Activation by Gene Amplification of pitB, Encoding a Third Phosphate Transporter of Escherichia coli K-12

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Two systems for the uptake of inorganic phosphate (P_i) in Escherichia coli, PitA and Pst, have been described. A revertant of a pitA pstS double mutant that could grow on P_i was isolated. We demonstrate that the expression of a new P_i transporter, PitB, is activated in this strain by a gene amplification event.

Transport of inorganic phosphate (P_i) across the cytoplasmic membrane of Escherichia coli is mediated by the PitA protein and Pst system. PitA, which transports metal phosphates (28) and is constitutively expressed (17), is driven by the proton motive force (PMF) (18, 28). The Pst system, which transports P_i at the expense of ATP (6, 9), is composed of a periplasmic P_i-binding protein (PstS), two integral membrane proteins (PstC and PstA), and an ATP-binding protein (PstB) (24). The genes encoding these four proteins constitute an operon (24), together with phoU, which encodes a protein not required for P_i transport (25). Under P_i limitation, the expression of the Pst system, which is produced at a basal level under P_i-replete conditions, is further induced. Like, for example, phoA, which encodes the periplasmic enzyme alkaline phosphatase (26), the pst-phoU operon is part of the pho regulon, which is under the control of a two-component regulatory system consisting of the proteins PhoB and PhoR (29). Furthermore, the Pst system appears to be involved in regulation, since mutations in the genes of the pst-phoU operon generally result in constitutive expression of the pho regulon (29–31). The exact mechanism by which the Pst system controls the expression of the pho regulon is not known. To study the role of PstS in regulation, we attempted to isolate mutants with mutations in the membrane components of the Pst system that can transport P_i in the absence of the PstS protein. In one of the mutants obtained, a new P_i transporter, PitB, appeared to be expressed.

Construction of a pitS pitA double mutant. A pstS pitA double-mutant strain is expected to behave as an organic phosphate auxotroph (22), but previously described pstS pitA double mutants, such as strain C86, appear to take up P_i and to grow on P_i as the sole source of phosphate (reference 30 and data not shown). Western blotting revealed that the PstS protein was produced in this strain, although at much lower levels than in its parental strain, K10, grown under P_i limitation (data not shown). To characterize the pstS mutation in strain C86, a DNA fragment was amplified by PCR. The amplified fragment was considerably larger than the expected 1.4 kb, which was found when strains MC4100 and K10 were analyzed (Fig. 1A). An enlarged PCR fragment was also found in another pitA pstS strain, C78 (Fig. 1A). Sequencing of the PCR fragment from strain C86 revealed the presence of an IS2 element in the promoter region of pstS (Fig. 1B), whereas no other mutation was found in the pstS gene or the other genes of the pst-phoU operon. Hence, strain C86 and probably also strain C78 contain a Pst system, which is expressed at a lower level due to the insertion of an IS2 element in its promoter.

We decided to construct a new pitA pstS double mutant. Strain K10 carries an uncharacterized pitA mutation (30). Sequencing of a PCR fragment containing this pitA allele revealed a single point mutation with respect to the wild type (4), resulting in a Gly220Asp substitution in the phosphatase domain of PitA. A pstS::kan mutation was constructed by ligating a kanamycin resistance cassette from pUC18K (13) into the PvuI site of pSN5182 (15). The resulting plasmid, pSL15, was digested with NruI and BamHI, and the 5.8-kb DNA fragment carrying the pstS::kan allele was used to transform recBC sbcB strain AM1095 (8), to disrupt the chromosomal pstS gene. A pstS::kan derivative of strain K10, designated CE1485, was subsequently constructed by P1 transduction (14). Western blotting (Fig. 2A, lane 2) confirmed the absence of the PstS protein in this strain, which failed to grow on P_i, as the sole source of phosphate (Fig. 3A), whereas growth on glycerol 3-phosphate (G3P) was not affected (results not shown).

Furthermore, the efficiency of the cells in taking up 32P_i was drastically reduced (Fig. 3B). Whereas PhoU expression was induced in strain K10 under low-P_i (LP_i) conditions, it was detected in strain CE1485 after growth in high-P_i (HP_i) medium (Fig. 2), indicating that the pho regulon is constitutively expressed. Furthermore, this result shows that the kanamycin resistance cassette in pstS has no polar effect on the expression of the downstream genes in the pst operon. Alkaline phosphatase assays (25) confirmed the constitutive expression of the pho regulon in CE1485 (data not shown).

Isolation of a pseudorevertant of pitA pstS strain CE1485. When CE1485 was plated on synthetic medium plates (25) with P_i as the sole source of phosphate, revertants appeared after overnight incubation. One of these revertants, designated...
Thus, a mutation in pitB and PitB are 81% identical in their amino acid sequences. Inspection of the genome database (4) which are located at min 67.9 and 66.9, respectively, on the chromosomal map (16). A DNA fragment containing pitB was rescued by conjugation using a series of Hfr strains carrying Tn10 derivatives as donors (21). The wild-type allele was 37% cotransducible with the pitA10::Tn10 marker and 32% cotransducible with the nupG511::Tn10 marker, which are located at min 67.9 and 66.9, respectively, on the chromosomal map (16). Inspection of the genome database (4) revealed a pitA homolog, designated pitB, in this region. PitA and PitB are 81% identical in their amino acid sequences. Thus, a mutation in pitB might be responsible for the restoration of growth of CE1485 on P\textsubscript{i}.

The suppressor mutation in strain CE1487 was mapped by conjugation using a series of Hfr strains carrying Tn10 selection markers and by P1 transduction with an ordered set of transposon insertion mutants as donors (21). The wild-type allele was 37% cotransducible with the metC162::Tn10 marker and 32% cotransducible with the nupG511::Tn10 marker, which are located at min 67.9 and 66.9, respectively, on the chromosomal map (16). Inspection of the genome database (4) revealed a pitA homolog, designated pitB, in this region. PitA and PitB are 81% identical in their amino acid sequences. Thus, a mutation in pitB might be responsible for the restoration of growth of CE1485 on P\textsubscript{i}.

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To determine whether the pitB gene of the pseudorevertant CE1487 indeed encodes a functional P\textsubscript{i} transporter, a 2.2-kb DNA fragment containing pitB and 360 bp of upstream DNA was amplified by PCR using primers pitB8 (5'-CGACCCAATAA ACGGGAATCG) and pitB10 (5'-GGGATGATCGACT GG-3'). The PCR product was ligated into HindIII-digested pUC18 (33). Introduction of the resulting plasmid, pSL39, into the pitA pitS strain CE1485 restored growth on synthetic HP\textsubscript{i} plates. To inactivate pitB on the chromosome, a gentamicin resistance cassette from pBSL142 (2) was inserted into the Mmu site in pitB on pSL39, yielding pSL37. AM1095 was transformed with EcoRl- and HindIII-digested pSL37, resulting in pitB::Gm mutant CE1490, and the mutation was transferred to pseudorevertant strain CE1487 by P1 transduction. The resulting strain, CE1491, failed to grow on synthetic HP\textsubscript{i} plates. Thus, the PitB protein is responsible for the growth of strain CE1487 on P\textsubscript{i}, as the sole source of phosphate.

Characterization of the pitB mutation in strain CE1487. Sequencing the 2.2-kb DNA fragment containing pitB did not reveal any mutation. Therefore, a major chromosomal DNA rearrangement, possibly caused by the presence of an IS5 element near pitB (4) (Fig. 4A), could have affected gene expression. In Southern hybridizations with a pitB probe (probe 2, Fig. 4A), the expected 8.6-kb BamHI and 8.1-kb ClaI fragments were detected in the chromosomal DNA of strain CE1485 (Fig. 5A). In contrast, a much larger BamHI fragment and, besides the expected 8.1-kb fragment, an additional ClaI fragment of approximately 6.5 kb reacted with the probe in the DNA of CE1487 (Fig. 5A). These new hybridizing fragments gave much stronger signals than those obtained with the DNA of strain CE1485 (Fig. 5A), although equal amounts of DNA were used, suggesting that a DNA rearrangement took place in CE1487, resulting in the amplification of a DNA segment.

To determine the extent of the DNA rearrangement in strain CE1487, additional hybridizations were performed with probes 1 and 3 (Fig. 4A), which were obtained after PCR amplification of the chromosomal segments. With probe 3, the expected 15.8-kb BamHI and 8.1-kb ClaI fragments were detected in the genomic DNA of both strains with equal intensities (Fig. 5B), indicating that gene 0304 was not implicated in the DNA rearrangement. With probe 1, the large BamHI fragment and the 6.5-kb ClaI fragment, which were also detected...
with the pitB probe, gave strong hybridization signals with DNA from strain CE1487 but were not detected in CE1485 DNA (data not shown). Therefore, like pitB, the O230 gene appears to be amplified in strain CE1487.

To resolve the exact extent of the amplified DNA fragment, PCRs were performed with the primers shown in Fig. 4B. Only the primer combinations pr5-pr2, pr5-pr3, and pr5-pr4 yielded fragments when chromosomal DNA of strain CE1487 was used as the DNA template but not with DNA from strain CE1485. Sequencing of the PCR fragments revealed the fusion point to be located within the pitB-gsp intercistronic region (Fig. 4C).

The amplified fragment begins with the sequence GGAAGG TCCGAACAGTCCT from the IS5 element and ends again in the promoter region of pitB, making it 6.4 kb long (Fig. 4B). Both the increased copy number of pitB (19) and the presence of the IS5 element in the pitB promoter region after the DNA rearrangement could be responsible for the increased expression of pitB in the pseudorevertant strain. Such an IS5-mediated activation of gene expression has been described previously, for example in the cryptic bgl operon of E. coli K-12 (20).

Transport characteristics of PitB. To compare the characteristics of PitA- and PitB-mediated Pi transport, pitB and pitA were PCR amplified with primer couples pitB17 (5'-CGGATCCTTAAATCAATTGGC-3') and pitB18 (5'-CGGATCCTTAAATTCAATTGGC-3') and pit1 (5'-CGGATCCTTACAGGAACTGGC-3') and pit2 (5'-CGGATCCTTACAGGAACTGGC-3'), respectively, and cloned in pJF118EH (7) under tac promoter control. Introduction of the resulting plasmids, pSL41 and pSL42, respectively, but not of vector pJF118EH enabled pitA pitB pstS strain CE1491, even without isopropyl-β-D-thiogalactopyranoside, to grow on Pi as the sole source of phosphate and to take up 33Pi (data not shown). To determine the PMF dependency of PitB-

![FIG. 3. Growth and 33Pi uptake. (A) Growth curve of pitA mutant strain K10 (E), pitA pstS double-mutant CE1485 (h), and the pseudorevertant CE1487 (†). Cells were grown overnight in Luria broth supplemented with G3P, pelleted, and resuspended in HEPES-buffered synthetic medium (25) supplemented with 0.5% glucose and 660 μM KH2PO4. Growth was monitored for 7 h. (B) Uptake of 33Pi by cells of strains K10 (E), CE1485 (h), and CE1487 (†). Cells were grown in Luria broth supplemented with 20 mM glucose and 1 mm G3P to an optical density at 660 nm (OD660) of approximately 0.9, washed, and resuspended in a solution of 20 mM potassium piperezine-N,N'-bis(2-ethanesulfonate) (PIPES) (pH 7.0)–10 mM MgSO4. These cells were stored on ice, and, within 2 h, transport assays were performed at 30°C with 50 μM 33Pi-labeled potassium phosphate as described previously (27). The experiments were repeated three times with essentially the same results, and data from a representative experiment are shown.](http://jb.asm.org/)

![FIG. 4. (A and B) Maps of the pitB chromosomal region of strains CE1485 (A) and CE1487 (B). Only the relevant BamHI, ClaI, and EcoRI sites are depicted. At the top of panel A, the probes used for Southern hybridization are indicated. At the top of panel B, the arrowheads indicate PCR primers with the following sequences: pr1, 5'-GGAAGGATCCTTAAATCAATTGGC-3'; pr2, 5'-CGGATCCTTAAATCAATTGGC-3'; pr3, 5'-CGGATCCTTAAATCAATTGGC-3'; pr4, 5'-CGGATCCTTAAATCAATTGGC-3'. The numbers between the restriction sites indicate the lengths of the fragments in kilobases. (C) Nucleotide sequence of the pitB-gsp intercistronic region. Coding sequences for the glutathionylspermidine synthetase (Gsp) gene and PitB proteins are indicated in bold italics and boxed. Dashed arrows indicate inverted repeats, which may function as the transcriptional terminator of the gsp gene. Putative -35 and -10 sequences of the pitB promoter are indicated. The insertion in strain CE1487 of the amplified DNA fragment containing IS5-pitB is indicated.](http://jb.asm.org/)

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mediated P\textsubscript{i} transport, \(P_i\) transport was studied in right-side-out membrane vesicles prepared from strain CE1491 carrying pSL41 or pSL42 as described previously (10). Like PitA-out membrane vesicles prepared from strain CE1491 carrying pitA in air-saturated 50 mM potassium PIPES (pH 7.0)–10 mM MgSO\textsubscript{4}, diluted to a final protein concentration of 0.1 to 0.5 mg of protein/ml uptake was inhibited by valinomycin and nigericin, which selectively dissipate the transmembrane potential (\(\Delta\psi\)) and transmembrane pH gradient (\(\Delta p\text{H}\)), respectively (Fig. 6). Apparently, PitB activity is dependent on both components of the PMF. In addition, the initial velocities of \(^{32}\text{P}_i\) uptake were determined over the first 30 s of linear uptake at \(P_i\) concentrations between 4 and 320 \(\mu\text{M}\) and the \(K_m\) value of PitB was determined by direct fitting of the data to the Michaelis-Menten equation (data not shown). The apparent \(K_m\) for \(P_i\), found, 39 \(\mu\text{M}\), is close to the reported value for PitA, 24 to 38 \(\mu\text{M}\) (17, 32).

Besides \(P_i\), arsenate is transported by PitA (3). Consequently, pitA mutants are resistant to arsenate. To investigate whether PitB can transport arsenate, various strains were streaked on plates containing synthetic medium (25) supplemented with 660 \(\mu\text{M}\) \(K_2\text{HPO}_4\), 1 mM G3P, and 10 mM arsenate. Whereas the pitA mutant strains K10 and CE1485 were able to grow on this medium, growth of the PitB-expressing pseudorevertant strain CE1487 was greatly impaired (data not shown), indicating that PitB is able to transport arsenate.

In conclusion, we demonstrated that expression of a cryptic homolog of pitA, PitB, can be activated in vivo by a DNA rearrangement involving DNA amplification and insertion of IS\textsubscript{5} in the promoter region and that this gene encodes a \(P_i\) transporter with similar characteristics to PitA. Interestingly, a screening of 34 completely sequenced genomes (http://www.ncbi.nlm.nih.gov/COG) revealed that several other bacteria, including, for example, *Pseudomonas aeruginosa*, contain more than one PitA homolog.

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