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An analysis of multifactorial influences on the transcriptional control of *ompF* and *ompC* porin expression under nutrient limitation

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Expression of the major outer-membrane porins in *Escherichia coli* is transcriptionally controlled during nutrient limitation. Expression of *ompF* was more than 40-fold higher under glucose limitation than under nitrogen (ammonia) limitation in chemostat cultures at the same growth rate. In contrast, *ompC* expression was higher under N limitation. The basis of regulation by nutrient limitation was investigated using mutations affecting expression of porin genes. The influence of *cyaA*, *rpoS*, *ackA* and *pta*, as well as the two-component *envZ-ompR* system, was studied under glucose and N limitation in chemostat cultures. A major contributor to low *ompF* expression under N limitation was negative control by the RpoS sigma factor. RpoS levels were high under N limitation and loss of RpoS resulted in a 19-fold increase in *ompF* transcription, but little change was observed with *ompC*. Lack of RpoS under glucose limitation had a lesser stimulatory effect on *ompF* expression. Porin production was minimally dependent on EnvZ under N limitation due to OmpR phosphorylation by acetyl phosphate. Evidence obtained with *pta* and *ackA* mutants suggested that the acetyl phosphate level also regulates porins independently and indirectly via RpoS and other pathways. *pta-envZ* double mutants had a residual level of porin transcription, implicating alternative means of OmpR phosphorylation under nutrient limitation. Another critical factor in regulation was the level of cAMP, as a *cyaA* mutant hardly expressed *ompF* under glucose limitation but boosted *ompC*. In addition, the role of DNA-binding proteins encoded by *hns and himA* was tested under glucose limitation: the *hns* mutation reduced the glucose-limitation peak, but the *himA* mutation suppressed the *hns* effect, suggesting a complex web of interrelationships between the DNA-binding proteins. Indeed, multiple inputs and no single regulator were responsible for the high peak of *ompF* expression under glucose limitation.

Keywords: chemostat culture, *Escherichia coli* outer-membrane, RpoS, cAMP, acetyl phosphate

INTRODUCTION

Nutrient availability is a major factor in bacterial survival and proliferation (Nystrom, 1998). Reduced growth rate resulting from nutrient limitation invokes a number of regulated changes in bacterial outer-membrane composition (Lugtenberg *et al*., 1976; Overbeeke & Lugtenberg, 1980; Sterkenburg *et al*., 1984). Porin proteins control the permeability of the outer membrane and nutrient limitation strongly and differentially regulates porin expression (Overbeeke & Lugtenberg, 1980; Liu & Ferenci, 1998). Aside from nutrient limitation, porin levels are also sensitive to a wide variety of environmental parameters including osmolarity, temperature, pH, growth phase and cell density (Pratt *et al*., 1996; Buckler *et al*., 2000). The mechanism of porin regulation is particularly complex and many factors are involved in porin transcriptional and translational control (Pratt *et al*., 1996). The best-understood input

Abbreviations: AcP, acetyl phosphate; *D*, dilution rate; H-NS, histone-like DNA-binding protein; IHF, integration host factor.

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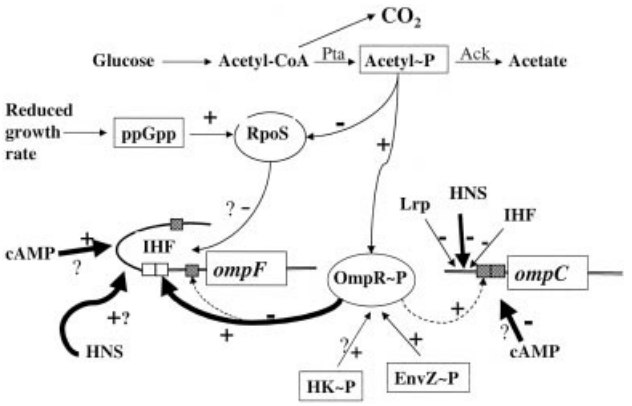

Fig. 1. Transcriptional regulation of porin expression in *E. coli*. The promoters of the *ompF* and *ompC* genes include binding sites for OmpR-P with high and low affinity (open and shaded boxes). High *ompF* expression requires low OmpR-P levels, whereas high *ompC* expression and repressed *ompF* requires high OmpR-P (dashed lines; Pratt *et al.*, 1996). Additional regulatory factors can influence porin regulation by affecting the level of OmpR-P; for example, EnvZ, AcP or alternative histidine kinases (HK-P). A second way of affecting regulation is by demonstrated interactions with the respective promoters; for example, IHF at both promoters, H-NS and Lrp at the *ompC* promoter. A third category of regulators which have no defined molecular mechanism includes RpoS and cAMP (indicated by question marks). Under nutrient limitation with reduced growth rates, ppGpp (Gentry *et al.*, 1993) and the level of AcP influence the level of RpoS (Hengge-Aronis, 2000). Stimulatory and inhibitory effects are designated by (+) and (−), respectively. The major influences operating under glucose limitation are denoted by thick arrows.

into controlling porin transcription involves EnvZ and OmpR (Fig. 1). OmpR phosphorylation is essential for porin transcription: a high level of OmpR-P stimulates *ompC* and represses *ompF*, and a low level of OmpR-P induces *ompF* transcription (Kenney *et al.*, 1995; Lan & Igo, 1998; Matsubara & Mizuno, 1999). EnvZ plays a major role in OmpR phosphorylation and dephosphorylation (Russo & Silhavy, 1991; Tokishita & Mizuno, 1994; Kenney, 1997). High osmolarity produces more OmpR-P by increasing EnvZ phosphorylation, while low osmolarity induces the activity of EnvZ phosphatase, resulting in dephosphorylated OmpR-P (Slauch *et al.*, 1988). Porin regulation is, however, not totally dependent on EnvZ (Forst *et al.*, 1988). Several alternative histidine kinase donors (Matsubara & Mizuno, 1999), and some high-energy molecules including acetyl phosphate (AcP), phosphoramidate and carbamyl phosphate can phosphorylate OmpR (McCleary *et al.*, 1993). Aside from EnvZ and OmpR, many global regulators such as alternative sigma factor (σ^s; Pratt *et al.*, 1996), histone-like DNA-binding protein (H-NS; Atlung & Ingmer, 1997), integration host factor (IHF; Goosen & Van de Putte, 1995) and AcP (Heyde *et al.*, 2000) all regulate porin expression at the transcriptional level. Another signal affecting porin regulation by unknown pathways is cAMP (Scott & Harwood, 1980). Additional regulatory circuits affect the control of OmpF translation through MicF (Suzuki *et al.*, 1996), but translational control will not be considered in this study.

Nutrient limitation, resulting in reduced growth rates, launches a wide range of adaptations in both physiological and regulatory circuits. Nutrient limitation enhances nutrient-scavenging ability by improving outer-membrane permeability and inducing high-affinity cytoplasmic-membrane transport systems (Ferenci, 1996, 1999). It also triggers dramatic changes in the intracellular pool of metabolites or the 'metabolome' (Tweeddale *et al.*, 1998). Signalling molecules such as polyphosphate (Kornberg *et al.*, 1999), ppGpp (Gentry *et al.*, 1993), cAMP (Notley-McRobb *et al.*, 1997), AcP (McCleary *et al.*, 1993), anhydroglucitol (Shiga *et al.*, 1999), and endoinducers such as galactose and maltotriose (Death & Ferenci, 1994; Ferenci, 1996) are all influenced by nutrient limitation. Also, global regulators such as σ^s (Notley & Ferenci, 1996; Teich *et al.*, 1999) are affected by nutrient limitation and in turn affect expression of a large number of genes (Hengge-Aronis, 1999).

We previously reported that porin expression was strongly and differentially regulated by nutrient limitation (Liu & Ferenci, 1998). Expression of *ompF* in *Escherichia coli* was much higher under glucose limitation than under growth limitation by N source and peaked sharply at glucose concentration below 1 μM, while *ompC* expression was higher under N limitation and repressed by glucose limitation. The expression of both *ompF* and *ompC* was shown to be absolutely dependent on OmpR, and loss of *ompR* abolished *ompF* and *ompC* transcription under both glucose and N limitation (Liu & Ferenci, 1998).

A chemostat culture approach was used to study porin regulation in this work because it permits reproducible conditions for nutrient limitation. Briefly, the cultures were grown at a dilution rate of $D = 0{\cdot}3\ h^{-1}$, corresponding to doubling times of approximately 2·5 h. Cultures growing at $D = 0{\cdot}3\ h^{-1}$ express a hunger response with a partial elevation in RpoS and associated cellular changes (Ferenci, 1999). This dilution rate was chosen because it is the optimum for *ompF* expression in glucose-limited continuous cultures (Liu & Ferenci, 1998). In N-limited continuous cultures, the low ammonium ion concentration limits growth whereas the glucose concentration remains in the millimolar range. The glucose-excess conditions of N-limited cultures have different metabolic and regulatory consequences; for example, acetate production is high in N-limited, but low in glucose-limited chemostats (el-Mansi & Holms, 1989). In contrast, cAMP levels are low in N-limited but high in glucose-limited cultures (Notley-McRobb *et al.*, 1997). Also, as shown in this study, RpoS levels are higher under N limitation than under glucose limitation. The influence of these and other factors on porin regulation is considered below.

Porin regulation under nutrient limitation is complex and poorly understood. The simplest working hypoth-

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Table 1. Bacterial strains used in this study

Strain	Relevant genotype	Source/reference
BW2800	MC4100/F′(*lacI*^{q1}, *lacZ*::Tn5, *lacY*$^{+}$, *lacA*$^{+}$)	Ferenci & Stretton (1989)
BW3301	MH513 *rpoS*::Tn*10*	This study
BW3302	MH225 *rpoS*::Tn*10*	This study
BW3303	MH513 *ompR*::Tn*10*	This study
BW3304	MH225 *ompR*::Tn*10*	This study
BW3305	MH513 *hns*::*neo*	This study
BW3306	MH225 *hns*::*neo*	This study
BW3308	MH513 *himA*Δ*82tet*	This study
BW3309	MH225 *himA*Δ*82tet*	This study
BW3319	BW3305 *rpoS*::Tn*10*	This study
BW3320	BW3306 *rpoS*::Tn*10*	This study
BW3321	BW3305 *himA*Δ*82*::*tet*	This study
BW3322	BW3306 *himA*Δ*2*::*tet*	This study
BW3323	MC4100 *rpoS*::Tn*10*	This study
BW3329	BW3301 *hns*::*neo*	This study
BW3343	MH513 *envZ60*::Tn*10*	This study
BW3345	MH225 *envZ60*::Tn*10*	This study
BW3346	BW3343 *ackA*::Tn*phoA′-9*	This study
BW3357	MH513 *ackA*::Tn*phoA′-9*	This study
BW3358	MH225 *ackA*::Tn*phoA′-9*	This study
BW3366	BW3358 *envZ60*::Tn*10*	This study
BW3367	BW3358 *rpoS*::Tn*10*	This study
BW3368	BW3357 *rpoS*::Tn*10*	This study
BW3381	DY330 *cyaA*::*tet*	This study
BW3382	DY330 *pta*::*kan*	This study
BW3383	MH513 *cyaA*::*tet*	This study
BW3601	MH513 *pta*::*kan*	This study
BW3602	MH225 *pta*::*kan*	This study
BW3603	BW3345 *pta*::*kan*	This study
BW3605	MH225 *cyaA*::*tet*	This study
BW3607	BW3343 *pta*::*kan*	This study
BW3608	BW3601 *rpoS*::Tn*10*	This study
BW3609	BW3602 *rpoS*::Tn*10*	This study
CP724	*ackA*::Tn*phoA′-9*	Shin & Park (1995)
CU221	CSH26 Δ(*pro–lac*) *ara thi hns*::*neo*	Yamada *et al.* (1991)
CV1008	F^{-} *ara thi* Δ*lac pro ilvIH*::*Mudl1734 lrp*::Tn*10*	M. Freundlich*
DY330	W3110 Δ*lacU169 gal490 λcl857* Δ(*cro–bioA*)	Yu *et al.* (2000)
HN1641	W3102 (*galK2*) *supO strR himA*Δ*82tet*	H. Nash*
JB100	HfrG6 *malT*^{C}*-1 ompR*–Tn*10*	Brass *et al.* (1984)
JMS6210	*envZ60*::Tn*10 recA111*::*Kan*	Slauch *et al.* (1988)
MC4100	F^{-} *araD139* Δ(*argF–lac*)*U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB530 rbsR*	Casabadan (1976)
MC4100 Δfis	MC4100 Δfis::cat	H. Nash
MH225	MC4100 Φ(*ompC–lacZ*$^{+}$)10-25	Hall & Silhavy (1981)
MH513	MC4100 *araD*$^{+}$Φ(*ompF′–lacZ*$^{+}$)16-13	Hall & Silhavy (1981)
ZK1171	W3110 Δ*lacU169 tna-2 rpoS*::Tn*10*	Huisman & Kolter (1994)

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esis to explain the nutrient-limitation data was that the induction of *ompF* under glucose limitation was due to the low level of OmpR-P (optimal for *ompF*, and not ideal for *ompC*; Lan & Igo, 1998). The repression of *ompF* and induction of *ompC* by N limitation is then explicable by the high level of OmpR-P. To test this simple hypothesis, the studies described below explored regulation under nutrient limitation by detailed analysis

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of the influence of the two-component *envZ-ompR* system. As shown in Fig. 1, other factors such as AcP, *rpoS*, *cyaA* (via cAMP) and DNA-binding proteins such as H-NS and IHF influence porin expression, so these inputs were also investigated. The hypothesis that OmpR phosphorylation was the central factor in porin regulation proved to be only partly substantiated, and we provide evidence of the importance of AcP, cAMP and RpoS in porin gene expression under nutrient limitation.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. P1 transduction was used to construct mutants (Miller, 1972). The media and growth conditions for glucose- and ammonia-limited chemostats and batch culture were as previously described (Death *et al.*, 1993; Liu & Ferenci, 1998). The total salt concentration was approximately 100 mM, so provided a high-osmolarity environment.

Construction of *cyaA*::*tet* and *pta*::*kan* insertions. The insertion of antibiotic-resistance cassettes in the *cyaA* and *pta* genes was accomplished using the method of Yu *et al.* (2000). Briefly, linear *tet* and *kan* cassettes were amplified by PCR from strains BW3303 and BW2800 respectively, and recombined into the *cyaA* and *pta* genes in DY330 (Yu *et al.*, 2000) after electroporation. The cassettes in the resulting drug-resistant strains BW3381 and BW3382 were checked by PCR using primers flanking the target gene. The size of the gene with insertions was compared with the size of the original gene on a 1% agarose gel. After confirmation of the insertion, the mutated gene was picked up by P1 phage and transferred to the recipients (shown in Table 1).

For construction of the *cyaA*::*tet* mutation, the primers used to amplify *tet* were 5′-CCTATTCCTGGGAATACCCGAA-CCCACGTCCAAGAGGGTCATTATATTTCG-3′ and 5′-GACGTGATTGGTTTCAACCTGCACTGACAGGACT-CGACATCTTGGTTACCG-3′. For construction of the *pta*::*kan* mutant, the primers used to amplify *kan* were 5′-CGACTATCGTGCGTGCGAACTCTTCCACCACTATG-GACAGCAAGC GAACCG-3′ and 5′-GCACAGATAGCG-GCTGCTTTAACGGTACGCGTCAGAAGAACTCGTCA-AGAAG-3′.

β-Galactosidase and RpoS protein assays. The β-galactosidase activity of *lacZ* fusions of bacteria grown in the chemostat for 3 d was assayed, as described by Miller (1972), with results expressed in Miller units. The β-galactosidase activities presented in Table 2 and Table 3 were the mean of three to five separate chemostat culture results. The amount of RpoS was analysed by Western blotting. The procedures for protein extraction, SDS-PAGE separation, electrophoretic transfer and immunodetection of RpoS were as previously described (Liu *et al.*, 2000), and as specified in the legends.

RESULTS AND DISCUSSION

OmpR and EnvZ influence on porin expression under nutrient limitation

As previously reported (Liu & Ferenci, 1998), the transcriptional activator OmpR was needed for porin expression under nutrient limitation. OmpR control of porin gene transcription by osmolarity involves phosphorylation and dephosphorylation by EnvZ (Pratt *et al.*, 1996). The contribution of EnvZ to expression under nutrient limitation, but at constant osmolarity, was therefore analysed in the presence of an *envZ60*::Tn*10* mutation under both glucose and N limitation (Table 2). Under glucose limitation, disruption of *envZ* partially decreased expression of both *ompF–lacZ* and *ompC–lacZ* transcriptional fusions. Nevertheless, there was still significant EnvZ-independent transcription of *ompF* and *ompC* under glucose limitation, above that seen with the very low level in the *ompR*::Tn*10* mutant in glucose- and N-limited chemostats (Table 2).

Even more interestingly, the expression of *ompF* and *ompC* in *envZ* mutants was as high, or higher, under N limitation than found with wild-type *envZ*. The high residual *ompC* expression in *envZ* mutants suggested there was a considerable level of OmpR phosphorylation under glucose-rich conditions in the absence of EnvZ. The absence of EnvZ-dependent dephosphorylation may also have contributed to elevated OmpR-P levels. These results confirm that OmpR is a central regulator (as shown in Fig. 1) under both glucose and N limitation, but the role of EnvZ may not be essential under glucose-rich conditions.

RpoS influence on porin expression

The sigma factor RpoS has a negative effect on *ompF* expression in stationary phase (Pratt *et al.*, 1996), so its role under steady-state nutrient limitation was tested. Table 2 shows the influence of an *rpoS*::Tn*10* mutation on the transcription of *ompF* and *ompC* under glucose and N limitation. Disruption of the *rpoS* gene caused enhanced expression of *ompF* transcription under both glucose and N limitation, in line with negative control by RpoS (Fig. 1). The effect was particularly marked under N limitation, with a remarkable 19-fold increase at $D = 0{\cdot}3\ h^{-1}$. This result suggested that RpoS levels must be particularly high under N limitation to repress *ompF* expression to such a large extent in the wild-type. The quantity of RpoS under N limitation was not previously assayed but is shown in the Western blot in Fig. 2. The level of RpoS protein under glucose and N limitation shows that growth rate as well as nutrient availability affect the concentration of this protein. Data obtained from densitometer scans showed that N limitation produced more RpoS protein than glucose limitation, with fourfold more RpoS protein detected at $D = 0{\cdot}3\ h^{-1}$ than under glucose limitation. Evidence from mutants and the protein assays together suggest that the level of RpoS had a significant repressive effect on *ompF* transcription in N-limited environments.

A mutation in *rpoS* did not influence *ompC* expression nearly as much as that of *ompF* under either glucose or N limitation. Glucose limitation gave little difference in *ompC* expression at $D = 0{\cdot}3\ h^{-1}$. Under N limitation, there was a small increase in the *rpoS* mutant, possibly due to competition between sigma factors when RpoS is high in the wild-type (Farewell *et al.*, 1998). The *ompC*

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Table 2. Transcriptional regulation of *ompF* and *ompC* under glucose and nitrogen limitation growing at 0·3 h^{-1} dilution rate

Strain	Relevant genotype	β-Galactosidase activity ± SD	
		Glucose limitation	Nitrogen limitation
MH513	*ompF–lacZ*	2294 ± 195	51 ± 3
BW3303	MH513 *ompR*	6·8 ± 1·1	6 ± 1·8
BW3343	MH513 *envZ60*	592 ± 16	60 ± 2
BW3357	MH513 *ackA*	2636 ± 181	1378 ± 42
BW3346	MH513 *ackA envZ60*	2125 ± 27	1297 ± 56
BW3301	MH513 *rpoS*	2559 ± 186	964 ± 83
BW3368	MH513 *ackA rpoS*	2244 ± 42	1268 ± 56
BW3601	MH513 *pta*	973 ± 14	178 ± 8
BW3607	MH513 *envZ60 pta*	280 ± 72	73 ± 6
BW3608	MH513 *pta rpoS*	2426 ± 24	745 ± 19
BW3383	MH513 *cyaA*	65 ± 5	60 ± 4
BW3305	MH513 *hns*	390 ± 83	118 ± 27
MH225	*ompC–lacZ*	116 ± 33	170 ± 41
BW3304	MH225 *ompR*	7 ± 2	6·6 ± 1·7
BW3345	MH225 *envZ60*	31 ± 10	241 ± 5
BW3358	MH225 *ackA*	546 ± 11	603 ± 58
BW3366	MH225 *ackA envZ60*	428 ± 44	686 ± 60
BW3302	MH225 *rpoS*	130 ± 23	366 ± 40
BW3367	MH225 *ackA rpoS*	462 ± 14	898 ± 13
BW3602	MH225 *pta*	515 ± 85	422 ± 41
BW3609	MH225 *pta rpoS*	598 ± 64	686 ± 54
BW3603	MH225 *envZ60 pta*	58 ± 20	24 ± 2
BW3605	MH225 *cyaA*	638 ± 18	520 ± 23
BW3306	MH225 *hns*	2100 ± 80	877 ± 24

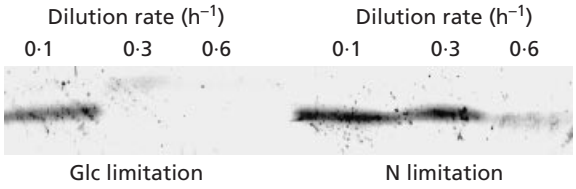

Fig. 2. Levels of RpoS under nutrient limitation. Immunoblotting using anti-RpoS antibodies was performed as described by Liu *et al.* (2000). Equivalent numbers of bacteria from glucose- and N-limited chemostats were extracted by the SDS-boiling method, from chemostats run at the dilution rates shown. Consistent with earlier studies (Notley & Ferenci, 1996; Teich *et al.*, 1999), the RpoS in glucose-limited chemostats was elevated significantly at the slowest growth rate. The N-limited cultures had 4-fold and 3·6-fold higher levels, as determined by densitometry (Liu *et al.*, 2000), than glucose-limited chemostats at $D = 0·3$ and 0·6 h^{-1}, respectively. Protein levels were quantitated from densitometer scans using ImageQuant software in three separate blots, each containing samples from separate chemostat cultures of MC4100 growing at dilution rates of 0·1, 0·3 and 0·6 h^{-1}.

results are consistent with the view that *rpoS* has a much stronger role in *ompF* than in *ompC* regulation. The mechanism by which RpoS affects transcription from the *ompF* promoter more than from *ompC* is not known.

The dual importance of AcP in porin expression

Given the high level of acetate metabolism in N-limited cultures (el-Mansi & Holms, 1989), and the possibility of AcP-dependent OmpR phosphorylation (Kenney *et al.*, 1995), the role of acetate metabolism in porin regulation was investigated. The production of AcP from glucose is controlled by two enzymes: phosphotransacetylase (product of gene *pta*) and acetate kinase (product of *ackA*) (Fig. 1). Mutation of *pta* reduces AcP production from glucose and mutation of *ackA* prevents AcP breakdown to acetate. AcP was shown to influence the control of porin expression under low-pH conditions (Heyde *et al.*, 2000). To clarify the importance of AcP in porin regulation, the influence of *ackA* and *pta* mutations on *ompF* and *ompC* expression was assayed under glucose and N limitation.

The *ackA* mutation blocking AcP breakdown strongly influenced porin expression under both glucose and N limitation. The expression of *ompC* (dependent on high OmpR-P levels) was increased about four- to fivefold under both glucose and N limitation (Table 2). Under N limitation, the increase was in addition to the rise with *rpoS*, as shown with the *ackA rpoS* double mutant. This was not surprising, since RpoS does not influence *ompC* directly. Less predictably, the *ackA* mutation also enhanced the expression of *ompF* under both glucose

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and N limitation. The effect on *ompF* expression under glucose limitation was relatively small, but the mutation of *ackA* increased *ompF* expression under N limitation 27-fold.

Given that high OmpR-P levels (derived from extra AcP) should result in high *ompC* but low *ompF* expression, the increased *ompF* expression under N limitation due to the *ackA* mutation cannot be explained purely through OmpR-P levels. The *ackA* results indicated that the accumulation of AcP must have altered control of other factors such as RpoS (which is involved in control of porin regulation; Table 2); AcP is already known to negatively affect RpoS levels (Bouche *et al*., 1998). A comparison of the results in Table 2 between *rpoS* and *ackA* mutants shows that the pattern of expression of *ompF* in these mutants is similar under both glucose and N limitation. This resemblance suggests that high *ompF* expression with high AcP is an indirect effect of reduction of RpoS levels. To see if the two mutations were epistatic, the influence of further introducing the *rpoS* mutation into the *ackA* strains was examined (Table 2). The double mutation of *ackA* and *rpoS* resulted in almost the same expression of *ompF* as that of the *rpoS* single mutation under glucose limitation. A complication is that the *ackA* mutation had a greater effect on *ompF* expression under N limitation than the *rpoS* mutation; this leaves open the possibility that AcP levels in porin regulation influence another factor besides RpoS.

To clarify the contribution of EnvZ and AcP to OmpR-P formation, an *envZ ackA* double mutant was constructed and *ompF*/*ompC* expression retested. The additional mutation in *envZ* had little influence on *ompF* and *ompC* expression in either glucose- or N-limited cultures. These results suggest EnvZ may be not an essential contributor to OmpR phosphorylation, particularly under N limitation, and AcP has a significant role in the phosphorylation of OmpR leading to high *ompC* expression. Nevertheless, the loss of OmpR-P phosphatase in an *envZ* mutant could also contribute to high OmpR-P levels in the double mutant.

Influence of a *pta* mutation on porin expression

In contrast to the *ackA* mutation, the *pta* mutation blocks AcP synthesis from glucose. The influence of decreased AcP on porin expression was checked with the *pta* mutants shown in Table 2.

The *pta* mutation resulted in a reduction of *ompF* expression under glucose limitation. Two explanations for this decrease were possible. The first possibility was that OmpR-P is lowered due to lack of AcP so there is insufficient OmpR-P for *ompF* expression. This was unlikely because *ompC* expression, requiring higher OmpR-P, was actually higher in the *pta* mutant. The second possibility was that lack of AcP in the *pta* mutant led to higher levels of RpoS and hence repression of *ompF* (Fig. 1). This is more likely, given that an *rpoS* mutation restored *ompF* expression in the *pta rpoS* double mutant. The properties of the *pta* mutants suggest AcP has a dual regulatory role under nutrient limitation, via both OmpR and RpoS.

The higher *ompC* expression in the *pta* mutant was unexpected and not explicable solely in terms of AcP acting as a phosphoryl donor to supply OmpR-P. The increase was entirely dependent on *envZ*-mediated OmpR phosphorylation, as the *pta envZ* double mutant had low ompC expression (Table 2). The *pta* mutation by itself must somehow indirectly change porin regulation through unknown mechanisms. We speculate that a lack of AcP may increase EnvZ-kinase activity to explain our results.

Under N limitation, expression of *ompF* was increased by the *pta* mutation. This was possibly due to the reduced availability of AcP lowering the OmpR-P level closer to that optimal for *ompF* expression; however, this is not the case because, surprisingly, the *pta*::*kan* mutation significantly increased *ompC* expression in both glucose- and N-limited cultures. Hence, a level of OmpR-P stimulating *ompC* expression should be too high for elevated *ompF* expression. The lack of AcP should also lead to higher RpoS levels, leading to *ompF* repression. More likely, the lack of AcP has more complex indirect effects than the ones considered so far. For example, indirect (and as yet unexplored) effects on other components in Fig. 1 or on DNA supercoiling (Graeme-Cook *et al*., 1989) could explain complex patterns of *ompF* and *ompC* expression changes.

The expression of *ompF* was also checked with *pta envZ* double mutants, in which the absence of OmpR phosphorylation by AcP and EnvZ should lead to low porin expression. Compared to a *pta* single mutation, the *pta envZ* double mutant reduced *ompF* expression under both glucose and N limitation but there was still at least 10-fold higher expression than seen with the *ompR* mutant. In contrast, the expression of *ompC* showed a greater decrease under both glucose and N limitation, as would be expected from a greater need for high OmpR-P levels than for *ompF*.

Influence of adenylate cyclase on porin expression

An early study implicated cAMP in porin regulation (Scott & Harwood, 1980). Given that cAMP levels are much higher under glucose than N limitation (Death & Ferenci, 1994), the role of *cyaA* under nutrient limitation was investigated. Unfortunately, *crp* mutants could not be studied here as they did not grow well under glucose limitation except at slow dilution rates (results not shown).

The lack of cAMP in the *cyaA* mutant led to a drastic drop of *ompF* expression under glucose limitation, but not N limitation. This result was in line with the relative amounts of cAMP under the two forms of limitation. It also underlined the significance of cAMP to the large peak of *ompF* expression under glucose limitation.

Expression of *ompC* was increased significantly in the *cyaA* mutant under both glucose and N limitation. This was consistent with earlier findings in batch culture,

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Table 3. Contribution of DNA-binding proteins to porin transcription under glucose limitation at 0·3 h^{-1} dilution rate

Strain	Relevant genotype	β-Galactosidase activity ± SD
MH513	*ompF–lacZ*	2294 ± 195
BW3308	MH513 *himA*	2136 ± 27
BW3305	MH513 *hns*	390 ± 83
BW3319	MH513 *hns rpoS*	445 ± 31
BW3321	MH513 *himA hns*	1609 ± 47
MH225	*ompC–lacZ*	116 ± 33
BW3309	MH225 *himA*	476 ± 108
BW3306	MH225 *hns*	2100 ± 80
BW3320	MH225 *hns rpoS*	1700 ± 62
BW3322	MH225 *himA hns*	2050 ± 123

showing that the addition of cAMP repressed *ompC* expression (Scott & Harwood, 1980; Thomas & Booth, 1992). The mechanistic connection between cAMP and porin expression is still poorly understood, as there is no obvious role of cAMP/Crp in porin transcription directly (Pratt *et al.*, 1996). The influence of cAMP on porin expression may be indirect and through other factors such as OmpR or RpoS. cAMP/Crp can bind to the promoter region of the *ompB* operon and can both positively and negatively affect expression from the *ompR/envZ* promoter (Huang *et al.*, 1992). It is unlikely that mutation of *cyaA* decreases OmpR (and therefore decreases OmpR-P) because expression of *ompC* increased under both conditions, and the increased *ompC* requires high levels of OmpR-P (Slauch & Silhavy, 1989). The simplest explanation for the decrease in *ompF* and increase in *ompC* expression in the absence of cAMP is that a lack of cAMP induces OmpR and results in more OmpR-P, and the high level of OmpR-P represses *ompF* and induces *ompC*. Another possible contributor was that decreased cAMP increases RpoS levels (cAMP has been shown to negatively regulate *rpoS* transcription; Lange & Hengge-Aronis, 1994). The *cyaA* mutation could affect *ompF* expression via elevated levels of RpoS.

DNA-binding proteins and their influence on porin expression under glucose limitation

The DNA-binding protein H-NS and several other nucleoid-associated proteins, such as HU, IHF, Fis and Lrp act as global regulators in the control of porin gene expression (Tsui *et al.*, 1988; Ferrario *et al.*, 1995; Suzuki *et al.*, 1996; Painbeni *et al.*, 1997). Also, H-NS has a strong influence on RpoS levels (Hengge-Aronis, 1999), which could in turn influence porin expression as indicated above. Therefore, mutants with insertions in these genes were checked for porin gene transcription to test whether they influenced the very high level of *ompF* expression under glucose limitation (Table 3). The Lrp and Fis inactivation mutants had little effect on regulation by nutrient limitation, so are not considered here (results not shown).

The DNA-binding protein IHF is implicated in porin control because it binds upstream of the *ompB*, *ompF* and *ompC* promoters, resulting in decreased transcription from each of these genes (Ramani *et al.*, 1992). The influence of *himA* on porin transcription was studied and the result is shown in Table 3. The mutation of *himA* had surprisingly little effect on *ompF*, but resulted in a slight increase of *ompC* expression. This suggested that IHF repression of *ompF* was essentially absent under glucose limitation whereas it was still influencing the *ompC* promoter.

Role of H-NS in porin regulation

An *hns* mutation had a huge positive effect on *ompC* expression, as was expected from the known influence of H-NS on *ompC* transcription, i.e. by binding to the intergenic region between *ompC* and *micF*, resulting in repression of both genes (Suzuki *et al.*, 1996). More surprisingly, there was a marked reduction in *ompF* expression with the *hns* mutant. Unlike the situation with *ompC*, no *in vitro* evidence exists to indicate that H-NS has a binding site in the *ompF* promoter region and positively regulates *ompF* transcription. A possible explanation for this reduction could have been increased RpoS repression with an indirect influence of *hns* on *ompF* transcription. H-NS inhibits *rpoS* expression at both translational and RpoS stability levels (Lange & Hengge-Aronis, 1994). Therefore, an *rpoS* mutation was introduced into the *hns* mutant. The double mutant did not have restored high levels of *ompF* expression, ruling out the involvement of RpoS. The complex nature of the *hns* effect on *ompF* was suggested by the observation that the double mutation of *himA* and *hns* restored *ompF* expression close to the level found in the *himA* single mutation. These results indicate that the *hns* loss did not cause reduced *ompF* expression directly; more likely, HNS may result in elevated IHF or reduce *ompF* expression in some unclear way.

Conclusions

The complex web of interactions affecting porin regulation under nutrient limitation is outlined in Fig. 1. RpoS, AcP and cAMP were all significant inputs in differentiating glucose- and N-limited cultures. RpoS was a significant factor in regulation, particularly under N limitation, where its level was especially high. AcP contributed to regulation as a phosphoryl donor to OmpR, bypassing the EnvZ requirement under N limitation. A secondary role of AcP was in controlling RpoS levels, indirectly influencing *ompF* expression. The *cya*A product was essential for *ompF* expression under glucose limitation and was confirmed as playing a significant role in the repression of *ompC*. Lastly, the *hns* and *himA* mutations implicated the significance of histone-like proteins in maintaining the wild-type levels of porin production under nutrient limitation. Each of the above involvements leads to unanswered questions

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as to the molecular mechanisms of these effects. Indeed, the means whereby RpoS, cAMP and H-NS influence *ompF* expression without an obvious interaction with the promoter remains a puzzle and a target of future research. In addition, other factors not considered here, such as DNA supercoiling, could affect transcription (Graeme-Cook *et al*., 1989). There are other unresolved puzzles as well; for example, the elevated expression of both *ompF* and *ompC* in the *ackA* mutant is not easily explicable via the model in Fig. 1.

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