# 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 AS2*phoR* is MC4100 *phoR*/λpΦ-(*phoA-lac*). The Φ(*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 Φ(*ompF-lac*) and MH225 Φ(*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
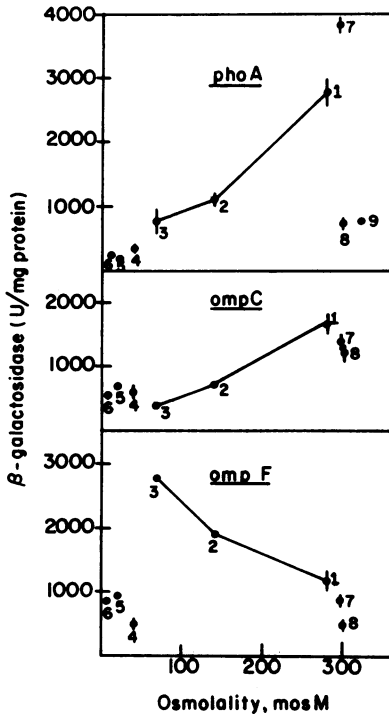
FIG. 1. Variation in gene expression with osmolarity and medium composition. Operon fusion strains were grown as described in the text. Specific activity of the β-galactosidase produced from Φ(phoA-lac), Φ(ompC-lac) or Φ(ompF-lac) is plotted against the osmolarity of each growth medium. Vertical bars represent the standard deviation of two to five independent growth experiments. The points representing dilutions of M63-glycerol medium are connected by a line. Media: 1, M63-glycerol; 2, 0.5-strength M63-glycerol; 3, 0.25-strength M63-glycerol; 4, NB; 5, 0.5-strength NB; 6, 0.25-strength NB; 7, M63-glucose; 8, TSB; 9, NB-0.3 M sucrose.

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). Osmolality 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 μmol 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 $KH_2PO_4$, 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 [Φ(phoA-lac)], production of β-galactosidase is a measure of transcriptional activity at the phoA promoter. Strain AS2phoR contains both an intact phoA gene and a Φ(phoA-lac) operon fusion introduced on a lambda phage (13). The constitutive levels of β-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 β-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 β-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, β-galactosidase activity was 2,887 U/mg. When the osmolarity was reduced to 70 mosM by dilution, the Φ(phoA-lac) level fell to 788 U/mg. The same experiment was performed with Φ(ompF-lac) and Φ(ompC-lac) for comparison

(Fig. 1). The Φ(*ompC-lac*) response to dilution paralleled that for *phoA*, although the *phoA* response was larger in magnitude. The Φ(*ompF-lac*) response to dilution was in the opposite direction, β-galactosidase activity increased when the medium was diluted. The direction of *ompF* and *ompC* variation with osmolarity shown in Fig. 1 agrees with results from experiments in which osmolarity was increased (9, 12, 15). For *phoA*, as well as for *ompF* and *ompC*, variations in osmolarity produce a corresponding change in gene expression in minimal medium of constant chemical composition (Fig. 1).

The high level of *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 ~320 mosM, and measured Φ(*phoA-lac*) levels. The β-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 ~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 *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 *phoA* expression in TSB was only 25% of that in the minimal medium.

Complex medium also repressed the elevation in *ompF* levels that was associated with reduced osmolarity of minimal medium (Fig. 1). Φ(*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 *phoA* expression, cells were grown in M63-glucose (medium 7). *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 *phoA* gene expression in complex TSB medium.

*ompF* expression was reduced in media containing glucose (media 7 and 8), as might be expected from the observation of cAMP involvement in *ompF* regulation (14). The combination of glucose content and osmoregulation of *ompF* and *ompC* can probably account for the predominance of *ompC* over *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 Φ(*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 *ompF* over *ompC* in NB was enhanced as cells approached the stationary phase.

Osmolarity can signal large changes in expression of three *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 *phoA* to the high levels attained in isomolar minimal media (media 1 and 7). *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 μ, 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.

TABLE 1. Osmolality of complex and minimal media

| Medium | Osmolality[a] (mosM/kg) |
| --- | --- |
| NB | 43 |
| TSB | 307 |
| M63-glucose | 300[b] |
| M63-glycerol[c] | 320 |

[a] Osmolality (milliosmoles per kilogram) is approximately equal to osmolarity (milliosmoles per liter) in dilute solution.

[b] Calculated from M63-glycerol by difference in sugar osmolarity.

[c] The glycerol concentration in this medium was 42 mM. Since *E. coli* is freely permeable to glycerol (1), this sugar does not contribute to the osmolarity sensed by the cell, and the effective osmolarity of this medium is only 278 mosM.

LITERATURE CITED

1. **Bovell, C. R., L. Packer, and R. Helgerson.** 1963. Permeability of *Escherichia coli* to organic compounds and

inorganic salts measured by light scattering. Biochim. Biophys. Acta **75**:257–266.

2. **Echols, H., A. Garen, S. Garen, and A. Torriani.** 1961. Genetic control of repression of alkaline phosphatase in *E. coli.* J. Mol. Biol. **3**:425–438.

3. **Epstein, W., and S. G. Schultz.** 1965. Cation transport in *Escherichia coli.* V. Regulation of cation content. J. Gen. Physiol. **49**:221–234.

4. **Granett, S., and M. Villarejo.** 1982. Regulation of gene expression in *Escherichia coli* by the local anesthetic procaine. J. Mol. Biol. **160**:363–367.

5. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the outer membrane porin proteins of *Escherichia coli* K-12. J. Mol. Biol. **146**:23–43.

6. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. J. Mol. Biol. **151**:1–15.

7. **Herzenberg, L. A.** 1959. Studies on the induction of β galactosidase in a cryptic strain of *Escherichia coli.* Biochim. Biophys. Acta **31**:525–539.

8. **Kawaji, H., T. Mizuno, and S. Mizushima.** 1979. Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12. J. Bacteriol. **140**:843–847.

9. **Kennedy, E. P.** 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. **79**:1092–1095.

10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

11. **Lugtenberg, B., R. Peters, H. Bernheimer, and W. Berendson.** 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli.* Mol. Gen. Genet. **147**:251–256.

12. **Ozawa, Y., and S. Mizushima.** 1983. Regulation of outer

13. **Sarthy, A., S. Michaelis, and J. Beckwith.** 1981. Use of gene fusions to determine the orientation of gene *phoA* on the *Escherichia coli* chromosome. J. Bacteriol. **145**:293–298.

14. **Scott, N. W., and C. R. Harwood.** 1980. Studies on the influence of the cyclic AMP system on major outer membrane proteins of *Escherichia coli* K12. FEMS Microbiol. Lett. **9**:95–98.

15. **van Alphen, W., and B. Lugtenberg.** 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli.* J. Bacteriol. **131**:623–630.

16. **Villarejo, M., and C. Ping.** 1978. Localization of the lactose permease protein(s) in the *E. coli* envelope. Biochem. Biophys. Res. Commun. **82**:935–942.

17. **Wanner, B. L., R. Kodaira, and F. C. Neidhardt.** 1977. Physiological regulation of a decontrolled *lac* operon. J. Bacteriol. **130**:212–222.

18. **Wanner, B. L., and P. Latterell.** 1980. Mutants affected in alkaline phosphatase expression: Evidence for multiple positive regulators of the phosphate regulon in *Escherichia coli.* Genetics **96**:353–366.

19. **Wanner, B. L., A. Sarthy, and J. Beckwith.** 1979. *Escherichia coli* pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. J. Bacteriol. **140**:229–239.

20. **Wurtzel, E., M.-Y. Chou, and M. Inouye.** 1982. Osmoregulation of gene expression. I. DNA sequence of the *ompR* gene of the *ompB* operon of *Escherichia coli* and characterization of its gene product. J. Biol. Chem. **257**:13685–13691.

membrane porin protein synthesis in *Escherichia coli* K-12: *ompF* regulates the expression of *ompC.* J. Bacteriol. **154**:669–675.