Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1092–1095, February 1982
Biochemistry

Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*

(periplasmic space/fixed anions/membrane phospholipids/outer membrane)

EUGENE P. KENNEDY

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

Contributed by Eugene P. Kennedy, November 9, 1981

ABSTRACT **The membrane-derived oligosaccharides (MDO) of *Escherichia coli* are periplasmic constituents containing 8–10 glucose units in a highly branched structure, linked by β 1–2 and β 1–6 bonds [Schneider, J. E., Reinhold, V., Rumley, M. K. & Kennedy, E. P. (1979) *J. Biol. Chem.* 254, 10135–10138]. The MDO are multiply substituted with *sn*-1-phosphoglycerol residues (derived from membrane phosphatidylglycerol) and with *O*-succinyl ester residues and, thus, are highly anionic. Experiments in this paper offer evidence that the biosynthesis of MDO is an important aspect of osmoregulation in *E. coli*. Cells grown in medium of low osmolarity (*ca.* 50 mosM) synthesize 16 times more MDO than those grown in the same medium with 0.4 M NaCl. In cells grown in medium of low osmolarity, it appears that MDO is the principal source of fixed anion in the periplasmic space and, thus, acts to maintain the high osmotic pressure and Donnan membrane potential of the periplasmic compartment. Regulation of MDO synthesis in response to changes in osmolarity of the medium appears to occur at the genetic level because the synthesis of new protein is needed to permit the production of MDO at high rates after shift of cells to medium of low osmolarity.**

In 1973, van Golde *et al.* (1) discovered that the turnover of membrane phospholipids in *Escherichia coli* is related to the biosynthesis of a hitherto-unrecognized class of cell constituent—the membrane-derived oligosaccharides (MDO). MDO of *E. coli* are a family of closely related substances containing about 8–10 glucose units/mol in a highly branched structure, joined by β 1–2 and β 1–6 linkages (2). They are variously substituted with *sn*-1-phosphoglycerol and phosphoethanolamine residues derived from the membrane phospholipids. Some species also contain *O*-succinyl ester residues, adding to their net negative charge.

MDO are localized in the periplasmic space of *E. coli* (3). This fact, together with their high abundance, wide occurrence in Gram-negative organisms, and relation to the metabolism of membrane lipids, suggests an essential function localized at the cell surface. The present paper reports evidence that the synthesis of MDO is an important aspect of osmoregulation in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains. The strains of *E. coli* used in this work are listed in Table 1.

Medium. Cells were grown at 37°C with vigorous aeration on a rotary shaker in low osmolarity medium containing KH_2PO_4 (1 mM), $(NH_4)_2SO_4$ (1.5 mM), $MgCl_2$ (0.08 mM), $FeSO_4$ (0.5 mg/liter), and thiamine (2 mg/liter). The medium was supplemented with Difco Casamino acids (5 g/liter) and adjusted to pH 7 with Tris free base. The osmolarity of this medium (medium I), measured by the method of freezing-point depression, was 73 mosM. The osmolarity of the medium was increased by the addition of various salts or sucrose as indicated; in most experiments, [2-^{3}H]glycerol (0.5 mM; 400,000 cpm/μmol; New England Nuclear) was also added.

Table 1. *E. coli* strains

Strain	Relevant genotype	Source
DF 214	*pgi zwf*	D. Fraenkel
BB 26-36	*plsB*	R. Bell
A 324	Wild-type	S. Luria

Measurement of Synthesis of MDO. Cultures (5 ml) of cells in this medium with [2-^{3}H]glycerol as tracer were shaken until growth ceased, which was usually after 18 hr because of limitation of the carbon source, at cell densities of about 5–9 × 10^8 per ml. To each culture, 1 ml of bovine serum albumin (50 g/liter) was added, followed by 1 ml of trichloroacetic acid (300 g/liter). The tubes were thoroughly agitated with a Vortex mixer. The precipitate, containing labeled lipids, was removed by centrifugation. A sample (5 ml) of each supernatant, containing labeled MDO, was shaken vigorously with 100 mg of Norit A charcoal in a tightly closed tube on a mechanical shaker. The labeled MDO is adsorbed on the charcoal under these conditions. The charcoal was washed by centrifugation three times with 3 ml of water. The MDO was then eluted from the charcoal by two successive extractions with 1 ml of 15% (vol/vol) pyridine (in water) for 5 min on the mechanical shaker. A portion (usually 0.2 ml) of the combined pyridine extracts was assayed for radioactivity as a measure of the synthesis of MDO from glycerol.

MDO is the principal labeled constituent of the charcoal-adsorbable, pyridine-extractable fraction after growth of cells on [2-^{3}H]glycerol in medium of moderate osmolarity, but the small amounts of labeled non-MDO material may constitute a significant fraction of the reduced amount of radioactivity found in this fraction after growth on medium of high osmolarity. Therefore, in some experiments the pyridine extract was analyzed further by chromatography on Sephadex G-25, from which MDO emerges in a well-defined peak (1).

EXPERIMENTAL RESULTS

Osmolarity of the Medium Regulates the Synthesis of MDO. Strain BB 26-36 (*pls*B) requires exogenous glycerol or glycerophosphate for growth (4). Because *sn*-1-phosphoglycerol residues derived from phosphatidylglycerol are major constituents of MDO, it is convenient to determine the amounts of synthesis of MDO by measuring the radioactivity of the MDO fraction after growth of cells on [2-^{3}H]glycerol. (Only glycerol residues are labeled from [2-^{3}H]glycerol because of the loss of tritium

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: MDO, membrane-derived oligosaccharide(s).

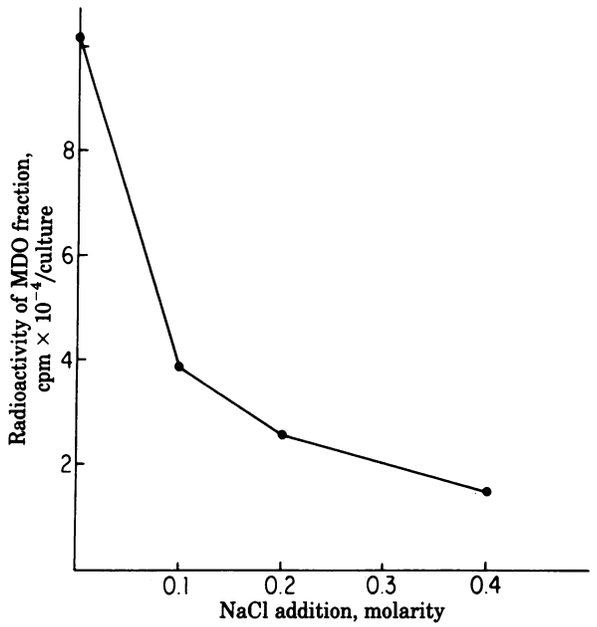

FIG. 1. Regulation of synthesis of MDO by osmolarity of the medium. Duplicate cultures of strain BB 26-36 were grown out in 5 ml of medium I containing 0.5 mM [2-^{3}H]glycerol (400,000 cpm/μmol), with varying additions of NaCl as indicated. Growth under these conditions is limited by the amount of Casamino acids used as source of nitrogen and carbon. Addition of NaCl made no consistent difference in final cell density attained (0.20–0.26 mg of protein per ml). The amount of [2-^{3}H]glycerol converted to MDO in each culture was measured by extraction of the entire culture with trichloracetic acid, adsorption on charcoal, and elution with aqueous pyridine.

during metabolism of glycerophosphate to dihydroxyacetone phosphate.)

When cultures of BB 26-36 were grown in media adjusted to various osmolarities by the addition of NaCl, the levels of labeled MDO recovered were strikingly reduced (Fig. 1) with increasing osmolarity of the medium.

The effect of added NaCl in the medium in reducing the amounts of MDO synthesized was not a specific effect of this salt. Essentially the same result was observed when the osmolarity of the medium was increased with other salts or with sucrose (Table 2).

The labeled MDO derived from cells grown on medium of high or low osmolarity was further analyzed by chromatography on Sephadex G-25 (Fig. 2). In the extract from cells grown on medium of low osmolarity, the labeled MDO comprised 88% of the total radioactivity of the extract. In contrast, labeled MDO represented only 37% of the label in the pyridine extract

Table 2. Effect of increased osmolarity from various solutes on the synthesis of MDO

Culture No.	Culture Conditions	Radioactivity of MDO fraction, cpm × 10^{-4}
1	No additions	9.07
2	NaCl, 0.4 M	1.70
3	K_2SO_4, 0.27 M	2.40
4	$(NH_4)_2SO_4$, 0.27 M	2.59
5	Sucrose, 0.6 M	1.53

Cells of strain BB 26-36 were grown in duplicate cultures of 5 ml each in medium I containing 0.5 mM [2-^{3}H]glycerol, as in the experiment of Fig. 1. Further amounts of solutes were added as indicated. The radioactivity of the MDO fraction was estimated as in Fig. 1. Values shown are averages of duplicates that agreed within 5%.

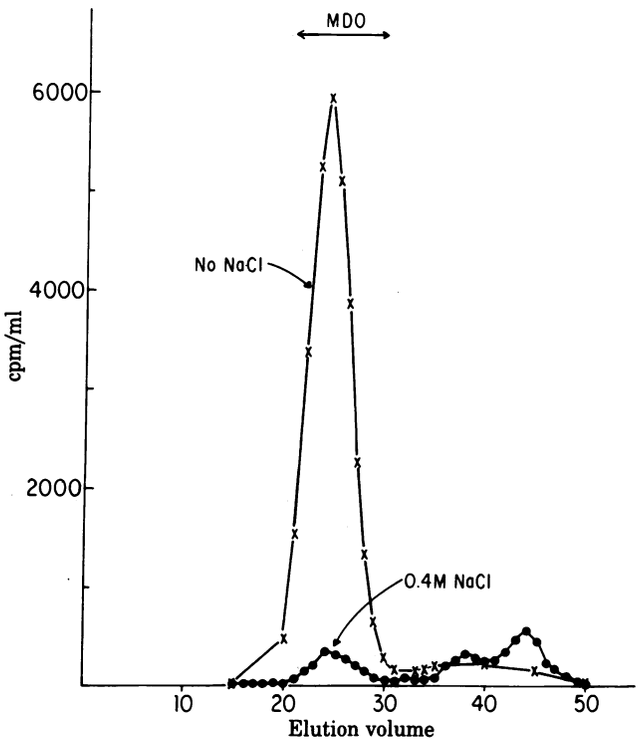

FIG. 2. Chromatography of labeled MDO fractions on Sephadex G-25. A sample (1.0 ml) of the pyridine extract derived from the cultures described in Fig. 1, grown either with no added salt or with 0.4 M NaCl, was chromatographed on a column of Sephadex G-25 (Pharmacia). The column was 1 cm^2 in cross section and 45 cm in height and had been equilibrated with 0.15 M ammonium acetate in 7% (vol/vol) aqueous propanol. The column was eluted with the same buffer at the rate of 14 ml/hr. Fractions (1.0 ml) were collected and counted.

derived from cells grown on medium of high osmolarity. Thus, the effect of osmolarity in regulating MDO synthesis was even more dramatic than is shown in Fig. 1. The synthesis of MDO in cells grown in low osmolarity was 16 times higher than in cells grown in medium with added 0.4 M NaCl, when corrected for non-MDO contaminants as in Fig. 2.

Other Strains. Regulation of MDO synthesis by the osmolarity of the medium is not peculiar to strain BB 26-36. Strains A 324, DF 214, and *E. coli B* gave essentially identical results.

Mechanism of Regulation. If some enzyme essential for the biosynthesis of MDO is inhibited as a result of growth in medium of high osmolarity, cells grown on medium of low osmolarity and then transferred to high osmolarity should abruptly reduce the rate of synthesis of MDO. This hypothesis was tested in cells of strain DF 214 (*pgi zwf*). This strain cannot carry out either the synthesis or catabolism of glucose and is, therefore, very useful for measurement of the rate of synthesis of glucose-containing MDO.

Four cultures of strain DF 214 (5 ml each) were grown to a cell density of $\approx 3 \times 10^8$ per ml in medium I supplemented with 25 mM sodium gluconate. One pair of cultures was treated with 0.5 ml of 4.4 M NaCl to bring the final concentration of added NaCl to 0.4 M. All four cultures were further incubated until the cell number of each had doubled. At this point, each culture was pulse-labeled with 0.2 mM [6-^{3}H]glucose (2×10^6 cpm/μmol) for 5 min to determine the rate of synthesis of MDO. Each culture was next treated with an equal volume of ethanol to extract the labeled MDO. The extract was taken to dryness on a rotary evaporator, the residue was resuspended in 2 ml of water, and a portion was analyzed by chromatography on Sephadex G-25, as in the experiment of Fig. 2.

Cultures 1 and 2, grown entirely in the absence of added salt, incorporated an average of 34,000 cpm in MDO during the period of labeling, or 3.4 nmol of labeled glucose per min/mg of protein. Cultures 3 and 4, grown for the last doubling with 0.4 M NaCl added, incorporated 19,000 cpm, or 1.7 nmol of labeled glucose per min/mg of protein.

It appears that medium with high osmolarity does not inhibit synthesis of MDO, but growth in such medium reduces the specific activity of the cells by dilution.

As a further test of this hypothesis, in a converse experiment, cells of DF 214 grown in medium I containing 0.4 M NaCl were transferred to medium I containing no added salt but with or without added chloramphenicol (50 μg/ml). After 1 hr in the low osmolarity medium in the absence of chloramphenicol, cells of strain DF 214 incorporated glucose into MDO at the high rate of 4.7 nmol of glucose per min/mg of protein. Cells similarly treated but in medium containing chloramphenicol were less than 1/10th as active in MDO synthesis.

The synthesis of new protein in medium of low osmolarity, prevented by the addition of chloramphenicol, is apparently needed for the synthesis of MDO at maximum rates.

DISCUSSION

In 1973, Munro and Bell (5) reported that the rate of turnover of membrane phospholipids in *E. coli* is related to the osmolarity of the medium in which the cells are growing. The rate of turnover is much increased as the osmolarity of the medium is reduced. These workers were not aware of the existence of MDO nor of the relation of these oligosaccharides to the metabolism of membrane lipids. In 1977, Schulman and Kennedy (6) found that the turnover of membrane phospholipids in *E. coli* is principally, although not entirely, the result of the transfer of their polar headgroups to MDO. The present work now offers an explanation of the findings of Munro and Bell (5) and leads to the hypothesis that the synthesis of MDO is an important aspect of osmotic regulation in *E. coli* and other Gram-negative organisms.

Cells of *E. coli* and other Gram-negative bacteria contain two distinct aqueous compartments: the cytoplasm, contained within the inner membrane, and the *periplasmic space*, contained between the inner and outer membranes. The growth of cells requires that the cytoplasm contain essential constituents, such as ions, amino acids, nucleotides, sugars etc., which in cells grown in conventional mineral medium have a total concentration of about 300 mosM (7). This value cannot be greatly reduced if cells are to continue to live and grow. From their fundamental study of the periplasmic space in *E. coli* and *Salmonella typhimurium*, Stock *et al.* (7) concluded that the inner, cytoplasmic membrane has little mechanical rigidity. When the cells are grown in medium of low osmolarity, the cytoplasmic membrane will swell unless prevented from doing so by the osmolarity of the periplasmic space. Stock *et al.* (7) offered evidence that the osmolarity of the periplasmic space is in fact about the same as that of the cytoplasm. This requires that, during growth in medium of minimum osmolarity, the periplasm also has an osmolarity higher than that of the medium. This fact in turn points to the existence of a high concentration of fixed anions in the periplasmic compartment, amounting to about 150–200 meq/g (dry weight) of cells. The authors attempted to account for this fixed negative charge in terms of known negatively charged components of the cell envelope, such as periplasmic proteins, peptidoglycan, and lipopolysaccharide, but concluded that a large fraction remained unidentified.

The present study offers evidence that the major component of the fixed anion in the periplasmic space is MDO. A 5-ml culture grown in minimum osmolarity, containing 2.5 mg (dry weight) of cells, incorporated 90,000 cpm of [2-^{3}H]glycerol (0.225 μmol) into MDO. The average MDO molecule contains three phosphoglycerol residues per mole with a total net negative charge of 5, which amounts to 150 μeq of net negative charge per gram of dry weight. Under these conditions, MDO comprises almost 7% of the dry weight of the cells.

It is now proposed that the negatively charged MDO and their counter-ions constitute the principal source of the osmotic pressure in the periplasmic space of cells growing in medium of low osmolarity. Thus, regulation of their rate of synthesis is an important element in the regulation of osmolarity. When cells are grown in a medium containing essential nutrients plus a solute such as 0.3 M sucrose, which is permeable to the outer membrane but not to the inner membrane, there is little net hydrostatic pressure on the inner membrane because the contents of the cytoplasm are also ≈300 mosM. Under these conditions, there is no need for the cell to increase the osmolarity of the periplasmic space; in fact, the synthesis of MDO is minimal. In contrast, for cells growing in a medium with nutrients and other solutions in total concentration of 50 mosM, with the cytoplasm containing constituents totaling 300 mosM, the imbalance would cause a pressure on the inner membrane of about 6.4 atm (1 atm = 1.013×10^5 Pa). To prevent the membrane from swelling, this must be countered by increasing the osmotic pressure of the periplasmic space by the synthesis and excretion of substances impermeable to the outer membrane. Under these conditions, synthesis of MDO occurs at maximal rates. Although periplasmic proteins and other macromolecules also undoubtedly contribute to the regulation of the osmolarity of the periplasm, because of their high molecular weights their contribution must be relatively limited.

The structure of MDO molecules is in many ways particularly suitable for their function in the regulation of osmolarity of the periplasm. With molecular weights in the range of about 2200–2600, they are ordinarily impermeable to the outer membrane, a property obviously essential for their postulated function. Their highly branched structure and multiple subtitution with phosphoglycerol and succinate residues allow them to maintain a high anionic charge per residue of glucose.

The presence of fixed anions in the periplasmic space leads to the development of a Donnan membrane potential across the outer membrane, negative on the inside as demonstrated by Stock *et al.* (7). This membrane potential leads to an unequal distribution of cations across the membrane, with the concentration of hydrogen ions and other cations being higher in the periplasmic space than in the medium. The magnitude of the Donnan membrane potential is a function of the ratio of the concentrations of fixed anion to permeable anion, and is, therefore, greatest in cells grown in medium of low osmolarity—a condition in which the MDO content of the periplasm is high and the concentration of anion permeable to the outer membrane is low. The Donnan potential across the outer membrane may be an important aspect of the adaptation of cells to medium of low osmolarity.

The following working model is offered for the regulation of the biosynthesis of MDO and its relation to osmoregulation. When the total osmolarity of the periplasmic space falls below that of the cytoplasm, the difference in turgor pressure, tending to cause the inner membrane to swell, is detected by an *osmotic sensor* presumably localized in the inner membrane. (This osmotic sensor, as discussed below, either could be specific for the regulation of MDO synthesis or could be a more general sensor, regulating more than one system that responds to changes in osmolarity). The increase in turgor pressure gen-

erates a signal that increases the synthesis of some essential protein(s). The synthesis of new protein must be required because MDO synthesis is not stimulated by a shift to medium of lower osmolarity if the synthesis of new protein is prevented by the addition of chloramphenicol. The protein(s) whose synthesis thus is required may be enzyme(s) needed for the biosynthesis of MDO or may act as regulator(s) of these enzymes. When MDO synthesis is activated to maximal levels by growth in medium of minimal osmolarity, synthesis at high rates continues after transition to medium of high osmolarity. Thus, the enzymes of MDO synthesis are not inhibited by the conditions of growth in high osmolarity; rather their activity seems to be reduced simply by dilution during growth in the new medium. Regulation is, thus, primarily at the genetic level.

In their important studies, Epstein and his collaborators have shown that potassium transport plays a vital role in osmoregulation in *E. coli* (8, 9). Laimins *et al.* (9) found that the activity of the high-affinity Kdp system, one of two transport systems for potassium in *E. coli*, responds to the osmolarity of the medium. An increase in osmolarity at constant concentration of potassium in the medium increased the expression of the *kdp* operon. These investigators suggested that the product of the *kdpD* gene, tentatively identified as a membrane-bound protein with a molecular weight of 90,000, may exist in either of two conformations. When the osmolarity of the medium is increased, the turgor pressure on the inner membrane, reflecting the differential between the osmolarity of the cytoplasm and the external medium, is decreased. It is proposed that under these conditions the membrane-bound KdpD protein assumes a conformation that allows it to interact with a promoter region of the *kdp* operon, increasing its transcription.

The osmolarity of the medium also strikingly affects the proportionate amounts of two major outer membrane proteins, OmpC and OmpF, products of the *ompC* and *ompF* gene, respectively (10–12). In their model for the regulation of the synthesis of these outer membrane proteins, Hall and Silhavy (13) suggest that the product of a gene designated *envZ* [previously designated *perA* (14)] plays an essential role. It is proposed that the *envZ* gene product is an envelope protein that senses the external environment and then transduces a cytoplasmic signal, which determines the expression of the desired outer membrane protein. The signal provided by the *envZ* gene regulates the dimerization of a cytoplasmic, bifunctional regulatory protein, product of the *ompR* gene. The OmpR protein, in one of its two possible forms, in turn regulates the expression of the *ompC* and *ompF* genes. Mutations in the *envZ* gene are pleiotropic, affecting a number of periplasmic and outer membrane proteins. Thus, in the model of Hall and Silhavy (13), an osmotic sensor is postulated to regulate more than one system. In contrast, the model of Laimins *et al.* (9) suggests that the osmotic sensor regulating the Kdp system directly regulates that system. In view of the large number of changes in cell structures and composition involved in osmoregulation, models postulating general osmotic sensors that coordinately regulate more than one system seem perhaps more economical, but much further work is obviously needed to understand the molecular basis of osmoregulation.

The author acknowledges the skilled technical assistance of Edward McSweegan. This research was supported by Grants GM19822 and GM 22057 from the National Institute of General Medical Sciences.

1. van Golde, L. M. G., Schulman, H. & Kennedy, E. P. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1368–1372.
2. Schneider, J. E., Reinhold, V., Rumley, M. K. & Kennedy, E. P. (1979) *J. Biol. Chem.* **254**, 10135–10138.
3. Schulman, H. & Kennedy, E. P. (1979) *J. Bacteriol.* **137**, 686–688.
4. Bell, R. M. (1974) *J. Bacteriol.* **117**, 1065–1076.
5. Munro, G. F. & Bell, C. A. (1973) *J. Bacteriol.* **116**, 257–262.
6. Schulman, H. & Kennedy, E. P. (1977) *J. Biol. Chem.* **252**, 4250–4255.
7. Stock, J. B., Rauch, B. & Roseman, S. (1977) *J. Biol. Chem.* **252**, 7850–7861.
8. Epstein, W. & Laimins, L. A. (1980) *Trends Biochem. Sci.* **5**, 21–23.
9. Laimins, L. A., Rhoads, D. B. & Epstein, W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 464–468.
10. Taylor, R. K., Hall, M. N., Enquist, L. & Silhavy, T. J. (1981) *J. Bacteriol.* **147**, 255–258.
11. van Alphen, W. & Lugtenberg, B. (1977) *J. Bacteriol.* **131**, 623–630.
12. Kawaji, H., Mizuno, T. & Mizushima, S. (1979) *J. Bacteriol.* **140**, 843–847.
13. Hall, M. N. & Silhavy, T. J. (1981) *J. Mol. Biol.* **151**, 1–15.
14. Wanner, B. L., Sarthy, A. & Beckwith, J. (1979) *J. Bacteriol.* **140**, 229–239.