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EFFECTS OF BETAINE ON THE ULTRASTRUCTURE OF SALT-TREATED *ESCHERICHIA COLI*

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ABSTRACT. The ultrastructural changes have been studied in *Escherichia coli* 1130B-2ATCC (*E. coli*) grown on nutrient agar medium, medium + 0.8 M NaCl and medium + 0.8 M NaCl + 0.001 M betaine. Each dish was inoculated with 10^{-7} bacteria and allowed to grow for 72 hours. Colony counts in the media was, medium alone (83), medium + NaCl was (18) and medium + NaCl + betaine was (48), indicating that nearly three times as many colonies grew in the presence of betaine in the NaCl medium, than those grown in the NaCl medium alone. Electron micrographs of sectioned *E. coli* cells grown on normal medium displayed a cell wall composed of outer membrane and cytoplasmic membrane enclosing a periplasmic space. The nucleoid was centrally located and contained fine DNA fibrils. Numerous ribosomes were present in the electron-dense cytoplasm. The cell division was achieved by septum formation. Sectioned cells grown on NaCl medium displayed many vesicles being pinched off from the cell surface. The outer membrane and the cytoplasmic membrane were disrupted at several sites, resulting in loss of cellular contents. The cytoplasm in some sections became electron-lucent, devoid of ribosome and contained dense bodies and membrane whorls. In other cells the cytoplasm appeared fragmented into small masses. In longitudinal sections of the cells the nucleoid material appeared in thick DNA fibrils, which in transverse sections was seen as a dark, round clump. The cell division was arrested, non-dividing cells were elongated and displayed aberrant mesosomes, vesicles and bulges. Sectioned cells grown on NaCl-betaine medium appeared normal. Their outer and cytoplasm membranes were intact and enclosed a periplasmic space. The nucleoid material appeared as fine DNA fibrils. The cytoplasm was electron-dense and rich in ribosomes. This study suggests that betaine acts as an effective osmoprotectant for *E. coli* offsetting NaCl-induced osmotic stress by maintaining the integrity and stability of its cell constituents.

Keywords: *Escherichia coli*, outer membrane, cytoplasmic membrane, periplasmic space, nucleoid, ribosomes, vesicles, aberrant mesosomes, betaine

All organisms depend upon maintaining the consistency of their internal environment in different environmental conditions. Organisms have devised different mechanisms to cope with changes in their internal water, which is essential for survival (Yancey, 2005).

It is well know that high concentrations of sodium salts in the external medium exert toxic effects on microorganisms, plants and animals, retarding their growth and respiration, which can cause death (Heilbrunn, 1952; Lanyi, 1979; Yancey, *et-al.*, 1982). Organic substances such as free amino acids, sugars, methylamines and urea act as osmolytes in salt-stressed bacteria, plants and animals exposed to high salinity (Rudulier *et al.*, 1984; Yancey, 1982; 1985).

Certain species of non-halophilic bacteria exposed to high salt concentration in growth medium, respond by increasing intracellular concentration of amino acids like free proline or glutamate. In the presence of exogenously added amino acids to the salty growth-medium, accumulation of these metabolites is elevated, stimulating bacterial growth and respiration (Britten & McClure, 1962; Brown & Stanley, 1972; Measures, 1975; Csonka, 1979). Recently it has been shown that accumulation of trehalose is induced in NaCl-treated water-stressed cyanobacterium, *Nostoc punctiforme* (Yoshida & Sakamoto, 2009).

The aim of the present study was to investigate ultrastructural changes in the NaCl-treated *E. coli* and to demonstrate if exogenous betaine added to the NaCl medium counteracts deleterious effects

* Our esteemed colleague and co-investigator Dr. Duncan T. Kennedy passed away after completion of this study.

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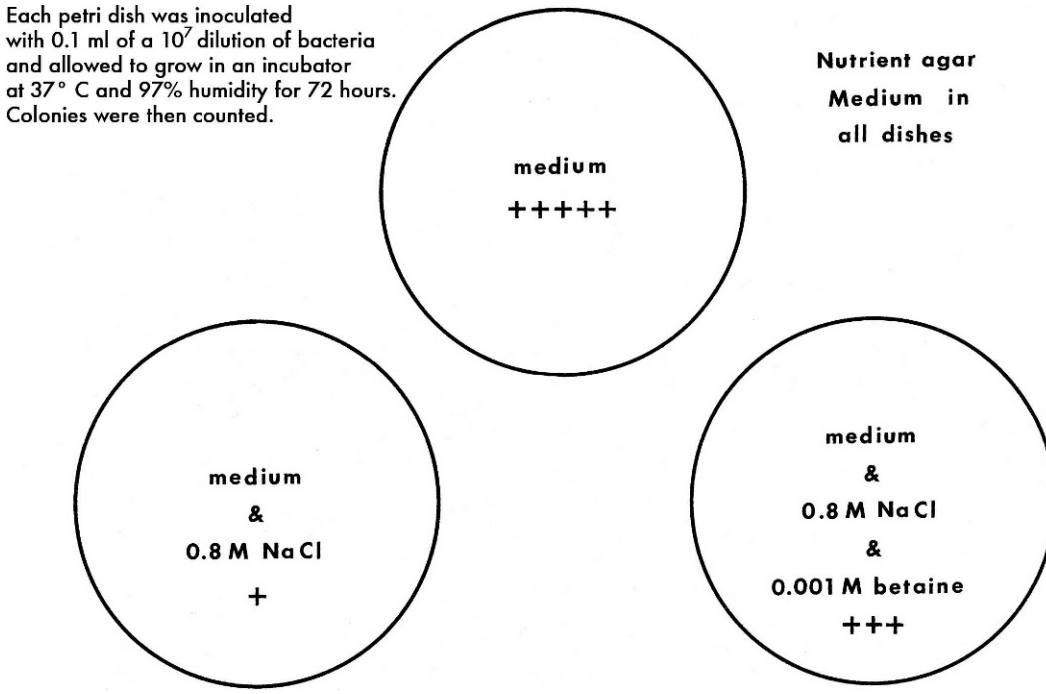

Figure 1.—A diagrammatic representation of numbers (+) of *E. coli* colonies grown in petri dishes on normal growth medium, medium + NaCl and medium + NaCl + betaine.

of high-salt medium and stabilizes the cellular constituents.

METHODS

Escherichia coli 1130B-2ATCC was grown on nutrient agar medium (Difco Labs, Detroit, MI, USA), medium + 0.8 M NaCl and medium + 0.8 M NaCl + 0.001 M betaine (glycine betaine) (Sigma Chemical Company, St. Louis, MO. USA). Each petri dish was inoculated with 10^7 bacteria and allowed to grow in an incubator at 37° C and 97% humidity for 72 hours to achieve equilibrium with the media. Colonies were counted.

For electron microscopic study, *E. coli* colonies were fixed for 15 minutes in the following mixture: 1part 8% glutaraldehyde, 1 part 4% osmic acid and 2 parts 0.1 M phosphate buffer (pH 7.2). The material was washed in several changes of phosphate buffer, dehydrated in an ethanol series to propylene oxide and embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA, USA). Polymerization was carried out at 60° C overnight. Thin sections were cut on a Porter-Blum MT-2 ultra-microtome (ThermoFisher Scientific, Waltham, MA, USA). Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi transmission electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Growth of E. coli in media.—The number of *E. coli* colonies grown on normal growth medium was five times the number of colonies that grew on the medium containing 0.8 M NaCl. Colony count (mean of two replicates) in the media was medium alone (83), medium + NaCl (18) and medium + NaCl + betaine (48), indicating that nearly three times as many colonies grew on the medium containing 0.8 M NaCl and 0.001 M betaine than those grown on the medium containing 0.8 M NaCl alone (Fig. 1).

Electron microscopic observations.—Electron micrographs of sectioned *E. coli* cells grown on normal medium measured about 3.0 um in length and 0.7 um in width. The cell wall was composed of an outer membrane, a distinct cytoplasmic (inner) membrane enclosing a periplasmic space.

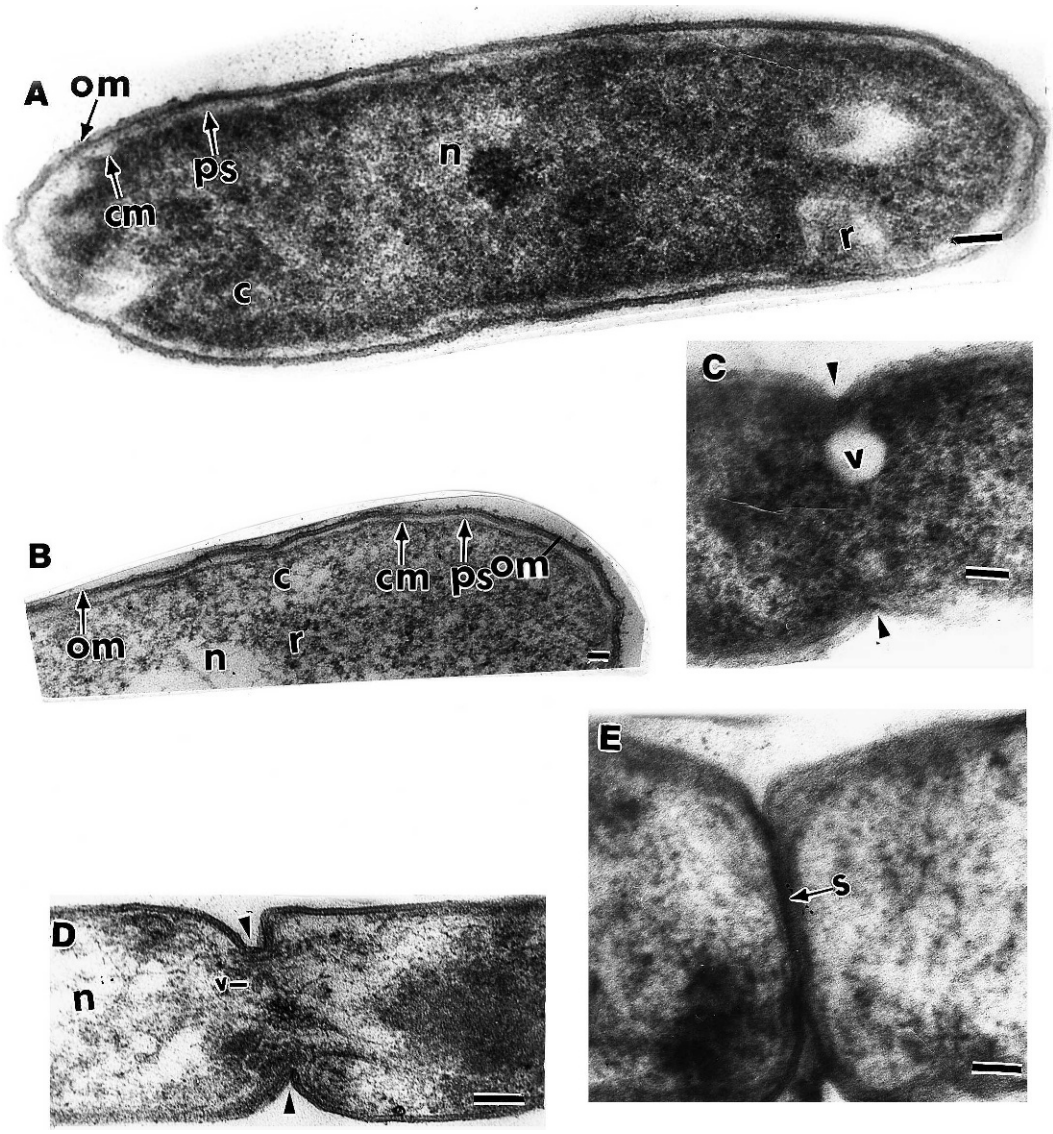

Figure 2.—Electron micrographs of sectioned *E. coli* in normal medium. A and B represent longitudinal sections of the rods displaying outer membrane (om), periplasmic space (ps) and cytoplasmic membrane (cm) constituents of the cell wall, nucleoid (n) and electron-dense cytoplasm (c) containing numerous ribosomes (r). C, D and E show progression (arrowheads) of cell division. n, nucleoid; s, septum; v, vesicles. Bar A–E, 0.1 μm.

The periplasmic space contained fine filamentous material, which probably represent peptidoglycan layer. The nucleoid displaying fine DNA fibrils occupied the central zone of the cells. The cytoplasm appeared electron-dense and contained numerous ribosomes (Figs. 2A, 2B).

The cell division was achieved by septum formation initiated by a bilateral shallow invagination in the middle of the cell. The invagination became progressively deeper toward the center of the cell and vesicles appeared at the division site (Figs. 2C, 2D). The septum was apparently formed by inward invagination of the cytoplasmic membrane and peptidoglycan layer with later contribution from the outer membrane (Fig. 2E).

Electron micrographs of sectioned *E. coli* cells grown on NaCl containing medium revealed drastic changes in all cell constituents. The nucleoid in longitudinal sections displayed thick

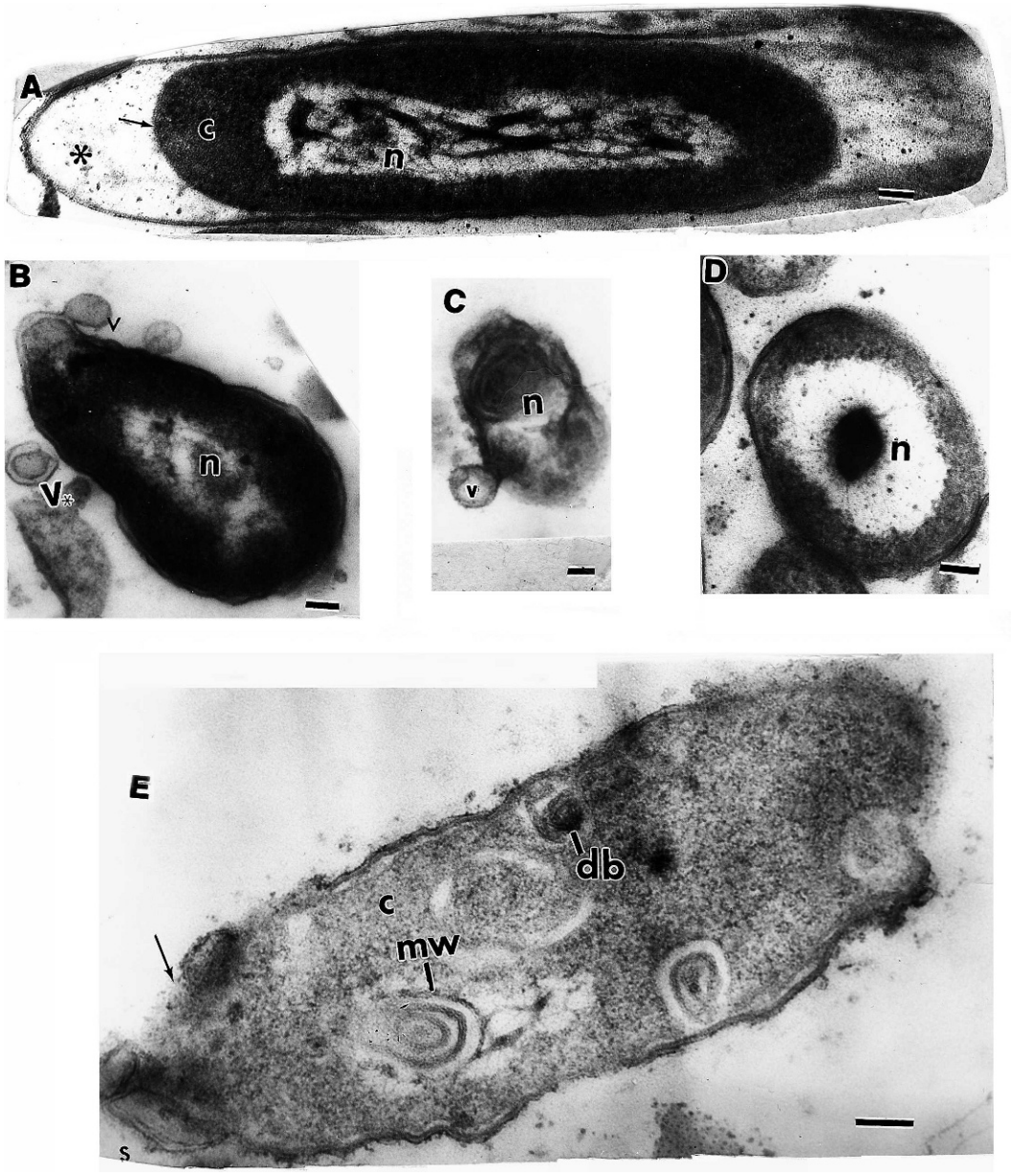

Figure 3.—Electron micrographs of sectioned *E. coli* in medium + NaCl. A represents a longitudinal section showing nucleoid (n) with thickened DNA fibrils and cytoplasm (c) separating from the cell wall (arrow) creating a clear space at the pole (*). B, C and D are transverse sections displaying dark, round nucleoid (n) and vesicles (v) extruding from the outer surface. Note the vesicle in Fig 3B is bounded by a double membrane (v*). E is a longitudinal section of an *E. coli* cell displaying a break in the cell wall (arrow) and electron-lucent cytoplasm (c) containing a dense body (db) and membrane whorls (mw). Bar A–E, 0.1 μm.

DNA fibrils (Fig. 3A), which in transverse sections appeared as a dark round clump (Figs. 3B, 3C, 3D). The cytoplasm was separated from the cell wall creating a clear space at the poles (Fig. 3A). In some cells the cytoplasm became electron-lucent devoid of ribosomes and displayed dense bodies and membrane whorls (Fig. 3E). In other cells the cytoplasm was broken into small masses and the cytoplasmic membrane was displaced toward the interior of the cell (Fig. 4A).

Figure 4.—Electron micrographs of *E. coli* cells in medium + NaCl *continued*. A represents a longitudinal section of a rod showing cytoplasm broken into dark masses (c). Displacement of the cell membrane (cm) into the cell interior is noteworthy. B represents transverse sections of empty looking rods with broken cell walls (arrows) and many vesicles (v) in close association with the external surface of the cells. C represents an elongated non-dividing cell showing vesicles (v) and bulges (b) at the external surface. m, aberrant mesosome; n, nucleoid. Bar A–C, 0.1 μm.

The cells exposed to NaCl containing medium displayed numerous vesicles with an average size of 100 nm, pinched off from the outer surface into the external space (Figs. 3B, 3C, 4B, 4C). In many cells the outer and cytoplasmic membranes appeared broken with the loss of cell contents into the external space (Figs. 3E, 4B). Cell division was arrested, non-dividing cells were elongated and displayed aberrant mesosomes and bulges near the center of the cells (Fig. 4C).

Most of the *E. coli* cells grown on medium containing 0.8 M NaCl + 0.001 M betaine appeared normal. They displayed intact outer membrane and cytoplasmic membrane enclosing a periplasmic space and centrally placed nucleoid containing fine DNA fibrils (Fig. 5A). The cytoplasm appeared electron-dense and contained numerous ribosomes (Figs. 5A, 5B, 5E). The increase in the number of *E. coli* colonies in the NaCl + betaine medium (Fig 1) apparently occurred due to division of rods by septum formation.

DISCUSSION

This study has shown that the number of *E. coli* colonies grown on a growth medium containing 0.8 M NaCl and 0.001 M betaine were nearly three times more than the number of colonies grown on the medium containing 0.8 M NaCl alone. This observation suggests that betaine exerts a strong osmoprotecting effect, stimulating *E. coli* growth under salt-induced osmotic stress.

Electron micrographs of sectioned *E. coli* cells grown on normal medium showed the cell wall composed of outer and cytoplasm membranes enclosing a periplasmic space, the nucleoid containing fine DNA fibril and the cytoplasm containing numerous ribosomes. Similar ultrastructural features have been described in gram-negative bacteria by other investigators (Jensen & Park, 1967; Murray *et al.*, 2002). The cell division is achieved by septum formation. The septum is formed by a process apparently similar to that described by other authors (Burdett & Murray, 1974; Weigand, Vinci & Rothfielf, 1976). In the recent years it has been proposed that multi-protein Tol-Pal complex in gram-negative bacteria plays a physiological role in completion of cell division (Gerding *et al.*, 2007, Yeh *et al.*, 2010).

High NaCl concentrations in the external environment have been known to cause harmful effects on bacteria, plants and animals, retarding their growth and causing death (Heilbrunn 1952; Lanyi, 1979). Electron micrographs of sectioned *E. coli* cells grown on NaCl containing medium revealed deleterious changes in all cell constituents. The nucleoid displaying thickened DNA fibrils, which is seen as a dark clump in transverse sections, is apparently due to the osmotic loss of water from the cells. The drastic changes in the nucleoid and accompanying lack of ribosomes indicate a loss of protein synthesis. The presence of numerous vesicles at the external surface of NaCl- treated cells suggests changes in permeability of the outer and cytoplasmic membranes, possibly from alterations in the Tol-Pal protein complex. In the gram-negative bacteria, Tol-Pal protein complex is also implicated in maintaining outer membrane integrity (Cascales *et al.*, 2007; Yeh, 2010). In *E. coli* Tol-Pal mutants, electron micrographs clearly demonstrate the presence of vesicles at the cell surface, their formation has been attributed to a major defect in the outer membrane (Bernadac *et al.*, 1998).

In the present study of NaCl-treated cells failed to divide, the non-dividing cells were elongated and displayed aberrant mesosomes and bulges. Disruption of the outer and cytoplasmic membranes apparently has adverse effects on the framework of cell wall, transport of metabolites and energy production resulting in cell death.

Certain amino acids such a proline glutamate and betaine are known to protect cells against salt-induced osmotic stress and dehydration (Yancey *et al.*, 1982 Rudulier *et al.*, 1984). Britten & McClure, (1962), reported that in *E. coli* levels of intracellular proline were elevated in direct proportion to increases in osmolality of the medium in the presence of externally added proline. Exogenous proline has also been shown to offset the inhibitory effect of high NaCl in *Salmonella typhimurium* (Csonka, 1979). Furthermore, it has been demonstrated that proline transport into the cell-free membrane vesicles of *E. coli* is enhanced on exposure to media of high osmolality (Kaback & Deuel, 1969).

Glycine betaine, a small N-trimethylated amino acid, is widely distributed in nature and serves as an osmolyte, protecting cells from salt-induced osmotic stress (March, 1992; Rudulier *et al.*, 1984; Yancey, 2005). In marine invertebrates, betaine balances the osmotic pressure of the blood with that of the surrounding sea water (Baldwin, 1964). Halophilic plants growing in

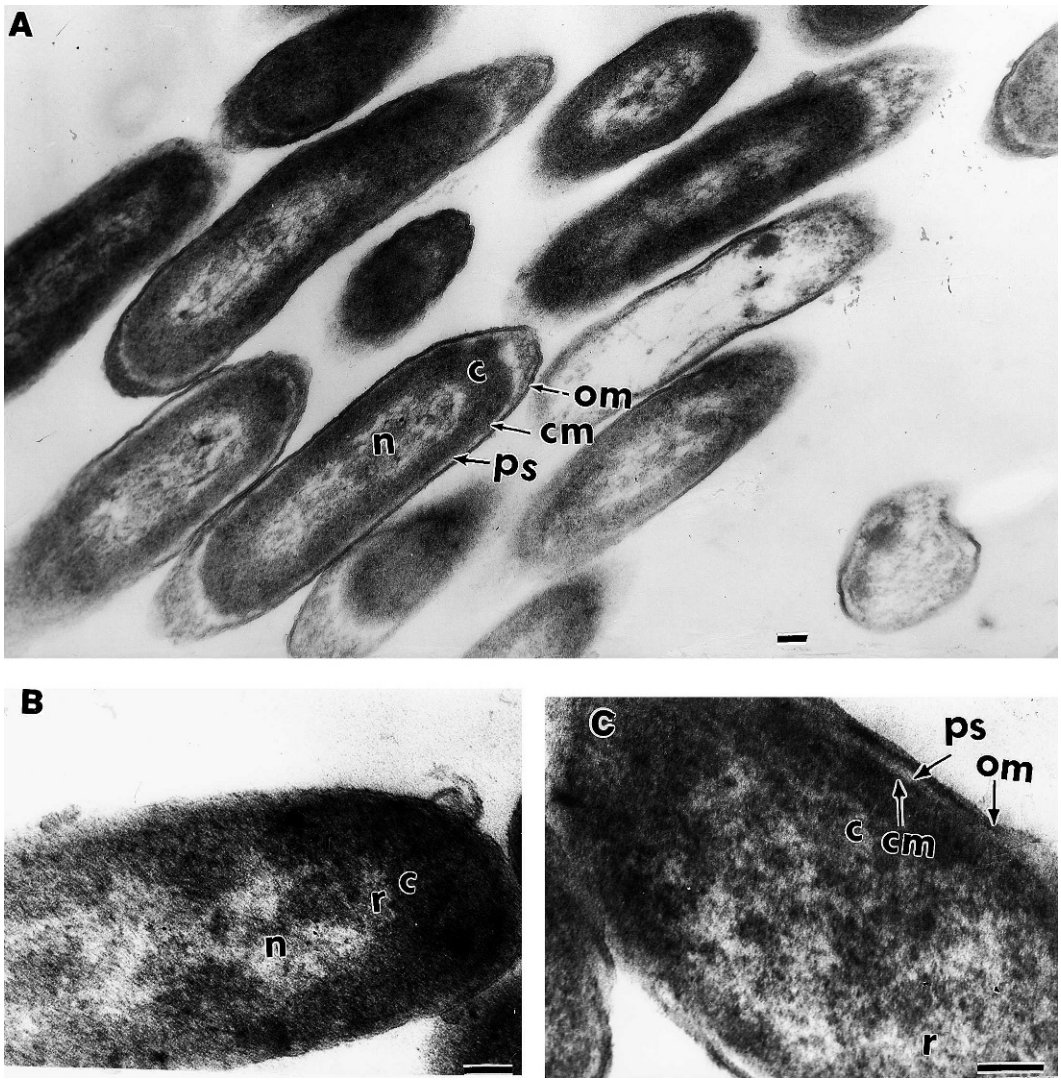

Figure 5.—Electron micrographs of *E. coli* sectioned cells in medium + NaCl + betaine. A represents a low magnification field showing normal looking rods. c, cytoplasm; cm, cytoplasmic membrane; om, outer membrane; n, nucleoid; and ps, periplasmic space. B and C are higher magnification images of parts of the cells, showing outer membrane (om), cytoplasmic membrane (cm), periplasmic space (ps) and numerous ribosomes (r) in the electron-dense cytoplasm (c) - n, nucleoid. Bar A–C 0.1 μm.

environments, in which the NaCl concentration fluctuates widely, increase synthesis of glycine betaine in response to salt stress, correlating with salt resistance (Rains & Valentine, 1979; Storey & Wyn Jones, 1975). Cyanobacteria strains with low salt tolerance synthesize and accumulate trehalose and or sucrose, and strains with the highest salt tolerance accumulate glycine betaine or glutamate betaine (Mackey et al., 1984; Yashide & Sakamoto, 2009).

In moderately halophilic bacterium Ba_1, the intracellular level of glycine betaine is directly proportional to salt-stress in salinities of 0.5 to 3.0 M NaCl, suggesting its role as a strong osmoregulatory solute (Risk, *et al.*, 1982). In addition Synechocystis DUN52, a halophilic cyanobactrium utilizes glycine betaine as a major osmolyte (Mohammed, 1983). Glycine betaine also stimulates the growth rate of enteric bacteria, *Klebsiella pneumoniae*, *Salmonella*

typhimurium & *E coli* in high-salt media and this stimulatory effect has been found to be far greater than that in proline, (Rudulier & Bouillard, 1983). Rudulier, *et al.*, (1984), have shown that growth of *E. coli* in NaCl medium is enhanced when supplemented with 0.001 M glycine betaine, which even in such a low concentration protects the bacterium from salt-stress. Furthermore, exogenous glycine betaine stimulates E. coli growth in the high-salt media by active transport into the cells driven by an electrochemical gradient, (Penroud & Rudulier, 1985).

In the present study, sectioned *E. coli* cells grown on NaCl-betaine containing medium appeared normal, displaying intact and distinct outer membrane and cytoplasmic membrane enclosing a periplasmic space. The centrally located nucleoid contained fine DNA fibrils. The electron-dense cytoplasm contained numerous ribosomes. These observations suggest that betaine acts as an effective osmoprotactant for NaCl-stressed *E. coli* by offsetting salt-induced osmotic stress. Organic molecules such as betaine are referred to as compatible solutes since they protect cells of organisms by counterbalancing perturbation of intracellular macromolecules (Yancey, 2005).

In conclusion this study has shown that *E. coli* growth is retarded in a NaCl containing medium, but its growth is stimulated in a NaCl-betaine containing medium. Ultrastructural features of normal *E. coli* cells including the outer and cytoplasmic membrane and enclosed periplasmic space, the nucleoid and ribosomes in the cytoplasm are severely altered and/or destroyed in a NaCl containing medium, but are restored to their normal form in a NaCl-betaine containing medium. The findings of this study suggest that yields of crops grown in soils with increasing salinity could be increased by adding small quantities of osmolytes like betaine to the irrigating water to protect soil microbes essential to plants, from salt-induced stress.

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LITATURE CITED

Baldwin, E. 1964. An Introduction to Comparative Biochemistry 4th Edition. Cambridge University Press, London.

Bernadac, A. et al. 1998. *Escherichia coli* Tol-Pal mutants form outer membrane vesicles. Journal of Bacteriology 180:4872–4878.

Britten, R.J. & F.T. McClure. 1962. The amino acid pool in *Escherichia coli*. Bacteriological Reviews 26:292–335.

Brown, C.M. & S.O. Stanley. 1972. Environment mediated changes in the cellular content of the ''pool'' constituents. Applied Chemical Biotechnology 22:363–389.

Burdett, I.D.J. & R.G.E. Murray. 1974. Septum formation in *Escherichia coli*; characterization of septum cell division. Journal of Bacteriology 119:303–324.

Cascales, E.A. et al. 2002. Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. Journal of Bacteriology 184: 754–759.

Csonka, C.N. 1979. The role of L-proline in response to osmotic stress in *Salmonella typhimurium*: Selection of mutants with increased osmotic tolerance as strains which overproduce proline. Pp. 35–51. *In Genetic Engineering of Osmoregulation*: Impact on plant production for food, chemicals and energy (Rains, D.W., Valentine, R.C. & A. Hollander, eds.). Planum Press, New York.

Gerding, M.A. 2007. The trans-envelope Tol-Pal complex is part of the cell division machinery and required for outer membrane invagination during cell constriction in *E. coli*. Molecular Microbiology 63:1008–1025.

Heilbrunn, L.V. 1952. An Outline of General Physiology. W. B. Saunders Company, Philadelphia.

Jensen, W.A. & K.B. Park. 1967. Cell Ultrastructure. Wadsworth Publishing Company, Belmont, CA.

Kaback, H.R. & T.F. Deuel. 1969. Proline uptake by disrupted membrane preparation from *E. coli*. Archives of Biochemistry & Biophysics 132:188–129.

Lanyi, J.K. 1979. Salt Tolerance in Microorganisms. Pp. 218–233. *In Biosaline Concept*: An approach to the utilization of underexplored resources (Hollander, A., et al. eds.). Planum Press, New York.

Mackay, M.A., R.S. Morton & L.J. Borowitzka. 1984. Organic osmoregulatory solutes in cyanobacteria. Journal of General Microbiology 130: 2177–2191.

March, J. 1992. Advanced Organic Chemistry: Reactions, Mechanisms and Structure, 4th Edition. John Wiley & Sons, New York.

Measures, J.C. 1975. Role of Amino acids in osmoregulation of non-halophilic bacteria. Nature 257:398–400.

Mohammed, F.A., R.H. Reed & W.D.P. Steward. 1983. The halophilic cyanobacterium Synechocystis DUN52 and its osmotic responses. FEMS Microbiology Letters 16:287–290.

Murray, P.R. et al. 2002. Medical Microbiology 4th Edition. Mosby, St. Louis.

Penroud, B. & D. Le. Rudulier. 1985. Glycine betaine transport in *Escherichia coli:* osmotic modulation. Journal of Bacteriology 161(1):393–401.

Rains, D.W. & R.C. Valentine. 1979. Biological Strategies for Osmoregulation. Pp. 1–5. *In Genetic Engineering of Osmoregulation*: Impact on plant production for food, chemicals and energy (Rains, D.W., Valentine, R.C. & A. Hollander, eds.). Planum Press, New York.

Risk, M. et al. 1982. Osmoregulatory mechanisms of the mesohalophilic bacterium Ba_1, Pp. 539–544. *In Biosaline Research*; A Look to the Future (Pietro, A.S. ed.). Planum Press, New York.

Rudulier, D. Le & L. Bouillard. 1983. Glycine betaine, an osmotic effector in Klebsiella pneumoniae and other members of the Enterobacteriaceae. Applied & Environmental Microbiology 46(1):152–159.

Rudulier, D. Le. et al. 1984. Molecular Biology of osmoregulation. Science 224:1064–1068.

Storey, R. & R.G. Wyn Jones. 1975. Betaine and choline levels in plants and their relationship to NaCl stress. Plant Science Letters 4:161–168.

Weigland, R.A., K.D. Vinci & L.I. Rothfield. 1976. Morphogenesis of the bacterial division septum: A new class of septum-defective mutants. Procedures of the National Academy of Science USA 73: 1882–1886.

Yancey, P.H. et al. 1982. Living with water stress: Evolution of osmolyte systems. Science 217: 1214–1222.

Yancey, P.H. 2005. Commentary: Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology 208: 2819–2830.

Yeh, Y.C. et al. 2010. The *Caulobacter* Tol-Pal complex is essential for outer membrane integrity and the positioning of polar localization factor. Journal of Bacteriology 192:4847–4859.

Yoshida, T. & T. Sakamoto. 2009. Water-stress induced trehalose accumulation and control of trehalase in the cyanobacterium *Nostoc punctiforme* IAM M-15. Journal of General and Applied Microbiology 55:135–145.

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