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NOTES

Membrane-Derived Oligosaccharides Affect Porin Osmoregulation Only in Media of Low Ionic Strength

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Gram-negative bacteria grown under conditions of low osmolarity accumulate significant amounts of periplasmic glucans, membrane-derived oligosaccharides (MDO) in *Escherichia coli* and cyclic glucans in members of the family *Rhizobiaceae*. It was reported previously (W. Fiedler and H. Rotering, J. Biol. Chem. 263:14684–14689, 1988) that *mdoA* mutants unable to synthesize MDO show a number of altered phenotypes, among them a decreased expression of OmpF and an increased expression of OmpC, when grown in a Bacto Peptone medium of low osmolarity and low ionic strength. Although we confirm the findings of Fiedler and Rotering, we find that the regulation of OmpF and OmpC expression in *mdoA* mutants is normal in cells grown on other low-osmolarity media, eliminating the possibility that MDO itself might control porin expression. Our data suggest that a certain minimal ionic strength in the periplasm is needed for normal porin regulation. In media containing very low levels of salt, this may be contributed by anionic MDO.

Membrane-derived oligosaccharides (MDO) of *Escherichia coli* and the analogous cyclic glucans of members of the family *Rhizobiaceae* are representatives of a type of cell constituents, the periplasmic glucans, widely distributed in gram-negative bacteria (20). The synthesis of MDO in *E. coli* and of cyclic glucans in the *Rhizobiaceae* is a striking example of osmoregulation, being about 10-fold higher during growth in a medium of low osmolarity than in a medium of high osmolarity (11, 15).

The cyclic glucans are thought to play an important but poorly understood role in the interaction of species of *Rhizobium* and *Agrobacterium* with specific plant hosts (1, 6, 18). Recent reports have suggested a number of important functions for MDO in *E. coli*, including roles in cell-to-cell signaling, chemotaxis, lysis induction by bacteriophages, regulation of capsular polysaccharide synthesis, and the osmoregulation of outer membrane protein expression (3, 9). It is the last of these processes which we address in this report.

E. coli K-12 has two major outer membrane pore proteins, or porins, OmpF and OmpC. Synthesis of these porins is regulated in response to the osmolarity of the external medium by a two-component regulatory system consisting of the products of the *ompB* locus, OmpR and EnvZ (7, 8, 22). OmpR is a cytoplasmic DNA-binding protein which binds to sites upstream of the promoters of both porin genes (17). EnvZ, on the other hand, is an integral membrane protein (4, 13) and is proposed to be a sensor of external osmolarity, mediating its effect through OmpR (21). However, while EnvZ is clearly involved in the osmoregulation of porin gene expression, the means by which it senses the osmolarity of the medium are not yet known.

Fiedler and Rotering (3) observed altered expression of the porins OmpF and OmpC in the presence of a mutation at *mdoA*. They suggest that this effect is consistent with a model in which the EnvZ protein detects the presence or absence of MDO in the periplasm and uses this information as an indication of the osmolarity of the external medium, regulating expression of the porins in response. By this model, when MDO is present (i.e., in low osmolarity) EnvZ would direct the preferential synthesis of OmpF, while when MDO is absent (in high osmolarity or in the presence of an *mdoA* mutation), it should direct the synthesis of OmpC instead.

To test this hypothesis, we analyzed the osmoregulation of OmpF and OmpC in the presence of three different *mdoA* mutations (*mdoA1*, *mdoA200*::Tn*10*, and *mdoA202*::Tn*5*) in three different genetic backgrounds (parent strains MC4100, MA1008, and RZ60).

All bacterial strains used in this study are listed in Table 1. Cells were usually grown in A medium (10), which was prepared as a 2× stock and was sterilized by autoclave. Prior to use, 50 ml of this stock was diluted with an equal volume of sterile water or of 0.88 M sucrose (Boehringer Mannheim) as appropriate and then filter sterilized. Membranes were isolated from cells grown in A medium (low osmolarity) or A medium containing 0.44 M sucrose (high osmolarity). A portion (0.1 ml) of an overnight culture grown in the appropriate medium was subcultured into 10 ml of the same medium and grown to early to mid-log phase (optical density at 600 nm = 0.3 to 0.4). Cells were harvested by centrifugation, drained well, and frozen in a dry ice-ethanol bath. Membranes were then isolated as described in reference 16. These membrane fractions were analyzed by electrophoresis on gels of 12% polyacrylamide and 6 M urea.

All three *mdoA* mutations (*mdoA1*, *mdoA200*::Tn*10*, and *mdoA202*::Tn*5*) are known to block the synthesis of MDO at an early step of the pathway (2). However, under the culture conditions employed in these experiments, which we noted to be slightly different from those of Fiedler and Rotering (3),

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TABLE 1. Strains of *E. coli* K-12

Strain	Genotype	Source or reference
MA1008	*lacZ43 pyrC46 thi-1 relA1 spoT1*	CGSC[a] 5154
NFB200	MA1008 *mdoA200*::Tn*10*	E. P. Kennedy
NFB702	MA1008 *mdoA202*::Tn*5*	12
TA1008	MA1008 *mdoA1*	3
MC4100	F⁻ *araD139 lacU169 rpsL150 relA1 thiA flbB5301*	M. J. Casabadan
FR193	MC4100 *mdoA200*::Tn*10*	This study[b]
OG108	MC4100 *mdoA202*::Tn*5*	This study[b]
OG115	MC4100 *mdoA1*	This study[b]
RZ60	*dgk-6*	19
RZ60-4	RZ60 *mdoA200*::Tn*10*	This study[b]
OG110	RZ60 *mdoA202*::Tn*5*	This study[b]
OG128	RZ60 *mdoA1*	This study[b]
SG477	MC4100 *envZ22*(Am)	5
FR808	SG477 *mdoA200*::Tn*10*	This study[b]

[a] *E. coli* Genetic Stock Center

[b] Transduction experiments were carried out with phage P1 *vir* as described by Miller (14). Tetracycline-resistant colonies were selected in the presence of 20 μg of tetracycline per ml. Kanamycin-resistant colonies were selected in the presence of 25 μg of kanamycin sulfate per ml.

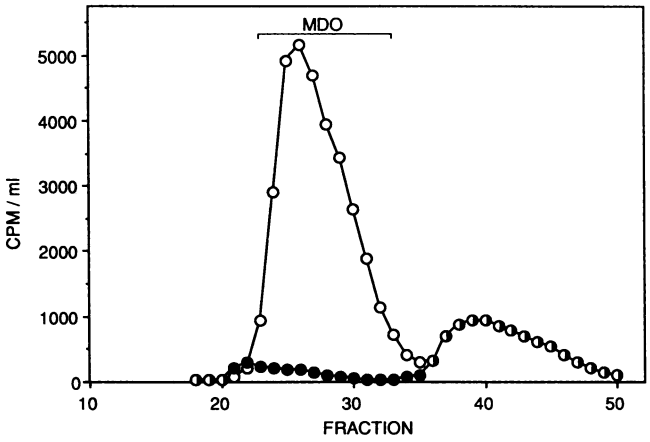

FIG. 2. Chromatography of labeled MDO fractions on Sephadex G-25. Samples (1 ml) of neutralized trichloroacetic acid extracts derived from cultures of MC4100 (○) and FR193 (●) were chromatographed on a column of Sephadex G-25. The column was 1 cm^2 in cross section and 52 cm in height and had been equilibrated with 0.15 M ammonium acetate in 7% (vol/vol) aqueous propanol. The column was eluted with the same buffer at a rate of 15 ml/h. Fractions (1 ml) were collected and analyzed for radioactivity.

none of these mutations significantly affects the osmoregulation of OmpF or OmpC in any of the backgrounds tested (Fig. 1). There are differences in porin expression among the various genetic backgrounds used, but in every case, increasing the osmolarity of the medium by adding 0.44 M sucrose leads to increased OmpC and decreased OmpF production, regardless of the mutational state of *mdoA*.

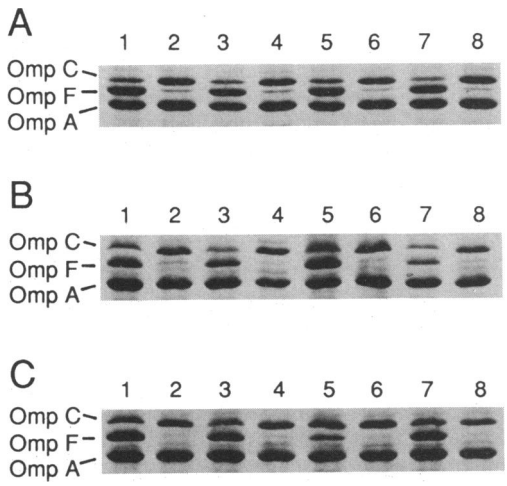

FIG. 1. Osmoregulated porin expression in *mdoA* mutants. Membrane fractions were prepared as described in the text from strains grown either in A medium (lanes 1, 3, 5, and 7) or A medium supplemented with 0.44 M sucrose (lanes 2, 4, 6, and 8). The samples were analyzed on a 12% polyacrylamide gel containing 6 M urea and stained with Coomassie blue. The positions of the outer membrane proteins OmpC, OmpF, and OmpA are indicated on the left. OmpA is used as an internal standard. (A) MC4100 background. Lanes: 1 and 2, MC4100; 3 and 4, OG115 (*mdoA1*); 5 and 6, FR193 (*mdoA200*); 7 and 8, OG108 (*mdoA202*). (B) MA1008 background. Lanes: 1 and 2, MA1008; 3 and 4, TA1008 (*mdoA1*); 5 and 6, NFB200 (*mdoA200*); 7 and 8, NFB702 (*mdoA202*). (C) RZ60 background. Lanes: 1 and 2, RZ60; 3 and 4, OG128 (*mdoA1*); 5 and 6, RZ60-4 (*mdoA200*); 7 and 8, OG110 (*mdoA202*).

Thus, osmoregulation occurs normally even in the absence of MDO production.

To ensure that these strains actually harbor *mdoA* mutations, the production of MDO was assayed directly. Cultures (5 ml) of cells were grown at 37°C in a medium containing 1% Bacto Peptone, 50 mM NaCl, 20 μg of uracil per ml, and 2 μg of thiamine per ml, with 0.1 mM [2-^{3}H]glycerol (1.6×10^7 cpm) as a tracer, until growth ceased after about 18 h. Cells were harvested and were washed twice with fresh culture medium. After they had been resuspended in 1 ml of 1% bovine serum albumin, 110 μl of 30% trichloroacetic acid was added. Suspensions were vortexed and incubated for 1 h at room temperature. After centrifugation, the trichloroacetic acid-soluble supernatants were neutralized by the addition of NH_4OH. An aliquot (1 ml) was analyzed further by chromatography on Sephadex G-25, from which MDO emerges as a well-defined peak (23). Figure 2 shows the results of this analysis for MC4100 and its *mdoA200*::Tn*10* derivative, FR193. The elution profiles of trichloroacetic acid-soluble cell extracts from Sephadex G-25 indicate that while MC4100 produces normal levels of MDO, FR193 clearly produces none. The same result was found for OG115 and OG108, which contain the *mdoA1* and *mdoA202*::Tn*5* alleles, respectively (data not shown).

The results described above demonstrate that porin expression is osmoregulated normally even in the absence of MDO. However, it remains at least a formal possibility that this osmoregulation in the absence of MDO is mediated not by EnvZ but by some alternative system which becomes active only when the MDO-EnvZ system breaks down. To address this possibility, double-mutant strains which carry both *mdoA* and *envZ* null alleles were constructed. If an alternative pathway is active in the absence of MDO, the additional absence of EnvZ should have no further effect. However, this is not what we found (Fig. 3). In *envZ*$^+$ strains (MC4100 and FR193), osmoregulation is normal regardless of the presence or absence of MDO, while in *envZ* null strains (SG477 and FR808), porin expression is severely reduced, again regardless of the presence or absence of

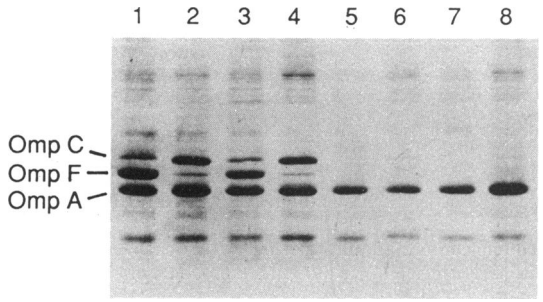

FIG. 3. Dependence of osmoregulated porin expression on *mdoA* and *envZ* alleles. Strains were grown in either A medium (lanes 1, 3, 5, and 7) or A medium supplemented with 0.44 M sucrose (lanes 2, 4, 6, and 8). Membrane fractions were analyzed as described in the legend to Fig. 1. Lanes: 1 and 2, MC4100; 3 and 4, FR193 (*mdoA200*); 5 and 6, SG477 [*envZ22*(Am)]; 7 and 8, FR808 [*mdoA200* and *envZ22*(Am)].

MDO. Thus, porin osmoregulation is fully dependent on the presence of functional EnvZ protein but not on the presence or absence of MDO.

These results are in apparent conflict with those reported earlier by Fiedler and Rotering (3). In an attempt to resolve this conflict, we decided to examine porin expression under exactly the conditions used in the earlier study. After growth on 0.5% Bacto Peptone (Difco) medium (3) containing 20 μg of uracil per ml and 2 μg of thiamine per ml, the wild-type strain MA1008 expresses a high OmpF/OmpC ratio (Fig. 4, lane 1), whereas the mutant strain TA1008 (*mdoA1*) has a much lower OmpF/OmpC ratio than is normal (Fig. 4, lane 6), confirming the results reported by Fiedler and Rotering (3). The addition of a small amount of salt (15 mM KCl) to the growth medium has no effect on the OmpF/OmpC ratio of the wild type (Fig. 4, lane 2) but restores the OmpF/OmpC ratio of the mutant (Fig. 4, lane 7) to the wild-type level and therefore compensates for the MDO defect in TA1008. When 300 mM KCl is added, OmpF amounts are reduced and amounts of OmpC are increased in both strains (Fig. 4, lanes 3 and 8), as expected for growth in a medium of such high osmolarity. The addition of 30 mM sucrose, in place of 15 mM KCl, to the medium has no detectable effect on the OmpF/OmpC ratio of the wild type (Fig. 4, lane 4). In the mutant, the presence of 30 mM sucrose does not compensate for an MDO defect on the OmpF/OmpC ratio (Fig. 4, lane 9). Thus, the effect of equiosmolar 15 mM KCl on the mutant

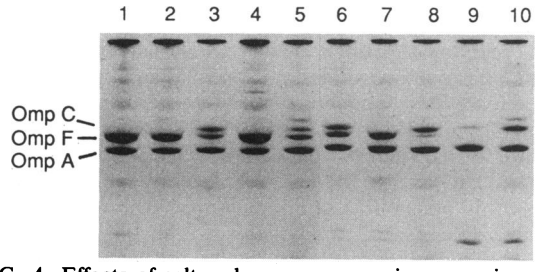

FIG. 4. Effects of salt and sucrose on porin expression. Membrane fractions of MA1008 and TA1008 (*mdoA1*) were prepared and analyzed as described in the legend to Fig. 1 after growth on Bacto Peptone (0.5%) or Bacto Peptone supplemented with KCl or sucrose. Lanes 1 to 5, MA1008; lanes 6 to 10, TA1008. Lanes: 1 and 6, no added salt or sucrose; 2 and 7, 15 mM KCl; 3 and 8, 300 mM KCl; 4 and 9, 30 mM sucrose; 5 and 10, 600 mM sucrose.

strain appears to be one of ionic strength, not osmolarity. The addition of 15 mM NaCl also restores the OmpF/OmpC ratio of the mutant to normal (data not shown), indicating that the observed effect is not specific for potassium. The total shutdown of OmpF synthesis (Fig. 4, lane 9) in the presence of 30 mM sucrose is surprising and causes an even more drastic difference between porin patterns of the wild-type strain and the *mdoA* mutant than that which is seen without any added osmolytes. The addition of 600 mM sucrose leads to increased OmpC expression in both strains (Fig. 4, lanes 5 and 10), as expected for growth in a medium of high osmolarity. We noted that at high osmolarity there is also a slight difference between porin patterns of the wild-type strain and the *mdoA* mutant (Fig. 4, lanes 3 versus 8 and lanes 5 versus 10) after growth on Bacto Peptone medium. However, it is hard to believe that MDO itself might be responsible for the expression of these different porin patterns, because at high osmolarity even wild-type strains make no or very little MDO (11). We presently do not know what causes these differences. Similar results were obtained with strain NFB200 (*mdoA200*) in place of TA1008 (data not shown).

Consistent with the results reported previously by Fiedler and Rotering (3), we have found that in a medium of very low osmolarity, mutations at the *mdoA* locus have a pronounced effect on porin production. However, our results indicate that under certain conditions under which these effects of *mdoA* mutations are observed, the addition of a small amount of salt to the growth medium restores the normal, reciprocal regulation of the porins in the mutant strains.

The fact that osmoregulation can occur normally, even when MDO synthesis has been abolished, effectively rules out any model in which EnvZ utilizes the presence or absence of MDO as an indication of the osmolarity of the growth medium. If MDO were indeed the signal recognized by EnvZ, then the cell should respond to the lack of MDO caused by an *mdoA* mutation as if it were growing under high-osmolarity conditions at all times. We have observed normal EnvZ-mediated osmoregulation in the presence of three different *mdoA* mutations in three different genetic backgrounds. Thus, we must conclude that EnvZ is sensing something other than MDO in order to regulate porin expression.

Under certain conditions, however, blocking MDO synthesis does have a significant effect on porin production. In the very low osmolarity and low-ionic-strength medium used by Fiedler and Rotering (3), mutations at *mdoA* cause an increase in OmpC production and a decrease in OmpF production. The addition of low concentrations of salt reverses this effect, but an equiosmolar concentration of sucrose does not. This unexpected result suggests that the ionic strength of the periplasm is of critical importance for EnvZ function in media of very low osmolarity. Under these conditions, MDO are produced in large amounts (about 5% of the dry weight of the cells), and the presence of these multiply charged molecules in the periplasm would provide a high degree of ionic strength. This ionic strength would be lost in an *mdoA* mutant, which could lead to misregulation of porin synthesis. At present, we cannot distinguish whether the effect of ionic strength on porin regulation is direct, as a component of the low-osmolarity signal recognized by EnvZ, or indirect, through other effects on cell physiology. This question must be addressed by future work.

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REFERENCES

1. **Abe, M., A. Amemura, and S. Higashi.** 1982. Studies on cyclic β-(1-2)glucan obtained from periplasmic space of *Rhizobium trifolii* cells. Plant Soil **64:**315–324.
2. **Bohin, J.-P., and E. P. Kennedy.** 1984. Mapping of a locus (*mdoA*) that affects the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. J. Bacteriol. **157:**956–957.
3. **Fiedler, W., and H. Rotering.** 1988. Properties of *Escherichia coli* mutants lacking membrane-derived oligosaccharides. J. Biol. Chem. **263:**14684–14689.
4. **Forst, S., D. Comeau, S. Norioka, and M. Inouye.** 1987. Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. J. Biol. Chem. **262:**16433–16438.
5. **Garrett, S., R. K. Taylor, T. J. Silhavy, and M. L. Berman.** 1985. Isolation and characterization of Δ*ompB* strains of *Escherichia coli* by a general method based on gene fusions. J. Bacteriol. **162:**840–844.
6. **Geremia, R. A., S. Cavaignac, A. Zorreguieta, N. Toro, J. Olivares, and R. A. Ugalde.** 1987. A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β-(1→2) glucan. J. Bacteriol. **169:**880–884.
7. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K12. J. Mol. Biol. **151:**1–15.
8. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K12. J. Mol. Biol. **146:**23–43.
9. **Hoeltje, J.-V., W. Fiedler, H. Rotering, B. Walderich, and J. van Duin.** 1988. Lysis induction of *Escherichia coli* by the cloned lysis protein of the phage MS2 depends on the presence of osmoregulatory membrane-derived oligosaccharides. J. Biol. Chem. **263:**3539–3541.
10. **Kawaji, H., T. Mizuno, and S. Mizushima.** 1979. Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12. J. Bacteriol. **140:**843–847.
11. **Kennedy, E. P.** 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79:**1092–1095.
12. **Lacroix, J.-M., I. Loubens, M. Tempete, B. Menichi, and J.-P. Bohin.** 1991. The *mdoA* locus of *Escherichia coli* consists of an operon under osmotic control. Mol. Microbiol. **5:**277–285.
13. **Liljestrom, P.** 1986. The EnvZ protein of *Salmonella typhimurium* LT-2 and *Escherichia coli* K-12 is located in the cytoplasmic membrane. FEMS Microbiol. Lett. **36:**145–150.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Miller, K. J., E. P. Kennedy, and V. N. Reinhold.** 1986. Osmotic adaption by gram-negative bacteria: possible role for periplasmic oligosaccharides. Science **231:**48–51.
16. **Morona, R., and P. Reeves.** 1982. The *tolC* locus of *Escherichia coli* affects the expression of three major outer membrane proteins. J. Bacteriol. **150:**1016–1023.
17. **Norioka, S., G. Ramakrishnan, K. Ikenaka, and M. Inouye.** 1986. Interaction of a transcriptional activator, OmpR, with reciprocally osmoregulated genes, *ompF* and *ompC*, of *Escherichia coli*. J. Biol. Chem. **261:**17113–17119.
18. **Puvanesarajah, V., F. M. Schell, G. Stacey, C. J. Douglas, and E. W. Nester.** 1985. Role for 2-linked-β-D-glucan in the virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **164:**102–106.
19. **Raetz, C. R. H., and K. F. Newman.** 1978. Neutral lipid accumulation in the membranes of *Escherichia coli* mutants lacking diglyceride kinase. J. Biol. Chem. **253:**3882–3887.
20. **Schulman, H., and E. P. Kennedy.** 1979. Localization of membrane-derived oligosaccharides in the outer envelope of *Escherichia coli* and their occurrence in other gram-negative bacteria. J. Bacteriol. **137:**686–688.
21. **Slauch, J. M., S. Garrett, D. E. Jackson, and T. J. Silhavy.** 1988. EnvZ functions through OmpR to control porin gene expression in *Escherichia coli* K-12. J. Bacteriol. **170:**439–441.
22. **van Alphen, W., and B. Lugtenberg.** 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. J. Bacteriol. **131:**623–630.
23. **van Golde, L. M. G., H. Schulman, and E. P. Kennedy.** 1973. Metabolism of membrane phospholipids and its relation to a novel class of oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **70:**1368–1372.