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 (23). 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 *pstS 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 a putative membrane-spanning segment 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 $^{33}P_i$ 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

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FIG. 1. Characterization of the *pstS* mutation in C86. (A) Amplification of the *pstS* gene and its promoter region by PCR using chromosomal DNA of strains K10, C86, C78, and MC4100 (5) and the primers *pst4* (5'-GAGTAATAATGGATGCC-3') and *pst5* (5'-CGGTGGTTAAAGCAGGC-3'). Strains K10 (*pitA10*), C86 (*pstS21* derivative of K10), and C78 (*pstS28* derivative of K10) were kindly provided by the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, Conn.). The positions of the molecular size markers are indicated at the left. (B) Position of the IS2 element in the promoter region of the *pstS* gene in strain C86. The two *pho* boxes, the -10 region and the Shine-Dalgarno sequence (SD), are indicated. The start of the coding region of *pstS* is indicated by an arrow.

CE1487, grew as efficiently as strain K10 in HP_i medium (Fig. 3A), and $^{33}\text{P}_i$ uptake was restored (Fig. 3B). Strain CE1487 was resistant to kanamycin, and the PstS protein could not be detected on Western blots (Fig. 2, lanes 3). Furthermore, sequencing of a PCR-amplified fragment revealed no other differences in the *pitA* gene besides the characterized single point mutation. Therefore, CE1487 is not a true revertant of strain CE1485 but classifies as a pseudorevertant.

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_i .

To determine whether the *pitB* gene of the pseudorevertant CE1487 indeed encodes a functional P_i transporter, a 2.2-kb DNA fragment containing *pitB* and 360 bp of upstream DNA

was amplified by PCR using primers *pitB8* (5'-CGACCATAACGGGAATCG) and *pitB10* (5'-GCGGTGATGAATCACTGG-3'). The PCR product was ligated into *HincII*-digested pUC18 (33). Introduction of the resulting plasmid, pSL39, into the *pitA pstS* strain CE1485 restored growth on synthetic HP_i plates. To inactivate *pitB* on the chromosome, a gentamicin resistance cassette from pBSL142 (2) was inserted into the *MluI* site in *pitB* on pSL39, yielding pSL37. AM1095 was transformed with *EcoRI*- 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_i plates. Thus, the PitB protein is responsible for the growth of strain CE1487 on P_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 *Bam*HI and 8.1-kb *Cla*I fragments were detected in the chromosomal DNA of strain CE1485 (Fig. 5A). In contrast, a much larger *Bam*HI fragment and, besides the expected 8.1-kb fragment, an additional *Cla*I 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 *Bam*HI and 8.1-kb *Cla*I fragments were detected in the genomic DNA of both strains with equal intensities (Fig. 5B), indicating that gene *O304* was not implicated in the DNA rearrangement. With probe 1, the large *Bam*HI fragment and the 6.5-kb *Cla*I fragment, which were also detected

FIG. 2. Expression of the PstS and the PhoU proteins in *pitA* mutant strain K10 (lane 1), *pitA pstS* mutant strain CE1485 (lane 2), and the pseudorevertant strain CE1487 (lane 3). Cells were grown in a peptone-based, phosphate-poor medium (11) supplemented with 0.5% glucose, 660 μM K_2HPO_4 , and 1 mM G3P (HP_i medium) (A) or with no K_2HPO_4 or G3P added (LP_i medium) (B). The alternative phosphate source G3P was omitted from the LP_i medium, since it may be degraded by alkaline phosphatase, thereby generating P_i . Although growth of CE1485 in the LP_i medium was very poor, enough cells could be collected for this analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12) and, for Western blotting (1), transferred to a nitrocellulose membrane (Schleicher & Schuell). Immunodetection was performed with polyclonal antisera directed against the P_i -binding protein (PstS) and PhoU. The positions of molecular size standard proteins are indicated on the left in kilodaltons.

FIG. 3. Growth and ^{33}P uptake. (A) Growth curve of *pitA* mutant strain K10 ($\circ$), *pitA pstS* double-mutant CE1485 ($\square$), and the pseudorevertant CE1487 ($\triangle$). 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 K_2HPO_4 . Growth was monitored for 7 h. (B) Uptake of ^{33}P by cells of strains K10 ($\circ$), CE1485 ($\square$), and CE1487 ($\triangle$). Cells were grown in Luria broth supplemented with 20 mM glucose and 1 mM G3P to an optical density at 660 nm (OD_{660}) of approximately 0.9, washed, and resuspended in a solution of 20 mM potassium piperazine-*N,N'*-bis(2-ethanesulfonate) (PIPES) (pH 7.0)–10 mM MgSO_4 . These cells were stored on ice, and, within 2 h, transport assays were performed at 30°C with 50 μM ^{33}P -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.

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 TCCGAACAAGTCCT 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 P_i transport, *pitB* and *pitA* were PCR amplified with primer couples pitB17 (5'-CGGAA TTCATGCTAAATTTATTTGTTG-3') plus pitB18 (5'-CGC GGATCCTTAAATCAACTGCAATGC-3') and pit1 (5'-CG GAATTCATGCTACATTTGTTTGC-3') plus pit2 (5'-CGC GGATCCTTACAGGAAGTCAAGG-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 P_i as the sole source of phosphate and to take up $^{33}P_i$ (data not shown). To determine the PMF dependency of PitB-

FIG. 4. (A and B) Maps of the *pitB* chromosomal region of strains CE1485 (A) and CE1487 (B). Only the relevant *Bam*HI, *Cla*I, and *Eco*RI 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'-GGAAGATCGATGCGCTGG-3'; pr2, 5'-CCATTACCAGCCTTGGGG-3'; pr3, 5'-GGGGAAATCTTCTCGGC-3'; pr4, 5'-GGATA TCGTCAGCGCGC-3'; pr5, 5'-CCTGTGTATATATCAAGGCC-3'; pr6, 5'-CAGGTAACGATGGTGC GG-3'; and pr7, 5'-CCTGCTCGGCACTCTCGG-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*), including the stop codon, 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.

FIG. 5. Southern blot analysis of chromosomal DNA of CE1485 and CE1487. Blots were hybridized with the *pitB* probe (A) and probe 3 (B) (see Fig. 4). DNA was digested with *Bam*HI or *Cla*I, and fragments were separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred from the gel to Hybond-N⁺ membranes (Amersham) with a vacuum blotter (Bio-Rad model 785). After transfer, the filter was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) for 5 min, and DNA was cross-linked by UV irradiation for 2 min. Labeling of the probes, hybridization, and detection were done with digoxigenin labeling and detection kits (Boehringer Mannheim). Hybridization and stringency washes were carried out at 68°C. The positions of molecular size standard DNA fragments are indicated in kilobases.

mediated P_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-mediated $^{33}P_i$ uptake (data not shown), PitB-mediated $^{33}P_i$ uptake was inhibited by valinomycin and nigericin, which selectively dissipate the transmembrane potential ($\Delta\psi$) and transmembrane pH gradient (ΔpH), respectively (Fig. 6). Apparently, PitB activity is dependent on both components of the PMF. In addition, the initial velocities of $^{33}P_i$ uptake were determined over the first 30 s of linear uptake at P_i concentrations between 4 and 320 μM and the K_m value of PitB was

FIG. 6. Uptake of $^{33}P_i$ in right-side-out membrane vesicles of strain CE1491 expressing *pitB* from plasmid pSL41. Membrane vesicles were diluted to a final protein concentration of 0.1 to 0.5 mg of protein/ml in air-saturated 50 mM potassium PIPES (pH 7.0)–10 mM $MgSO_4$. The membrane vesicles were preincubated for 3 min at 30°C with 2 μM pyrroloquinoline quinone in the absence (Δ) or presence ($\blacksquare$) of 20 mM glucose to generate the PMF and in the presence of glucose in combination with 2.5 μM valinomycin ($\square$) or 2.5 μM nigericin ($\blacktriangle$). Transport was initiated on addition of 50 μM $^{33}P_i$ -labeled potassium phosphate and analyzed by rapid filtration (27). The experiments were repeated twice with essentially the same results, and data from a representative experiment are shown.

determined by direct fitting of the data to the Michaelis-Menten equation (data not shown). The apparent K_m for P_i found, 39 μM , is close to the reported value for PitA, 24 to 38 μM P_i (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 μM K_2HPO_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 IS5 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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