JOURNAL OF BACTERIOLOGY, Oct. 2001, p. 5768–5771 Vol. 183, No. 19
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.19.5768–5771.2001

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

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Received 15 May 2001/Accepted 10 July 2001

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 external 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 *pstC*, 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 μM 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 *metC162*::Tn*10* 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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TABLE 1. Alkaline phosphatase activities of various *E. coli* strains[a]

Strain[b]	Relevant characteristics	Plasmid[c]	PhoA activity[d]
K10	*pitA10 relA1 spoT1*		16 ± 3
CE1485	K10 *pstS*::*kan*		2,180 ± 45
CE1491	CE1485 *pitB*::*gm*		2,070 ± 300
CE1493	CE1491 PitA$^+$		40 ± 4
CE1494	CE1491 PitA$^+$		25 ± 3
CE1495	CE1493 *pitB*$^+$ *metC*::Tn*10*		40 ± 3
CE1496	CE1494 *pitB*$^+$ *metC*::Tn*10*		23 ± 2
CE1497	CE1495 *pitA*::*gm*		1,570 ± 140
CE1498	CE1496 *pitA*::*gm*		1,300 ± 125
CE1491	CE1485 *pitB*::*gm*	pJF118EH	1,885 ± 280
CE1491	CE1485 *pitB*::*gm*	pSL41 (*pitB*)	42 ± 15
CE1491	CE1485 *pitB*::*gm*	pSL42 (*pitA*)	100 ± 10
CE1487	CE1485 PitB$^+$		80 ± 10
CE1492	CE1487 Δ(*pstSCAB-phoU*)::*cam*		2,940 ± 170
MG1655			15 ± 2
CE1500	MG1655 *pitA*::*gm pstS*::*kan*	pJF118EH	1,690 ± 110
CE1501	MG1655 *pitA*::*gm* Δ(*pstSCAB-phoU*)::*cam*	pJF118EH	2,080 ± 150
CE1500	MG1655 *pitA*::*gm pstS*::*kan*	pSL41 (*pitB*)	48 ± 5
CE1501	MG1655 *pitA*::*gm* Δ(*pstSCAB-phoU*)::*cam*	pSL41 (*pitB*)	1,645 ± 230

[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 *Pvu*I site approximately in the middle of the *pstS* gene. Strains carrying this mutation do not produce the P_i-binding protein, as was verified by Western blotting (9).

[c] Vector plasmid pJF118EH (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.

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 (HP_i) and low-phosphate (LP_i) media, and alkaline phosphatase activity was determined. Indeed, high activity was measured after growth of these strains in LP_i medium (Fig. 2). These results demonstrate that the requirement for PstS in P_i regulation of the *pho* regulon can be substituted by PitA activity.

Wild-type *pitA* can restore P_i regulation in a *pstS* mutant. To investigate whether expression of a wild-type *pitA* gene can restore P_i regulation in a *pstS* mutant, the mutant *pitA* allele in CE1491 was replaced with the wild-type gene by P1 transduction with *zhf-5*::Tn*10* strain CAG18450 (13) as the donor. A Tet^r transductant that could grow on P_i as a phosphate source was designated CE1499. Even though colonies of this strain were blue on XP-containing L broth plates, quantative 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 LP_i medium (Fig. 2). The relatively weak repression of alkaline phosphatase activity in strain CE1499 after growth under P_i-replete conditions may explain why pseudorevertants with a compensatory mutation in

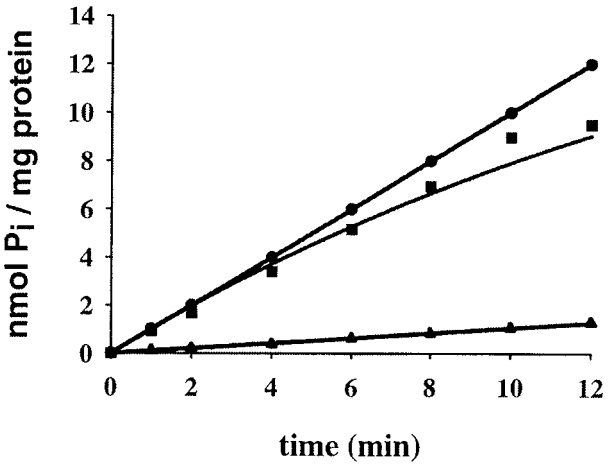

FIG. 1. Uptake of $^{33}P_i$ by cells of strains CE1491 (▲), CE1493 (●), and CE1494 (■). Growth of cells and uptake experiments were performed essentially as described previously (9). The experiments were repeated twice with essentially the same results, and the data from one of these experiments are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

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_i 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 HP_i and complex medium (Fig. 2 and Table 1, respectively), and *phoA* expression could be induced when cells were grown in LP_i medium (Fig. 2). These results demonstrate that the activity of the wild-type PitA transporter can substitute for PstS in P_i regulation.

PitB expression also restores P_i regulation in a *pstS* mutant. Because *pitA pstS* strain CE1487 had recovered the ability to

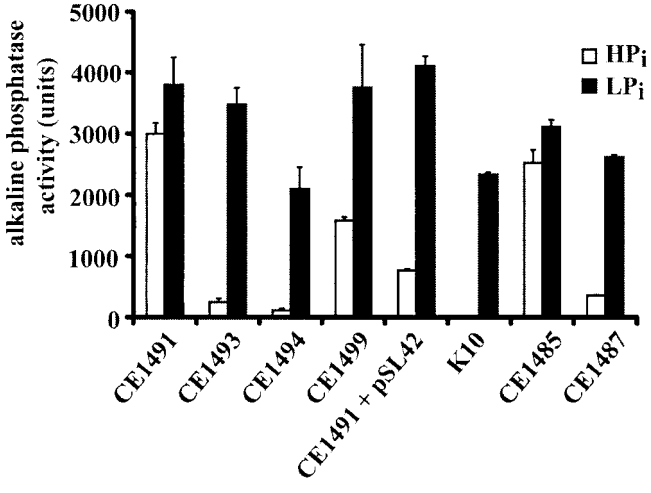

FIG. 2. Alkaline phosphatase activities in K10 and its derivatives grown in HP_i or LP_i medium. Cells were grown overnight in the peptone-based HP_i and LP_i media (10) as described previously (9). The alkaline phosphatase activities were determined and are expressed as in Table 1. The data represent averaged results of three or four independent experiments, and standard deviations are given.

grow on P_i 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_i medium, and it could be induced by growth in LP_i medium (Fig. 2). Furthermore, alkaline phosphatase was constitutively expressed in strain CE1491, a *pitB*::*gm* derivative of CE1487, directly demonstrating that the regained P_i control on *phoA* expression in the *pstS* mutant CE1487 is due to the expression of *pitB* and not to a secondary mutation. In addition, introduction of plasmid pSL41, carrying the *pitB* gene, into this strain resulted in a drastic reduction of alkaline phosphatase expression under HP_i 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 *pstS* in the P_i regulation of the *pho* regulon.

Other *pst* genes are still required for P_i regulation in the absence of PstS. To investigate whether the other genes of the *pst* operon are still required for P_i control of the *pho* regulon in the absence of PstS, a strain was constructed in which all genes of the entire *pstSCAB-phoU* locus were deleted. For this purpose, plasmid pSN507 (2) was digested with *Mun*I and *Sna*BI, thereby removing a DNA segment encompassing approximately the 3′ half of *pstS*, the entire *pstC*, *pstA*, and *pstB* genes, and approximately two-thirds from the 5′ end of *phoU*, and ligated with a Cam^r cassette (1). The resulting plasmid was digested with *Eco*RI, and the 6.4-kb DNA fragment with the Δ*pstSCAB-phoU* mutation was used to transform *recBC sbc* strain AM1095 (8). One Cam^r transformant, which expressed alkaline phosphatase constitutively and did not produce PstS 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 P_i control of the *pho* regulon even when PitB is active and that the P_i 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 K10. 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 P_i 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 *pstS*::*kan pitA*::*gm* and Δ(*pstSCAB-phoU*)::*cam pitA*::*gm* derivatives of MG1655, designated CE1500 and CE1501, respectively (Table 1). The subsequent introduction of plasmid pSL41 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 P_i control of the *pho* regulon in the absence of PstS, but the products of other genes of the *pst* operon remain required for this control.

Our observation that the P_i regulation of the *pho* regulon can be restored in the absence of the P_i-binding protein by increased PitA or PitB activity is very unexpected in the light of previous publications (11, 21, 24). The results demonstrate that P_i signaling via the Pst system can occur in the absence of PstS and challenge the view that extracellular rather than intracellular P_i concentrations control the expression of the *pho* regulon. It is conceivable that PhoU associated with the Pst system has direct access to P_i that enters the cell via this system. This P_i might modify PhoU, or, alternatively, PhoU may convert a proportion of the P_i into a metabolic compound (21), and either action may repress the autokinase activity of PhoR. P_i that enters the cell via PitA or PitB may be channeled into other metabolic routes or may not be available to PhoU, because it remains associated with divalent cations (19). However, when intracellular P_i increases due to overproduction of PitA or PitB, it may become accessible to PhoU.

We thank Paul Schoondermark for constructing the Δ(*pstSCAB-phoU*)::*cam* strain CE1489. We are indebted to The Netherlands Culture Collection of Bacteria (NCCB) for providing us with plasmids and strains.

This research was supported by the Life Sciences Foundation (ALW), which is subsidized by The Netherlands Organization for Scientific Research (NWO).

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