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Characterization of PitA and PitB from *Escherichia coli*

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Escherichia coli **contains two major systems for transporting inorganic phosphate (P_i). The low-affinity P_i transporter (*pitA*) is expressed constitutively and is dependent on the proton motive force, while the high-affinity Pst system (*pstSCAB*) is induced at low external P_i concentrations by the *pho* regulon and is an ABC transporter. We isolated a third putative P_i transport gene, *pitB*, from *E. coli* K-12 and present evidence that *pitB* encodes a functional P_i transporter that may be repressed at low P_i levels by the *pho* regulon. While a *pitB*$^+$ cosmid clone allowed growth on medium containing 500 μM P_i, *E. coli* with wild-type genomic *pitB* (*pitA* Δ*pstC345* double mutant) was unable to grow under these conditions, making it indistinguishable from a *pitA pitB* Δ*pstC345* triple mutant. The mutation Δ*pstC345* constitutively activates the *pho* regulon, which is normally induced by phosphate starvation. Removal of *pho* regulation by deleting the *phoB-phoR* operon allowed the *pitB*$^+$ *pitA* Δ*pstC345* strain to utilize P_i, with P_i uptake rates significantly higher than background levels. In addition, the apparent K_m of PitB decreased with increased levels of protein expression, suggesting that there is also regulation of the PitB protein. Strain K-10 contains a nonfunctional *pitA* gene and lacks Pit activity when the Pst system is mutated. The *pitA* mutation was identified as a single base change, causing an aspartic acid to replace glycine 220. This mutation greatly decreased the amount of PitA protein present in cell membranes, indicating that the aspartic acid substitution disrupts protein structure.**

Escherichia coli contains at least two major systems for transporting inorganic phosphate (P_i). The low-affinity inorganic phosphate transporter (Pit) is dependent on the proton motive force for energy and is constitutively expressed (30, 31, 49). When P_i is plentiful, this is the major uptake system for phosphate, with a reported apparent K_m (K_m^{app}) of 25 μM (30) to 38 μM (50) in whole cells and 11.9 μM in membrane vesicles (43). If the external P_i concentration is below the millimolar range, the high-affinity phosphate-specific transport (Pst) system is induced. This has a K_m^{app} of around 0.2 μM (30, 50). The Pst system is a complex of four proteins, including a periplasmic binding protein, which is energized by ATP and belongs to the ABC transporter family (7, 15, 47). The *pst* operon contains five genes under *pho* regulon control (1, 40, 41), which induces a range of genes when the phosphate supply is limited. Both the Pit and Pst systems are highly specific for P_i (30). Another two transporters accept P_i as a low-affinity analogue for either glycerol-3-phosphate (*glpT*) (18) or glucose-6-phosphate (*uhpT*) (29, 53), but in the absence of Pit and Pst activity, these latter two systems cannot support cell growth when supplied with P_i (38).

Divalent cations, such as Mg^{2+} or Ca^{2+}, were shown elsewhere to be essential for Pit activity (32), and experiments by van Veen et al. (43, 44) indicate that Pit forms a soluble neutral metal phosphate ($MeHPO_4$) complex which is symported with a proton. This is supported by the recent identification of a *pitA* mutant that accumulates reduced amounts of zinc(II), conferring resistance to toxic external concentrations of zinc (4). Efflux and homologous exchange of metal phosphate can occur under particular conditions, but there is no mixed exchange of metal phosphate for P_i, glycerol-3-phosphate, or glucose-6-phosphate (43). Interestingly, Beard et al. (4) suggest that PitA may also play a role in Zn^{2+} efflux when the ion reaches highly toxic external concentrations.

The Pit transport system was first reported by Willsky et al. (49) when mutations in the Pst system of several *E. coli* K-12 strains revealed a second P_i transporter. When a *pst* mutation was introduced into strain K-10, there was no measurable P_i transport, indicating that Pit is nonfunctional. This lesion was isolated as an α-glycerol-3-phosphate (G3P) auxotroph and mapped at min 78.51 on the *E. coli* genome (37, 38) and is called *pitA* (Swiss Protein P37308). Since these studies were done, the sequencing of the *E. coli* genome has revealed the presence of another putative phosphate transporter gene, designated *pitB,* at min 67.44 (5) (Swiss Protein P43676). This gene has 75% sequence identity to *pitA.* If *pitB* is also a functional phosphate transporter, it may have contributed to the kinetic values and substrate specificities determined in earlier studies. These studies used strains in which the Pst system was repressed and/or mutated (30, 44, 50). Alternatively, the *pitB* gene may not encode a P_i transporter, as *cysP* is a sulfate permease from *Bacillus subtilis* which has some sequence identity with the Pit family of transporters but not with sulfate transporters (24).

Elvin et al. (10) located and cloned the *pitA* gene, overexpressed it, and partially purified the protein. This paper describes the cloning of *pitB* and the demonstration that *pitB* is a P_i transporter which appears to be repressed or inactivated by the *pho* regulon. In addition, the *E. coli* K-10 *pitA* mutation is identified, and the kinetic parameters of the PitA and PitB proteins are investigated.

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TABLE 1. Description of *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics and/or background strain	Construction or reference
Plasmids		
pAN686	*pitA*$^+$	*Cla*I/*Sph*I fragment from pCE27 (10) in pBR322
pAN920	*pitA*$^+$ (opposite orientation from pAN686)	*Sal*I/*Bam*HI fragment from pAN686 in pBR322
pAN656	*pitB*$^+$; long upstream region (1,403 nucleotides)	*Cla*I/*Bam*HI fragment from AN2538 in pBR322
pAN1116	*pitB*$^+$; short upstream region (206 nucleotides)	*Ssp*I/*Cla*I fragment from pAN656 in *Eco*RV/*Cla*I sites of pBR322
pAN1243	*pitA1*	PCR product from AN3066 in pBR322
pAN1244	*pitA* G220D mutation	Site-directed mutagenesis of *pitA*$^+$ in pBR322
Strains		
AN248	*pit*$^+$ *pst*$^+$ K-12 strain	30
AN3066	*pitA1* Δ*pstC345 srl*::Tn*10 recA*	47
AN3901	*pitB*::Cat^r derivative of JC7623	By recombination
AN3902	*pitA1 pitB*::Cat^r Δ*pstC345*	P1 AN3901 × AN3020
AN3926	*pitB*::Cat^r Δ*pstC345*	P1 AN3901 × AN2537
AN4080	*pitA1 pitB*::Cat^r	P1 K-10 × AN3902
AN4081	*pitA1* Δ*pstC345* Δ(*phoB-phoR*) Kan^r	P1 ANCH1 × AN3020
AN4085	*pitA1 pitB*::Cat^r Δ*pstC345* Δ(*phoB-phoR*) Kan^r	P1 ANCH1 × AN3902
ANCH1	Δ(*phoB-phoR*) Kan^r	54
Strains with plasmids		
AN3514; pBR322 control	*pitA1* Δ*pstC345*	pBR322/AN3066
AN3135; *pitB*$^+$ (long upstream)	*pitA1* Δ*pstC345*	pAN656/AN3066
AN3171; *pitA*$^+$	*pitA1* Δ*pstC345*	pAN686/AN3066
AN3531; *pitA*$^+$ (opposite orientation)	*pitA1* Δ*pstC345*	pAN920/AN3066
AN3937; *pitA1* PCR from AN3066	*pitA1* Δ*pstC345*	pAN1243/AN3066
AN3938; *pitA* G220D	*pitA1* Δ*pstC345*	pAN1244/AN3066
AN3903; pBR322 control	*pitA1 pitB*::Cat^r Δ*pstC345*	pBR322/AN3902
AN3904; *pitA*$^+$	*pitA1 pitB*::Cat^r Δ*pstC345*	pAN920/AN3902
AN3905; *pitB*$^+$ (short upstream)	*pitA1 pitB*::Cat^r Δ*pstC345*	pAN1116/AN3902

MATERIALS AND METHODS

Bacterial strains. Strains and plasmids are listed in Table 1.

Cosmid cloning and sequencing of *pitB*. Chromosomal DNA was prepared from a *recA* derivative of K-12 strain AN2537 (Δ*pstC345*) (39) and partially digested with *Hin*dIII to generate fragments of approximately 20 kb. These fragments were ligated into the cosmid cloning vector pHC79 and packaged into λ heads with extracts prepared from the lysogen-induced strains BHB2690 (prehead donor) and BHB2688 (packaging protein donor) (33). Packaged cosmids were adsorbed to AN3020 (*pitA* Δ*pstC345*) (47), a strain which shows no growth on minimal medium supplemented with 500 μM inorganic phosphate (P_i medium) (10). The mixture was spread onto Luria-Bertani plates containing 100 μg of ampicillin (AMP)/ml and 1 mM G3P. Either G3P or glucose-6-phosphate may be used as the phosphate source for *pit pst* strains, which cannot utilize P_i for growth (38). AMP-resistant colonies were replica plated onto P_i medium, and colonies were screened for growth in the absence of G3P, which indicated the presence of a functional P_i transport gene. Cosmid DNA was prepared from one of these isolates. Restriction analysis identified an approximately 3-kb *Cla*I/*Bam*HI fragment that encoded a protein which complemented the *pit* mutation in AN3020, enabling growth of this strain on P_i medium. This DNA fragment was subcloned into the M13 vector mp18 and sequenced. The sequence is identical to the subsequently published *pitB* sequence (5). The *pitA* gene from plasmid pCE27 (10) was also sequenced and was found to be identical to the published sequence (37) (Swiss Protein P37308).

Preparation of cells for uptake. Cells were grown and prepared as described in the work of Rosenberg et al. (30), with the addition of 5% Luria-Bertani medium to the overnight growing medium. AMP (50 μg/ml) was added where appropriate. All cells were washed three times, and suspensions were stored at 4°C for less than 30 min.

Measurement of phosphate uptake. Measurement of $^{33}P_i$ (NEN) uptake by cells was carried out under conditions in which P_i uptake was linear over time. These conditions were 1 to 15 μM P_i (PitA) or 10 to 100 μM P_i (PitB) over a 25-s period as previously described (30), except that the phosphate-free buffered "uptake" medium was pH 6.6 or 7.0 and the magnesium concentration was 1.8 or 10 mM. Data from kinetic experiments were analyzed by nonlinear regression using the Michaelis-Menten equation and the Graphpad Prism program.

Genetic techniques. Plasmid DNA was prepared using the Magic Minipreps DNA purification system (Promega). Site-directed mutagenesis was carried out using the Amersham Sculptor in vitro mutagenesis system and PitA- or PitB-specific oligonucleotide primers. The nucleotide sequence at each mutation was checked by DNA sequencing. Transductions using phage $P1_{kc}$ were performed as previously described (28).

The *pitA* and *pitB* genes were amplified from genomic DNA, which was supplied by using approximately 0.2 μl of a single AN3066 colony mixed in 100 μl of water. The *pit* gene sequences were amplified by PCR using nucleotide primers complementary to the 5′ and 3′ regions flanking the open reading frames (ORFs), beginning with approximately 40 upstream nucleotides and ending just after the translation termination codons. *Bam*HI sites were included at the distal ends of each primer (Table 2). The thermostable *Pfu* DNA polymerase was used in order to minimize introduction of errors by PCR (23). A longer *pitB*$^+$ PCR product including 319 nucleotides upstream of the ORF was also prepared. The PCR products were purified using the Wizard PCR Purification system (Promega) and cloned into M13mp18, and several isolates of each were sequenced.

Inactivation of genomic *pitB*. The chloramphenicol resistance gene from pBR328 was excised with the restriction endonucleases *Aat*II and *Sau*I, 5′ overhangs were filled in, and 3′ overhangs were digested by T4 DNA polymerase 1; then the gene was ligated into the *Stu*I site in *pitB* in pAN656 to create pAN1193. Genomic replacement of wild-type *pitB* was carried out by transforming pAN1193 into the *recB21 recC22 sbcB15 sbcC201* mutant strain JC7623 as pre-

TABLE 2. Primers for amplification of *E. coli pit* genes

Sequence	Primer
pitA[a]	5′ CTGAGGATCCGCCGCGTTCATGTCCT
	3′ TGACGGATCCGTTTTGGTGCGTACGATTACAG
pitB[a]	5′ GTCAGGATCCATGCGTCCGTTCGTAAATTC
	3′ CGCCGGATCCGGGCATTTTCAGGAAG
pitB[b]	5′ AGAGGGATCCTGAACCGTTAATTG
	3′ CACTGGATCCGGTGTTGGTTGATG

[a] Includes about 40 upstream nucleotides.
[b] Includes 319 upstream nucleotides.

viously described (26). The insertion of *pitB*::Cat was checked by PCR. P1 transduction was then used to transfer *pitB*::Cat into *pstC* (AN2537) and *pitA pstC* (AN3020) strains. Recipients were selected by resistance to 50 μg of chloramphenicol/ml. Successful removal of wild-type *pitB* was confirmed by PCR. The rapid spray alkaline phosphatase assay (6) showed that alkaline phosphatase was derepressed under high-phosphate conditions (Luria-Bertani medium plus 1 mM G3P), indicating the presence of Δ*pstC345*.

Inactivation of the *pho* regulon. The *phoB-phoR* operon deletion from ANCH1 (54) was transduced into AN3020 and AN3902 and selected for by resistance to kanamycin. Inactivation of the *pho* regulon was confirmed by a negative result with the rapid spray alkaline phosphatase assay from cells grown on Luria-Bertani medium.

Isolation of membrane fraction. Two-liter cultures of *E. coli* strains were grown to stationary phase in Luria-Bertani medium plus 34 mM glucose, 100 μg of AMP/ml, and 1 mM G3P, as required. The membrane fraction was prepared by passing the cells through a Sorvall Ribi cell fractionator, followed by centrifugation and ammonium sulfate precipitation, as previously described (9). The protein concentration of each sample was determined.

Polyclonal antipeptide antibody. The PitA peptide ARIHLTPAEREKKDC (from A188 to D201) and the equivalent sequence in PitB, DRIHRIPEDRK KKKC (from D188 to K201), are from an extramembranous loop in the putative folded structure. Rabbits were immunized with the PitA peptide coupled to a lysine core matrix via a C-terminal cysteine (22). The multiple antigen peptide system conjugate was dissolved in phosphate-buffered saline (PBS) and emulsified with an equal volume of Freund's adjuvant (12), and 200 μg was injected subcutaneously. Standard sampling and injecting protocols were followed (16). Sera were isolated by centrifugation, and initial positive responses determined by enzyme-linked immunosorbent assay (11) were screened by Western blotting of the sera against the membrane fractions of AN3903 (*pitA pitB*), AN3904 (*pitB*), and AN3905 (*pitA*). This showed that there was no cross-reactivity between PitA antibody and the PitB protein. The PitB peptide was attached to Imject maleimide-activated keyhole limpet hemocyanin (Pierce) according to the manufacturer's instructions. The conjugate was partially dissolved in dimethyl sulfoxide with sonication and then diluted with 1 volume of PBS. Injections and screenings were carried out as previously described for the PitA peptide.

Purification of antipeptide antibody. Sera containing PitA antipeptide antibody were not purified. The PitB equivalent was isolated from sera by immunoaffinity purification (SulfoLink kit; Pierce) following a two-stage ammonium sulfate precipitation (16) and dialysis against PBS. The synthetic peptide column was prepared according to the manufacturer's instructions, and the dialyzed PitB antipeptide antibody solution was passed through the column three times.

Western blots. The membrane fraction of various *E. coli* strains was solubilized at 150 μg (PitA Western blots) or 1 mg (PitB Western blots) of protein per ml. Electrophoresis was performed according to the method of Laemmli (20) using a sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred onto a polyvinylidene difluoride membrane by electroblotting. After blocking in 10% milk powder–10 mM Tris-HCl (pH 7.5)–0.9% NaCl (blocking buffer), the polyvinylidene difluoride membrane was incubated with either polyclonal antipeptide PitA antibody diluted 1/500 in blocking buffer or polyclonal PitB antibody diluted 1/100. Alkaline phosphatase conjugated with goat anti-rabbit immunoglobulin (DAKO) was applied at a 1/1,500 (PitA) or a 1/1,000 (PitB) dilution in blocking buffer. The blot was immunostained with Western Blue stabilized alkaline phosphatase substrate (Promega).

RESULTS

Cloning of *pitA* and *pitB*. The *pitA* and *pitB* genes from *E. coli* strain K-12 were isolated and sequenced in this laboratory as described in Materials and Methods, and the ORFs and putative promoter regions were identical to the published sequences of *pitA* at nucleotides 3635272 to 3636771 (37) and *pitB* at nucleotides 3132887 to 3134386 (5) on the *E. coli* K-12 genome. The ORFs of *pitA* and *pitB* contain 1,497 nucleotides each, with 75% identity in the nucleotide sequences and 81% identity in the deduced amino acid sequences. Topological models suggest 10 putative transmembrane helices, with most sequence variation occurring in the putative hydrophilic loop regions.

Confirmation of *pitA* and *pitB* translation start codons. To identify the start codons, attempts were made to overexpress *pitA* or *pitB* and purify the protein products. However these attempts were hindered by the toxicity of the overexpressed genes (unpublished data). Instead, the translation starts were identified by functional analysis of alleles carrying mutated start codons (34). Both *pitA* and *pitB* have an ATG codon at the beginning of a 1,497-nucleotide ORF, and the nucleotide sequence similarity between the two DNA fragments exists only over this ORF region, suggesting that these ATG codons may initiate translation of the Pit genes. These putative ATG start codons were changed to GTG and CTG, causing a progressive drop in the P_i uptake activity of these mutants (Table 3). Changing an ATG start codon to GTG or CTG would be expected to progressively reduce expression of these proteins. In addition, two termination codons inserted in frame only 13 nucleotides after the putative ATG start of *pitB* completely abolished all P_i uptake activity. *pitA* has a second in-frame ATG codon 232 nucleotides after the putative ATG start codon, but termination codons placed before (at 118 nucleotides) or after this methionine also abolished all uptake activity. The combination of the above results indicates that translation of the *pit* genes commences at the beginning of the identified ORFs.

TABLE 3. Phosphate uptake activity of PitA and PitB with altered putative start codons

Putative start codon	Comparison with wild-type P_i uptake activity (%)	
	PitA[a]	PitB[b]
Vector	<0.5	<0.5
ATG	100 ± 1.6	100 ± 2.7
GTG	32.4 ± 0.6	8.4 ± 1.0
CTG	3.9 ± 0.1	4.4 ± 0.5

[a] Assayed at 6 μM P_i. Values are shown with standard errors of the means. n = 6; 100% = 45 nmol of P_i min^{-1} mg (dry weight)$^{-1}$.

[b] Assayed at 50 μM P_i. Values are shown with standard errors of the means. n = 3; 100% = 55 nmol of P_i min^{-1} mg (dry weight)$^{-1}$.

Kinetic parameters of PitA and PitB. To characterize the kinetic properties of PitA and PitB with respect to P_i uptake, AN3066 cells expressing either protein from the plasmid vector pBR322 were grown, deprived of phosphate, and assayed as described in Materials and Methods. The background strain AN3066 (*pitA1* Δ*pstC345*) does not grow in minimal medium supplemented with 500 μM P_i (P_i medium) but will grow with the addition of G3P. Both *pitA* and *pitB* can support cell growth when expressed on plasmids in AN3066. The PitA protein has a K_m^{app} about 10-fold lower than that of PitB on pAN656 in whole cells (Table 4). While the V_{max}^{app} for PitA was relatively stable, PitB V_{max}^{app} values were variable, as indicated by the high error values. Magnesium concentration and pH were altered to allow comparison with kinetic parameters measured by other researchers. While the K_m^{app} values remained similar under these conditions, the V_{max}^{app} was lower for both PitA and PitB when the magnesium concentration was increased from 1.8 to 10 mM and the pH was increased from 6.6 to 7.0 (Table 4).

Interestingly, removing 1,196 nucleotides upstream of the *pitB* gene altered PitB's kinetic parameters. Decreasing the length of upstream DNA of *pitB* from 1,403 to 206 nucleotides in plasmid pAN1116 unexpectedly decreased the K_m^{app} five-

TABLE 4. Kinetic parameters for PitA and PitB

Plasmid[a]	Phenotype	Assay condition		K_m^{app} [b] (*n*)	V_{max}^{app} [b] (*n*)
		pH	[Mg] (mM)		
pAN686	*pitA*$^+$	6.6	1.8	1.9 ± 0.1 (5)	58 ± 4 (5)
		7.0	10.0	1.9	39
pAN656	*pitB*$^+$ long[c]	6.6	1.8	28.6 ± 1.4 (5)	33 ± 9 (5)
		7.0	10.0	28.1 ± 1.0 (3)	17 ± 3 (3)
pAN1116	*pitB*$^+$ short[d]	7.0	10.0	6.0 ± 0.5 (3)	67 ± 2 (3)

[a] Host strain is AN3066 for all experiments.
[b] K_m^{app} values are shown as micromolar concentrations with standard errors of the means. V_{max}^{app} values are shown as nanomoles of P_i minute^{-1} milligram (dry weight)$^{-1}$ with standard errors of the means.
[c] 1,403 upstream nucleotides.
[d] 206 upstream nucleotides.

fold, while the V_{max}^{app} increased fourfold and became less variable, as shown by the decrease in error values (Table 4). To determine if this increase in PitB activity correlated with the amount of PitB protein produced, Western blotting was carried out. Polyclonal antipeptide PitB antibody was used to measure the expression of PitB protein in the membrane fractions of strains which produce various levels of PitB activity. (The relevant plasmids are listed in Table 4.) There was no cross-reactivity with the PitA protein (Fig. 1, lane 5). Negligible PitB protein expression was found for the *pitB*$^+$ *pitA1* Δ*pstC345* strain containing genomic *pitB*, which does not grow on P_i medium. The presence of plasmid pAN656 (*pitB*$^+$ plus 1,403 upstream nucleotides) resulted in low levels of PitB protein in the cell membranes, while large amounts of PitB were produced when the upstream *pitB* DNA was decreased to 206 nucleotides in pAN1116. While this Western blot showed that the higher P_i uptake activity from pAN1116 correlated with increased PitB protein expression, the increase was much

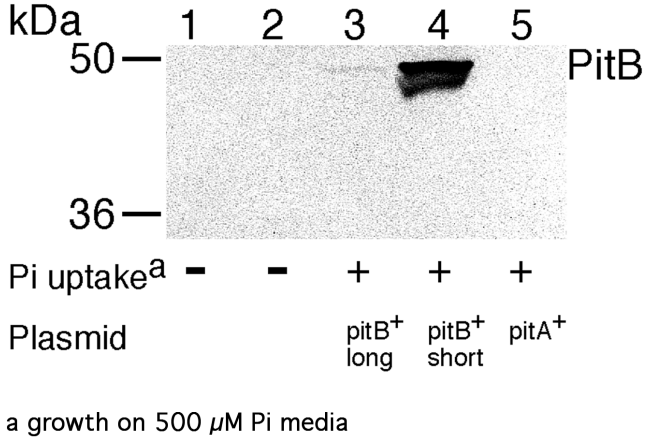

FIG. 1. Expression of PitB protein in different strains. Shown is a Western blot of purified polyclonal antipeptide PitB antibody against the membrane fraction of the indicated *pitA1* Δ*pstC345* strains (*pitB* genotype of the chromosome and plasmid). Lane 1, AN3903 (*pitB*, vector pBR322); lane 2, AN3514 (*pitB*$^+$, vector pBR322); lane 3, AN3135 (*pitB*$^+$, plasmid *pitB*$^+$ with 1,403 upstream nucleotides); lane 4, AN3905 (*pitB*, plasmid *pitB*$^+$ with 206 upstream nucleotides); lane 5, AN3904 (*pitB*, plasmid *pitA*$^+$). Strains in lanes 2 and 3 have wild-type *pitB* on the genome, which produces no P_i uptake activity.

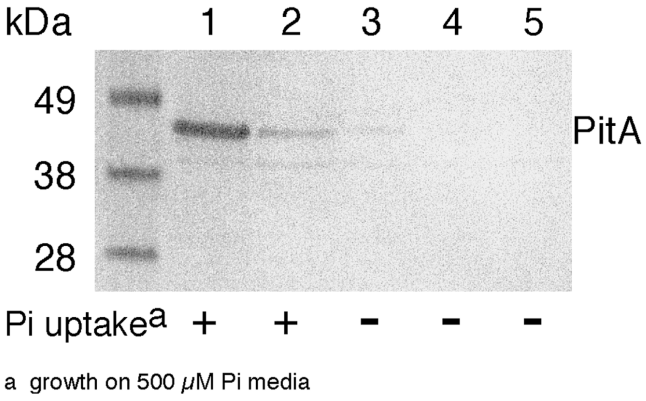

FIG. 2. PitA protein expression from the AN3066 *pitA1* mutation. Shown is a Western blot of polyclonal antipeptide PitA antibody against the membrane fraction of the indicated strains. All plasmids are in the AN3066 (*pitA1*) background unless otherwise stated. Lane 1, pAN920 (*pitA*$^+$); lane 2, AN248 (genomic *pitA*$^+$); lane 3, pBR322 (vector control); lane 4, pAN1243 (*pitA1* PCR from AN3066); lane 5, pAN1244 (*pitA* G220D).

greater than the fourfold elevation in V_{max}^{app} noted in uptake experiments. This variation may be due to the different growth conditions used. P_i uptake assays were carried out on cells grown in minimal medium, while the cells used in Western blotting were cultured in Luria-Bertani medium (Materials and Methods). However, these experiments do indicate that the excision of 1,196 nucleotides upstream of *pitB* enhanced PitB protein expression from plasmid pAN1116, elevating PitB P_i uptake and increasing the transporter's affinity for P_i. The deleted region contains a large stem-loop sequence which may terminate transcription read-through from the plasmid (52). Whether increased transcription of the *pitB* gene was due to the removal of this stem-loop or due to interference with regulation at the *pitB* promoter was not investigated further.

Identification of the *E. coli* K-10 Pit mutation in phosphate transport. While *E. coli* K-10 has been characterized as deficient in Pit activity (49), the mutation has not been defined. Copies of *pitA* and *pitB* nucleotide sequences from the K-10 strain AN3066 were made by PCR. DNA sequencing revealed that the *pitB* ORF and the 319 upstream nucleotides are wild type. The *pitA* sequence contains a point mutation of G to A at nucleotide 658 in the ORF of the published sequence, which creates an amino acid change of G220D in the coding sequence (*pitA1*). To check that this single base change could account for the loss of Pit function in K-10, site-directed mutagenesis was used to recreate this mutation in a K-12 *pitA*$^+$ gene. Both sequences were cloned into pBR322 and then transformed into AN3066, which is deficient in P_i transport (*pitA pstC*). Neither the mutant genomic *pitA* from AN3066 nor the site-directed *pitA*(G220D) allowed growth on P_i medium. This indicates that the single point mutation found in the AN3066 *pitA* is sufficient to create a nonfunctional Pit gene. Western blotting on the membrane fraction of whole cells, using polyclonal antipeptide PitA antibody, showed the level of expression of PitA protein inserted in the cell membrane (Fig. 2). While cells containing a wild-type *pitA* plasmid showed a strong band representing PitA protein (lane 1), cells containing the AN3066 genomic *pitA* (lane 4) or the site-directed *pitA*(G220D) (lane 5) had low

levels of protein similar to the *pitA* mutant background strain (lane 3). Therefore, the G220D mutation seems to affect insertion of the protein into the membrane.

Functional characterization of PitA and PitB. Plasmids containing either the *pitA* or *pitB* gene conferred P_i uptake activity on strain AN3066, which contains *pitA1* Δ*pstC345* and a wild-type *pitB* gene sequence. These assays show that a functional P_i transporter can be produced from the *pitB* gene when it is located on a plasmid but not when it is present only on the AN3066 genome. However, P_i uptake activity from the AN3171 strain, previously attributed to *pitA*, could in fact result from an interaction between the plasmid's PitA protein and the PitB protein expressed from the genome. To explore this further, a *pitA*$^+$ *pitB pstC* mutant was created by inserting a chloramphenicol resistance gene into the *pitB* coding region of a *pstC* strain. This strain (AN3926) grew on P_i medium, indicating that the PitA protein can transport P_i in the absence of PitB. Wild-type *pitA* or *pitB* plasmids could also restore growth on P_i medium when transformed into a *pitA pitB pstC* triple mutant strain (AN3902). Hence, both PitA and PitB can transport P_i independently of each other.

Effect of the *pho* regulon on *pitB* activity. The discovery that *E. coli* K-10, which lacks Pit activity (49), contains *pitB* which is wild type for the coding region and at least 319 upstream nucleotides poses an interesting problem. *pitB* was cloned due to the ability of the 3-kb fragment to complement for P_i uptake when in vector pHC79 (Materials and Methods), and P_i uptake assays confirmed that a functional P_i transporter was encoded on this fragment. Therefore, genomic *pitB* may be under regulation that is disrupted when the gene is placed on a plasmid.

In all experiments described so far, P_i uptake by PitA or PitB was isolated from P_i transport mediated by the Pst system by assaying strains which contain a Pst system deletion (Δ*pstC345*). This Pst deletion also constitutively induces the *pho* regulon (8), which is normally activated only under conditions of phosphate limitation. The *pho* regulon regulates a series of genes associated with phosphate transport and utilization. It is possible that PitB may be down-regulated by the *pho* regulon. Thus, a constitutive *pho* regulon could repress PitB activity at any phosphate concentration in the strains which we have used to assay Pit transport. To test this possibility, the *phoB-phoR* operon, which controls the *pho* regulon, was deleted from the *pitB*$^+$ strain AN3020 (*pitB*$^+$ *pitA pstC*), which did not grow on P_i medium, and the *pitB pitA pstC* triple mutant, which lacks *pitB* and was also unable to grow on P_i medium. Deletion of *phoB-phoR* enabled both strains to grow on P_i medium, indicating that both strains had one or more transport systems which were active in the absence of the *pho* regulon. However, the triple mutant, which contains no Pit or Pst transporters, had P_i uptake that was not significantly above background levels, while the *pitB*$^+$ strain, AN4081, had a significant rate of P_i transport (Table 5). The fact that the control strain was able to grow on P_i medium but had no measurable P_i uptake may be attributed to the difference in P_i concentrations used in these experiments. Growth was assessed at 500 μM P_i, while P_i uptake was measured at 20 μM P_i. Thus, the growth of the control strain may be due to the presence of a transport system which has a lower affinity for P_i than that of either PitA or PitB. As the *pho* regulon controls a large number of genes involved in P_i assimilation (up to 137 proteins by two-dimensional gel analysis [42]), more than one system may be involved. The genomic PitB exhibited P_i transport of 4.4 nmol of P_i min^{-1} mg (dry weight)$^{-1}$ at 20 μM P_i. By comparison, P_i uptake by PitB expressed from pAN656 was 5 to 25 nmol of P_i min^{-1} mg (dry weight)$^{-1}$ at 20 μM P_i, and PitB on pAN1116, which produced greater protein expression and a lower K_m^{app}, had P_i uptake rates of 46 to 59 nmol of P_i min^{-1} mg (dry weight)$^{-1}$ at 20 μM P_i (results not shown). Thus, these experiments show that chromosome-encoded *pitB* is active in the absence of the *pho* regulon.

TABLE 5. Effect of the *pho* operon on PitB inorganic phosphate uptake activity

Strain	Relevant genotype	Alkaline phosphatase activity[a]	Growth on minimal medium with source of phosphate:		Phosphate uptake[d]
			P_i and G3P[b]	P_i[c]	
AN3066	*pitB*$^+$ *phoB*$^+$ *phoR*$^+$	+	+	−	0.6 ± 0.1
AN4081	*pitB*$^+$	−	+	+	4.4 ± 0.3
AN4085		−	+	+	0.9 ± 0.2

[a] Rapid spray assay on cells grown on Luria-Bertani medium.
[b] 500 μM P_i and 1 mM G3P.
[c] 500 μM P_i.
[d] Assayed at 20 μM P_i. Values are shown with standard errors of the means. $n = 4$. Values are nanomoles of P_i minute^{-1} milligram (dry weight)$^{-1}$.

DISCUSSION

We have shown that *E. coli* contains two *pit* genes encoding proteins able to transport inorganic phosphate. *pitA* has previously been characterized, but we have shown that *pitB* also encodes a functional P_i transporter. Previously, Pit activity was reported to be constitutive (30, 49). While *pitA* appears constitutive, *pitB* may be regulated by the amount of available P_i. Our data suggest that *pitB* repression/inhibition is mediated through the *pho* regulon, since deletion of the *phoB-phoR* operon activated P_i uptake by *pitB*. The *pho* regulon is normally induced under conditions of P_i limitation, and P_i-dependent activation requires an interaction between the sensor protein PhoR and the Pst transport system. PhoR phosphorylates the transcriptional regulator PhoB, which then binds to a *pho* box consensus sequence within the promoter of genes in the *pho* regulon (46). However, the *pho* regulon of the Δ*pstC345* strain used in our experiments is active at all P_i concentrations (8). The *pitB*$^+$ strain AN3066 (*pitA1* Δ*pstC345*) was unable to grow on P_i medium until the *phoB-phoR* operon was deleted. The rate of P_i uptake obtained from PitB expressed from the genome was higher than the control rates and was similar to the lower rates produced from plasmid pAN656. P_i uptake by *pitB* on plasmids pAN656 and pAN1116 may be caused by the increased copy number of *pitB* titrating out *pho* regulon inhibition. Thus, our experiments show that genomic *pitB* encodes a functional P_i transporter repressed/inactivated by the *pho* regulon, either directly through PhoB or via another pathway controlled by the *pho* regulon. The most likely explanation is that PhoB represses the *pitB* gene because this has already been observed for a *pitB*-like *pit* gene in *Rhizobium meliloti* using a *pit*::*lacZ* fusion. The *R. meliloti pit* gene was repressed under conditions of P_i limitation, but repression was relieved in

a *phoB* mutant (2). As in *E. coli*, mutating the Pst transport system equivalent (*phoCDET*) caused derepression of the *pho* regulon, repressing *pit* at all P_i concentrations. Transforming a *phoC R. meliloti* strain with plasmid-borne *pit* increases cell growth on P_i medium to normal levels, suggesting that the repression can be overcome by supplying multiple copies of the gene. Only a two- to threefold increase in *pit* expression was needed to suppress the *phoC* phenotype (3).

While most studies on the *pho* regulon in *E. coli* have focused on the activation of genes, there is mounting evidence that the *pho* regulon may also repress some genes involved in phosphate assimilation. Two-dimensional protein experiments have shown that the *pho* regulon induces about 118 proteins and represses around 19 proteins (all with pIs of less than 7) under conditions of P_i limitation (42). More specifically, Willsky and Mallamy (51) have shown that two proteins which are repressed under P_i-limiting conditions are not repressed in *phoB* or *phoR* strains under the same conditions. Smith and Payne (36) propose that these are the periplasmic peptide binding proteins OppA and DppA.

Putative *pho* box sequences have been identified within the promoter regions of the *R. meliloti orfA-pit* operon and the *E. coli opp* and *dpp* operons (3, 36). However, while genes activated by the *pho* regulon have a *pho* box located 10 bases upstream from the −10 region (reference 27 and references therein), those operons which may be inhibited by the *pho* regulon have *pho* boxes either upstream of or overlapping the proposed −35 region. It has been suggested previously that this atypical positioning may reflect the negative regulation by PhoB (3, 36). While the *pitB* promoter region contains several putative *pho* box sequences in atypical positions, *pitA* also has similar sequences in the equivalent locations. Any potential *pho* box involved in negative regulation should be unique to *pitB*, as *pitA* is not repressed by the *pho* regulon. The binding sites for PhoB repression have not been characterized for any gene, so other DNA motifs may be involved. Thus, binding studies will be needed to locate any PhoB interactions with the *pitB* gene.

PitB's change in K_m^{app} indicates that a different mode of enzyme activity is also occurring in the protein expressed from the shortened plasmid pAN1116. An inhibitor which interacts with the PitB protein may be diluted out by the increased PitB expression, altering the K_m^{app}. Alternatively, higher concentrations of PitB may allow the protein to form a complex of monomers with a more effective mechanism. Although most secondary transporters are thought to function as monomers, this is not always the case. The sodium proton antiporter from *E. coli* has been crystallized as a dimer (48) and exists in the cytoplasmic membrane as a homo-oligomer (14). Glutamate transporters from the human brain have been shown previously to form dimers and trimers (17). There are now several examples of transporters that have dual affinity for their substrate and/or two mechanisms (13, 19, 21, 35, 55). Further investigation is needed to determine if this applies to PitB.

It is also possible that PitA undergoes similar changes in substrate affinity when protein expression is increased. Western blotting indicates that *pitA* expression is greatly elevated by placing it on a plasmid (Fig. 2), and our K_m^{app} of around 2 μM for pAN686 is significantly lower than the 11.9 to 38 μM range obtained by researchers using genomic Pit (30, 43, 50). However, the previously reported K_m^{app} values for Pit have been measured under conditions which make it impossible to attribute activity to *pitA* and/or *pitB*.

The K-10 *pitA* lesion was identified as a replacement of glycine 220 with aspartic acid, disrupting membrane insertion of the PitA protein. While it is not surprising that replacement of glycine, a small uncharged amino acid, with an aspartic acid could disrupt membrane insertion of the protein, it was unexpected that this relatively stable lesion was caused by a single point mutation. However, recreation of this mutation by site-directed mutagenesis produced identical behavior.

Our results indicate that PitA is likely to be active under a greater variety of conditions than is PitB. This situation of multiple P_i transporters with overlapping activities has been reported previously for *Saccharomyces cerevisiae* (25) and *Neurospora crassa* (45).

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