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# Genetic and Physiological Regulation of Intrinsic Proteins of the Outer Membrane of *Salmonella typhimurium*

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Four major outer membrane polypeptides, accounting for approximately 20% of the total protein of the outer membrane of *Salmonella typhimurium*, were induced by growth in minimal medium. The polypeptides were tightly bound membrane components. Physiological and genetic evidence indicates that the four polypeptides fall in two separate regulation groups. Synthesis of one of these groups was coordinately regulated by the concentration of iron in the medium, and a mutant strain has been identified in which there is constitutive synthesis of this group of major outer membrane proteins.

The cell envelope of a typical gram-negative bacterium contains two membranes, the inner (cytoplasmic) and outer membranes (12). Many physiological functions are associated with the large number of proteins located in the inner membrane, and it is well established that the protein composition of the inner membrane can be modified in response to a variety of environmental and physiological stimuli, as shown, for example, by the induction of specific transport systems (5). In contrast, the outer membrane contains a much smaller number of proteins present in relatively large amounts.

In this report, we show that four major outer membrane polypeptides accounting for approximately 20% of the outer membrane protein of *Salmonella typhimurium* fall in two separate regulation groups that differ in their mode of physiological control. Synthesis of one of these groups is regulated by the concentration of iron in the medium, and a mutant strain has been isolated in which there is constitutive expression of this group of outer membrane proteins.

The demonstration that synthesis of a large fraction of the major outer membrane proteins is genetically and physiologically regulated suggests that these proteins play an active role in the physiology of the cell, possibly by participating in the translocation of iron and other nutrients across the outer membrane.

(A portion of this material has been presented [R. L. Bennett, J. Lopes, and L. Rothfield, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P85, p. 155]).

## MATERIALS AND METHODS

**Organisms and media.** *S. typhimurium* G30 (*galE*$^-$) and R18 (*galE*$^-$, *strA*$^R$), a mutant derived from G30, have been described (7). Strain SA536 (Hfr K6, *serA13*) was obtained from K. Sanderson. The enriched medium was proteose peptone-beef extract (15); minimal medium (M9, reference 3) contained 22 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 18 mM $NH_4Cl$, 8.5 mM NaCl, 1.9 mM $MgSO_4$, and 0.1 mM $CaCl_2$, supplemented with either 0.4% D-glucose, D-galactose, or glycerol; solid media contained 1.5% agar. Conditioned medium was prepared by growth of strain G30 in M9-glucose until the optical density at 600 nm reached 0.8 to 1.0 units. Cells were removed by centrifugation at 16,300 × *g* for 40 min, and the supernatant (conditioned medium) was decanted into sterile glassware and used without further treatment.

**Envelope isolation.** Exponentially growing cells were collected by centrifugation at 16,300 × *g* for 30 min at 4 C, suspended in cold 0.15 M NaCl, and pelleted again by centrifugation at 27,000 × *g* for 20 min. Cells were suspended in TE buffer [0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 8.0] and disrupted by sonication or by passage at 18,000 lb/in$^2$ through a French pressure cell prechilled to 4 C. Whole cells were then removed by centrifugation at 1,100 × *g* for 20 min. The envelope fraction was collected by centrifugation at 39,000 × *g* for 2 h, after which the pellets were suspended in TE buffer and the centrifugation was repeated. The pellets were suspended in TE buffer and stored at −20 C. Treatment of the cell envelope with detergents (1%) or chaotropic agents in TE buffer was for 1 h at 37 C followed by centrifugation at 105,000 × *g* for 1 h to separate the solubilized material from the particulate residue. The supernatant was dialyzed against 20 mM Tris-hydrochloride, 2 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS), pH 8.0, before preparation for electrophoresis.

**Membrane fractionation.** Cells were grown in 1 liter of enriched medium or M9-glycerol medium. (Glycerol was used as the carbon source to induce

expression of L-α-glycerophosphate dehydrogenase, a marker enzyme for inner membrane.) Inner and outer membranes were separated according to the procedure of Osborn et al. (12), except that the membranes were not washed before layering on the sucrose density gradient.

**Analytical methods.** L-α-glycerophosphate dehydrogenase activity was measured in an incubation mixture containing 0.1 M Tris-hydrochloride (pH 7.3), 0.01 M NaCN, 30 μg of 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) monotetrazolium bromide per ml, 10 μg of phenazine methosulfate per ml, and 0.05 M DL-α-glycerophosphate. Portions of the pooled fractions containing 10 to 150 μg of protein were assayed. The increase in absorbance at 550 nm at room temperature was corrected for the increase in absorbance in the absence of substrate. Phospholipase activity (combined activities of phospholipase A and lysophospholipase) was measured as described by Osborn et al. (12). Iron uptake was measured during growth of cells in M9-glucose medium containing 0.06 μCi of $^{59}FeCl_3$ per ml (New England Nuclear Corp.). At intervals, 1 ml of the cell suspension was centrifuged for 6 min in a Brinkman Microfuge, and the $^{59}Fe$ remaining in the supernatant was determined using a Nuclear Chicago gamma counter. Protein was assayed by the method of Lowry et al. (8), and iron was measured by the method of Carter (2).

**Electrophoresis.** The SDS-polyacrylamide gel system of Neville (11) was used. The running gel and lower reservoir were buffered with 0.42 M Tris-hydrochloride, pH 9.18. The running gel was 11% (wt/vol) acrylamide and 0.1% *N',N'*-methylenebisacrylamide, and the stacking gel was 3% acrylamide plus 0.2% *N',N'*-methylenebisacrylamide. Samples containing 50 to 100 μg of protein in 0.02 ml of TE buffer were mixed with 0.01 ml of 0.05 M Tris-hydrochloride (pH 6.8), 1% (wt/vol) SDS, 0.002 M EDTA, 0.14 M 2-mercaptoethanol, 10% (wt/vol) glycerol, with 0.2% bromophenol blue added as the tracking dye. This solution was heated to 100 C for 3 min before it was layered on the gel. Slab gels (9-cm running gel, 1-cm stacking gel) were used for unlabeled preparations. After electrophoresis, gels were immersed in a solution of 0.05% Coomassie blue in methanol-water-acetic acid (5:4:1). The gels were stained by overnight incubation at 37 C and destained electrophoretically (E-C Apparatus Corp., St. Petersburg, Fla.).

Tubular gels (11-cm running, 1.5-cm stacking) were used for electrophoresis of labeled proteins. The gels were frozen and thawed before fractionation (Aliquogel fractionator, Gilson Medical Electronics, Middleton, Wis.). Each 1-mm fraction was incubated in 0.5 ml of 1% SDS for 2 h at 37 C before addition of scintillation fluid.

**Isolation of recombinants.** Strain SA536 (Hfr) was grown in 10 ml of enriched medium in a 250-ml flask with slow shaking. Strain R18 ($F^-$) was grown in enriched medium with vigorous aeration. The Hfr was mixed with a 10-fold excess of $F^-$, and the mixture was incubated for 4 h without shaking. This mixture was diluted and plated on M9-galactose medium containing 200 μg of streptomycin per ml. After 2 days, recombinants appeared at a frequency of $5 \times 10^{-4}$ colony-forming units per Hfr. (This low frequency of recombination was characteristic of strain R18 and may reflect an abnormality of the outer membrane structure in this strain.) Because these matings were allowed to continue for 4 h before plating on the selective medium, the recombinants isolated were possible siblings. A total of 240 recombinants, including progeny from two independent matings, was cloned on M9-galactose plates before scoring for the excretion of ribonuclease (19). Clones that did not excrete ribonuclease but did express OM-1 to -3 constitutively (see below) were recovered from both matings.

## RESULTS

**Induction of outer membrane proteins.** Physiological regulation of the synthesis of outer membrane proteins was first suggested by the observation that cells grown in minimal medium contained several envelope polypeptides that were not present in cells grown in enriched medium (Fig. 1 and 2a). The present paper is concerned with four of these polypeptides (OM-1, -2, -3, and -4).

When inner (cytoplasmic) and outer membranes of the cell envelope were analyzed separately (Fig. 3), OM-1, -2, -3, and -4 were seen primarily in the outer membrane fraction. Thus, within the limits of resolution of the membrane separation technique, OM-1 to -4 appear to be outer membrane proteins. In the Neville SDS-gel electrophoretic system, polypeptides OM-1, OM-2, and OM-3 had apparent molecular weights of 82,000, 79,000, and 77,000, respectively, and OM-4 had an apparent molecular weight of 45,000. Labeling experiments with radioactive arginine (Fig. 1) indicated that OM-1, -2, and -3 accounted for approximately 13%, and OM-4 accounted for 8%, of the outer membrane protein in cells grown in minimal medium, confirming that these polypeptides are major components of the outer membrane. Thus, of the nine major peptides found in the outer membrane of cells grown in minimal medium, four (OM-1 to -4) appeared to be repressed by growth in enriched medium.

Similar differences in electrophoretic pattern between induced and uninduced cells were obtained when the envelope polypeptides were solubilized in 0.3% SDS at 70 C and 100 C, and in 1 or 3.6% SDS at 100 C. When cells grown in enriched medium and cells grown in minimal medium were mixed before cell disruption and isolation of the envelope fraction, the gel pattern was identical to that seen when purified envelopes were mixed just before preparation for electrophoresis. These results indicate that the differences in gel patterns reflect true dif-

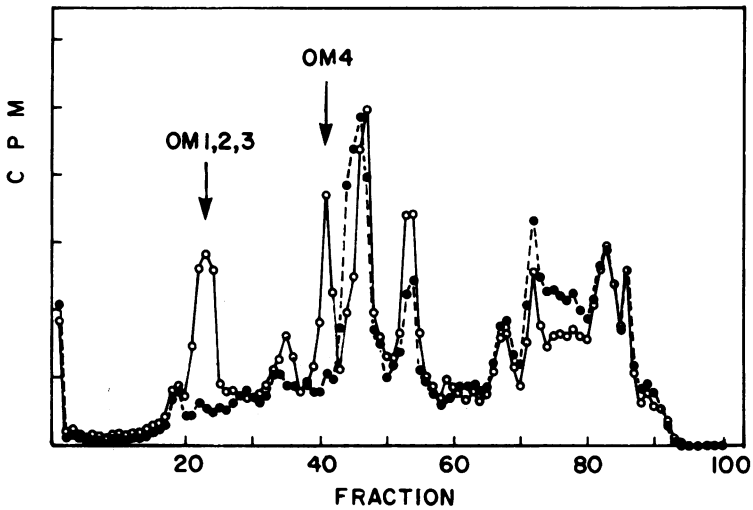


FIG. 1. *Cell envelope proteins of cells grown in minimal and enriched media. Strain G30 was grown to mid-exponential growth phase in 20 ml of M9-glucose. 15 μCi of [$^{14}$C]arginine (15 μCi/mg) was added, and growth was continued for 60 min to achieve steady-state labeling. A parallel culture of strain G30 was grown in enriched medium and was labeled by addition of 200 μCi of [$^{3}$H]arginine for 60 min. Cells were collected, and cell envelopes were prepared. The cell envelope preparations were mixed, treated with SDS, and subjected to electrophoresis as described in Materials and Methods. The peak labeled OM1,2,3 includes three bands (OM-1, -2, and -3) that are resolved on stained gels (see Fig. 2a for comparison). Symbols: (○) [$^{14}$C]arginine; (●) [$^{3}$H]arginine.*

ferences in the protein compositions of the cell envelopes rather than artifacts of solubilization or of modification during preparation of the cell envelope fractions.

**De novo synthesis of (OM-1 to -4).** Evidence that the new polypeptides resulted from de novo synthesis rather than from modification of preexisting proteins was obtained by labeling the cells with radioactive leucine before the shift from enriched to minimal medium. When the prelabeled cells were shifted to minimal medium, there was no detectable appearance of label in OM-1 to -4, although synthesis of the new polypeptides was clearly seen when the labeled leucine was added after the shift. When the reverse experiment was carried out by prelabeling cells in minimal medium and shifting to enriched medium without radioactive precursor, there was no apparent loss of labeled OM-1 to -4 relative to the other labeled cell envelope proteins. The appearance of OM-1 to -4 was prevented by addition of chloramphenicol at the time of shift to minimal medium. These experiments indicate that the appearance of OM-1 to -4 results from de novo synthesis of the polypeptides.

**Independent regulation of OM-1 to -3 and OM-4.** The first evidence that OM-1, -2, -3, and -4 are regulated by at least two separate control systems came from isolation of a constitutive mutant (strain R18) in which OM-1 to -3 were fully expressed both in enriched and in minimal medium (Fig. 2). In contrast, OM-4 was regulated normally, being induced only in minimal medium. We conclude that R18 carries a mutation in a gene that controls the inducibility of OM-1 to -3 without affecting OM-4, providing strong evidence that the two groups of proteins are regulated independently.

Strain R18 originally was isolated as a mutant that excreted ribonuclease and other periplasmic proteins into the medium and is therefore presumed to be defective in the barrier function of the outer membrane (7). This defect does not appear to result from a defect in the lipopolysaccharide (LPS) of this strain. R18 remains sensitive to phages C21 and T4 and resistant to phage P22, indicating that the LPS of R18 is similar to the LPS of its parent G30. In addition, LPS purified from R18 has the expected acceptor activity for the in vitro addition of galactose catalyzed by the enzyme uridine 5'-diphosphate-galactose: lipopolysaccharide-α,3-galactosyl transferase (J. Lopes and L. I. Rothfield, unpublished data).

Studies of genetic recombinants between R18 and SA536, a wild-type Hfr strain, showed that the mutation responsible for constitutive expression of OM-1 to -3 in R18 was unrelated to the mutation causing leakage of periplasmic proteins. Of 12 recombinant clones that did not excrete the periplasmic ribonuclease, five still

constitutively expressed OM-1 to -3 when grown in enriched medium. Therefore, one mutant phenotype, the excretion of ribonuclease, was removed by recombination, whereas the second mutant phenotype, constitutive expression of OM-1 to -3, was unaffected, indicating that the two phenotypes resulted from separate and independent mutations. The isolation of the constitutive strain (R18) among the periplasmic leaky mutants therefore appears to have resulted from a fortuitous second mutation in this strain.

**Mechanism of induction of OM-1 to -3.** Examination of the temporal sequence of induction of the four proteins showed that OM-4 appeared within one doubling after the shift to minimal medium, whereas induction of OM-1 to -3 was delayed until the cells had undergone several rounds of division. This further indicated that the two groups of proteins were regulated by different mechanisms and suggested the possibility that induction of OM-1 to -3 might be due to depletion of a component of the minimal medium.

This idea was supported by studies of induction in fresh medium and in medium that previously had been conditioned by supporting the growth of cells to high cell density. When cells were inoculated into fresh minimal medium, expression of OM-1 to -3 was delayed until the cells had grown to a density of approximately $4 \times 10^8$ cells per ml. In contrast, when cells were inoculated into conditioned medium, OM-1 to -3 were induced even at low cell densities ($<1 \times 10^8$ cells/ml); dilution of conditioned medium with fresh medium prevented the induction at low cell density. These results were consistent with a model in which OM-1 to -3 were normally repressed by a component present in enriched and, to a lesser extent, in minimal medium,

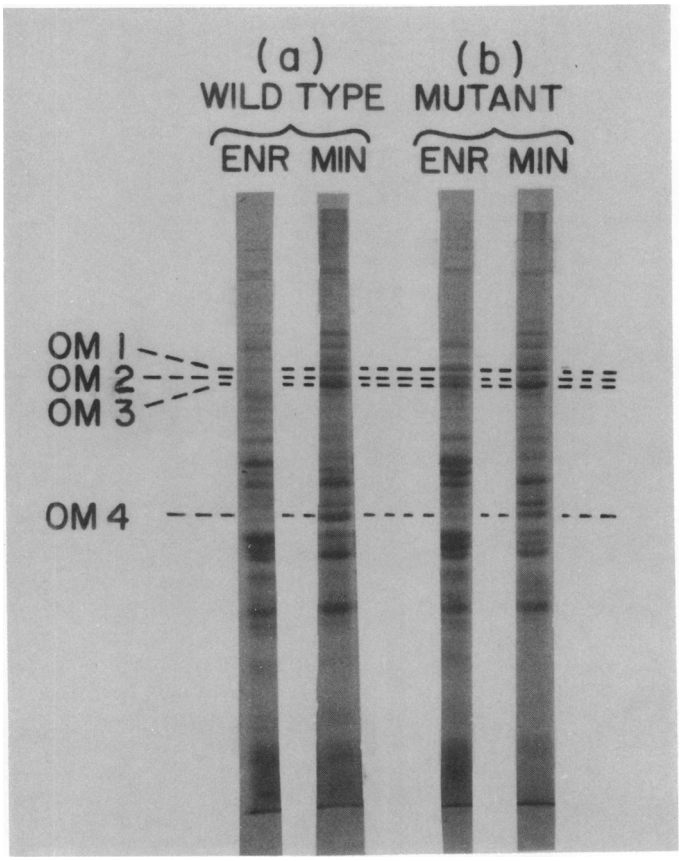


FIG. 2. *Cell envelope proteins of strains G30 and R18. (a) Strain G30 (wild type) was grown to mid-exponential growth phase in enriched medium (ENR) and in M9-glucose medium (MIN). Cell envelopes were prepared and subjected to electrophoresis as described in Materials and Methods. (b) Strain R18 (mutant) was treated in the same way.*

with induction occurring when the concentration of this component fell to low levels during growth in minimal medium.

This led to the discovery that the expression of OM-1 to -3 was regulated by the concentration of iron in the medium. When a variety of components that might have been present in limiting amounts in minimal medium were added to the growth medium, it was found that 1.8 $\mu$M $FeSO_4$ was effective in preventing induction of OM-1 to -3 (Fig. 4). As expected, induction of OM-4 was not affected by addition of iron to the medium. OM-1 to -3 were not repressed by addition of $CaCl_2$, $MnCl_2$, or $ZnCl_2$, or by supplemental nitrogen sources (adenosine, mixtures of amino acids) or by replacing glucose with other carbon sources (glycerol, lactate).

Chemical analysis showed that M9 medium prepared in this laboratory contains 0.75 $\mu$M iron, presumably from iron contamination of water and other components of the medium. The relation of concentration of iron to derepression of OM-1 to -3 was determined by inoculating cells into minimal medium containing $^{59}Fe^{3+}$ and removing samples at intervals for analysis of iron uptake and for appearance of OM-1 to -3. The three polypeptides were first detected in the cell envelope when the $^{59}Fe$ concentration fell to 50% of the original level, suggesting that the threshold concentration of iron for derepression of OM-1 to -3 is approximately 0.4 $\mu$M. No attempt was made to determine whether $Fe^{2+}$ or $Fe^{3+}$ was directly responsible because of the difficulty in controlling reduction of $Fe^{3+}$ to $Fe^{2+}$ in the medium and in the cells.

**Nature of the induced proteins.** Polypeptides OM-1 to -4 were tightly bound to the cell envelope. Complete solubilization occurred

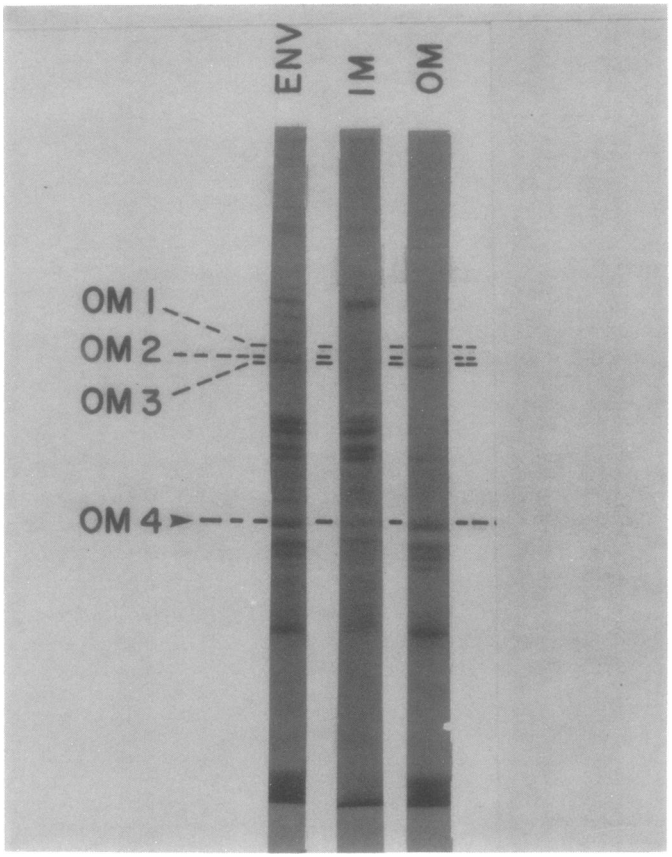


FIG. 3. *Comparison of inner and outer membranes. Strain G30 was grown in M9-glucose medium as in Fig. 2. Cell envelope (ENV), inner membrane (IM), and outer membrane (OM) fractions were prepared and were subjected to electrophoresis as described in Materials and Methods. The ratios of lipase to glycerophosphate dehydrogenase activities in the three fractions were: ENV, 1.4:1; IM, 0.2:1; OM, 14.2:1.*

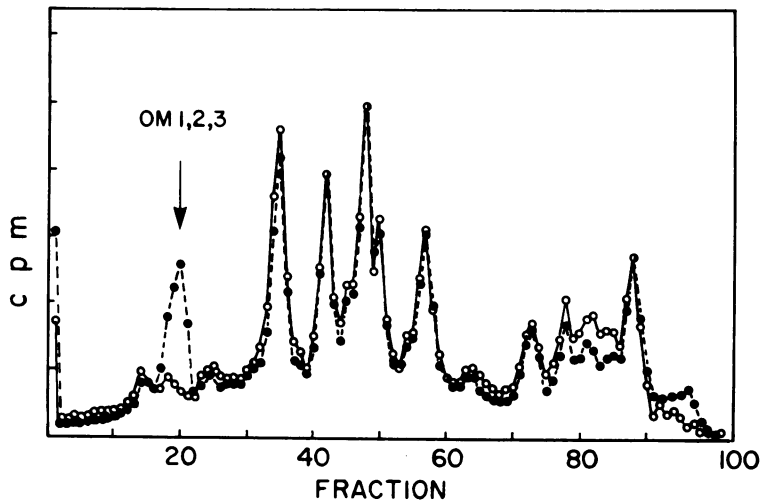


FIG. 4. *Effect of iron on expression of OM-1 to -3. Strain G30 was grown to mid-exponential growth in 20 ml of M9-glucose containing 6.5 μM ferrous sulfate. The culture was labeled with [$^{14}$C]arginine as described in Fig. 1, and cell envelopes were prepared. A parallel culture was treated identically, except that ferrous sulfate was omitted and [$^{14}$C]arginine was replaced by [$^{3}$H]arginine (0.15 mCi, 0.15 mCi/mg). Cell envelope preparations were mixed, treated with SDS, and subjected to electrophoresis as described in Materials and Methods. Symbols: (○) [$^{14}$C]arginine; (●) [$^{3}$H]arginine.*

only after treatment of the cell envelope with SDS or with 6 M guanidinium thiocyanate, a powerful chaotropic agent (10). The proteins were not released by treatment of the cell envelope with the weaker neutral detergents Triton X-100 or Alfonic (all major envelope proteins are resistant to solubilization by these detergents). Treatment with 6 M guanidinium hydrochloride resulted in partial (<40%) solubilization. Thus, release of the proteins was seen only with agents that are among the most effective presently available in their ability to disrupt hydrophobic bonds, suggesting that hydrophobic interactions probably play a major role in the association of these proteins with other membrane components.

## DISCUSSION

The observation of physiological and genetic regulation of outer membrane polypeptide synthesis indicates that the spectrum of intrinsic proteins of the outer membrane can be modified in response to changing needs of the cell. Differences in outer membrane polypeptides from cells grown under different conditions have been observed in another strain of *S. typhimurium* (1) and in *Escherichia coli* (16). These independent observations indicate that regulation of outer membrane proteins is widespread in gram-negative bacteria. This in turn suggests that the role of this membrane is not purely structural and that it may provide a variety of functions that are useful in the physiology of the total organism.

The finding that synthesis of OM-1 to -3 is specifically repressed by addition of iron to the medium suggests that these polypeptides are in some way involved in iron metabolism. The fact that they are intrinsic proteins suggests that they may participate in transport of iron across the outer membrane barrier. If this is correct, induction of the proteins when the external concentration of iron falls to low levels would serve a useful role in promoting entry of this essential nutrient. In fact, there is indirect evidence for participation of an outer membrane component in iron metabolism or transport in another gram-negative bacterium, *E. coli*, where *tonA,b* mutants appear to be defective in iron transport (6, 17). Because the *tonA,B* gene products also appear to be phage receptor sites (6), they probably are outer membrane components and therefore may well be involved in iron transport across the outer membrane. Other outer membrane proteins appear to function in the transport of vitamin B12 (4) and maltose (14). Thus, there may be several transport systems located in the outer membrane, contrary to the usual veiw that specific bacterial transport systems are restricted to the cytoplasmic membrane.

The possible relationship of OM-1 to -3 to the synthesis, degradation, or translocation of enterobactin (13), a low-molecular-weight compound that is involved in iron assimilation in *S. typhimurium*, is unknown. Similarly, nothing

is known about the relationship of these proteins to the *tonA,B* gene products of *E. coli*, although it should be noted that the *tonA* protein is not repressed by iron (9).

Precise definition of the structural and functional roles of the two groups of inducible outer membrane proteins described in this paper will require more study. As discussed above, it is likely that OM-1 to -3 will prove to be involved in iron transport or metabolism but the fact that the inducible polypeptides comprise such a substantial fraction of the total outer membrane proteins suggests that they may be involved in more than one function that is important to the life of the cell.

## ACKNOWLEDGMENTS

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