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Isolation and Characterization of *Enterobacter cloacae* Mutants Which Are Defective in Chemotaxis toward Inorganic Phosphate

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***Enterobacter cloacae* IFO3320 is attracted to P_i when cells are starved for P_i. Two Tn*1737*KH-induced mutants, which were constitutive for alkaline phosphatase, failed to exhibit P_i taxis even under conditions of P_i limitation. Both of the mutant strains exhibited normal chemotactic responses to peptone, suggesting that they are specifically defective in P_i taxis. Cloning and sequence analysis showed that the TN*1737*KH insertions were located in either the *pstA* or *pstB* genes which encode the channel-forming proteins of the P_i-specific transport (Pst) system in *E. cloacae*. These results suggest that the *E. cloacae* Pst system is required for P_i chemoreception.**

Since inorganic phosphate (P_i) is an essential constituent in bacteria, chemotaxis toward P_i presumably gives bacteria a selective advantage in microbial communities. However, bacterial P_i taxis has been reported only with *Pseudomonas aeruginosa* (5). Enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* do not exhibit P_i taxis even under conditions of P_i limitation (7). Although experimental evidence for P_i taxis is limited to *P. aeruginosa*, the specificity of chemoreceptors for P_i appears to be relatively high (5). No other phosphorus compounds have been shown to elicit responses similar to those for P_i. From competition experiments, it has also been suggested that the chemoreceptors for P_i are different from those for L-amino acids (5).

P_i taxis in *P. aeruginosa* is induced by P_i limitation (5). However, P_i taxis is not under the control of PhoB and PhoR, which act as a transcriptional regulator for the *pho* regulon (6). Our previous study showed that the chromosomal *phoB* and *phoR* mutants, which failed to induce alkaline phosphatase (AP) synthesis under P_i limitation, were fully induced for P_i taxis by P_i limitation (6). *P. aeruginosa* PhoU and the components of the P_i-specific transport (Pst) system are also involved in the regulation of P_i taxis (13).

Up to now, virtually nothing has been known about the P_i chemoreceptor. Mutants which are specifically defective in P_i taxis have not been isolated from *P. aeruginosa*. In the present paper, we report that *Enterobacter cloacae* IFO3320 exhibits P_i taxis under conditions of P_i limitation and that Tn*1737*KH-induced mutants, which are constitutive for AP synthesis, are specifically defective in P_i taxis. Furthermore, we describe genetic evidence that the Pst system is required for exhibition of P_i taxis in *E. cloacae*.

E. cloacae strains were grown in either 2× YT medium (14) or H_5 medium at 28°C. H_5 medium contained 2 g of glucose per liter, 1 g of $(NH_4)_2SO_4$ per liter, 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter, 0.1 g of KCl per liter, 0.01 g of $CaCl_2 \cdot 2H_2O$ per liter, 0.001 g of $FeCl_3$ per liter, 5 mM K_2HPO_4, and 40 mM *2*-[*4*-(*2*-hydroxyl)-*1*-piperazinyl] ethanesulfonic acid (HEPES [pH 7.0]). For P_i limitation, cells grown overnight were inoculated (a 3% inoculum) into H_0 medium, which was prepared by omitting K_2HPO_4 from H_5 medium, and incubated with shaking at 28°C.

Chemotaxis assays were carried out by the computer-assisted capillary assay method as described previously (12). The chemotaxis buffer contained 50 mM HEPES (pH 7.0), 1 mM $MgCl_2$, and 0.5 g of glucose per liter. Conjugation of *E. coli* donor strain DH5α (14), harboring Tn*1737*KH vector pMT6121 (16), and *E. cloacae* IFO3320 (Institute for Fermentation, Osaka, Japan) was performed as previously described (16). AP activities of *E. cloacae* strains were determined as described previously (5). Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligation, and agarose gel electrophoresis (14). *E. cloacae* was transformed by electroporation (11). The upstream region of the *pstA* gene was cloned from *E. cloacae* IFO3320 by the inverse PCR technique (14). Two primers, PST1 (sense primer, 279 bp downstream of the stop codon of *phoU*) and PST2 (antisense primer, 155 bp upstream of the stop codon of *phoU*), were used for PCR. PST1 was 5′-CTCTACATTGACGTTCATCGCTT TTGCCGC-3′, and PST2 was 5′-AGTACGCTTGGGATC GTACGTGAATCTTCC-3′.

When *E. cloacae* IFO3320 was grown in H_0 medium, the cells exhibited P_i taxis (Fig. 1). No positive response to P_i was detected with cells grown in H_5 medium. The strength of the chemotactic response to P_i was dependent on the concentration of P_i in the capillary. The concentration-response curve showed a peak at 1 mM P_i, and the lowest concentration needed to elicit an observable response (the threshold concentration) was approximately 5 μM (data not shown). *E. cloacae* IFO3320 was weakly attracted to pyrophosphate (PP_i), ATP, and 2-glycerophosphate when the cells were starved for P_i. However, no chemotactic response was observed with 3-phosphoglycerol. P_i-starved cells were also attracted to arsenate (AsO_4^{3-}). P_i competitively inhibited the response to AsO_4^{3-}, showing that both P_i and AsO_4^{3-} are likely detected by the same chemoreceptors (data not shown).

We previously showed that *P. aeruginosa* mutants, which were constitutive for AP synthesis, exhibited P_i taxis even under conditions of P_i excess (5). To examine whether a similar phenomenon is seen with *E. cloacae* IFO3320, AP constitutive mutants of *E. cloacae* were isolated after Tn*1737*KH mutagen-

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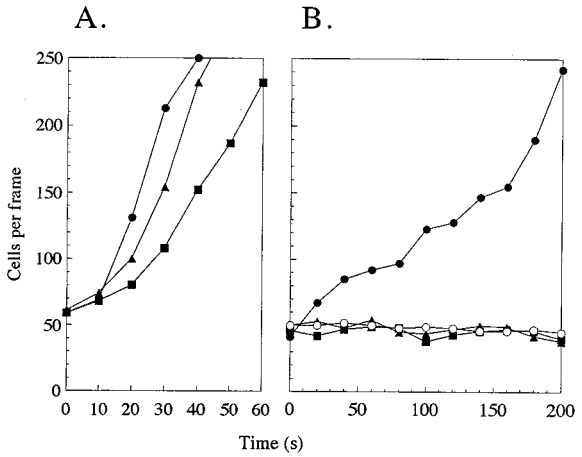

FIG. 1. Chemotaxis by *E. cloacae* cells toward 0.1% peptone (A) and 1 mM P_i (B). ●, P_i-starved IFO3320; ○, P_i-sufficient IFO3320; ▲, P_i-starved AP1; ■, P_i-starved AP2. P_i-sufficient cells were grown in H_5 medium. P_i-starved cells were grown in H_0 medium. Digital image processing was used to count the number of bacteria accumulating toward the mouth of the capillary. One videotape frame was analyzed at each time point, and the chemotactic response is presented as the number of bacteria per videotape frame as described previously (12).

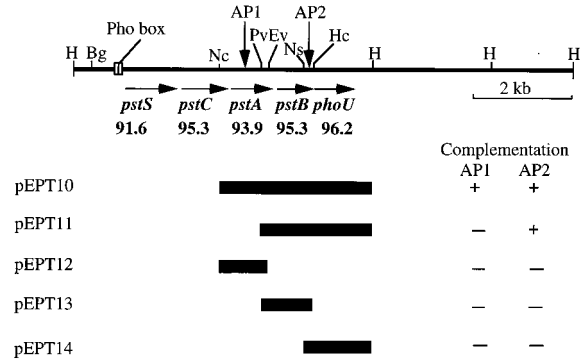

FIG. 3. Restriction map of the chromosomal region containing the *E. cloacae* *pst* operon and its subclones. The locations and orientations of *pstS*, *pstC*, *pstA*, *pstB*, and *phoU* are indicated by horizontal arrows below the restriction map. Numbers below the horizontal arrows are percentage of amino acid identity of the *E. cloacae* gene products with the *E. coli* counterpart, respectively. Vertical arrows above the restriction map show the insertion sites of Tn*1737*KH in AP1 and AP2. The abilities of recombinant plasmids to complement the mutations of AP1 and AP2 are indicated on the right. Restriction sites: Bg, *Bgl*II; B, *Bam*HI; Ev, *Eco*RV; H, *Hin*dIII; Nc, *Nco*I; *Nsp*I; Pv, *Pvu*II. Only restriction sites used to construct recombinant plasmids are indicated.

esis. Two Tn*1737*KH-induced mutants, designated AP1 and AP2, formed blue colonies on 2× YT plates supplemented with 5-bromo-4-chloro-3-indolyl-phosphate and were selected for further study. AP1 and AP2 showed high levels of AP activity even under conditions of P_i excess (data not shown). Chemotaxis assays, however, revealed that both AP1 and AP2 were defective in P_i taxis even under conditions of P_i limitation (Fig. 1). AP1 and AP2 exhibited normal chemotaxis toward peptone. They were also normal in chemotaxis toward L-amino acids, including L-serine, L-aspartate, and L-methionine (data not shown). These results suggest that AP1 and AP2 are specifically defective in P_i taxis.

The transposon insertion sites in AP1 and AP2 were identified by using the Km^r marker of Tn*1737*KH (Fig. 2). *E. cloacae* AP1 chromosomal DNA was first digested with *Bam*HI and hybridized with a digoxigenin-labeled 4.2-kb *Bam*HI-*Eco*RI fragment from Tn*1737*KH. A 14-kb *Bam*HI fragment, which was hybridized with the DNA probe, was cloned into pBR322 (3) to generate pEPT01. The 14-kb insert of pEPT01 was digested with various restriction enzymes, and the fragments were subcloned into pUC118 (17). *E. coli* MV1184 (17) was then transformed with these subclones. A recombinant plasmid, which had a 10.1-kb *Eco*RI-*Bam*HI fragment, was isolated from these transformants and designated pEPT02.

Nucleotide sequence analysis of the pEPT02 insert revealed that the 10.1-kb *Eco*RI-*Bam*HI fragment contained part of an open reading frame (*pstA*) and two whole open reading frames (*pstB* and *phoU*). A computer-assisted search revealed the putative polypeptides encoded by the *pstB* and *phoU* genes had amino acid sequences 95 and 96% identical to those of *E. coli* PstB and PhoU, respectively. The deduced amino acid sequence of the *pstA* gene was 97% identical to the 186 C-terminal amino acids of *E. coli* PstA. The insertion of Tn*1737*KH had taken place at a site 560 bp upstream of the stop codon of the *pstA* gene (data not shown). A similar analysis was performed with the Tn*1737*KH insertion site of AP2. The Tn*1737*KH insertion site was identified at a site 14 bp upstream of the stop codon of the *pstB* gene.

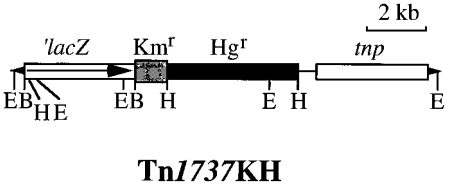

FIG. 2. Restriction map of Tn*1737*KH (16). Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. Km^r, kanamycin resistance gene; Hg^r, mercury resistance gene.

In *E. coli*, the products of the *pstS*, *pstC*, *pstA*, and *pstB* genes are required for the P_i-specific transport, and these genes, together with the *phoU* gene, form the *pst* operon (18). To confirm the presence of the *E. cloacae pstS* and *pstC* genes, the DNA sequences upstream of the *pstA* gene were cloned. A 5.9-kb *Hin*dIII fragment of IFO3320 chromosomal DNA, which hybridized to the *E. coli pstS* and *pstC* genes, was subjected to inverse PCR with primers PST1 and PST2. A 5.5-kb PCR product was then cloned into pUC118 and sequenced. Nucleotide sequence analysis showed the presence of two additional open reading frames (*pstS* and *pstC*) and part of the *pstA* gene (data not shown). The predicted products of the *pstS* and *pstC* genes had amino acid sequences 91 and 95% identical to those of *E. coli* PstS and PstC, respectively. Overall, *E. cloacae* PstA was 93% identical to *E. coli* PstA. *pstS* was preceded by a well-conserved Pho box sequence (CTGTCA TAAAACTGTCAT) (10).

Complementation analysis was performed to confirm that the *pstA* and *pstB* genes are required for P_i taxis. A 3.1-kb *Nco*I-*Hin*dIII fragment, which contained the *E. cloacae pstA*, *pstB*, and *phoU* genes, was cloned into broad-host-range vector pMMB66EH (4) in the same orientation as the *tac* promoter. The resulting plasmid, designated pEPT10, could restore the ability of AP1 and AP2 to exhibit P_i taxis and repress AP synthesis in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (Fig. 3 and 4). A series of subclones of pEPT10, designated pEPT11 to pEPT14 (Fig. 3), was also constructed by cloning the *pstA*, *pstB*, or *phoU* gene into pMMB66EH in the same orientation as the *tac* promoter. In the presence of IPTG, pEPT11 complemented the mutation of AP2 but not AP1. Neither pEPT12 nor pEPT13 restored the abilities of

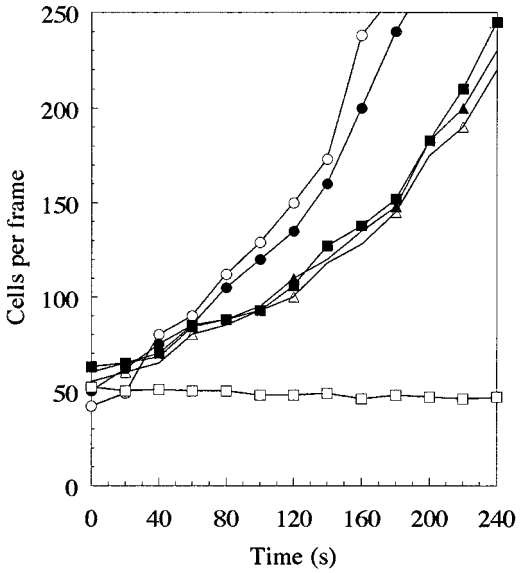

FIG. 4. Chemotaxis by P_i-starved cells of *E. cloacae* toward 1 mM P_i. See the legend to Fig. 1 for the chemotaxis assay method. No chemotactic response to P_i was observed with AP1(pEPT12), AP2(pEPT12), AP1(pEPT13), AP2(pEPT13), AP1(pEPT14), and AP2(pEPT14). ○, AP1(pEPT10); ●, AP2(pEPT10); □, AP1(pEPT11); ■, AP2(pEPT11); △, AP1(pEPT15); ▲, AP2(pEPT15).

AP1 and AP2 to respond to P_i. pEPT14 harboring the entire *phoU* gene did not complement the mutations of AP1 and AP2. These results confirm that the *pstA* and *pstB* genes, as well as the *phoU* gene, are required for P_i taxis. The fact that pEPT12 and pEPT13 failed to complement the mutations of AP1 and AP2 could be explained by the polarity effects due to Tn*1737*KH insertion.

To test whether the mutations of AP1 and AP2 can be complemented by the *E. coli* genes, plasmid pEPT15, which was constructed by cloning the *E. coli pstA*, *pstB*, and *phoU* genes (1) into pMMB66EH in the same orientation as the *tac* promoter, was introduced into AP1 and AP2. In the presence of IPTG, both AP1(pEPT15) and AP2(pEPT15) could show P_i taxis as well as AP synthesis under conditions of P_i limitation (Fig. 4).

In the present study, we found that *E. cloacae* IFO3320 exhibits P_i taxis when the cells are starved for P_i. This is unexpected, because enteric species *E. coli* and *S. typhimurium*, closely related to *E. cloacae*, are not attracted to P_i (7). The present data, however, convincingly show that *P. aeruginosa* is not unique in its ability to exhibit P_i taxis. Up to now, virtually nothing has been known about the chemoreceptor for P_i. It was not possible to isolate *P. aeruginosa* mutants, which are specifically defective in P_i taxis, by swarm assay techniques. Previously, we expected that *P. aeruginosa* PstS, a periplasmic P_i-binding protein, might be the chemoreceptor for P_i. The fact that the periplasmic glucose-binding protein has been identified as the glucose chemoreceptor of *P. aeruginosa* led to this hypothesis (15). However, we found that *P. aeruginosa* mutants lacking PstS showed P_i taxis, regardless of whether the cells were starved for P_i (8). We also recently found that the mechanism of P_i taxis by *P. aeruginosa* is dependent on methyl-accepting chemotaxis proteins (MCPs). The *P. aeruginosa cheB* mutant was proved to be defective in P_i taxis. However, we do not know whether *E. cloacae* senses P_i through MCPs. The *cheR* and *cheB* genes have not been cloned from *E. cloacae.*

Our previous data showed that *P. aeruginosa* mutants lacking the Pst system exhibit P_i taxis even under conditions of P_i excess (6, 13). In contrast, the present work indicated that the *E. cloacae* Pst system is absolutely required for exhibition of P_i taxis. *E. cloacae* mutant strains AP1 and AP2, which contain a Tn*1737*KH insertion in either the *pstA* or *pstB* gene, failed to exhibit P_i taxis. Since AP1 and AP2 were specifically defective in P_i taxis, it is possible that the *E. cloacae* Pst system serves as a chemoreceptor for P_i taxis. However, it is unlikely that PstS alone is sufficient for the P_i chemoreception in *E. cloacae*. We could detect PstS from both AP1 and AP2 grown in H_0 medium by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). It is also possible that the *E. cloacae* Pst system can cause an MCP-independent chemotactic signal, as in the case of the phosphoenol pyruvate-dependent phosphotransferase system (2). Alternatively, the *E. cloacae* Pst system may exert a positive control on P_i taxis. The Pst complex may activate expression of an unidentified gene encoding P_i chemoreceptor.

E. cloacae possesses a Pst system which is very similar to that in *E. coli*. The components of the *E. cloacae* Pst system showed striking homologies (>90% identical amino acids) to the *E. coli* counterparts (1). In addition, we observed that plasmid pEPT12, containing the *E. cloacae pstA* gene, could restore the ability of the *E. coli pstA* mutant to repress AP synthesis under conditions of P_i excess (data not shown). However, despite their striking homologies, *E. coli* does not show P_i taxis. A plasmid which carries the entire *E. cloacae pst* operon did not render P_i taxis to *E. coli* (9). Unknown proteins other than the Pst complex may be required for exhibition of P_i taxis.

Nucleotide sequence accession number. The nucleotide sequence of the entire *pst* operon has been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession no. D89963.

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