spherical; in 0.1 M NaCl plus 0.02 M $MgCl_2$, at least 90% of the cells became phase-pale spheres. In 0.1 M $MgCl_2$, all cells became spherical in a few minutes. Cells in 0.5 M NaCl, examined after 2 hr, were still rod-shaped but less phase-dense than intact cells.

The fine structure of cells treated as in experiment 1, Table 1, was examined. Though the

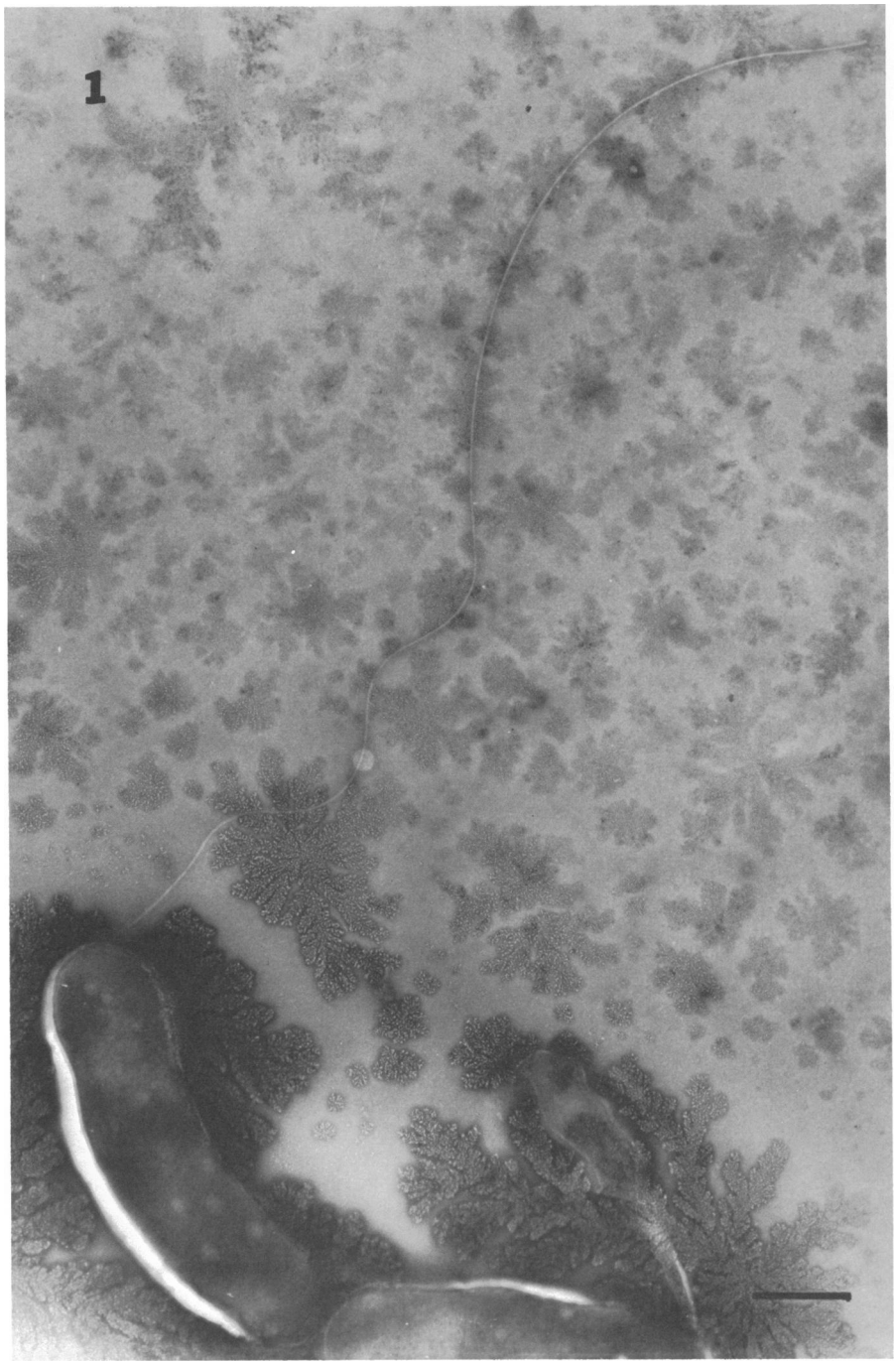

FIG. 1. *Negatively stained preparation of intact cell dried in "all salts" solution showing a long single polar flagellum. Bar is 0.5 μm.*

characteristic wall and membrane structure of intact cells was retained in 0.5 M NaCl plus 0.1 M $MgCl_2$ (Fig. 3A), suspension in more dilute solutions led to a loss of definition of the cell membrane as a triple-layered structure (Fig. 3B), followed by distortion and fragmentation of the wall (Fig. 3C).

When cells were suspended in 0.5 M NaCl

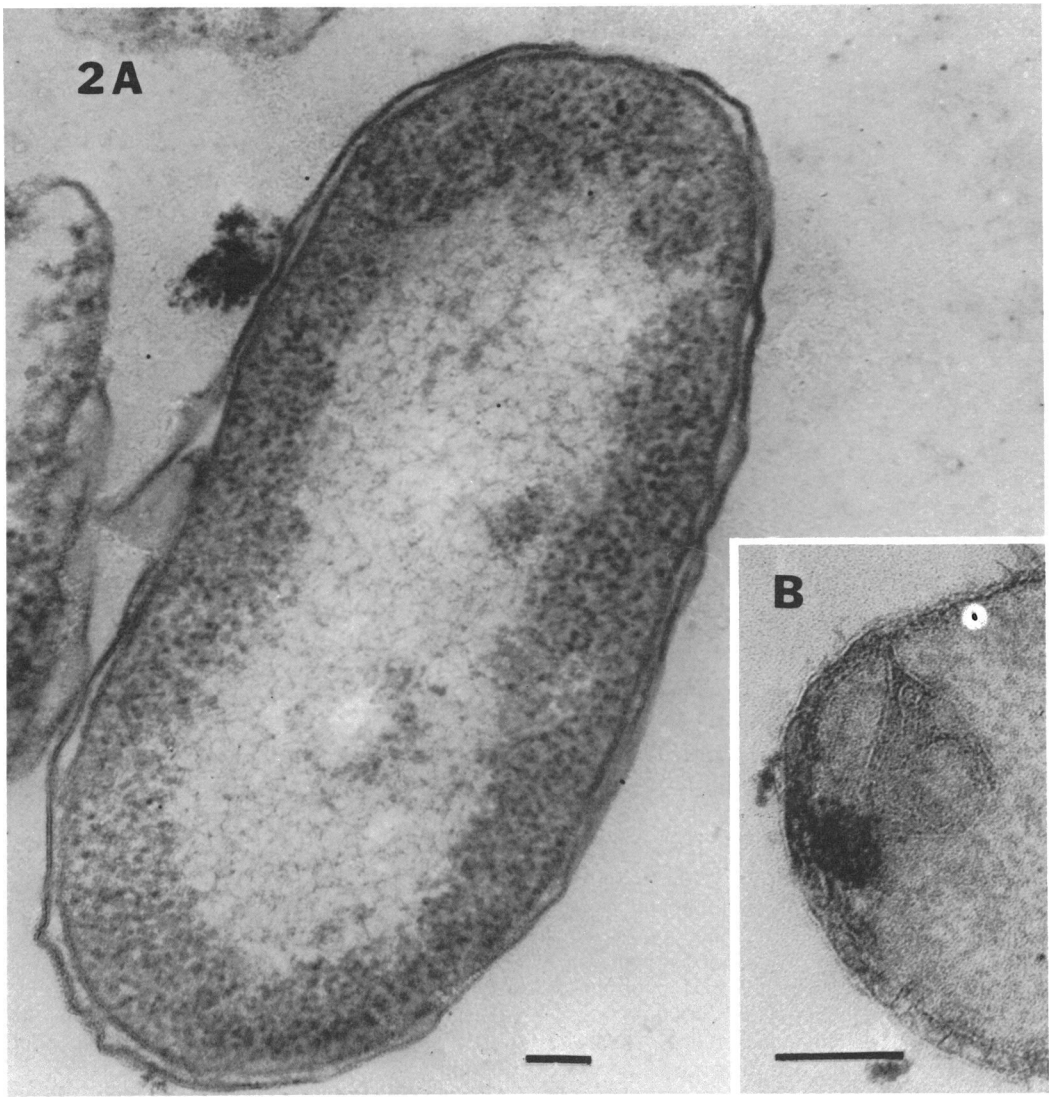

FIG. 2. *Thin sections of the marine psychrophile. A, Longitudinal, showing wall and membrane. B, Section with a mesosome. Transverse strands between wall and membrane were seen in some preparations. Bars are 0.1 μm.*

alone, the cell wall appeared as a detached but distinct triple layer, and only a faint suggestion of the cytoplasmic membrane remained (Fig. 4A and 4B). The nuclear region was poorly defined. Cells suspended in 0.1 M NaCl were transformed to a collection of fragments and vesicles (Fig. 4C). These are very similar to those obtained from cells suspended in distilled water (Fig. 6A), except that the latter had a smaller proportion of walls or wall fragments with continuous smooth structure. Few traces of the cytoplasmic membrane remained in the material sedimented after lysis in 0.1 M NaCl or distilled water.

In 0.1 M $MgCl_2$, a better preservation of the cell membrane was obtained than in 0.5 M NaCl (Fig. 5A). Even in 0.02 M $MgCl_2$, cellular integrity was better preserved than in 0.1 M NaCl alone (Fig. 4C and 5B).

Particles released on lysis. It was observed earlier that, when cells were lysed in distilled water, a good deal of cellular material was released in a form not sedimentable by centrifuging for 10 min at 8,000 × *g* but sedimentable after 60 min at 100,000 × *g* (14). Debris from cells lysed in distilled water (Fig. 6A) sedimented at 15,000 × *g* for 20 min. On centrifuging at 27,000 to 30,000 × *g* for 20 min, more material sedimented, similar in appearance to that shown in Fig. 6A.

TABLE 1. *Changes accompanying lysis of the marine psychrophile*

Experimental conditions[a]	Turbidity (%)[b] after				Release of ultraviolet-absorbing substances[c]	DNA remaining (%)[d]	RNA remaining (%)[d]
	15 min	60 min	120 min	180 min			
Experiment 1							
"All salts"	100	100	100		0.24		
0.5 M NaCl plus 0.1 M $MgCl_2$	108	106	106		0.30		
0.4 M NaCl plus 0.08 M $MgCl_2$	83	83	81		0.27		
0.3 M NaCl plus 0.06 M $MgCl_2$	86	86	86		0.49		
0.1 M NaCl plus 0.02 M $MgCl_2$	55	38	32		2.56		
0.5 M NaCl	81	71	30		2.38		
0.3 M NaCl	65	40	13		2.51		
0.1 M NaCl	22	12	10		2.89		
0.1 M $MgCl_2$	51	49	46		1.22		
0.06 M $MgCl_2$	51	46	42		1.67		
0.02 M $MgCl_2$	42	30	27		2.13		
Water	15	10	10		3.00		
"All salts," 37 C	88	49	46				
Experiment 2							
"All salts"	100	100	98	96	0.28	100	100
0.5 M NaCl plus 0.1 M $MgCl_2$	107	106	106	104	0.43	94	94
0.5 M NaCl	96	91	81	47	2.65	80	57
0.1 M $MgCl_2$	50	46	46	45	2.41	93	67
Water	24	19	19	18	3.95	53	47
"All salts," 37 C	88	61	60	60	4.55	43	25

[a] All incubations at 0 C unless otherwise stated. A 40-hr culture was used for experiment 1 and a 60-hr culture for experiment 2.

[b] "One-hundred per cent turbidity" equals that found in "all salts" solutions. The turbidity (absorbance at 660 nm) for experiment 1 was 0.590 (corresponding to 0.86 mg of bacteria per ml) and for experiment 2, 0.480 (0.68 mg of bacteria per ml).

[c] Expressed as absorbance of supernatant fractions at 260 nm per mg of bacteria per ml (read after four- to five-fold dilutions).

[d] The amounts of DNA and RNA in intact cells (in "all salts" solution) is taken as 100%. Cells contained 0.300 mg of RNA per mg (dry weight) of cells and 0.070 mg of DNA per mg (dry weight) of cells.

After 60 min at 65,000 × *g*, an assortment of particles sedimented (Fig. 6B–6D). No further material sedimented after 60 min at 100,000 × *g*.

The 65,000 × *g* sediment included debris and different-sized vesicles. The larger vesicles were 150 to 250 nm in diameter. Many vesicles only 12.5 to 15 nm in diameter were also observed. A few of these (shown especially in Fig. 6B) had a "doughnut" appearance. In others of the same size, only a suggestion of such a structure could be seen. Though the "doughnuts" are perhaps the most interesting structures found, they occur rarely (usually only a few per sample), and we have still not succeeded in obtaining large numbers of them regularly from each batch of cells.

As noted above (Fig. 3C, 4A, and 5A), exposure to 0.5 M NaCl alone, 0.1 M $MgCl_2$ alone, or 0.1 M NaCl plus 0.02 M $MgCl_2$ led to characteristic changes in the structure of cells sedimenting at 15,000 × *g*. When supernatant fractions from the first two treatments were centrifuged at 27,000 × *g* for 20 min, no material sedimented. Centrifuging at 65,000 × *g* for 60 min sedimented very small amounts of material resembling the fragments and vesicles shown in Fig. 6D. No material could be sedimented from the 15,000 × *g* supernatant fraction remaining after treatment with 0.1 M NaCl plus 0.02 M $MgCl_2$.

Temperature- and acid-induced lysis. When cells were held for 2 hr at 37 C in "all salts" solution, considerable structural deterioration took place, as suggested by the fall in turbidity (Table 1) and by the appearance of cells in thin section (Fig. 7). Even in cells that retained their shape, the wall was degraded, and much of the wall material was liberated as vesicles. The cytoplasmic membrane was rarely observed as a three-layered structure.

Cells lysed at *p*H 5.0 in "all salts" solution retained their rod shape. In thin section, the cell wall was distorted, and the cell membrane was less well defined than that of the intact cell, though some of the trilaminar structure remained (Fig. 8).

Cell surface structure and its changes during lysis. Replicas of unfixed cells dried in the cold

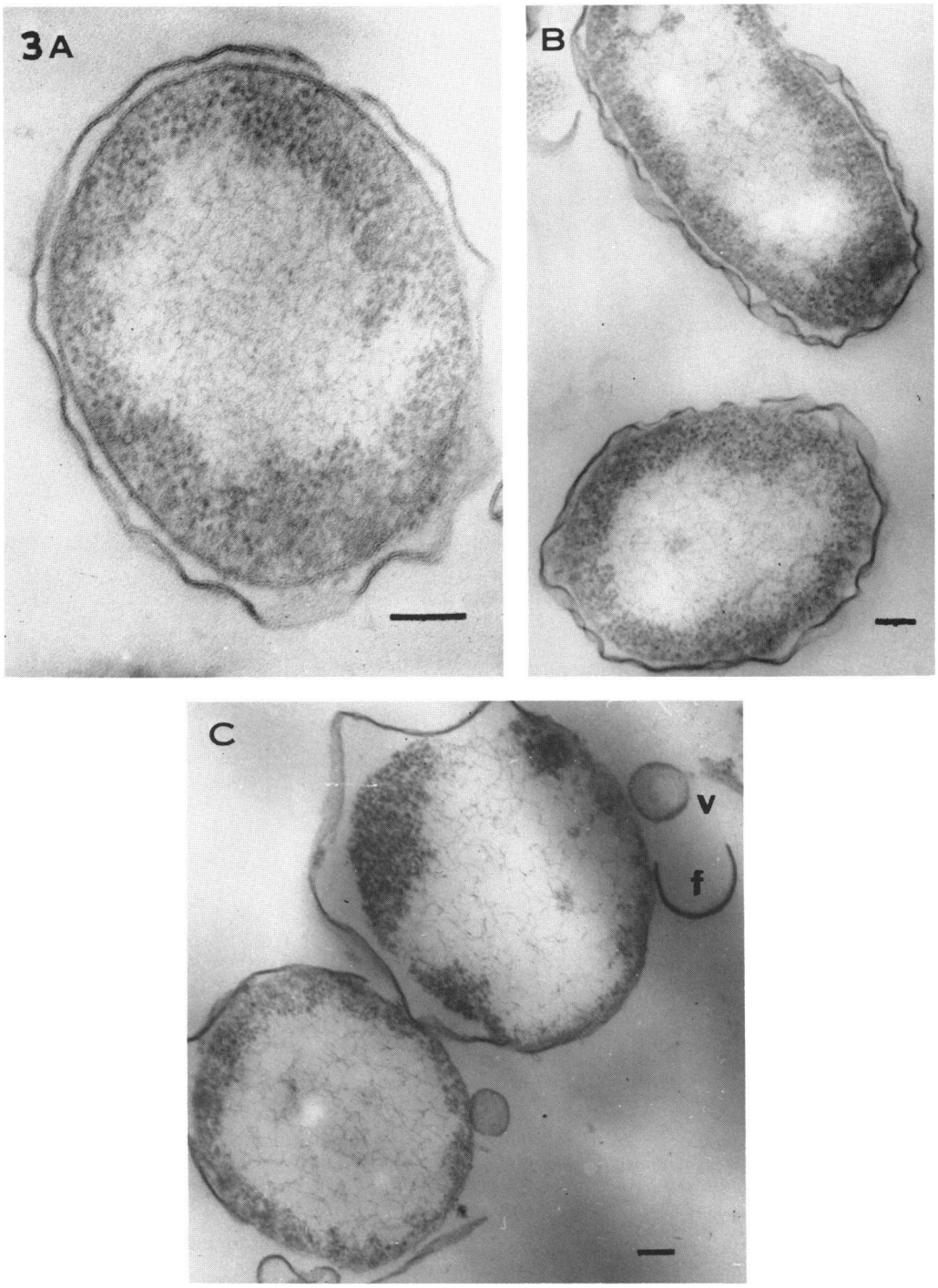

FIG. 3. *Thin sections of cells. A, Suspended in 0.5 M NaCl plus 0.1 M $MgCl_2$, showing characteristic trilaminar wall and membrane structure. B, Suspended in 0.3 M NaCl plus 0.06 M $MgCl_2$. Cell membrane can be faintly distinguished as a trilaminar structure, and fewer fibrils remain in the nucleoid. C, Suspended in 0.1 M NaCl plus 0.02 M $MgCl_2$. Trilaminar membrane structure has disappeared. Walls are broken and fragments (f) and vesicles (v) thought to originate from the walls, are seen. Bars are 0.1 μm.*

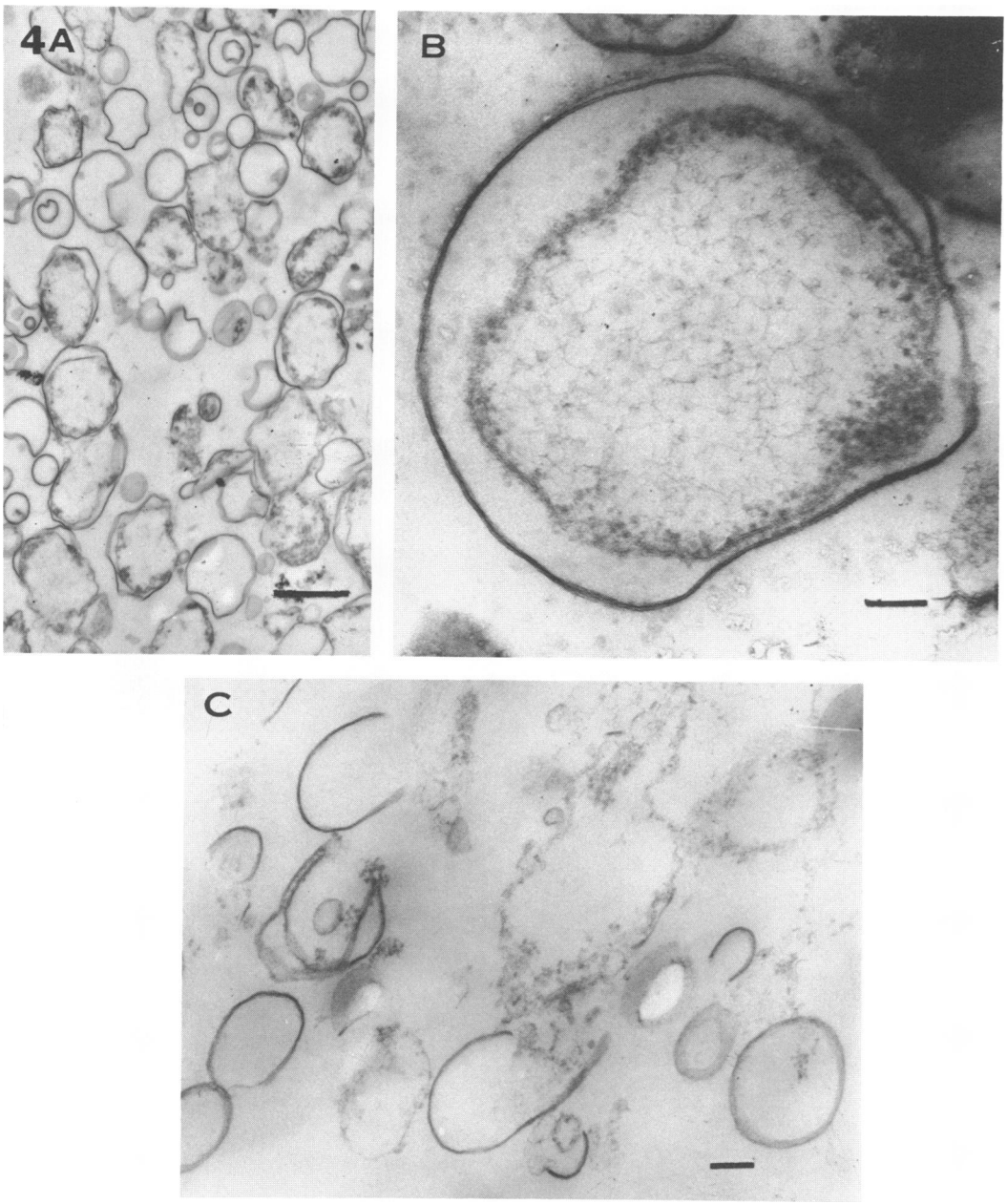

FIG. 4. *Thin sections of cells suspended in NaCl alone. A, Cells in 0.5 M NaCl. Bar is 1.0 μm. B, Cell from the 0.5 M NaCl preparation at higher magnification, showing a continuous trilaminar cell wall and short segments of the cell membrane. Bar is 0.1 μm. C, Cells suspended in 0.1 M NaCl showing fragments with a continuous smooth structure. Bar is 0.1 μm.*

revealed a matted surface texture. On lysis in distilled water, the surface was considerably disrupted, a result to be expected considering the appearance of such cells in thin section (Fig. 6A). Blebs, about 200 nm in diameter, appeared on the cell surface. Lysis at *p*H 5 caused some wrinkling of the surface, which seemed otherwise intact. Lysis at 37 C led to quite irregular contours and a rugged surface.

DISCUSSION

It was suggested earlier that this organism should be classified as a *Serratia* species because of its pigmentation, fatty acid, and phosphatide

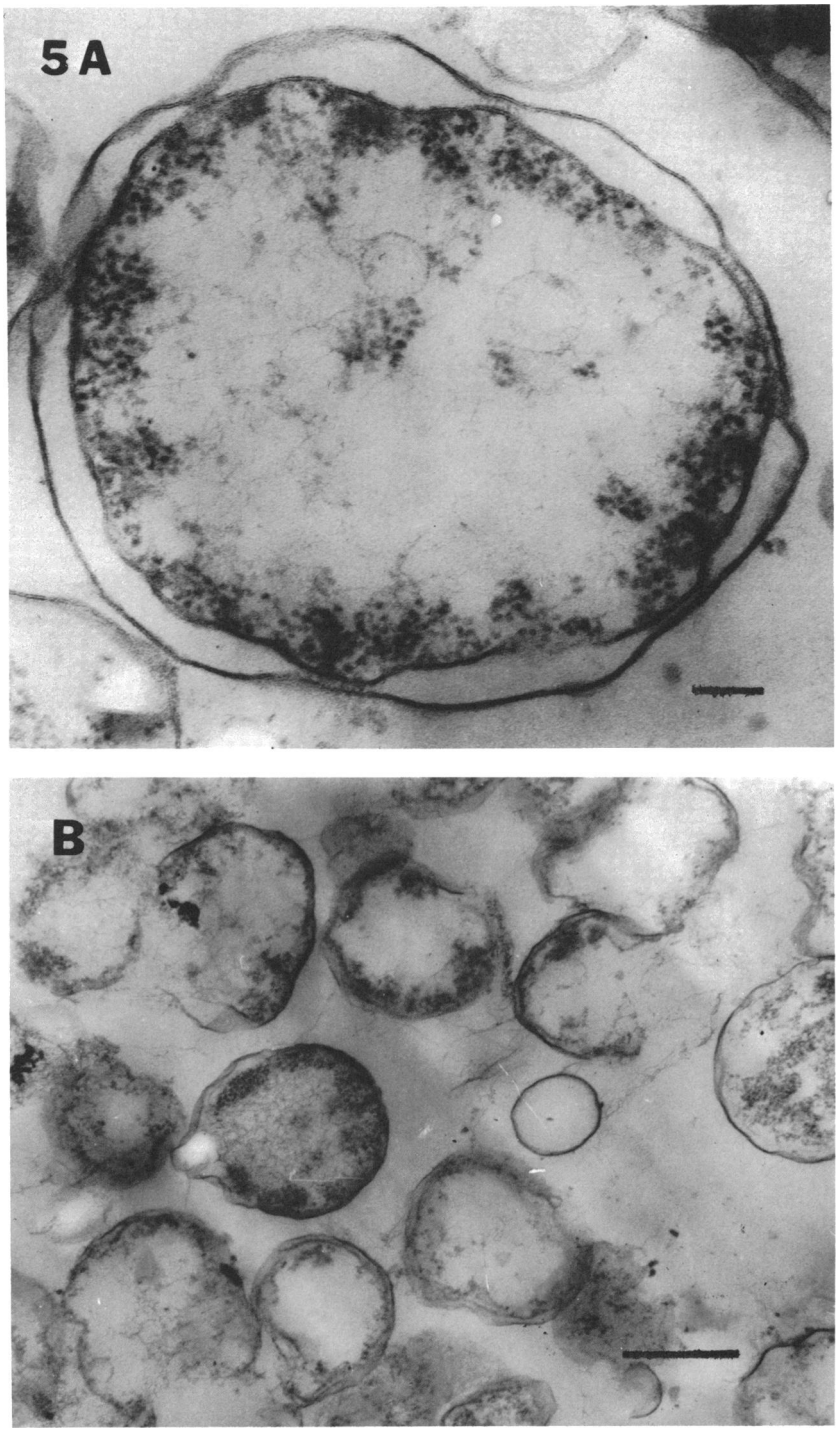

FIG. 5. *A, Thin section of a cell suspended in 0.1 M $MgCl_2$. Cell membrane has retained almost all of its trilaminar structure (compare with cells in 0.5 M NaCl, Fig. 4B). Bar is 0.1 μm. B, Thin section of cells suspended in 0.02 M $MgCl_2$. Structure is better preserved than in a fivefold greater Na^+ concentration (Fig. 4C). Bar is 0.5 μm.*

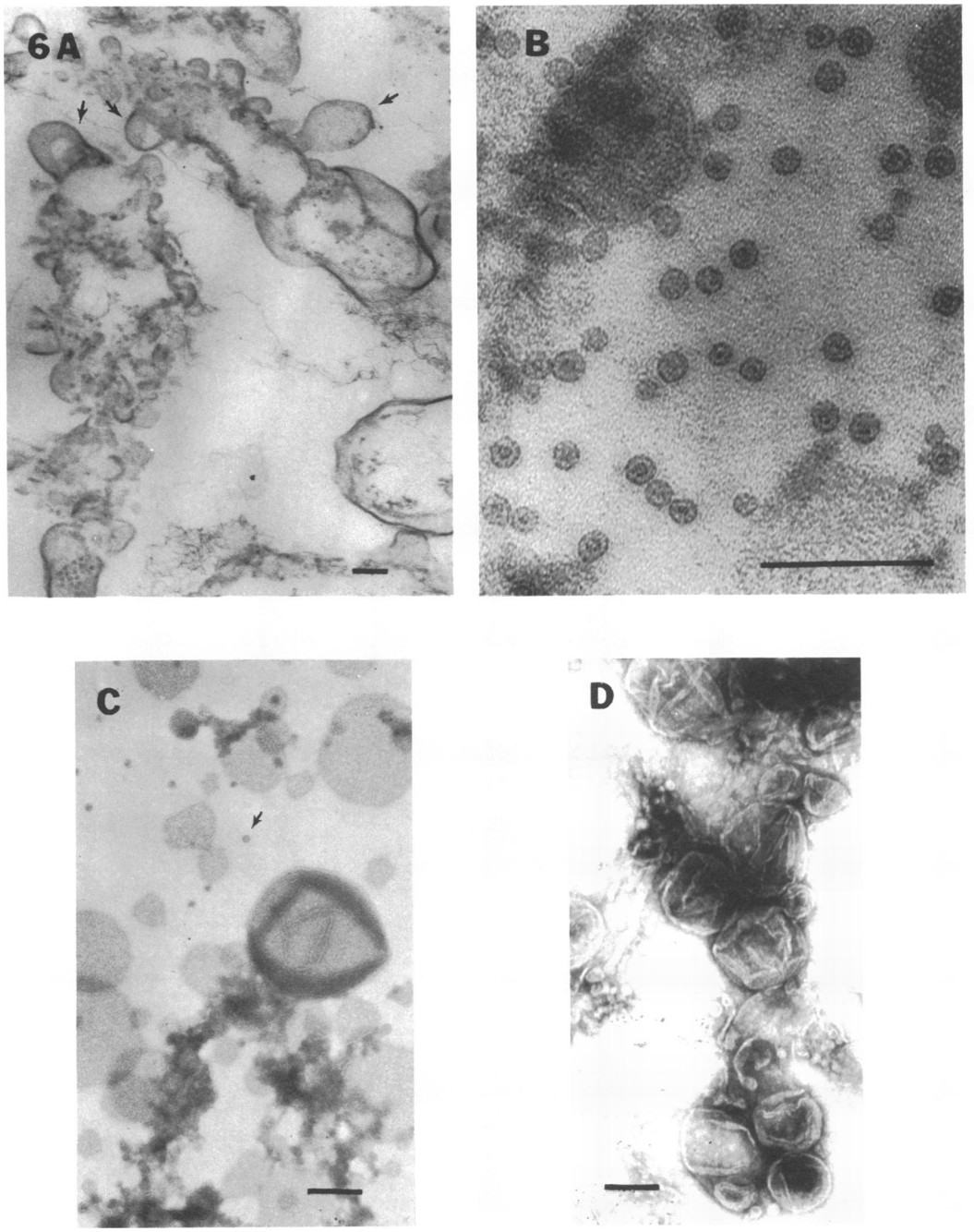

FIG. 6. *Effect of distilled water on cell structure. A, Thin section of lysed cells sedimented at 15,000 × g; cell wall fragments seem to be re-forming into vesicles (arrows). Little if any suggestion of the cell membrane remains. B to D, Material sedimenting at 65,000 × g and stained in cold aqueous 1% uranyl acetate; B, 12.5 to 15.0 nm vesicles exhibiting a "doughnut" shape; C and D, larger vesicles (150 to 250 nm), including a few "doughnut-shaped" vesicles (arrow). Bars are 0.1 μm.*

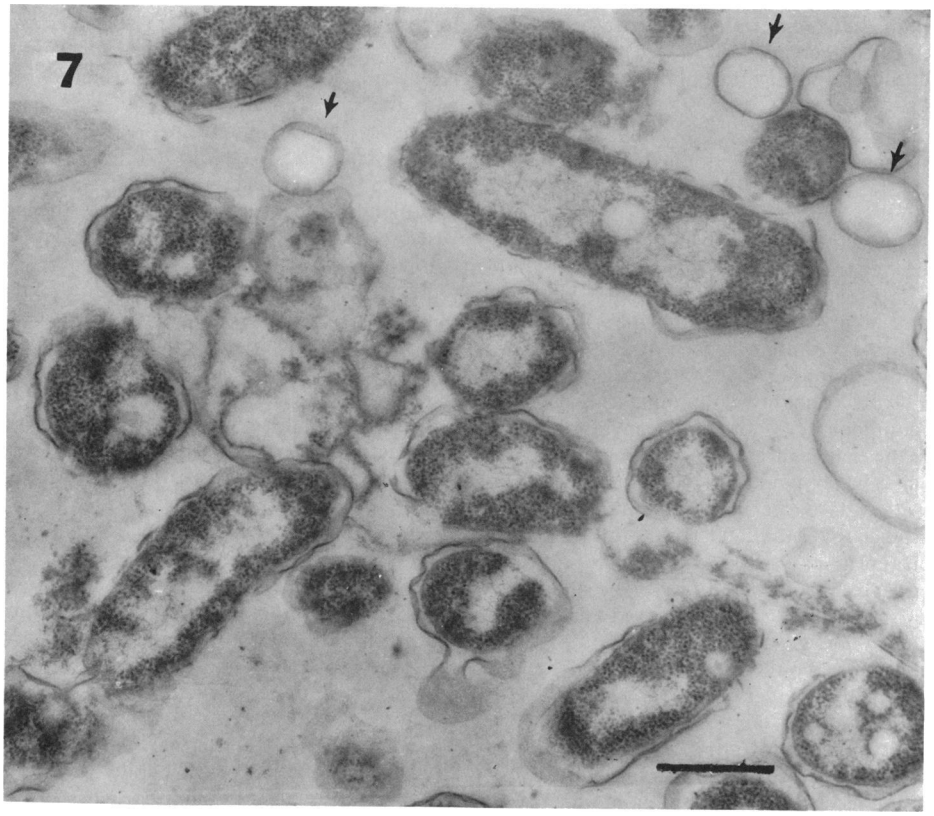

FIG. 7. *Thin section of cells suspended in "all salts" solution and held at 37 C for 2 hr, during which time considerable structural deterioration occurred. Some vesicles (arrows), possibly from cell walls, appear. Even in cells that do not appear extensively degraded, most of the trilaminar membrane structure is lost. Bar is 0.5 μm.*

composition (11, 12). Because it has only a single polar flagellum, this classification is unsuitable.

For stability, the red psychrophile needs low temperatures, *p*H values near neutrality, and both monovalent and divalent cations (11, 14, 18). In contrast to other marine bacteria, its structure is poorly maintained by monovalent salts and even more poorly by nonionic solutes (14, 17). Its sensitivity to changes in the environment may be the cause of the difficulties we experienced in fixing cells for thin-section examination. To demonstrate fine structure, we used formaldehyde as a fixative, because the more standard glutaraldehyde plus OsO_4 fixation seldom yielded a satisfactory resolution of wall and membrane structure.

Since a rather high concentration of formaldehyde (0.5 M) was used to fix cells, it seemed possible that formaldehyde might also be exerting an osmotic effect. It was found previously, however, that, although cells were stable in 0.1 M $MgCl_2$ plus 0.5 M NaCl, raising the NaCl concentration to 1.0 M did not lead to any discernible morphological changes (14). Experiments were also carried out in which 0.5 M sucrose or glycerol was added to cells suspended in "all salts" solution. No changes in turbidity, release

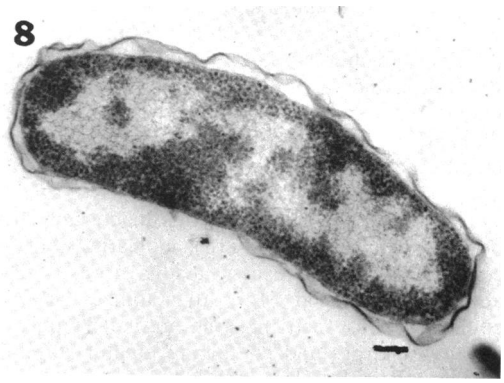

FIG. 8. *Thin section of cell lysed at pH 5.0 in "all salts" solution. A few breaks appear in the distorted cell wall, and a faint suggestion of the trilaminar cell membrane remains. Bar is 0.1 μm.*

of UV-absorbing material, or appearance under phase contrast were observed. We think it very unlikely, therefore, that formalin acts in any way except as a fixative.

Cells are surrounded by a triple-layered cell wall and a well-defined cytoplasmic membrane, characteristic of certain other gram-negative terrestrial and marine bacteria (6, 7, 10). No structures appeared in electron micrographs that could be regarded as specific to psychrophilic bacteria. Recent work has shown that psychrophilic and nonpsychrophilic marine vibrios are structurally very similar to each other (13).

In many gram-negative bacteria, a densely stained layer, probably containing the peptidoglycan components of the cell, can be observed between the wall and cytoplasmic membrane (9, 19). Such a layer could not be demonstrated by staining the B-16 marine pseudomonad extensively studied by MacLeod et al. (6, 7), and it does not appear in our red psychrophile. Possibly this is due to the unusual fixing method used. However, in parts of a few cells fixed and stained with glutaraldehyde-OsO_4-uranyl acetate, walls and membranes could be clearly seen, but no dense intermediate layer was seen.

Though both Na^+ and Mg^{2+} are needed for cell stability, these ions appear to act differently on different layers of the cell. Thin sections show that both ions are capable of maintaining wall integrity, whereas the cell membrane is best stabilized by Mg^{2+} and loses its characteristic trilaminar structure in NaCl alone. The fact that $MgCl_2$ is better able to prevent leakage of UV-absorbing compounds and loss of nucleic acids than a fivefold greater concentration of NaCl (Table 1) further supports our interpretation of a structural role of Mg^{2+} ions on the cell membrane. In contrast, Mg^{2+} ions were previously thought to be involved in wall rather than in membrane structure in marine and terrestrial gram-negative bacteria (6, 14).

The isoelectric point of this bacterium lies between *p*H 2.0 and 2.5 (18), indicating that its cell walls contain excess acidic groups. The ability of Na^+ and Mg^{2+} to stabilize the wall could be due to their masking mutually repulsive negative charges and conferring greater stability to the cell wall. This masking action has been suggested to account in part for the ionic requirements of extremely halophilic bacteria (1, 2, 4, 16).

Previous work showed that, when cells were lysed in distilled water, particles containing most of the cell lipid phosphorus and hexosamine were released. The morphology of these particles has now been studied. In addition to amorphous debris, different-sized vesicles were found. The larger ones (150 to 250 nm in diameter) were as large as parts of the wall pinched off from water-lysed cells (Fig. 6A). They also compared well with the blebs on the surface of such cells and are believed to originate in the cell wall. Many vesicles only 12.5 to 15 nm in diameter were also observed. Since much of the cell wall remained unbroken or formed large vesicles, the smaller vesicles are thought to have originated in the cytoplasmic membranes.

Particles containing hexosamine and lipid phosphorus are also released when cells are broken mechanically in NaCl plus $MgCl_2$ medium (14). Preliminary electron microscopy of these revealed that they resemble the collection of particles released on distilled water lysis. Either method of breaking down cellular structure seems to lead to extensive fragmentation of the outer layers of the cell.

Our results show that the fine structure of the outer layers of these bacteria is extraordinarily susceptible to environmental change. Mitochondrial and other cell membranes retain their "unit-membrane" (trilaminar) structure even after most of the lipid is extracted with acetone (9). The trilaminar structure of red blood cell membranes remains after digestion with phosphatidase C (20). In contrast, much more gentle treatment of the red psychrophile can lead to the disappearance of trilaminar structure (Fig. 3C, 4A, 4B, and 7). Quite possibly, the structure of these membranes depends on a delicate balance of charges between protein and lipid molecules. Such a balance could be disturbed either by a change in the ionic environment or removal of the charged portions of phosphatides by enzymic action. These changes could lead not only to disappearance of trilaminar structure but also to fragmentation and release of parts of the walls and membranes as vesicles.

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