Enhanced Utilization of Phosphonate and Phosphite by Klebsiella aerogenes

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Klebsiella aerogenes ATCC 9621 was able to utilize phosphonates (Pn), including aminoethylphosphonate, ethylphosphonate, methylphosphonate (MPn), and phosphonoacetate, and inorganic phosphite (Pp) as sole sources of phosphorus (P). The products of the phn gene cluster were absolutely required for Pn breakdown and Pp oxidation to inorganic phosphate (Pi) in this organism. To determine if K. aerogenes ATCC 9621 could be engineered to enhance the utilization of Pn and Pp, a multicopy plasmid, pBI05, which carried the entire phn gene cluster, was introduced into this strain. Despite the increased dosage of the phn genes, K. aerogenes ATCC 9621(pBI05) could utilize only up to 1.1-fold more Pn and Pp than did the control strain with the parent vector alone. These results suggested that Pn, which was generated from Pp and Pp, might limit further utilization of these P compounds. Consequently, to convert the resulting Pi to polyphosphate (polyP), the plasmid pKP28, which carried the K. aerogenes ppk gene (which encodes polyP kinase), was introduced into K. aerogenes ATCC 9621(pBI05). Overexpression of the ppk gene in K. aerogenes ATCC 9621(pBI05, pKP28) resulted in a 2.5-fold increase in Pi utilization over that of the control strain. This recombinant strain also accumulated approximately sixfold more Pi than did the control strain when the cells were grown with MPn as a sole source of P.

Phosphorus (P) compounds are major contaminants in industrial and municipal effluents (13). Inorganic phosphate (Pi) is recognized as one of the major nutrients contributing to the eutrophication of lakes, bays, and other natural bodies of water (13). Treatment of inorganic phosphate (Pi) associated with eutrophication of lakes, bays, and other natural bodies of water is recognized as one of the major nutrients contributing to the very narrow market for Pt, it is necessary to oxidize Pp to Pi used to adjust the pH and stabilize metal ions. Because of the of high concentrations of organic acids (15).

The E. coli phn locus, which comprises 14 genes (named alphabetically from phnC to phnP) in an operon with a single promoter, is required for utilizing Pn and Pp (2, 12). PhnC, PhnD, and PhnE probably comprise a periplasmic binding protein-dependent Pn transporter which also transports Pp, Pp esters, and Phn (12). PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, and PhnM are likely to be components of a C-P lyase pathway or C-P lyase enzyme complex which catalyzes C-P bond cleavage and Pi oxidation (2). Two additional proteins, PhnF and PhnO, appear to be regulatory proteins. The expression of the E. coli phn genes is known to be activated under conditions of Pp limitation (2, 12).

Previously, we examined a variety of bacterial species for their ability to utilize Pn and Pp as sole sources of P (14). After examining the bacterial growth on Pn and Pp, we found that Klebsiella aerogenes ATCC 9621 was able to grow well in minimal medium containing AEPn, EPPn, MPn, phosphonoacetate (PpAc), or Pi as a sole source of P (14). The growth rate of K. aerogenes ATCC 9621 was threefold higher than that of E. coli IFO13168 (B strain) in the medium with Pp. Therefore, for further study, we have also cloned the entire phn gene cluster from K. aerogenes ATCC 9621 by using E. coli phnCDE as a DNA probe (2).

The present work was undertaken to determine if K. aerogenes ATCC 9621 could be engineered to enhance the utilization of Pn and Pp, as sole sources of P by increasing the dosage of the phn genes. In the course of this study, we found that Pn, which was released from Pn and Pp, apparently limited further utilization of these P compounds. Therefore, to enhance the conversion of the resulting Pp to polyP, we employed the K. aerogenes ppk gene, which encodes polyphosphate (polyP) kinase (PPK) (9); this enzyme catalyzes the formation of polyP from ATP.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli MV1184 (16) and K. aerogenes ATCC 9621 (14) were grown on 2X YT medium (16). Plasmid pQF50, which carried the
promoterless lacZ gene, was described previously (9). pKP01 is a derivative of pUC119 (Pharmacia) containing the K. aerogenes ppk and its own promoter (9). pBR322 (16) and pSTV28 (Takara Shuzo Co., Ltd., Kyoto, Japan) were used as cloning and expression vectors. To construct pBR322MC, a 56-bp EcoRI-HindIII fragment containing a multiple cloning site of pUC118 (Takara Shuzo Co., Ltd.) was ligated to pBR322 which had been cut with EcoRI and HindIII. Plasmid pUC4K (Pharmacia) carrying a kanamycin resistance (Kmr) gene cassette was used for disrupting the chromosomal phn gene cluster. Antibiotics used for the selection of transformants were 50 mg of ampicillin, 50 mg of kanamycin, and 20 mg of chloramphenicol per liter. Standard procedures were used for plasmid preparations, restriction enzyme digestion, ligation, and agarose electrophoresis (16).

Disruption of the chromosomal phn gene cluster. Plasmid pPI04, which carried a 14-kb BglII-SmaI fragment containing the entire K. aerogenes phn gene cluster, was described previously (14). pPI04 was digested with EcoRI and ligated with a 1.2-kb EcoRI site-flanked Kmr gene cassette from pUC4K to construct pPI02.2. K. aerogenes ATCC 9621 was then transformed with pPI02.2 by electroporation, and Kmr transformants were selected on YT medium containing kanamycin. Plasmid pPI05, which carried the entire K. aerogenes phn gene cluster, was confirmed by Southern hybridization (16).

Pn and Pt uptake experiments. K. aerogenes cells were grown in 2X YT medium with shaking at 37°C for 7 h, inoculated (1% inoculum) into T0, minimal medium (7) containing either Pn or Pt as a sole source of P. The cultures were then incubated for 24 h under the same conditions, and samples were taken at intervals for the determination of growth (measured as optical density at 600 nm [OD600]) and P concentrations. Pn were measured by ammonium peroxidisulfate digestion (120°C, 30 min) followed by Pi measurement (14) by an ascorbic acid method (9). Pn was analyzed with a high-performance liquid chromatography system with an IC-Anion-SW column (4.6 by 50 mm; Tosoh Co., Ltd., Tokyo, Japan). The total P content of K. aerogenes cells was determined as described previously (9).

PolyP analysis. PolyP was extracted from bacterial cells with a 4 M guanidine thiocyanate solution and bound to silicate glass powders (Gene Clean kit II; Funakoshi Co., Ltd., Tokyo, Japan). The glass powders binding polyP were washed with an ethanol-NaCl solution, and then polyP was recovered with distilled water from the glass powder solution (1). PolyP was determined as described by Crooke et al. (3). The ability to serve as a substrate for the PKP-catalyzed conversion of [14C]ADP to [14C]ATP was used to quantitate levels of polyP. The amount of [14C]ATP was determined by thin-layer chromatography followed by visualization by an image analyzer (BAS1000; Fuji Co., Ltd., Tokyo, Japan).

Chemicals. EPn, and dimethylphosphite (DMP) were from Nacalai Tesque, Inc. (Kyoto, Japan), and [14C]ADP was from Dai-ichi Chemical Co. (Tokyo, Japan).

RESULTS AND DISCUSSION

Pn and Pt utilization. There are two Pn degradation pathways which are commonly referred to as the phosphonatase and the C-P lyase pathways (11). They differ in regard to their substrate specificities and mechanism of C–P bond fission. Interestingly, E. coli degrades Pn solely by the C-P lyase pathway, whereas Salmonella typhimurium appears to contain genes only for the phosphonatase pathway (5). The phn genes, whose products are involved in the C-P lyase pathway, have been cloned from K. aerogenes ATCC 9621 (14).

To determine if K. aerogenes ATCC 9621 possesses solely the C-P lyase pathway for utilizing Pn and Pt, we constructed the chromosomal phn mutant strain PHN1 using a Km gene cassette from pUC4K (Fig. 1). K. aerogenes PHN1 could not grow in T0 medium containing AEPn, EPn, MPn, PnAc, DMPn, or Pn as a sole source of P (Fig. 2). To further confirm the essentiality of the phn gene cluster, a 14-kb XbaI-KpnI fragment which carried the entire K. aerogenes phn gene cluster (14) was excised from pPI04 and cloned into pBR322MC (Fig. 1). K. aerogenes ATCC 9621(pPI05) could restore the ability of K. aerogenes PHN1 to grow on T0 medium containing either Pn or Pn as a sole source of P (Fig. 2). These results indicate that K. aerogenes ATCC 9621 possesses solely the C-P lyase pathway for utilizing Pn and Pn, as a sole source of P. To confirm the presence of Pn and Pn for sole sources of Pn or Pn uptake experiments were performed with K. aerogenes ATCC 9621 (pBR322MC), a control strain, and ATCC 9621(pPI05). However, despite the increased dosage of the phn gene cluster, K. aerogenes ATCC 9621(pPI05) removed only up to 1.1-fold
more Pₙ and Pₜ from T₀ medium than did ATCC 9621 (pBR322MC) (data not shown). Since both Pₙ breakdown and Pₜ oxidation could result in an elevated accumulation of cellular P, it is highly possible that Pₜ, which was released from Pₙ and Pₜ, limited further utilization of these P compounds. We also observed that the presence of Pₙ in the growth medium strongly inhibited the utilization of Pₙ and Pₜ by K. aerogenes ATCC 9621 (data not shown).

We scanned the nucleotide sequence upstream from the K. aerogenes phn gene cluster for the presence of a Pho box, the consensus sequence published by Kasahara et al. [CTGTCTATA(A,T)A(A,T)CTGTCA(C,T)] (6). Inspection of the promoter sequence revealed that the K. aerogenes phn gene cluster was preceded by a Pho box sequence (CTGTCATCA AACTGCGCT). There was a 14-of-18-bp match with the consensus Pho box sequence. We further confirmed that the promoter of the K. aerogenes phn gene cluster is activated under conditions of Pₙ limitation. A 2.1-kb Xbal-PstI fragment which carried the phn promoter was excised from pBI05 (Fig. 1) and inserted upstream from the promoterless lacZ gene (encoding β-galactosidase) in the vector pQF50 to construct pQF01. K. aerogenes ATCC 9621 was transformed with either pQF50 or pQF01, and the β-galactosidase levels were measured in K. aerogenes cells grown in T₀ medium with or without Pₙ. High enzyme levels were observed only with K. aerogenes ATCC 9621 (pQF01) cells grown in T₀ medium without Pₙ (data not shown).

Conversion of Pₙ and Pₜ to polyP. We attempted to place the phn gene cluster under the control of the tetracycline resistance (tet) promoter of pBR322 to express it constitutively. However, because of the absence of appropriate restriction sites for the phn gene cluster, this attempt has not yet been successful. As we have previously demonstrated (9), K. aerogenes is able to accumulate high levels of polyP. Therefore, it may be possible that the conversion of Pₜ to polyP, if enhanced, could improve the utilization of Pₙ and Pₜ in this organism.

Previously, we have cloned ppk from K. aerogenes ATCC 9621 (9). To enhance the conversion of Pₜ to polyP, a 2.2-kb EcoRI-HindIII fragment, which contained the K. aerogenes ppk gene and its own promoter, was excised from pKP01 (9) and cloned into a vector pSTV28 to construct pKP28 (Fig. 1). This recombinant plasmid was then introduced into K. aerogenes ATCC 9621 (pBI05) by electroporation. K. aerogenes ATCC 9621 (pBI05, pKP28) could remove 2.5-fold more Pₜ from T₀ medium containing 0.5 mM Pₚ than did the control strain ATCC 9621 (pBR322, pSTV28) (Fig. 3). Since the growth of K. aerogenes ATCC 9621 (pBI05, pKP28) was almost equivalent to that of the control strain, this recombinant strain accumulated approximately 2.1-fold more P (23 mg of P per g [dry weight] of cells) than did the control strain. The levels of polyP in K. aerogenes ATCC 9621 (pBI05, pKP28) were approximately 3.5 mg of P per g [dry weight] of cells after the 24-h incubation in T₀ medium containing 0.5 mM Pₜ (Fig. 4). However, the time course analysis of polyP accumulation revealed that the levels reached a maximum of 12 mg of P per g [dry weight] of cells around 6 h after the start of incubation (Fig. 5). No significant amount of polyP was observed with K. aerogenes ATCC 9621 (pBR322, pSTV28) and ATCC 9621 (pBI05, pSTV28). K. aerogenes ATCC 9621 (pBR322, pKP28) could accumulate polyP only when Pₜ was available (Fig. 4). K. aerogenes ATCC 9621 (pBI05, pKP28) also accumulated a maximum of 10% of its dry weight as polyP (33 mg of P per g [dry weight] of cells) when the cells were grown in T₀ medium containing 0.5 mM MPₙ as a sole source of P (Fig. 5). In this experiment, the total P content of K. aerogenes ATCC 9621 (pBI05, pKP28) reached a maximum of 62 mg of P per g (dry weight) of cells, or approximately sixfold more than that of the control strain with the parent vectors alone (data not shown).

We have examined, for the first time, the possibility of genetic improvement of Pₙ and Pₜ utilization in bacteria. The phn genes were essential for Pₙ and Pₜ utilization in K. aerogenes ATCC 9621, although their roles in Pₜ breakdown and Pₜ oxidation are specifically uncharacterized. In addition, it was
suggested that Pi, which was released from Pn and Pt, appeared to limit further utilization of Pn and Pt. In fact, we found that enhanced conversion of Pi to polyP could improve the utilization of Pn and Pt as sole sources of P in K. aerogenes ATCC 9621. It has been known that exopolyphosphatase plays an important role in the degradation of polyP to Pi (3, 8). Previously, we showed that the K. aerogenes ppx gene, encoding exopolyphosphatase, existed immediately downstream of the ppk gene without transcriptional termination (9). The chromosomal ppk mutant, which was constructed by inserting a Km' gene cassette into the wild-type gene, showed a reduced polyPase activity in K. aerogenes ATCC 9621 (data not shown). We also constructed a ppx ppk double mutant of K. aerogenes ATCC 9621 and introduced pKP28 into this mutant by electroporation. When this strain was grown in T0 medium with 0.5 mM Pn, it accumulated 12-fold more polyP than did ATCC

FIG. 3. Time course of OD600 (circles) and P concentration (squares) during growth of K. aerogenes ATCC 9621(pBR322, pSTV28) (A) and ATCC 9621(pBI05, pKP28) (B).

FIG. 4. PolyP accumulation in K. aerogenes strains. (A) Thin-layer chromatography of [14C]ATP generated by the PPK-catalyzed conversion of [14C]ADP. [14C]ADP and [14C]ATP were resolved with 1 M formic acid and 0.4 M LiCl as a solvent system and visualized with an image analyzer (BAS1000; Fuji Co. Ltd.). Lanes 1 and 5, K. aerogenes ATCC 9621(pBR322, pSTV28); lanes 2 and 6, K. aerogenes ATCC 9621(pBI05, pSTV28); lanes 3 and 7, K. aerogenes ATCC 9621(pBR322, pKP28); lanes 4 and 8, K. aerogenes ATCC 9621(pBI05, pKP28). Cultures were grown in T0 medium containing either Pi (lanes 1 to 4) or Pt (lanes 5 to 8) as a sole source of P. PolyP levels were determined with K. aerogenes cells sampled around 24 h after the start of incubation. (B) PolyP contents of K. aerogenes strains quantitated by the image analyzer. Error bars represent standard errors.
9621(pKP28) (data not shown). Consequently, we attempted to further improve the utilization of \( P_n \) and \( P_i \) in *K. aerogenes* by introducing both pBI05 and pKP28 into the *ppk ppx* double mutant. However, simultaneous introduction of pBI05 and pKP28 appeared to be detrimental to the *ppk ppx* double mutant, and this attempt has not yet been successful.

Our previous work demonstrated that high levels of \( P_i \) accumulation in *E. coli* were achieved by modifying the genetic regulation and increasing the dosage of the *E. coli* genes encoding PPK and the \( P_i \)-specific transport system (8). When \( P_i \) is available, the *E. coli* recombinants accumulated as much as 16\% of their dry weight as \( P_i \) (48\% as \( P_i \)), or approximately 10-fold more \( P_i \) than did the control strain. This level of \( P_i \) content was surprisingly high and even surpassed those of natural phosphorite deposits (typically 14\% as \( P_i \)), or approximately 10-fold more \( P_i \) than did the control strain when \( P_n \) was available as a sole source of \( P_i \). Further strain improvement would be possible if the functions of the *phn* gene products in *K. aerogenes* were specifically understood.

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