Osmoregulation of Alkaline Phosphatase Synthesis in *Escherichia coli* K-12

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Alkaline phosphatase, the phoA product, is synthesized constitutively in phoR mutants. This constitutive synthesis, which is independent of phosphate control, varies with changes in the osmolarity of the growth medium; phoA expression increases with increasing osmolarity. Maximum expression of the osmoregulated genes phoA, ompC, and ompF was achieved by osmotic manipulation of minimal medium; complex media repressed their expression.

*Escherichia coli* responds to changes in medium osmolarity by altering several properties: the internal K⁺ concentration (3), the amount of anionic oligosaccharides in the periplasmic space (9), and the relative amounts of the outer membrane porin proteins OmpF and OmpC (8, 12, 15). In medium of high osmolarity, ompC transcription is elevated and ompF expression is repressed. Both the absolute and relative amounts of OmpF and OmpC vary in media with differing chemical compositions (5, 11).

Porin expression is transcriptionally controlled by the ompR and envZ products (6, 20). Mutants altered in envZ do not make the OmpF porin and synthesize OmpC constitutively; these mutants do not respond to changes in the growth medium (6). envZ mutants are pleiotropic, affecting the synthesis of other envelope constituents as well. *PerA* mutations (19) map in envZ and dramatically reduce the amounts of both ompF and alkaline phosphatase, an envelope protein found in the periplasmic space. Wild-type cells treated with the local anesthetic procaine mimic the phenotype of envZ mutants; transcription of phoA and ompF is coordinately repressed, whereas ompC expression is unaffected (4). Alkaline phosphatase synthesis is normally induced by phosphate deprivation (2). Its structural gene, *phoA*, is a member of the phosphate regulon whose control is a complex, involving both positive and negative regulatory components in addition to the envZ (perA) effect (18). In strains with mutations in the regulatory gene *phoR*, alkaline phosphatase is produced constitutively and is independent of the phosphate concentration of the medium (2).

We investigated the effect of medium composition and osmolarity on the constitutive synthesis of alkaline phosphatase in a *phoR* mutant. We discovered that alkaline phosphatase synthesis is affected by changes in the medium osmolarity and that this affects *phoA* transcription. An additional factor, which could be the growth rate, also influences expression of *phoA*, *ompF*, and *ompC* expression.

All bacterial strains are derivatives of MC4100. Strain AS2phoR is MC4100 phoR/ΔphoA-lac. The Δ(phoA-lac) is an operon fusion in which β-galactosidase synthesis is under control of the *phoA* promoter. It was constructed as described by Sarthy et al. (13) and was kindly provided by P. J. Bassford, Jr. MH513 Δ(ompF-lac) and MH225 Δ(ompC-lac) were generously provided by T. H. Silhavy. Cultures were grown in a rotary shaker at 37°C in the indicated media overnight, diluted into the same medium to an absorbancy at 600 nm of 0.1, and grown to mid-exponential phase (absorbancy at 600 nm of 0.5 to 0.65). Medium 63 (M63) (7) was supplemented with 1 μg of thiamine per ml and 0.4% of either glycerol or glucose. Rich media contained 8 g of nutrient broth (NB) (Difco) per liter or 30 g of tryptic soy broth (TSB) (Difco) per liter.

Samples were removed for enzyme activity measurements by mixing 10-ml portions with chloramphenicol (final concentration, 125 μg/ml) and collecting cells by centrifugation. Alkaline phosphatase assays were performed immediately on cells suspended in 0.6 M Tris buffer, pH 8.2. For β-galactosidase assays, cell pellets were frozen at −70°C for 1 to 3 days, after which the pellets were thawed and the cells were permeabilized with toluene plus 1% Sarkosyl. This method efficiently permeabilizes cells that have been previously frozen, although it may be less effective for freshly harvested cells (17). Assays performed in this manner were highly reproducible, as indicated by the standard deviations shown in Fig. 1.

β-galactosidase activity was measured by hydrolysis of orthonitrophenyl β-galactoside, as previously described (16). Alkaline phosphatase
was measured as the rate of hydrolysis of 2 mM p-nitrophenyl phosphate in 0.6 M Tris, pH 8.2, at 37°C. Protein content was determined by protein analysis as described by Lowry et al. (10). Osmolarity was measured by freezing point depression, using an Advanced Instrument Osmometer.

Since OmpF and OmpC levels are strongly dependent on medium composition, we wanted to determine whether alkaline phosphatase activity was also influenced by environmental composition factors other than phosphate. When strain AS2phoR was grown on NB, the cells had a specific activity of 1.14 U of alkaline phosphatase per mg of protein, a low constitutive level. One unit is equivalent to 1 \mu mol nitrophenylphosphatase hydrolyzed per min at 37°C. AS2phoR grown on M63 glycerol, however, had 9 times more alkaline phosphatase activity (10.5 U/mg of protein) than the cells grown on NB. The ratio of M63-glycerol activity to NB activity was 9.2. The high enzyme level in M63-glycerol was not due to a residual response to phosphate deprivation of the phoR regulatory mutation. M63 medium (7) contains 0.1 M KH₂PO₄, a very high concentration of inorganic phosphate. When the isogenic phoR' strain was grown on M63-glycerol, alkaline phosphatase was completely repressed.

The differences in alkaline phosphatase activity between cells grown on these two media could reflect differences in transcriptional rates or in subsequent events leading to an active enzyme protein. In strains in which the lac operon fused to the promoter of phoA (\Phi(phoA-lac)), production of \beta-galactosidase is a measure of transcriptional activity at the phoA promoter. Strain AS2phoR contains both an intact phoA gene and a \Phi(phoA-lac) operon fusion introduced on a lambda phage (13). The constitutive levels of \beta-galactosidase produced from the fusion were medium dependent in a manner analogous to the authentic gene product, 333 U/mg of protein in NB and 2,887 U/mg of protein in M63-glycerol (or 8.5 times higher), where one unit is equivalent to 1 nmol of orthonitrophenyl \beta-galactoside hydrolyzed per min at 28°C. The close quantitative correspondence between the extent of variation of the authentic gene product (9-fold) and operon fusion \beta-galactosidase (8.5-fold) indicates that regulation occurs at the level of gene expression in response to some property of the medium.

Osmolarity is a factor known to trigger envZ regulation of ompF and ompC (8, 12, 15). We have measured the osmolarities of several complex and minimal media to discover whether osmolarity might be a hidden factor in medium-dependent expression of phoA, ompF, and ompC. Table 1 shows that M63-glycerol has seven times the osmolarity of NB. It is therefore possible that osmoregulation is the sole cause of the differences in phoA expression in these two media, with high osmolarity favoring phoA expression in a manner analogous to the ompC response.

To test this hypothesis, we varied osmolarity without changing the chemical composition of the medium. AS2phoR was grown on M63-glycerol, and dilutions of this medium to one-half or one-fourth strength with distilled water. phoA expression was very sensitive to medium osmolarity (Fig. 1). M63-glycerol and its two dilutions are media 1, 2, and 3, respectively. In the 280-mosM undiluted minimal medium, \beta-galactosidase activity was 2,887 U/mg. When the osmolarity was reduced to 70 mosM by dilution, the \Phi(phoA-lac) level fell to 788 U/mg. The same experiment was performed with \Phi(ompF-lac) and \Phi(ompC-lac) for comparison
(Fig. 1). The \( \Phi(\text{ompC-lac}) \) response to dilution paralleled that for \( \text{phoA} \), although the \( \text{phoA} \) response was larger in magnitude. The \( \Phi(\text{ompF-lac}) \) response to dilution was in the opposite direction, \( \beta \)-galactosidase activity increased when the medium was diluted. The direction of \( \text{ompF} \) and \( \text{ompC} \) variation with osmolarity shown in Fig. 1 agrees with results from experiments in which osmolarity was increased (9, 12, 15). For \( \text{phoA} \), as well as for \( \text{ompF} \) and \( \text{ompC} \), variations in osmolarity produce a corresponding change in gene expression in minimal medium of constant chemical composition (Fig. 1).

The high level of \( \text{phoA} \) expression in high osmolarity minimal medium does not occur in complex media of equivalent osmolarity (Fig. 1). We added 0.3 M sucrose to NB, raising the osmolarity to \( \sim 320 \) mosM, and measured \( \Phi(\text{phoA-lac}) \) levels. The \( \beta \)-galactosidase activity in the NB-0.3 M sucrose (medium 9) was increased to 762 U/mg from the 338 U/mg in unsupplemented NB (medium 4). This is far less than the \( \sim 3,000 \) U/mg predicted by extrapolation of the M63-glycerol dilution experiment, using osmolarity as the sole criterion for gene expression. Cells grown in TSB (medium 8) also had a lower level of \( \text{phoA} \) expression than predicted from a simple osmolarity hypothesis. The osmolarity of TSB is approximately the same as that of M63-glycerol (Table 1), yet \( \text{phoA} \) expression in TSB was only 25% of that in the minimal medium.

Complex medium also repressed the elevation in \( \text{ompF} \) levels that was associated with reduced osmolarity of minimal medium (Fig. 1). \( \Phi(\text{ompF-lac}) \) activity was 2,750 U/mg in one-quarter-strength M63-glycerol (medium 3) compared with only 617 U/mg in osmotically similar NB (medium 4).

The chemical composition of each medium must be considered in trying to interpret these observations. TSB contains glucose and NB and M63-glycerol do not. To determine whether glucose is responsible for repressing \( \text{phoA} \) expression, cells were grown in M63-glucose (medium 7). \( \text{phoA} \) reached 3,872 U/mg in this medium, higher than in M63-glycerol and five times higher than in isoosmolar TSB. Hence, neither osmolarity nor glucose content can account for the reduced level of \( \text{phoA} \) gene expression in complex TSB medium.

\( \text{ompF} \) expression was reduced in media containing glucose (media 7 and 8), as might be expected from the observation of cAMP involvement in \( \text{ompF} \) regulation (14). The combination of glucose content and osmoregulation of \( \text{ompF} \) and \( \text{ompC} \) can probably account for the predominance of \( \text{ompC} \) over \( \text{ompF} \) in TSB reported previously (5) and confirmed by our data.

In our experiments, expression of all three genes was low in NB (Fig. 1). Although \( \Phi(\text{ompF-lac}) \) levels were slightly greater in NB compared with those in TSB, the difference was not as great as that reported by Hall and Silhavy (5, 6). We found that the dominance of \( \text{ompF} \) over \( \text{ompC} \) in NB was enhanced as cells approached the stationary phase.

Osmolarity can signal large changes in expression of three \( \text{ompB} \)-regulated genes when cells are growing on minimal medium. The same signal brings far less response when growth is in complex media. Cells growing in TSB (medium 8) or NB-0.3 M sucrose (medium 9) were unable to induce \( \text{phoA} \) to the high levels attained in isomolar minimal media (media 1 and 7). \( \text{ompF} \) expression was also repressed when cells were growing in complex media. The metabolism of cells growing in complex media obviously differs from that in minimal medium in numerous ways. However, an important factor in differential gene expression may be growth rate. The growth rate \( \mu \), in doubling times per hour, was three to six times greater in complex media than in minimal media. The osmolarity response in minimal media, however, cannot be attributed to a growth rate difference. Growth rates in media 1 and 2 were identical; in medium 3 the rate was reduced by only 15%. Therefore, reduced gene expression may be associated with rapid growth and suggests that an additional level of regulation has been superimposed over the osmoregulation phenomenon.

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**LITERATURE CITED**

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