Regulation of Porin-Mediated Outer Membrane Permeability by Nutrient Limitation in *Escherichia coli*

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OmpF and OmpC porins were differentially regulated by nutrient limitation and growth rate in glucose- or nitrogen-limited chemostat cultures of *Escherichia coli*. Transcriptional and translational ompF fusions showed a sharp peak of expression under glucose limitation at $D = 0.3$ h$^{-1}$, with lower amounts at lower and higher growth rates. The peak of OmpR-dependent transcriptional stimulation of ompF under glucose limitation in minimal salts media was about 20-fold above nutrient excess levels and 3-fold higher than that achieved with low osmolarity. Analysis of outer membrane protein levels and results of growth competition experiments with porin mutants were consistent with the enhanced role of OmpF under glucose limitation, but not N limitation. In contrast, OmpC was the major porin under N limitation but was increasingly expressed under glucose limitation at very low growth rates approaching starvation, when OmpF was downregulated. In summary, outer membrane permeability under N-limited, sugar-rich conditions is largely based on OmpC, whereas porin activity is a complex, highly sensitive function of OmpF, OmpC, and LamB glycoporin expression under different levels of glucose limitation. Indeed, the OmpF level was more responsive to nutrient limitation than to medium osmolarity and suggested a significant additional layer of control over the porin-regulatory network.

Porin proteins control the permeability of polar solutes across the outer membrane of gram-negative bacteria like *Escherichia coli* (21). Optimal nutrient access is favored by larger porin channels as in OmpF protein (22) or solute-selective proteins like LamB glycoporin in the outer membrane (4). But high outer membrane permeability is a liability in less favorable circumstances, and access of toxic agents or detergents needs to be minimized through environmental control of outer membrane porosity and the increased proportion of smaller OmpC channels in the outer membrane. Normally, the total amount of OmpF and OmpC proteins is fairly constant, but the relative proportion of the two varies subject to factors such as osmolality of the medium (11, 12), temperature (16), the concentration of certain antibiotics (3), and growth phase (30). Medium with high osmolality, high temperature, or toxic ingredients favors the expression of OmpC, and medium of low osmolality and low temperature increases OmpF and diminishes the level of OmpC (28).

The best-understood input into controlling porin levels involves EnvZ and OmpR, which work together as regulators of ompF and ompC gene expression. EnvZ acts as the osmosensor to monitor the changes of external osmolarity to modify OmpR activity by phosphorylation and dephosphorylation (8). OmpR is the actual transcriptional activator of both porin genes (11, 20, 39). High osmolality results in more OmpR molecules that are phosphorylated, and low osmolality produces fewer phosphorylated OmpR (OmpR-P) (35). A low level of OmpR-P stimulates the transcription of the ompF gene, and a high level of OmpR-P activates the ompC gene and represses ompF (7, 19, 28, 29, 31). Other known factors regulating porins include some like integration host factor at the transcriptional level and others influencing ompF messenger translation through micF, which encodes an antisense RNA (reviewed in reference 28).

Much less clear than the above is the influence of nutrient concentration and nutrient limitation on porin expression. In nutrient-limited circumstances, bacteria need to scavenge molecules into the cell in order to maintain rapid growth. Outer membrane permeability becomes a bottleneck because the passive diffusion rate of sugar through porins inevitably drops in a linear fashion with decreasing nutrient concentration (5). *E. coli* is expert in adapting to the micromolar level of nutrients, and it would not be surprising that bacteria attempt to increase outer membrane permeability under hunger conditions. Previous studies indicate that outer membrane protein patterns are altered with nutrient limitation and growth rate in chemostat cultures limited by different nutrients. Continuous culture with glucose and nitrogen limitation of *Klebsiella aerogenes* NCTC418 changed the relative amounts of proteins with different types of nutrient limitation and with different growth rates (36). Low growth rates in chemostats also alter the antibiotic sensitivity profile of bacteria, presumably due to altered membrane permeability (2). In *E. coli* cultures, an early study found that glucose limitation strongly stimulated OmpF expression in chemostats at $D = 0.2$ h$^{-1}$ such that the ratio of porins to OmpA protein considerably increased and the OmpC level was low. But nitrate limitation caused less OmpF and more OmpC (26). Another indication of the sensitivity of porins to nutritional status was the finding that cyclic AMP (cAMP) in some (as yet undetermined) way affects the ratio of OmpF to OmpC (32).

One adaptive mechanism affecting outer membranes under glucose limitation is the tight growth rate- and glucose concentration-dependent induction of the LamB glycoporin (4). Given the published evidence listed above, it was unlikely that nonspecific porin expression remained constant under changing environmental nutrient levels, and this study provides a detailed picture of the regulation of the major outer membrane proteins. To study the control of major porins OmpF and OmpC under nutrient limitation, chemostat cultures with glucose or nitrogen limitation were used at various growth rates. Setting the dilution rate in a chemostat defines the growth rate as well as the steady-state nutrient concentration.
in the culture, with lower dilution rates resulting in lower nutrient levels. Three lines of investigation were adopted with the chemostat cultures, including studies with transcriptional and translational fusions, quantitation of outer membrane proteins, and growth competition experiments with strains lacking individual porins. The three approaches revealed a consistent but surprisingly complex pattern of regulation, particularly of OmpF porin levels.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study are listed in Table 1. P1 transduction (18) with P1crcl100 lysates grown on JB100 and NK6027 was employed to introduce ompR::Tn10 and metC162::Tn10 into MH513, MH225, MC4100, and BW2951 as recipients to create strains BW3303, BW3304, and BW3337 to BW3340 in Table 1.

Growth medium and culture conditions. The basal salts medium used in chemostats was minimal medium A (MMA) (18) supplemented with amino acids (40 mg/liter) where necessary. Glucose-limited feed medium consisted of 0.2% (wt/vol) glucose and 0.1% (wt/vol) ammonium sulfate. In N-limited feed medium, 0.03% (wt/vol) ammonium sulfate (or 0.006% [wt/vol] histidine) replaced 0.1% (wt/vol) ammonium sulfate, and 0.2% (wt/vol) glucose was present. Batch cultures used Luria-Bertani broth or nutrient broth and MMA containing 0.4% glucose limitation result in steady-state glucose concentrations ranging from below 10⁻⁷ M (at D = 0.1 h⁻¹) to above 10⁻⁶ M (at D = 0.6 h⁻¹) (24).

Under glucose limitation, the amount of OmpC protein was not drastically altered by growth rate, although it was slightly reduced at D = 0.3 h⁻¹ as determined from densitometry of gels such as that in Fig. 1 (result not shown). In striking contrast, the level of OmpF protein was greatly increased at D = 0.3 h⁻¹, but with lower levels at both low and higher dilution rates. The amount of OmpF in excess-glucose, exponential-phase batch culture in MMA was comparable to that found at D = 0.6 h⁻¹ (densitometric results not shown). The low level of OmpF in batch culture can be ascribed to the high osmolality of the minimal medium, which is over 100 mM with respect to salts and predictably shifted porin expression to OmpC. Nevertheless, the major increase in OmpF protein at D = 0.3 h⁻¹ was observed despite the high osmolality of the medium (Fig. 1). Indeed, expression was even higher in one-fifth-strength MMA at D = 0.3 h⁻¹ (unpublished results and ompF transcriptional fusion data below).

RESULTS

Effect of nutrient limitation on outer membrane protein patterns.

An E. coli K-12 strain wild type for outer membrane profile (strain MC4100) was cultured in chemostats with minimal salts media at three different growth rates either under glucose limitation or with nitrogen limitation in the presence of excess glucose. The cultures reached steady state and were sampled within 3 to 5 days, before mutational changes become evident. Figure 1 shows qualitative comparisons of outer membrane protein profiles under glucose and NH₃ limitation at dilution rates of 0.1, 0.3, and 0.6 h⁻¹ (corresponding to doubling times of 7, 2.5, and 1.1 h). These dilution rates with glucose limitation result in steady-state glucose concentrations ranging from below 10⁻⁷ M (at D = 0.1 h⁻¹) to above 10⁻⁶ M (at D = 0.6 h⁻¹) (24).

The basal salts medium used in chemostats was minimal medium A (MMA) (18) supplemented with amino acids (40 mg/liter) where necessary. Glucose-limited feed medium consisted of 0.2% (wt/vol) glucose and 0.1% (wt/vol) ammonium sulfate. In N-limited feed medium, 0.03% (wt/vol) ammonium sulfate (or 0.006% [wt/vol] histidine) replaced 0.1% (wt/vol) ammonium sulfate, and 0.2% (wt/vol) glucose was present. Batch cultures used Luria-Bertani broth or nutrient broth and MMA containing 0.4% (wt/vol) glucose and 0.1% (wt/vol) ammonium sulfate. Batch-cultured bacteria were grown at 37°C with constant shaking, and chemostat culture cells were grown as previously described (4).

Growth competition experiments. Experiments were performed with the chemostat media described above with (a) glucose limitation, (b) NH₃ limitation, and (c) histidine limitation at D = 0.3 h⁻¹. For each competition experiment, independent chemostat cultures of each strain were grown for 20 generations. The individual cultures were mixed in a 1:1 ratio prior to monitoring of competitive growth in the same medium at the same dilution rate as that in the inoculum cultures. Mixed cultures were sampled after 2, 4, 8, 24, 48, and 52 to 54 h, and the number of bacteria was determined by total plate counts (quadruplicate samples) as well as counts with different selection markers (e.g., on tetracycline-containing plates for strains carrying the metC162::Tn10 marker).

β-Galactosidase assay. The β-galactosidase activity of lacZ fusions was assayed by the method of Miller (18) and expressed in Miller units.

Outer membrane fractions and electrophoresis. The outer membrane protein fraction was prepared by disrupting cells in a French pressure cell and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15, 27). Cells from 70-ml chemostat cultures or 5-ml Luria-Bertani broth cultures were harvested by centrifugation, and the pellet was washed twice with 10 ml of 10 mM HEPES buffer (pH 7.4), resuspended in 10 ml of the same buffer, and broken by passage three times through a French pressure cell at 6,500 lb/in². The disrupted membrane was then pelleted by centrifugation at 35,000 × g for 1 h and resuspended in 200 μl of sample buffer (50 mM Tris-HCl, 4% sodium dodecyl sulfate, 16% glycerol, 0.048% bromophenol blue, 4.8% 2-mercaptoethanol), and samples were boiled for 5 min at 100°C and centrifuged for 10 min at 14,000 rpm in an Eppendorf microcentrifuge (model 5412) before electrophoresis. Proteins were separated on a 12% acrylamide gel by electrophoresis in the presence of 8 M urea (9), and protein bands were stained with 0.04% Coomassie blue R and destained with 10% acetic acid. OmpF and OmpC were identified by comparison with the protein profiles of control strains MH513(ompF-lacZ) and MH225(ompC-lacZ), respectively.

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<th>Strain</th>
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TABLE 1. Bacterial strains used in this study

FIG. 1. Outer membrane protein profile of E. coli K-12 wild-type strain MC4100 under glucose and NH₃ limitation at dilution rates of 0.1, 0.3, and 0.6 h⁻¹ (F⁻ C¹) and (F⁻ C⁻), respectively.
The amount of OmpC was high and relatively constant at different dilution rates under NH₃ limitation. In contrast to glucose limitation, the amount of OmpF protein was very low at all dilution rates under NH₃ limitation, with no significant difference among dilution rates. An interesting difference under N limitation was that the proportion of OmpA in the outer membrane was higher than that under glucose limitation at all three dilution rates (densitometric data not shown).

Transcriptional and translational regulation of porin synthesis under nutrient limitation. The protein composition of the outer membrane found in Fig. 1 indicated that at least OmpF porin was tightly regulated by nutrient limitation. To test this point, chemostat cultures were also used to study MC4100 derivatives with ompF-lacZ and ompC-lacZ transcriptional and translational fusions at different dilution rates.

Figure 2A shows the transcriptional expression of ompF under glucose limitation. In line with the protein increase shown in Fig. 1, there was a sharp peak of ompF expression under glucose limitation at $D = 0.3 \text{ h}^{-1}$, with lower expression at lower and higher growth rates. The peak was about 20-fold above nutrient excess levels (batch cultures with minimal salts medium and 0.2% glucose contained 170 Miller units of activity) and 3-fold higher than that achieved with low osmolarity (cultures in nutrient broth contained about 720 Miller units and those in fivefold-diluted MMA contained about 230 Miller units). The consistency of transcriptional expression with the protein pattern shown in Fig. 1 suggests a significant transcriptional control of OmpF protein amounts under glucose limitation.

Conditions of NH₃ limitation with excess glucose strongly repressed ompF expression at all dilution rates (Fig. 2A). Expression of MH513 decreased significantly to about 100 U, about 20-fold below the level under glucose limitation at $D = 0.3 \text{ h}^{-1}$ (close to that with high-osmolarity minimal medium batch cultures with 130 Miller units).

Expression of ompC in Fig. 2B showed less than a threefold change under glucose limitation at different dilution rates. The highest OmpC expression under glucose limitation was found at the lowest growth rates, and expression decreased from 300 Miller units at $D = 0.1 \text{ h}^{-1}$ to about 100 U at higher dilution rates ($D = 0.3$ to 0.7 h$^{-1}$), nearly equal to the expression in glucose-rich batch culture. Under NH₃ limitation, the expression of ompF increased about 1.5-fold at all dilution rates above that under glucose limitation. The higher transcription at low $D$ values was not evident in an absolute increase in OmpC protein amounts in Fig. 1, and so transcriptional regulation is not the only factor controlling OmpC levels.

To confirm the expression results with an independent set of transcriptional as well as translational fusions, we also tested the ompF-lacZ operon fusion construct in strain ST010 and ompF-lacZ protein fusion ST100 (13). These fusions are present as part of an θ lysogen and have a normal complement of porin genes. With these strains also, the transcriptional expression of ompF-lacZ gave a peak at $D = 0.3 \text{ h}^{-1}$ under glucose limitation (Fig. 2C), was slightly lower at $D = 0.1 \text{ h}^{-1}$, and was lowest at $D = 0.6 \text{ h}^{-1}$, consistent with the pattern in the other results.

The function of ompR in the expression of ompF and ompC under glucose limitation. Given the extensive change in ompF regulation under glucose limitation, we tested whether the high induction of ompF was dependent on the known transcriptional activator of porin genes, OmpR. Despite the very high level in Fig. 2, all of the expression was abolished in the presence of an ompR::Tnl0 mutation, as shown in Fig. 2A and B. Expression of both ompF and ompC under nutrient limitation was entirely OmpR dependent at all dilution rates.

The relative contribution of porins to outer membrane permeability. To clarify the physiological role of porins in bacteria under nutrient limitation, a series of competitive growth experiments under glucose or N limitation were set up between pairs of strains lacking particular porin proteins. The compared pairs contained additional selectable markers to aid in counting competing strains. To determine that the counting markers were indeed selectively neutral and not influencing growth, experiments were performed with reversed pairings of selectable markers and porin mutation combinations. To start competition experiments, bacteria were cultured in glucose- or N-limited chemostats separately. After 2 days of growth, the cultures were mixed in equal proportions. Competition was monitored in the same medium and at the same dilution rate. Under glucose limitation at $D = 0.3 \text{ h}^{-1}$, the wild-type strain MC4100 (ompC$^{-}$, OmpF$^{-}$, LamB$^{-}$) had a selective advantage over a mutant without OmpF porin, with the OmpF$^{-}$ strain being gradually washed out (Fig. 3A). In contrast, an OmpC$^{-}$ OmpF$^{-}$ strain was not significantly growth impaired under glucose limitation in competition with the OmpC$^{+}$ OmpF$^{+}$ strain (Fig. 3B). These results indicate that there was a significant difference in the contributions of OmpF and OmpC to glucose diffusion under glucose limitation, entirely consistent with the relative expression of these gene products under glucose limitation.

In competition experiments under NH₃ limitation or histidine limitation, loss of OmpF had little influence on the competitive ability of E. coli (Fig. 3C and D). The OmpC$^{-}$ mutant showed a slightly greater reduction in growth fitness under NH₃ limitation (Fig. 3E). Hence, the low level of OmpF shown in Fig. 1 above for N limitation was reflected in the low contribution of OmpF to NH₃ permeability, while OmpC is more important than OmpF under N limitation. The relatively small growth disadvantage seen with the OmpC$^{-}$ mutant under NH₃ limitation was unexpected, given the high OmpC expression under these conditions in wild-type bacteria, and so the exper-
Experiments were performed with strains containing the selectable metC::Tn10 marker (open symbols) and those without this marker (solid symbols). Experiments were with glucose and N limitation (with NH₃ or histidine) at a dilution rate of 0.3 h⁻¹. The same strains were tested in panels A, C, and D; the OmpF⁻ OmpC⁻ LamB⁻ strains MC4100 (●) and BW3340 (○), competing against OmpF⁻ strains BW3337 (□) and MH513 (■) under glucose (A), NH₃ (C), and histidine (D) limitation. The strains in panels B, E, and F were MC4100 (●) and BW3340 (○), competing against OmpC⁻ strains BW3338 (△) and MH513 (■) under glucose (B), NH₃ (E), and histidine (F) limitation. In panels G and H, the strains were the LamB⁺ OmpF⁻ strains MH513 (■) and BW3337 (○), competing against LamB⁻ OmpF⁻ strains BW2951 (▲) and BW3339 (●) under NH₃ and glucose limitation.

at D = 0.3 h⁻¹, the dilution rate giving the peak of OmpF expression in E. coli.

**DISCUSSION**

Earlier studies concluded that the outer membrane permeability of E. coli for glucose is determined by the major outer membrane porins OmpF, OmpC, and LamB (5, 21). Also known was that different environmental circumstances induce different proportions of these proteins (26). Our results extend these findings to show that each of these porins is optimally induced and functional under different levels of glucose limitation, with the expression of OmpF being particularly sensitive to nutrient level.

The expression of porins is most strikingly different when the environment of bacteria changes from a glucose-rich state to glucose limitation and eventually glucose depletion and starvation. The optimal scavenging ability of E. coli for glucose is specifically at micromolar medium concentrations of glucose, with lower transport affinity at excess or starvation levels of nutrient (4, 5). There is a sequential change in outer membrane protein composition with decreasing glucose concentrations and growth rate as schematically illustrated in Fig. 4. Yet it is not growth rate per se that controls porin levels but the nature of the limitation, as N limitation gives an entirely different pattern of porin expression, not only for OmpF and OmpC (shown here) but also for LamB (23). As shown in Fig. 4 and as based on published data (33), the glucose concentration in a chemostat drops with decreasing dilution rate (23).

Under glucose limitation, the earliest response (starting at above 10⁻⁷ M glucose) is induction of the LamB glycoporin, which is maximally induced at approximately 10⁻⁶ M medium glucose. It is only then that OmpF expression begins to increase markedly, between 10⁻⁸ and 10⁻⁷ M glucose. When sugar levels and growth rate are even lower, bacteria switch off both LamB and OmpF and go into a protective mode with the small OmpC channels being increasingly produced. At such low levels of glucose, at D = 0.1 h⁻¹, bacteria stop trying to optimize sugar scavenging strategies and switch to making protectants like trehalose and other stationary-phase responses (24).

Since the optimum for OmpF expression does not coincide with the induction of LamB under glucose limitation, there must be differences in the regulatory stimuli controlling these...
porins. LamB is induced by glucose limitation due to the higher levels of cAMP as well as of endogenous inducer (mal-totriose) at \(D = 0.6 \text{ h}^{-1}\) (23). OmpF expression may be affected by high cAMP levels (32), but the direct role of cAMP in porin regulation is still unexplained. In any case, cAMP levels continue to increase with decreasing dilution rate (25), but OmpF levels decrease below \(D = 0.3 \text{ h}^{-1}\). So at least one other input is required to explain the pattern.

The results with gene fusions indicated that the high OmpF expression at \(D = 0.3 \text{ h}^{-1}\) was transcriptionally regulated and entirely OmpR dependent. Previous results have identified low porin expression. The tentative conclusion is that high minimal medium (compared to 2,200 U in full-strength MMA) cose limitation needs more detailed definition. The only global effect by nutrient limitation, and their involvement under glucose limitation are considerable (unpublished results). It is also relevant that a further increase in OmpF expression is achievable by running chemostats at lower osmolarities and that the expression peaks at \(D = 0.3 \text{ h}^{-1}\) with MHS13 reaching about 2,600 U in one-fifth-strength minimal medium (compared to 2,200 U in full-strength MMA [result not shown]).

The above data suggests that the regulation by nutrient limitation is superimposed on the osmoregulation control of porin expression. The tentative conclusion is that high OmpF expression was not due to manipulation of OmpR-P levels but to some other factor(s) regulating transcription. One possibility is through some global, environmentally sensitive factor. Indeed, the regulation of outer membrane permeability is a complex process, involving several global regulators, such as RpoS (30), cAMP/Crp (32), MarA (3), and histone-like DNA binding proteins HNS (10, 38) and integration host factor (39), as well as Lrp (6). Several of these controls operate through micF (28) and so are unlikely to explain the current findings. However, many of these global regulators are potentially affected by nutrient limitation, and their involvement under glucose limitation needs more detailed definition. The only global regulator that has been investigated under chemostat conditions is RpoS (24), whose level increases significantly at \(D = 0.1 \text{ h}^{-1}\). RpoS-based regulation is therefore unlikely to be responsible for the OmpF peak at \(D = 0.3 \text{ h}^{-1}\), but RpoS may be involved in the OmpF replacement by OmpC at low dilution rates.

An interesting finding in this study was that NH₃-limited growth was not greatly impaired by the loss of any of the major individual porins, OmpF, OmpC, and LamB. Possibly NH₃ or NH₄⁺ ions (molecular weight, 17 or 18) are small enough or permeable enough to penetrate bilayers without porins being required. Alternatively, other channels may satisfy NH₃ or NH₄⁺ diffusion. For example, reconstituted OmpA protein is capable of forming diffusion pores in bilayers (37). Considering that the level of OmpA was particularly high under N limitation (Fig. 1), OmpA may satisfy an alternative channel requirement. Indeed, a previous study proposed a role for OmpA in amino acid permeability (17), but in vitro studies did not provide supporting evidence for this proposal (37).

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