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The *apeE* Gene of *Salmonella enterica* Serovar Typhimurium Is Induced by Phosphate Limitation and Regulated by *phoBR*

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Mutations in *apeR*, a regulatory locus of the outer membrane esterase *apeE* from *Salmonella enterica* serovar Typhimurium, were shown to be alleles of the *pstSCAB-phoU* high-affinity phosphate transport operon. Expression of *apeE* was induced by phosphate limitation, and this induction required the *phoBR* phosphate regulatory system.

The *apeE* gene of *Salmonella enterica* serovar Typhimurium encodes an outer membrane esterase (3, 4). The gene has been sequenced (3) and is similar to LipI, a secreted lipase from the insect pathogen *Photorhabdus luminescens* (15), and to EstA an outer membrane esterase from *Pseudomonas aeruginosa* (17). However, Southern analysis and sequence comparisons have shown that *apeE* is not present in *Escherichia coli* (3). In the initial identification of *apeE* Collin-Osdoby and Miller (4) isolated mutations in an unlinked locus that resulted in a 60-fold increase in *apeE* transcription. They named this regulatory locus *apeR* and suggested that it was a gene for a transcriptional repressor of *apeE*. No conditions were identified which affected *apeE* expression (4).

In this study we cloned the *apeR* gene and showed that *apeR* mutations are alleles of the *pstSCAB-phoU* operon that encodes a high-affinity phosphate transport system. We also showed that *apeE* expression is induced by phosphate limitation and that this induction is dependent on the *phoBR* regulatory system.

Cloning and identification of *apeR*. In an *apeR* wild-type background an *apeE*::*lacZYA* fusion makes about two Miller units of β-galactosidase and consequently forms white colonies on MacConkey agar. The *apeR1* mutation increases this to 225 U, and the colonies are red. To clone the *apeR* gene, the original Mu*d*I fusion in *apeE* was first replaced with a more stable Mu*d*I-1734 insertion (strain CAC13). Plasmid libraries containing random 8- to 12-kb fragments of *Salmonella* chromosomal DNA in the vector pBR328 (7) were screened for plasmids that complemented the MacConkey phenotype of an *apeR1* mutant. Three different plasmids were isolated that complemented both the *apeR1* and *apeR47*::Tn*5* mutations.

To demonstrate that these plasmids contained *apeR*, they were integrated into the chromosome of a *polA* strain by recombination between the cloned DNA on the plasmid and its homologous DNA on the *Salmonella* chromosome (6). Antibiotic resistance on the integrated plasmid was 82 to 84% phage P22 cotransducible with insertion *zic868*::Tn*10*, which had previously been shown to be 78% cotransducible with *apeR1*. This demonstrated that the cloned DNA was from the *apeR* region of the *Salmonella* chromosome.

Restriction enzyme analysis indicated that the three plasmids shared a 4.5-kb fragment (Fig. 1). Subcloning showed that DNA on both sides of an *Eco*RI site in the middle of this region was required for complementation. Four Tn*1000* insertions were isolated in the insert region of plasmid pAPR3. Two of these, located 3 kb apart, eliminated complementation. DNA sequence was obtained from the right end of the insert in pAPR3. This sequence was 93% identical to the last 261 bases of the *pstB* gene and the first 73 bases of the *phoU* gene in the *E. coli pstSCAB-phoU* operon. Alignment of the *E. coli* sequence with the restriction map and the Tn*1000* insertions indicated that the *apeR1* mutation must be an allele of *pstC*, *pstA*, or *pstB*. This is consistent with the equivalent map positions of *apeR* on the *Salmonella* chromosome (11) and *pstSCAB-phoU* operon on the *E. coli* chromosome (1).

Regulation of *apeE* by phosphate limitation and *phoBR*. The *pstSCAB-phoU* operon encodes a high-affinity phosphate transport system. Mutations in the *pst* operon often result in constitutive expression of genes that are induced by phosphate limitation. This is particularly true of members of the PhoBR regulon, such as the *E. coli phoA* gene (16). This led us to examine the effect of phosphate limitation on *apeE* induction. Strain CAC13, containing an *apeE*::*lacZYA* fusion, was grown in minimal morpholinepropanesulfonic acid medium with limiting phosphate (0.1 mM) or excess phosphate (2 mM) as previously described (8), and β-galactosidase activity was measured (10). Limiting phosphate induced *apeE* expression 140-fold, from 1.5 to 210 U of β-galactosidase (Table 1), explaining the effect of the *apeR (pst)* mutation.

To determine whether *apeE* is regulated by one of the known phosphate limitation regulatory genes, we used phage P22 transduction to construct isogenic strains that contained both the *apeE*::*lacZYA* fusion and mutations in either *ntrA (rpoN), phoP, katF (rpoS)*, or *phoBR*. β-Galactosidase activity was measured for these strains in both limiting and excess phosphate. As seen in Table 1, only the *phoBR* deletion significantly affected phosphate induction, completely eliminating induction by phosphate limitation, as well as the effect of the *apeR* mutation. This indicated that *apeE* is a previously uncharacterized member of the *phoBR* regulon.

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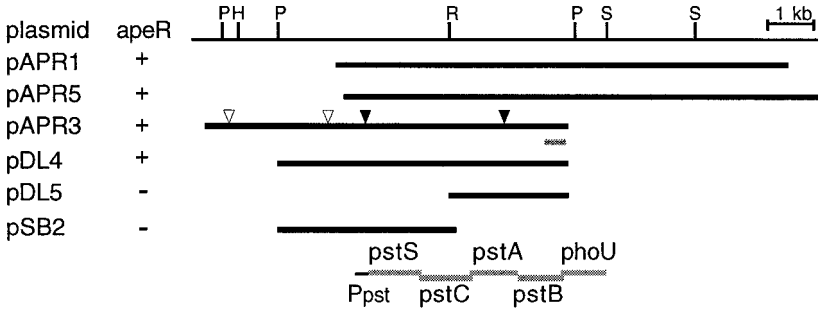

FIG. 1. *apeR* complementing plasmids. Bars indicate chromosomal DNA contained on the plasmids. Restriction enzyme sites are indicated as follows: H, *Hin*dIII; P, *Pvu*II; R, *Eco*RI; and S, *Sal*I. Triangles indicate Tn*1000* insertions: closed-triangle insertions eliminated complementation, open-triangle insertions did not. The *E. coli pstSCAB-phoU* operon is aligned with the restriction map based on DNA sequence determined from the right end of plasmid pAPR3.

Analysis of the *apeE* promoter region. The promoters of genes that are regulated by PhoB contain a PhoB binding site (PHO box) in the −35 region of the promoter. A consensus PHO box consists of two 7-bp direct repeats separated by a 4-bp AT spacer (16). Although there is no obvious consensus PHO box upstream of the *apeE* coding region, by using a less-stringent definition of the PHO box based on mutational analysis of the PhoB binding site by Makino et al. (9) and examination of other *phoBR* regulated genes (14), we have identified three potential half PHO boxes located 19 bp upstream of a likely −10 sequence (GATAAT) (Fig. 2).

To begin identifying regions of the *apeE* promoter that are required for phosphate control, we performed deletion analysis of the promoter region. We used the PCR to generate fragments with a constant 3′ end 1413 nucleotides downstream of the start of translation and various 5′ ends as indicated in Fig. 2. These fragments were inserted into the promoter detection vector pRS415 (13), where they control the expression of a promoter-less *lac* operon. Plasmid-containing strains of *S. enterica* strain TN1379 were grown in either 2 or 0.1 mM phosphate, and the β-galactosidase activity was determined. As shown in Fig. 2, successive removal of potential PHO box half-sites reduced phosphate induction. Subclone B, which included all three proposed PHO box half sites, was induced 27-fold by phosphate limitation. Subclone D, which contained two half-sites, was induced 19-fold, and subclone F, with one complete half-site, was induced only 8.6-fold. Subclones C, G, and E containing no intact half-sites were essentially uninducible. These results support the identification of the putative PHO boxes in the *apeE* promoter region. Further analysis, such as site-directed mutagenesis and DNA footprinting with PhoB protein, will be needed to confirm this. The differences in induction ratios between the plasmids and the chromosomal Mu*d*I-1734 insertion were probably caused by the increased copy number of the plasmids and cryptic promoter activity in the approximately 600 bp between the Mu*d*I-1734 insertion site and the 3′ end of the subclones.

Possible function of ApeE. As mentioned above, *apeE* is not present in *E. coli*, but a search of preliminary genomic sequence data showed that it is clearly present in the *S. enterica* serovars Typhi and Paratyphi A, as well as *S. enterica* serovar Typhimurium. Although this suggests that *apeE* could be involved in *Salmonella* virulence, it has not been identified in screens for *Salmonella* virulence factors. This is consistent with the report by Jiang et al. that a *phoBR* deletion itself failed to attenuate virulence (8), and therefore it is unlikely that PhoBR-regulated genes would either.

We have previously reported that *apeE* is required for the utilization of the model lipid substrate Tween 80 (polyoxyethylene sorbitan monooleate) and for the hydrolysis of methylumbelliferyl caprylate (3). Combined with the phosphate regulation data, this suggests that *apeE* could play an important role in the use of phospholipids as phosphate sources. The products of ApeE deacylation of phospholipids would be either *sn*-glycerol-3-phosphate or glycerophosphoryl diesters. In both *E. coli* and serovar Typhimurium these organic phosphate

TABLE 1. β-Galactosidase activity of *apeE*::*lacZ* fusions in limiting and excess phosphate

Strain	Genotype	β-Galactosidase activity[a] (Miller units) in:	
		2 mM phosphate	0.1 mM phosphate
CAC13	Δ*leuBCD485 apeE14*::Mu*d*I-1734	1.5	210
CAC47	Δ*leuBCD485 apeE14*::Mu*d*I-1734 *apeR1*	225	241
CAC49	Δ*leuBCD485 apeE14*::Mu*d*I-1734 Δ*phoB1*::*cat*	1.8	1.6
CAC50	Δ*leuBCD485 apeE14*::Mu*d*I-1734 Δ*phoB1*::*cat apeR1*	1.9	2.0
CAC39	Δ*leuBCD485 apeE14*::Mu*d*I-1734 *katF*::*bla*	1.0	272
CAC97	Δ*leuBCD485 apeE14*::Mu*d*I-1734 *phoP53*::Tn*10d*Tet	0.8	134
CAC99	Δ*leuBCD485 apeE14*::Mu*d*I-1734 *ntrA209*::Tn*10*	0.8	149

[a] Strains were grown overnight in morpholine propanesulfonic acid minimal medium with either 2 mM K_2HPO_4 and 0.06% glucose or 0.1 mM K_2HPO_4 and 0.4% glucose (8). The β-galactosidase activity was assayed as described by Miller (10). Each assay was performed in triplicate, and all strains were assayed at least twice.

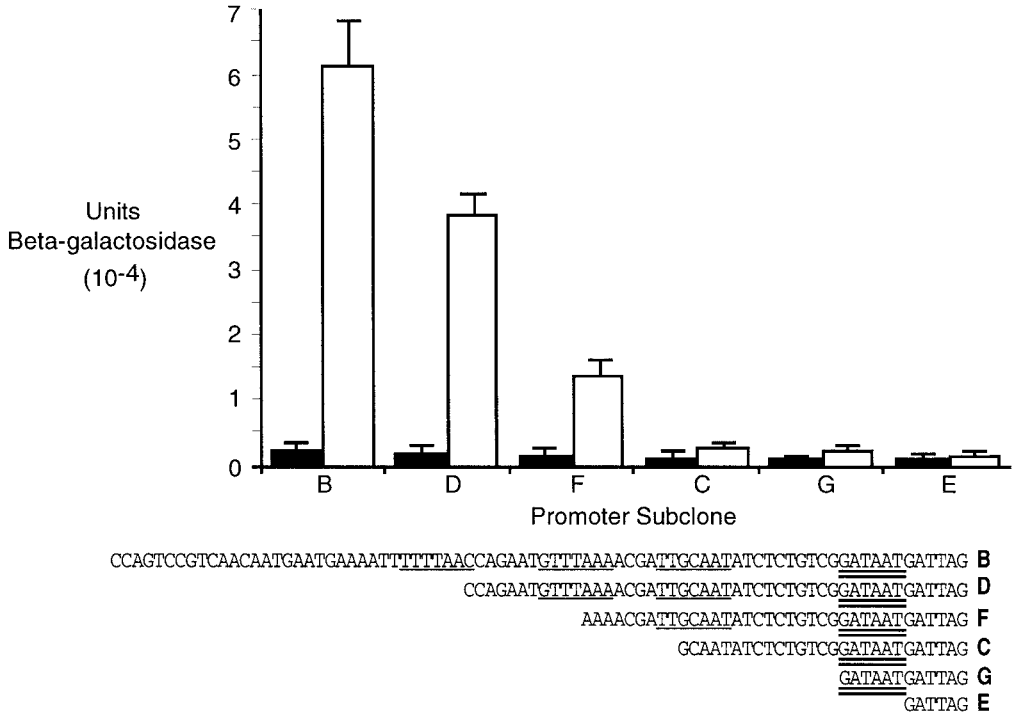

FIG. 2. Phosphate regulation of *apeE* promoter subclones. Plasmid strains were grown on 2 mM (solid bars) or 0.1 mM (open bars) phosphate, and β-galactosidase activity was assayed (10). Assays were done in triplicate, and each strain was assayed three times. The 5′ ends of the subclones are shown below the graph. Single underlines indicate PHO box half-sites, and the double underline indicates a potential −10 region.

sources are transported across the inner membrane by the PhoB-dependent Ugp transport system and so can be used as sole phosphate sources (2, 5, 12). Whereas *E. coli*, which lacks ApeE, uses PhoA to remove the phosphate from the phospholipid, *Salmonella* spp., which lack PhoA but have ApeE, would not need PhoA to use phospholipids as phosphate sources since the deacylated products would be transported by the Ugp system. Additional studies are needed to confirm this hypothesis.

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