Regulation of the pho Regulon of Escherichia coli K-12
Cloning of the Regulatory Genes phoB and phoR and Identification of Their Gene Products

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The regulatory genes phoB and phoR of the Escherichia coli K-12 pho regulon have been cloned using two types of selection. The genes were localized on one of the hybrid plasmids, pJP50, by analysis of mutant plasmids generated by Tn5 insertions. Restriction mapping of the other plasmid, pPR20, indicated that the gene order in this region of the chromosome is phoR phoR tsx. Analysis of plasmid-coded proteins in a minicell system showed that:

1. The products of phoB and phoR are proteins with apparent molecular weights of 30,000 and 47,000, respectively.
2. The synthesis of the phoR gene product is derepressed by phoR mutations.

The implications of this observation for the model of pho regulation are discussed.

1. Introduction

The PhoE protein of Escherichia coli K-12 is an outer membrane protein, which is induced by growth at limiting concentrations of inorganic phosphate (Overbeke & Lugtenberg, 1980; Tommassen & Lugtenberg, 1981). Like the OmpF protein and the OmpC protein the PhoE protein is involved in the formation of aqueous pores by means of which small hydrophilic solutes can pass through this membrane (for a recent review, see Nikaido, 1979). The protein is co-regulated with alkaline phosphatase and a number of other proteins inducible by phosphate limitation in a single regulon, designated the pho regulon (Argast & Boos, 1980; Tommassen & Lugtenberg, 1980).

At least three genes are involved in the regulation of the pho regulon, i.e. phoB (Bracha & Yagil, 1973) and phoR (Echols et al., 1961) at 9 minutes on the chromosomal map (Bachmann & Low, 1980) and phoM at 0 minutes (Wanner &
The phoB gene product acts as a positive regulator (Brickman & Beckwith, 1975; Yagil et al., 1975), whereas a dual role has been suggested for the phoR gene product: namely, as a repressor and as an activator in the presence and absence of phosphate, respectively (Garen & Echols, 1962; Wanner & Latterell, 1980). The phoM gene product acts as an activator that can partially replace the activator function of the phoR gene product (Wanner & Latterell, 1980). In addition, mutations in any of the genes phoS, phoT or pst result in the constitutive synthesis of the PhoE protein (Tommassen & Lugtenberg, 1980), alkaline phosphatase and the other proteins inducible by phosphate limitation (Morris et al., 1974; Willsky & Malamy, 1976). However, these genes play only an indirect role in the regulation, as they seem to play a direct role in the transport of inorganic phosphate (Willsky et al., 1973).

An essential step in the study of the multiple regulators in the expression of the pho regulon is to identify the products of the regulatory genes. In this paper we describe the cloning of the genes phoB and phoR and the identification of their products.

2. Materials and Methods

(a) Bacterial strains and growth conditions

All bacterial strains are derivatives of E. coli K-12. Their sources and relevant characteristics are listed in Table 1. The phoR18 derivatives of strains CE1107 and PC1232 were obtained from crosses of these strains with Hfr strain C9 by selection for thr+, leu+, rpsL transconjugants. The phoR18 derivatives were selected as alkaline phosphatase constitutive transconjugants. The recA56 derivatives of strains CE1174, CE1217 and AB1157 were obtained from crosses of these strains with strain PC1505 as a donor by selection for his+, rpsL transconjugants. The recA56 derivatives were selected as ultraviolet light-sensitive transconjugants. The recA56 derivatives were selected as ultraviolet light-sensitive transconjugants.

To make strain P2257 (tsx::Tn10), a random Tn10 integrate of P1848 was generated, using ANK561 (b221 c1857 c1::Tn10 o29 p80) (Kleckner et al., 1977), and individual TcR+ colonies were screened for resistance to phage T6. Strain P2257 was resistant to the phage, and this resistance was transduced 100% with TcR into P1848. Since the phoR+ and prot+ genes are 95% and 80% cotransducible with TcR, respectively, the Tn10 insertion is most likely located in the tsx gene.

Except noted, cells were grown overnight in tryptone/yeast broth (Lugtenberg et al., 1976) at 37°C under aeration. To select for ApR, CmR or KnR strains, concentrations of 50, 25, 25 and 10 μg/ml, respectively, of these antibiotics were applied. For growth of cells containing plasmid pACYC184 or its derivatives the medium was always supplemented with Cm.

(b) Genetic techniques

Conjugation (Havik et al., 1976) and transformation (Brown et al., 1979) were carried out as described previously. Sensitivity to bacteriophages was determined by cross-streaking.

(c) DNA techniques

Chromosomal DNA was isolated as described by Cosloy & Oishi (1973). Plasmid DNA was isolated by the cleared lysate procedure of Clewell & Helinski (1969), followed by CsCl/ethidium bromide isopycnic centrifugation. The alkaline-extraction procedure of Birnboim & Doly (1979) was used for rapid screening of plasmids.

† Abbreviations used: Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; TeR, ApR, CmR and KnR, resistance to these antibiotics; kb, 10^3 base-pairs.
CLONING OF THE GENES phoR AND phoR

Table 1

Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics†</th>
<th>Source, reference‡</th>
</tr>
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<tr>
<td>CE1107</td>
<td>f', thr leu thi pyrF thy ilvA his lacY argG tonA tsx rpsL cod dra vtp gluR ompB471</td>
<td>Lugtenberg et al. (1978)</td>
</tr>
<tr>
<td>C9</td>
<td>Hfr Cav, relA1 tonA22 pit-10 spoT1 ompF phoR18</td>
<td>A. Garen, via CGSC</td>
</tr>
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<td>CE1217</td>
<td>thr' leu' phoR18 derivative of CE1107</td>
<td>This study</td>
</tr>
<tr>
<td>CE1174</td>
<td>phoN200, phoB201 derivative of CE1107</td>
<td>Tommassen &amp; Lugtenberg (1980)</td>
</tr>
<tr>
<td>PC1505</td>
<td>Hfr KL16, thr ilv phx recA56</td>
<td>PC</td>
</tr>
<tr>
<td>CE1216</td>
<td>his' recA56 derivative of CE1174</td>
<td>This study</td>
</tr>
<tr>
<td>CE1220</td>
<td>his' recA56 derivative of CE1127</td>
<td>This study</td>
</tr>
<tr>
<td>AB1157</td>
<td>F', thr leu proA12 (del proA-phoE-gpt) his thr ilo phx recA56 derivative of CE1198</td>
<td>Adelberg</td>
</tr>
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<td>CE1218</td>
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<td>This study</td>
</tr>
<tr>
<td>PC1232</td>
<td>F', thr leu lacY gal mtl mal lam tonA phx rpsL azi minA minB</td>
<td>PC</td>
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<td>This study</td>
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<td>PC0031</td>
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<tr>
<td>W1485F-</td>
<td>thi</td>
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<td>P2257</td>
<td>tsx234 : : Tn10-14 derivative of P1848</td>
<td>This study</td>
</tr>
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</table>

† Genotype descriptions follow the recommendations of Bachmann & Low (1980).
‡ CGSC. E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn. (B. J. Bachmann, Curator); PC, Phabagen Collection, Department of Molecular Cell Biology, Section Microbiology, State University of Utrecht, Utrecht, The Netherlands.

Restriction endonucleases EcoRI, BamHI, HindIII, SalI, BglII, PstI, ClaI and SmaI were obtained from Boehringer Mannheim. KpnI was obtained from Bethesda Research Laboratories. The conditions for restriction endonuclease reactions were those proposed by the manufacturers. Analyses of plasmid DNA fragments were performed by electrophoresis in a 0.6% agarose slab gel (van den Hondel et al., 1979). HindIII- and EcoRI-generated fragments of bacteriophage λ DNA were used as molecular weight standards in gels. Bacteriophage T4 DNA ligase was a generous gift from P. Weisbeek. Ligation was performed as described by Tanaku & Weisblum (1975).

(d) Isolation of plasmids carrying an inserted Tn5 element

Insertions of the transposable Kn8-determining element Tn5 in plasmid pJP50 were obtained using phage λ b221 e1857 rec:: Tn5 (Berg, 1977). Cells of strain CE1218 bearing plasmid pJP50 were grown overnight at 37°C in brain/heart broth (Lugtenberg et al., 1976) supplemented with Cm and maltose (0.2%), collected by centrifugation and concentrated 3-fold in 0.9% NaCl, 20 mM-MgCl2. After 45 min of incubation at 37°C, λ:: Tn5 phage was added to 0-1 ml vol. of cells at a multiplicity of 10, the mixtures were incubated for 30 min at 30°C, then diluted 10-fold in tryptone yeast broth plus Cm and grown overnight at 30°C. Subsequently the cultures were diluted 1 in 100 in tryptone/yeast broth plus Cm, Kn and sodium citrate (0.1%), and grown overnight at 30°C. Kn8Cm8 cells were harvested and crude plasmid DNA was isolated (Birnboim & Doly, 1979) and used to transform phoN phoB strain CE1216 and phoR strain CE1220 selecting for Kn8Cm8 transformants. The colonies were screened for constitutive synthesis of alkaline phosphatase by spraying them with a solution of 20 mg p-nitrophenyl phosphate/ml (Tommassen & Lugtenberg, 1980). Transformants of phoN phoB strain CE1216 with an intact phoB gene on the plasmid produce alkaline
phosphatase constitutively, in contrast to transformants that contain a Tn5 insertion in the phoB gene on the plasmid. Transformants of phoR strain CE1220 with a Tn5 insertion in the phoR gene on the plasmid produce alkaline phosphatase constitutively, in contrast to transformants with an intact phoR gene on the plasmid.

(c) Analysis of protein synthesis in minicells

Techniques used for the analysis of plasmid-coded proteins in minicells have been described previously (Tommassen et al., 1982). In this paper several proteins are indicated by their apparent molecular weight ($\times 10^{-3}$) followed by the letter K.

3. Results

(a) Cloning of the genes phoB and phoR by direct selection for phoB

The rationale for cloning of the phoB gene was as follows. Mutant strains that lack the OmpF protein and the OmpC protein are sensitive to 3% sodium dodecyl sulphate, whereas expression of the PhoE protein in such a strain results in resistance to the detergent (Lugtenberg et al., 1978). The ompR phoB phoS strain CE1216 lacks the OmpC protein and the OmpF protein due to the ompB mutation and the PhoE protein due to the phoB mutation. A cloned phoB+ gene would complement the phoB mutation and thus allow constitutive expression of the phoE gene due to the presence of the phoS mutation. This would result in resistance to 3% sodium dodecyl sulphate.

For cloning the phoB gene, purified DNA of the prototrophic strain PC0031 was used as the source of phoB+ DNA. The miniplasmid pACYC184 was used as the cloning vector. This vector renders the cells resistant to Km and Tc. It has a unique site for restriction endonuclease BamHI in the Tc gene (Chang & Cohen, 1978). pACYC184 DNA and chromosomal DNA were mixed and digested with endonuclease BamHI. After inactivation of the endonuclease ligation was allowed using phage T4 DNA ligase. The ligated mixture was used to transform strain CE1216, selecting colonies that were simultaneously resistant to Km and sodium dodecyl sulphate. One such colony was found. This strain is sensitive to Tc, indicating that a BamHI-generated DNA fragment is cloned in the Tc gene. As it produced alkaline phosphatase constitutively and is sensitive to the PhoE-protein-specific phage TC45 (Chai & Foulds, 1978), it must contain the wild-type phoB+ allele. As the properties, TC45 sensitivity and alkaline phosphatase constitutivity, could be transferred to the phoS phoB strain CE1216 by transformation with the plasmid DNA, this phoB+ allele is located on the plasmid of this strain, designated as pJP50.

Since the phoR gene is located very close to phoB (Bachmann & Low, 1980), the possibility that the hybrid plasmid pJP50 might also contain the phoR gene was tested. phoR strain CE1220 was transformed with pJP50 and, in contrast to the parental phoR strain, all 25 CmR transformants tested did not produce alkaline phosphatase constitutively and were resistant to phage TC45. Therefore pJP50 also carries the phoR gene.

In order to determine a restriction-enzyme cleavage map, purified pJP50 was digested with several restriction endonucleases. Cleavage sites of different restriction enzymes were deduced from the sizes of restriction fragments of this
CLONING OF THE GENES *phoB* AND *phoR*

DNA obtained by digestions and suitable double digestions with the appropriate enzymes. A restriction map of pJP50 is presented in Figure 1. The position of the vector plasmid in this map was deduced from its known restriction map (Chang & Cohen, 1978). Unexpectedly, the plasmid contains only one BamHI site (Fig. 1). Therefore, one BamHI site must have been deleted during the cloning and ligation procedure. The size of the entire plasmid is 18.7 kb.

(b) Cloning of the genes *phoB* and *phoR* by selection for a nearby *Tc^R* gene

Since strain P2257 has a transposon Tn10 insertion in *tsx*, which is 95% cotransducible with *phoR*, it was thought that a Tc^R clone from P2257 DNA might carry *phoR* and *phoB*. The DNA of P2257 was digested with BamHI (1 unit/μg DNA) for 5 min at 37°C, and the partially digested DNA was ligated with the fully digested DNA of the plasmid vector pRSF2124, which carries an Ap^R gene (So et al., 1975). By transformation to strain P1848, a single Ap^R Tc^R transformant was obtained. The plasmid DNA was extracted from this strain and transformed into strains CE1216 and CE1220. Strain CE1216 became sensitive to phage TC45, while CE1220 became repressed for alkaline phosphatase production. The plasmid, designated pPR20, therefore carries *phoB* and *phoR*.

The purified pPR20 was subjected to single and double digestions with several restriction enzymes to obtain a map (Fig. 2). The piece of Tn10 in the plasmid was localized by reference to its known cleavage sites (Jorgensen *et al.*, 1979). The size of the entire plasmid is 43.8 kb. It has 14.8 kb of cloned DNA in common with pJP50.

(c) Localization of *phoB* and *phoR* on pJP50

In order to localize *phoB* and *phoR* on pJP50, transposon Tn5 insertion mutants were isolated. Cells of strain CE1218 carrying pJP50 were infected with λ::Tn5.
After selection for \( \text{Cm}^R \text{Kn}^R \) cells (see Materials and Methods) crude plasmid DNA was extracted and used to transform \( \text{pho}S \text{pho}B \) strain CE1216 and \( \text{pho}R \) strain GE1220 to resistance to both Cm and Kn. Transformants of strain CE1216 with a Tn5 insertion in the \( \text{pho}B \) gene of the plasmid and transformants of strain CE1220 with a Tn5 insertion in the \( \text{pho}R \) gene of the plasmid could be elected by screening for alkaline phosphatase synthesis. This procedure yielded five independent insertions in \( \text{pho}B \) and four in \( \text{pho}R \).

Plasmid DNA was extracted from these transformant strains and from seven other transformants which contain a Tn5 insertion in the plasmid outside the genes \( \text{pho}B \) and \( \text{pho}R \). The location of each insertion was determined by digesting the plasmids with \( \text{HindIII} \) and \( \text{BamHI} \). \( \text{HindIII} \) cuts the 5.7 kb Tn5 element twice, 1.1 kb from each end, whereas \( \text{BamHI} \) only cuts once, 2.7 kb from one end (Rothstein & Reznikoff, 1981). The sizes of the \( \text{HindIII} \) and \( \text{BamHI} \) restriction fragments of each derivative were used to locate each insertion precisely. The results, presented in Figure 3, show that the presumed insertions in \( \text{pho}R \) as well as those in \( \text{pho}B \) are clustered. Both genes are located very close to each other and therefore they might be parts of the same operon.

To investigate this possibility, the plasmids with insertions in the \( \text{pho}B \) gene were transformed to \( \text{pho}R \) strain CE1220 by selection for \( \text{Cm}^R \text{Kn}^R \) transformants. The alkaline phosphatase synthesis in all transformants was repressed, indicating that the plasmids with a Tn5 insertion in \( \text{pho}B \) contain a functional \( \text{pho}R \) gene. Similarly, plasmids with insertions in \( \text{pho}R \) were transformed to \( \text{pho}S \text{pho}B \) strain CE1216. All transformants produced alkaline phosphatase constitutively, showing that the plasmids with insertions in \( \text{pho}R \) still contain a functional \( \text{pho}B \) gene. Since transposon Tn5 insertions are usually strongly polar (Berg et al., 1980), these results indicate that \( \text{pho}B \) and \( \text{pho}R \) are not part of the same operon.
(d) Identification of the phoB and phoR gene products in minicells

In order to identify the products of the genes phoB and phoR, the synthesis of plasmid-coded proteins was studied in minicells isolated from strain PC1232. The results (Fig. 4A) show that, compared with minicells isolated from the strain carrying cloning vector pACYC184 (lane (a)), the minicells from the strain carrying the hybrid plasmid pJP50 produce at least six additional labelled polypeptides with apparent molecular weights of 100 K, 47 K, 46 K, 34 K, 33 K and 30 K (lane (b)). In minicells carrying pJP50 with a Tn5 insertion several additional labelled proteins are present, which are encoded by the transposon (e.g. Fig. 4A, lane (c)). Minicells containing plasmid pJP50 with any of the five Tn5 insertions in the phoB gene lack the labelled 30 K protein (e.g. lane (d)). Therefore, this 30 K protein must be the product of the phoB gene. Similarly, the labelled 47 K protein could not be detected in minicells containing Tn5 insertions in the phoR gene of pJP50 (e.g. Fig. 4A, lanes (e) and (f)). Therefore, this 47 K protein must be the product of the phoR gene. In minicells with pJP50 containing Tn5 insertion number 9 (Fig. 3) in the phoR gene, an additional labelled protein band with an apparent molecular weight of 42 K is visible (Fig. 4A, lane (f)). Therefore, this polypeptide must be a fragment of the phoR gene product. Since no fragments of the phoR gene product could be detected in minicells with pJP50 carrying any of the other Tn5 insertions in phoR, the direction of transcription of the phoR gene is probably as indicated in Figure 3. Since no fragment of the phoB gene product was observed in minicells containing pJP50 with any of the Tn5 insertions in phoB, the direction of transcription of the phoB gene could not be established.

The synthesis of plasmid-coded proteins was also studied in minicells isolated from the phoR mutant minicell-producing strain CE1198. The results (Fig. 4B) show that insertion of Tn5 in the phoR gene results in derepression of the synthesis of the phoB gene product (compare lanes (e) and (f) with (b) and (c)). Therefore, the phoR gene product seems to exert its function by repressing the expression of the phoB gene.
4. Discussion

We have cloned the regulatory gene phoR of the pho regulon by using the properties: (1) that expression of the outer membrane PhoE protein is dependent on the phoB+ gene product; and (2) that a strain that lacks all major outer membrane-pore proteins is sensitive to 3% sodium dodecyl sulphate. This procedure should also be applicable to cloning the structural genes for any of these
pore proteins as well as other regulatory genes like \( \text{phoM} \). The structural genes for major pore proteins of related organisms can probably also be cloned in this way, provided that they are expressed in \( E. \text{coli} \) K-12.

The \( \text{phoR} \) gene was found on the same DNA fragment as the \( \text{phoB} \) gene. Whereas no definite proof was available showing that \( \text{phoB} \) and \( \text{phoR} \) are two distinct cistrons (Brickman & Beckwith, 1975), the Tn\( 5 \)-insertion experiments now show that \( \text{phoB} \) and \( \text{phoR} \) are indeed two cistrons and, moreover, that they are probably not part of a single operon. Furthermore, reference of the data presented in Figure 3 to the pPR20 map (Fig. 2) shows that the gene order in this part of the chromosome is \( \text{phoB} \) \( \text{phoR} \) \( t\&x \), since the Tn10 insertion was obtained in \( P2257\)-inactivated \( t\&x \).

![Diagram](image)

**Fig. 5.** Model for \( \text{pho} \) regulation. \( R \), \( M \) and \( B \) are the products of the genes \( \text{phoR} \), \( \text{phoM} \) and \( \text{phoB} \), respectively. \( B \) is an activator, which is essential for expression of \( \text{phoE} \) and the other structural genes of the \( \text{pho} \) regulon. In the presence of sufficient inorganic phosphate (\( P_i \)), \( R \) is in the repressor form \( R^R \). \( P_i \) limitation converts \( R^R \) to the activator form \( R^A \) (Wanner & Latterell, 1980). Since the \( \text{phoR} \) gene product seems to repress \( \text{phoB} \) in the presence of \( P_i \), the simplest explanation for the regulation of \( \text{phoE} \) gene expression is the assumption that \( R^R \) and \( R^A \) regulate \( \text{phoB} \) expression. If \( R^R \) in the presence of sufficient \( P_i \) competes with the activator \( M \) for the same site on \( \text{phoB} \) DNA, the level of \( \text{phoR} \) expression will be low. Under conditions of \( P_i \) limitation, competition between \( R^R \) and \( M \) changes to co-operation between \( R^A \) and \( M \), resulting in high levels of \( \text{phoB} \) expression and thus in synthesis of the \( \text{PhoE} \) protein and the other proteins of the \( \text{pho} \) regulon. The essentials of this model have been proposed recently by Tommassen & Lugtenberg (1982).

The regulation of the \( \text{pho} \) regulon is very complicated, since at least three regulatory genes, namely \( \text{phoR} \), \( \text{phoR} \) and \( \text{phoM} \), seem to be involved (Wanner & Latterell, 1980). Our present results contribute the following information to the \( \text{pho} \) regulation process. Firstly, the products of the genes \( \text{phoB} \) and \( \text{phoR} \) have been identified as proteins with apparent molecular weights of 30,000 and 47,000, respectively (Fig. 4). Secondly, they show that the closely linked genes \( \text{phoB} \) and \( \text{phoR} \) are not part of the same operon. Thirdly, the observation that the synthesis of the \( \text{phoB} \) product is derepressed by mutations in the \( \text{phoR} \) gene is consistent with and supports part of our recently proposed model for \( \text{pho} \) regulation (see Fig. 5; and Tommassen & Lugtenberg, 1982). The identification of the products of genes \( \text{phoB} \) and \( \text{phoR} \) is essential for the purification of these proteins. Moreover, it allows us to test whether the interactions proposed in the model (Fig. 5) between these regulatory proteins and operator sequences on cloned \( \text{phoB} \) (this paper) and \( \text{phoE} \) DNA (Tommassen *et al.*, 1982) do indeed occur.
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