The Phosphate-Binding Protein of *Escherichia coli* Is Not Essential for \( P_i \)-Regulated Expression of the *pho* Regulon

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Disruption of *pstS* encoding the \( P_i \)-binding protein in *Escherichia coli* generally leads to the constitutive expression of the *pho* regulon. We demonstrate that \( P_i \)-controlled expression is restored when the activity of the \( P_i \) transporter PitA or PitB is increased. Apparently, *PstS* is not an essential component of the signal transduction pathway.

Growth of *Escherichia coli* under \( P_i \) limitation results in the induction of the *pho* regulon, which includes *phoA* encoding alkaline phosphatase (18) and the *pst* operon encoding a \( P_i \) transporter. This \( P_i \) transporter consists of a periplasmic \( P_i \)-binding protein (*PstS*), two integral membrane proteins (*PstA* and *PstC*), and an ATP-binding protein (*PstB*) (4, 16). Central to the regulation of the *pho* regulon is a two-component regulatory system encoded by the *phoBR* operon (21). In addition, the *Pst* system plays a role in \( P_i \) regulation, since mutations in any of the genes of the *pst* operon generally lead to constitutive expression of the *pho* regulon (21–23). This constitutive expression is not due to decreased intracellular phosphate levels, which were reported to be maintained at a high level under high-\( P_i \) conditions by a secondary \( P_i \) transporter, PitA (11, 24). Furthermore, the regulatory and transport roles of the *Pst* system could be uncoupled by specific amino acid substitutions in *PstC* or *PstA* (5, 6). Since periplasmic *PstS* binds \( P_i \) with high affinity, it could potentially act as the primary sensor of extracellular \( P_i \) (20). The interaction of \( P_i \)-loaded *PstS* with the membrane components of the *Pst* system might lead to a conformational change, which is sensed by the product of the fifth gene of the *pst* operon, PhoU. PhoU is not involved in \( P_i \) transport (15), but probably forms the regulatory link between the \( P_i \) transporter and the PhoBR system (20).

To study the role of *PstS* in signal perception, we wished to isolate missense mutations in *psta* or *pstc* that allow the *Pst* system to transport \( P_i \) in the absence of *PstS*. In a previous study, such mutants were not obtained, but a third \( P_i \) transporter, PitB, was discovered (9). In this study, we demonstrate that PitA or PitB activity can restore \( P_i \) regulation of the *pho* regulon in the absence of *PstS*.

**Pseudorevertants in pitA restore \( P_i \) regulation in a *pstS* mutant.** To study the role of *PstS* in \( P_i \) regulation of the *pho* regulon, we attempted to isolate mutants in *psta* or *pstd*, allowing the *Pst* system to transport \( P_i \) in the absence of *PstS*. Therefore, strain CE1491, which lacks all three known \( P_i \) transporters (Table 1), was mutagenized with ethylmethane sulfonic acid, and mutants that could grow on minimal medium plates (9) with 660 \( \mu \)M \( P_i \) as the sole source of phosphate were selected. Emanating from the idea that restored \( P_i \) transport via the *Pst* system might also restore \( P_i \) control of the *pho* regulon, the mutants obtained were tested for expression of alkaline phosphatase on *L* broth plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP) (3). Six of 300 mutants tested showed drastically reduced alkaline phosphatase activity, and two mutants, designated CE1493 and CE1494, were characterized in detail. Quantitative analysis showed that alkaline phosphatase activity was reduced after growth in complex medium almost to the level found in *pitA* *Pst*\(^{-}\) strain K10 (Table 1). Furthermore, uptake of \( ^{33}P_i \) was considerably improved compared to that in the parental strain, CE1491 (Fig. 1). However, after *P1* transductions with the revertants as donors and strain K10 as acceptor, all Kan\(^{r}\) transductants tested (50 in each case) failed to grow on \( P_i \) as a phosphate source, and alkaline phosphatase was highly expressed (data not shown), showing that the reversion is not closely linked to the *PstS::kan* mutation. Furthermore, since CE1493 and CE1494 were resistant to gentamicin, the *pitB::gm* mutation had not reverted. Hence, we considered the possibility that the *pitA* mutation had reverted. First, the *pitB::gm* allele was replaced with a wild-type *pitB* gene by *P1* transduction with the *mecC162::Tn10* strain CAG18475 (13) as the donor, resulting in strains CE1495 and CE1496 (note that a wild-type *pitB* gene gives a *PitB*\(^{-}\) phenotype) (9). Subsequently, a *pitA::gm* mutation was introduced. The resulting strains, CE1497 and CE1498, respectively, had lost the ability to grow on \( P_i \) (results not shown), showing that the reversion in strains CE1493 and CE1494 is linked to the *pitA* locus. Furthermore, they produced high levels of alkaline phosphatase (Table 1), demonstrating that the reduced alkaline phosphatase activity in strains CE1493 and CE1494 results from the same mutation and is not due to a secondary mutation (for example, in the *phoBR* genes). After PCR amplification and cloning in PCRII\(-\)TOPO (Invitrogen), the *pitA* alleles of the revertants were sequenced. A point mutation resulting in the substitution of Thr41 (which is highly conserved in a large superfamily of \( P_i \) transporters) (12) by Ile was found in both strains. The original *pitA* mutation of strain K10, which resulted in the Gly220Asp substitution (9), was retained, demonstrating that

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the revertants CE1493 and CE1494 carry a compensatory mutation in pitA, rather than a true reversion. To investigate whether the pho regulon can be induced in strains CE1493 and CE1494, the cells were grown in high-phosphate (HPi) and low-phosphate (LPi) media, and alkaline phosphatase activity was determined. Indeed, high activity was measured after growth of these strains in LPi medium (Fig. 2). These results demonstrate that the requirement for PstS in P; regulation of the pho regulon can be substituted by PitA activity.

Wild-type pitA can restore Pi regulation in a pstS mutant. To investigate whether expression of a wild-type pitA gene can restore Pi regulation in a pstS mutant, the mutant pitA allele in CE1491 was replaced with the wild-type gene by P1 transduction with zhf-5::Tn10 strain CAG18450 (13) as the donor. A Tetr transductant that could grow on P; as a phosphate source was designated CE1499. Even though colonies of this strain were blue on XP-containing L broth plates, quantitative analysis showed that the alkaline phosphatase activity was reduced compared to that of the parental strain CE1491, although not to the same extent as in the pseudorevertants CE1493 and CE1494 (Fig. 2). Alkaline phosphatase activity was induced again in strain CE1499 after growth in LPi medium (Fig. 2). The relatively weak repression of alkaline phosphatase activity in strain CE1499 after growth under Pi-replete conditions may explain why pseudorevertants with a compensatory mutation in pitA rather than true revertants were picked in the original mutant selection described in the previous paragraph. Probably true revertants were among the strains that could grow on the minimal medium plates with P; as the sole source of phosphate, but were blue on XP-containing L broth plates. However, introduction of plasmid pSL42, carrying pitA, into CE1491 resulted in severe repression of alkaline phosphatase synthesis in HPi and complex medium (Fig. 2 and Table 1, respectively), and phoA expression could be induced when cells were grown in LPi medium (Fig. 2). These results demonstrate that the activity of the wild-type PitA transporter can substitute for PstS in P; regulation.

\[ \text{PitB expression also restores Pi regulation in a pstS mutant.} \]

Because pitA pstS strain CE1487 had recovered the ability to

\[ \text{alkaline phosphatase activity (units)} \]

\[ \text{time (min)} \]

\[ \text{nmol P; / mg protein} \]

- FIG. 1. Uptake of \( ^{33}\text{P} \) by cells of strains CE1491 (\( \Delta \)), CE1493 (\( \Delta \)), and CE1494 (\( \Delta \)). Growth of cells and uptake experiments were performed essentially as described previously (9). The experiments were repeated twice with essentially the same results, and the data from one of these experiments are shown.

\[ \text{TABLE 1. Alkaline phosphatase activities of various E. coli strains} \]

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Plasmid</th>
<th>PhoA activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K10</td>
<td>pitA10 relA1 spoT1</td>
<td></td>
<td>16 ± 3</td>
</tr>
<tr>
<td>CE1485</td>
<td>K10 pstS::kan</td>
<td></td>
<td>2,130 ± 45</td>
</tr>
<tr>
<td>CE1491</td>
<td>CE1485 pitB::gm</td>
<td></td>
<td>2,070 ± 300</td>
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<tr>
<td>CE1493</td>
<td>CE1491 PitA^+</td>
<td></td>
<td>40 ± 4</td>
</tr>
<tr>
<td>CE1494</td>
<td>CE1491 PitA^-</td>
<td></td>
<td>25 ± 3</td>
</tr>
<tr>
<td>CE1495</td>
<td>CE1493 pitB^- metC::Tn10</td>
<td></td>
<td>40 ± 3</td>
</tr>
<tr>
<td>CE1496</td>
<td>CE1494 pitB^- metC::Tn10</td>
<td></td>
<td>23 ± 2</td>
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<tr>
<td>CE1497</td>
<td>CE1495 pit::gm</td>
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<td>1,570 ± 140</td>
</tr>
<tr>
<td>CE1498</td>
<td>CE1496 pit::gm</td>
<td></td>
<td>1,300 ± 125</td>
</tr>
<tr>
<td>CE1499</td>
<td>CE1493 pitB::gm</td>
<td>pJFl18EH</td>
<td>1,885 ± 280</td>
</tr>
<tr>
<td>CE1491</td>
<td>CE1493 pitB::gm</td>
<td>pSL41 (pitB)</td>
<td>42 ± 15</td>
</tr>
<tr>
<td>CE1492</td>
<td>CE1493 pitB::gm</td>
<td>pSL42 (pitA)</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>CE1487</td>
<td>CE1493 pitB^+</td>
<td></td>
<td>80 ± 10</td>
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<tr>
<td>CE1492</td>
<td>CE1487 ΔpstSCAB-phoU::c</td>
<td></td>
<td>2,940 ± 170</td>
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<tr>
<td>MG1655</td>
<td>mg</td>
<td></td>
<td>15 ± 2</td>
</tr>
<tr>
<td>CE1500</td>
<td>MG1655 pitA::gm pstS::kan</td>
<td>pJFl18EH</td>
<td>1,690 ± 110</td>
</tr>
<tr>
<td>CE1501</td>
<td>MG1655 pitA::gm ΔpstSCAB-phoU::c</td>
<td>pJFl18EH</td>
<td>2,080 ± 150</td>
</tr>
<tr>
<td>CE1500</td>
<td>MG1655 pitA::gm pstS::kan</td>
<td>pSL41 (pitB)</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>CE1501</td>
<td>MG1655 pitA::gm ΔpstSCAB-phoU::c</td>
<td>pSL41 (pitA)</td>
<td>1,645 ± 230</td>
</tr>
</tbody>
</table>

a Strains were grown overnight at 37°C in L broth supplemented with 1 mM glucose-3-phosphate. Addition of glucose-3-phosphate resulted in equal growth of all strains and did not influence alkaline phosphatase activities (data not shown).

b Strains K10 and MG1655 were obtained from the E. coli Genetic Stock Center, Department of Biology, Yale University, Conn. Strains CE1485, CE1491, and CE1487 were described previously (9), and all other strains were constructed in the present work. The pstS::kan mutation is an insertion of a kanamycin resistance cassette in the PnuI site approximately in the middle of the pstS gene. Strains carrying this mutation do not produce the P; binding protein, as was verified by Western blotting (9).

c Vector plasmid pJFl18EH (7) and plasmids pSL41 and pSL42 (9) have been described previously.

d The alkaline phosphatase activities were determined with para-nitrophenyl phosphate as a substrate (17). Alkaline phosphatase activity is expressed in units, which are defined as nanomoles of para-nitrophenol released per minute per optical density at 660 nm of cell culture. Values represent the averaged results of three or four independent experiments, and standard deviations are given.
grow on Pi, due to the expression by gene amplification of pitB (9), it was of interest to determine whether normal regulation of the pho regulon was regained in this strain as well. Indeed, the expression of alkaline phosphatase in strain CE1487 was significantly reduced compared to that in its parental strain CE1485 after growth in HP, medium, and it could be induced by growth in LP, medium (Fig. 2). Furthermore, alkaline phosphatase was constitutively expressed in strain CE1491, a pitB::gm derivative of CE1487, directly demonstrating that the regained Pi control on phoA expression in the pitS mutant CE1487 is due to the expression of pitB and not to a secondary mutation. In addition, introduction of plasmid pSLA1, carrying the pitB gene, into this strain resulted in a drastic reduction of alkaline phosphatase expression under HP conditions, whereas cells carrying the control plasmid pJF118EH still showed high enzyme activity (Table 1). Thus, like PitA activity, PitB activity can compensate for the absence of pitS in the Pi regulation of the pho regulon.

Other pit genes are still required for Pi regulation in the absence of PitS. To investigate whether the other genes of the pit operon are still required for Pi control of the pho regulon in the absence of PitS, a strain was constructed in which all genes of the entire pitSCAB-phoU locus were deleted. For this purpose, plasmid pSN507 (2) was digested with MunI and SnaBI, thereby removing a DNA segment encompassing approximately the 3' half of pitS, the entire pitC, pitA, and pitB genes, and approximately two-thirds from the 5' end of phoU, and ligated with a Cam- cassette (1). The resulting plasmid was digested with EcoRI, and the 6.4-kb DNA fragment with the ΔpitSCAB-phoU mutation was used to transform recBC sbc strain AM1095 (8). One Cam- transformant, which expressed alkaline phosphatase constitutively and did not produce PitS and PhoU as verified by Western blotting, was designated CE1489. The deletion was then transferred by P1 transduction to PitB+ strain CE1487. High levels of alkaline phosphatase activity were detected in the resulting strain, CE1492, after growth in complex medium (Table 1). This result demonstrates that PstCAB and/or PhoU is still required for Pi control of the pho regulon even when PitB is active and that the Pi signaling proceeds (at least in part) via the same pathway as in the wild-type strain.

Influence of the genetic background. All experiments described so far were carried out in the genetic background of strain K12. Although this strain is the classical strain for studies of the pho regulon, it carries relA and spoT mutations, which were recently shown to affect Pi regulation in other strains (14). To exclude the possibility that our results were influenced by this background, several mutations were transferred by P1 transduction into strains MG1655 and W3110, which do not carry spoT or relA mutations. As expected, alkaline phosphatase was produced constitutively in the pitS::kan pitA::gm and Δ(pitSCAB-phoU)::cam pitA::gm derivatives of MG1655, designated CE1500 and CE1501, respectively (Table 1). The subsequent introduction of plasmid pSLA1 containing pitB did not affect alkaline phosphatase activity in strain CE1501, but severely reduced this activity in strain CE1500 (Table 1). Similar results were obtained for the derivatives of strain W3110 (data not shown). Hence, also in different genetic backgrounds, PitB activity can compensate for the loss of Pi control of the pho regulon in the absence of PstS, but the products of other genes of the pit operon remain required for this control.

Our observation that the Pi regulation of the pho regulon can be restored in the absence of the P::binding protein by increased PitA or PitB activity is very unexpected in the light of previous publications (11, 21, 24). The results demonstrate that Pi signaling via the Pst system can occur in the absence of PstS and challenge the view that extracellular rather than intracellular Pi concentrations control the expression of the pho regulon. It is conceivable that PhoU associated with the Pst system has direct access to Pi that enters the cell via PitA or PitB, because it remains associated with divalent cations (19). However, when intracellular Pi increases due to overproduction of PitA or PitB, it may become accessible to PhoU.

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REFERENCES


