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Organization and Multiple Regulation of Histidine Utilization Genes in *Pseudomonas putida*

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The arrangement of the histidine utilization (*hut*) genes in *Pseudomonas putida* was established by examining the structure of a DNA segment that had been cloned into *Escherichia coli* via a cosmid vector. Southern blot analysis revealed that the restriction patterns of the *hut* genes cloned into *E. coli* and present in the *P. putida* genome were identical, indicating that no detectable DNA rearrangement took place during the cloning. Expression of the *hut* genes from a series of overlapping clones indicated the gene order to be *hutG-hutI-hutH-hutU-hutC-hutF*. The transcription directions of the different *hut* genes were determined by cloning the genes under control of the lambda p_L promoter. This showed that *hutF*, encoding formiminoglutamate hydrolase, was transcribed in a direction opposite to that of the other genes. Inactivation of the cloned *hut* genes by Tn*1000* insertion revealed that the *hut* genes were divided into three major transcriptional units (*hutF*, *hutC* [the repressor gene], and *hutUHIG*), but *hutG* may also be independently transcribed. When cloned individually with *hutC* on the same vector, *hutF* and *hutU* (which encodes urocanase) expression was induced by urocanate, indicating that these two genes each possess an operator-promoter element. Tn*1000* insertions (in the cloned genes) or Tn*5* insertions (in the *P. putida* genome) affecting the *hutI* or *hutH* gene only partially eliminated *hutG* expression. Furthermore, *hutG*, which specifies *N*-formylglutamate amidohydrolase, was regulated by the *hutC* product when the two genes were cloned on the same vector and expressed in *E. coli*. Therefore, *hutG* can be expressed independently from its own promoter, in keeping with earlier observations that *N*-formylglutamate amidohydrolase synthesis is not coordinated with that of urocanase and histidase and can be induced by *N*-formylglutamate or urocanate.

The histidine degradation pathway in *Pseudomonas* species consists of five reactions (8, 16), while four steps are required in most other organisms which have been studied, e.g., *Salmonella typhimurium* (18), *Klebsiella aerogenes* (17), *Bacillus subtilis* (6), and mammalian species (26). The enzymes and their genes required by *Pseudomonas putida* for the conversion of histidine to glutamate plus formate and ammonia are: histidase (*hutH*), urocanase (*hutU*), imidazolone propionate hydrolase (IPAase) (*hutI*), formiminoglutamate iminohydrolase (FIGLUase) (*hutF*), and formylglutamate amidohydrolase (FGase) (*hutG*). Genetic studies have indicated that the *hut* gene organization and regulation patterns are diverse in the aforementioned bacterial systems. In *B. subtilis* (15), *hut* genes are arranged as a single operon, with histidine serving as the inducer, while in *S. typhimurium* (25) and *K. aerogenes* (12), the *hut* genes are arranged into two operons whose expression is induced by urocanate. In *Pseudomonas testosteroni* and *P. putida*, all enzymes are induced by urocanate, while FGase is induced by its substrate, formylglutamate (FG), as well (9, 14). The *hut* genes from *S. typhimurium* and *K. aerogenes* have been expressed in *Escherichia coli* (4, 11, 22). Restriction and complementation analysis (2, 3) confirmed the order of the *Salmonella* and *Klebsiella hut* genes that had been revealed by previous genetic studies. A 16-kilobase (kb) *P. putida* DNA fragment containing all of the *hut* genes has been transduced into *E. coli* after cosmid ligation and subsequent packaging of bacteriophage in vitro (7). Activities of the Hut enzymes encoded on this pMC1 plasmid were detected readily in *E. coli*, which lacks its own genes for histidine dissimilation (27).

This study concerns the organization and regulation of the *hut* genes in *P. putida*, as analyzed through the construction of *hut* gene subclones and use of transposable element insertion mutagenesis. These experiments permitted determination of the arrangement of the *hut* genes, the direction and number of the *hut* transcriptional units, and the unique regulation of *hutG* expression of FGase.

MATERIALS AND METHODS

Bacterial strains. *P. putida* PRS1 (ATCC 12633) was the wild-type strain used to study the inducibility of Hut enzymes and to isolate Tn*5* insertion mutants.

E. coli strains used to construct subclones and insertion mutants were: RDP210 (F⁻ *lacY1 leuB6 thi-1 hsdR hsdM rpsL supE44*), RDP186 [F42 *lacI*/Δ(*lac-pro*) *thi-1 recA1 rpsE*], AB1157 (F⁻ *lacY1 galK2 xyl-5 mtl-1 ara-14 proA2 argE3 his-4 leuB6 thr-1 tsx-33 rpsL31 supE44 rpsL*), RDP145 (F⁻ *galK2 hsdR4 endA1 sbcB15 tonA rpsL gyrA recAB*), JM103 [F128 *lacI*^{q} *traD36*/Δ(*lac-pro*) *supE thi rpsL endA sbcB15 hsdR4*], and N4830 [F⁻ *sup his strA recA1 galOP3 ilvA* Δ8 (λ *c*I857 ΔBAM ΔH1)].

LB medium (21) was used as a rich medium for both *E. coli* and *P. putida* strains. Minimal A medium, consisting of 0.1% $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1.05% K_2HPO_4, 0.45% KH_2PO_4, with 0.4% succinate or 0.2% glucose added as the carbon source, was used for growth of *P. putida*. Medium 56 with glucose as the carbon source (21) was used to culture *E. coli* strains.

Recombinant plasmids. The plasmid pMC1, which encodes all *hut* genes from *P. putida* (7), was used to construct several subclones. Construction of pLH1, pLH2, and pMC4 were described by Consevage et al. (7). The plasmids pLH4 and pLH4a were prepared from pLH2 (14). Plasmid pLH17 contained a 3.7-kb *Sal*I fragment of pLH1 in pBR322.

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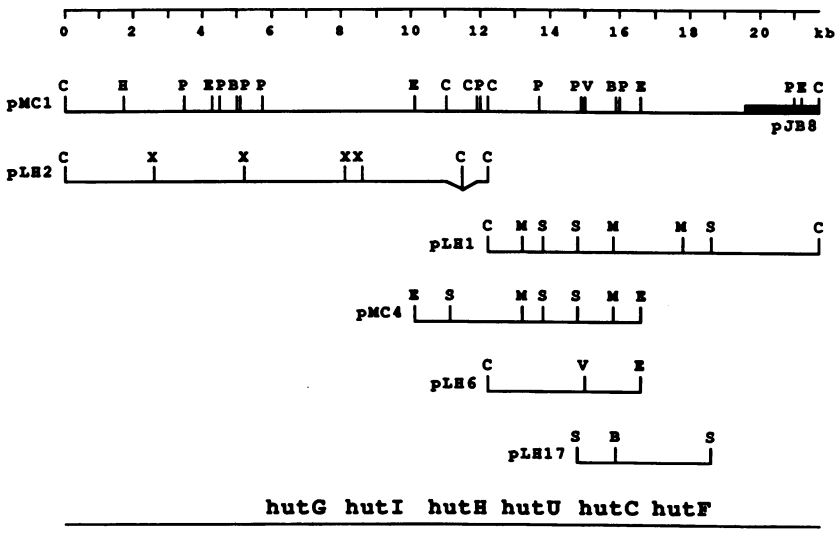

FIG. 1. Plasmids derived from pMC1. Restriction maps of pMC1 and its derivatives are shown. Some restriction sites were determined only on particular plasmids. The maps are arranged to reveal the positions with respect to pMC1. The map for pLH2 also illustrates the deletion of a 0.9-kb *Cla*I fragment. The vectors used in subcloning were pJB8 for pLH1, pBR322 for pLH2 and pLH17, pUC8 for pMC4 and pBEU1 for pLH6. Deduced arrangement of the *hut* genes is included at the bottom. The hatched area shown in pMC1 corresponds to the vector pJB8. Restriction enzyme sites are indicated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Sma*I; P, *Pst*I; S, *Sal*I; V, *Eco*RV; and X, *Xho*I.

Plasmid pLH6 was constructed in the following manner. A 4.1-kb fragment was isolated from pLH1 by *Eco*RI-*Cla*I digestion. The ends of the fragment were filled to form blunt ends by the Klenow fragment of DNA polymerase I (19) and then ligated with *Hin*dIII linkers. The resulting fragment was inserted into the corresponding site of pBEU1. The plasmids described above are shown in Fig. 1.

Plasmids pLH22 and pLH23 were constructed by inserting the 1.9-kb *Sma*I fragment from pLH1 into the *Hpa*I site of pPL-lambda in different orientations. To construct pLH10 and pLH11, a 2.9-kb fragment was isolated from pMC4 by *Sma*I-*Eco*RI digestion. The *Eco*RI end was filled to form a blunt end, and the fragment was then inserted into the *Hpa*I site of pPL-lambda. Plasmids pLH20 and pLH21 were constructed by inserting a 2.9-kb *Xho*I fragment of pLH2 into the *Xho*I site on pPL-lambda in different orientations; this *Xho*I site on the vector was newly created at the original *Hpa*I site by *Xho*I linker ligation. Plasmid pLH19 contained the 1.7-kb *Sal*I-*Eco*RI fragment from pLH1 inserted into the *Hpa*I site of pPL-lambda. Plasmid pLH24 was constructed by inserting the 2.9-kb *Xho*I fragment of pLH2 into the *Sal*I site of pLH19. Orientations of the inserts on the vector were determined by restriction analysis. The construction of the pPL-lambda derivatives described above are shown in Fig. 2.

Recombinant plasmids were transformed into *E. coli* host strains by the RbCl-$CaCl_2$ method (19). Plasmid DNA was isolated by the alkaline detergent method (19). The Tn*5*-containing plasmid pUW964 in *E. coli* HB101 was provided by N. J. Panopoulos, Department of Plant Pathology, University of California, Berkeley.

Purification of DNA fragments and ligation. Restriction enzyme-digested DNA fragments were separated by agarose gel electrophoresis (for ligation, ultrapure agarose [International Biotechnologies, Inc.] was used), and the sizes of the fragments were determined with markers of *Pst*I- or *Hin*dIII-digested lambda DNA. DNA fragments were isolated from gels by the procedure of Benson (1) and redissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. DNA fragments and desired vectors digested with appropriate restriction enzymes were mixed in a 10:1 ratio in 66 mM Tris hydrochloride, pH 7.5, containing 5 mM dithiothreitol, 1 mM ATP, and 5 mM $MgCl_2$, with a final DNA concentration of 10 to 100 μg/ml. A 10-μl volume of the DNA mixture was incubated with 400 U of T4 ligase (New England BioLabs, Inc.) at 16°C for 12 h.

Preparation of crude extracts. Cultures of *P. putida* or *E. coli* clones were grown with vigorous aeration to an A_{600} of 0.9 (8×10^8 cells per ml) and centrifuged at 4°C at 12,000 × *g* for 20 min. The pellet was suspended in 10 ml of 1% NaCl, recentrifuged as before, and finally suspended in 2 ml of 50 mM potassium phosphate, pH 7.5. Disruption was by sonic treatment (Branson Sonifier Model 140E) with a microprobe operated at half-maximal power for four 30-s treatments. Cell debris was removed after centrifugation at 24,000 × *g* for 30 min, and the supernatant was kept on ice until assays were performed.

Enzyme and protein assays. Histidase was assayed spectrophotometrically at 277 nm by the method of Rechler and Tabor (24). Urocanase was assayed as described by George and Phillips (10), except that the final volume was increased to 1.0 ml. FIGLUase was determined colorimetrically and IPAase was measured spectrophotometrically by the methods of Rao and Greenberg (23) and Kimhi and Magasanik (15), respectively. The FGase assay was described by Hu et al. (14). Protein concentration was analyzed by the method of Bradford (5). Specific activities are stated as micromoles of product formed per minute per milligram of protein under the conditions of each assay.

Insertion mutagenesis. Tn*1000* insertion mutagenesis was performed by the method of Guyer (13) with the modifications described by Consevage et al. (7).

Tn*5* insertion mutagenesis of *P. putida* PRS1 was conducted by the method of Weiss et al. (29) with minor modifications. The Tn*5*-containing suicide plasmid pUW964 (unable to replicate in PRS1) was introduced into PRS1 from *E. coli* HB101 by filter mating for 18 h at 28°C. PRS1 mutants with Tn*5* insertions into the chromosome were selected on

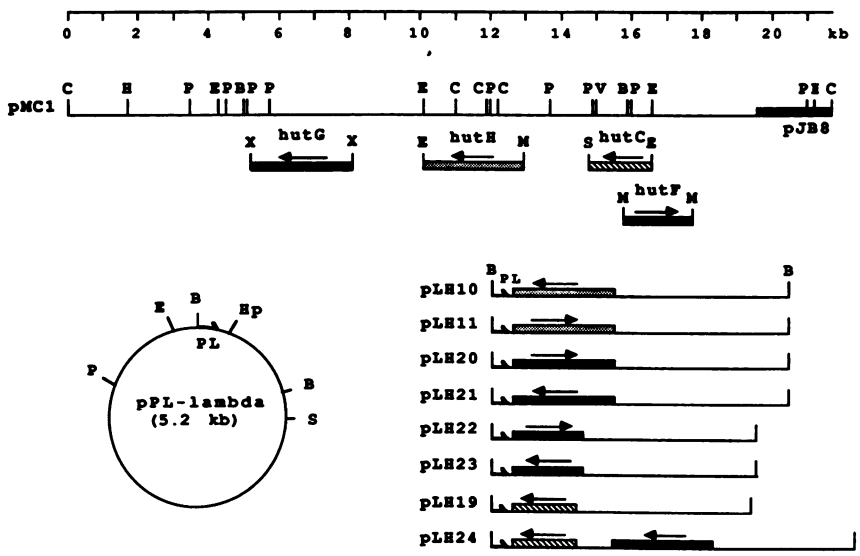

FIG. 2. Cloning of *hut* genes into pPL-lambda. The restriction maps of pMC1 and pPL-lambda are shown on the top and lower left portions of the figure, respectively. The fragments to be cloned into pPL-lambda are depicted with respect to their locations on pMC1. The transcriptional direction of *hutU* is from right to left on pMC1 (7), which is not shown on the figure. The locations of the fragments on pPL-lambda in the form linearized at the *Bam*HI site near the p_L promoter are shown on the lower right portion of the figure. The direction for expression from the p_L promoter is from left to right for the plasmid constructions illustrated in the lower right section of the figure. The large arrow associated with each fragment indicates a relative orientation but corresponds to the deduced direction of transcription. The *hutC* gene is transcribed from right to left on pMC1 and placed in the direction opposite to the p_L promoter in pLH19 and both *hutC* and *hutG* are oriented in the direction opposite to the p_L promoter in pLH24 (see lower right section). Restriction enzyme sites are indicated as follows: C, *Cla*I; H, *Hin*dIII; P, *Pst*I; E, *Eco*RI; B, *Bam*HI; V, *Eco*RV; S, *Sal*I; Hp, *Hpa*I; X, *Xho*I; M, *Sma*I.

LB plates containing 50 μg of kanamycin per ml. Insertions in the *hut* genes were identified by replica plating on minimal A medium with histidine as the carbon source. The phenotypes of the *hut* mutants were confirmed by direct assays of the Hut enzyme activities. The genomic location of the insertions was identified by Southern blot analysis (19). Both prehybridization and hybridization were carried out at 68°C in the buffer containing 6× SSC (1× SCC is 0.15 M NaCl plus 0.015 M sodium citrate) (19), 0.02 M Na_2HPO_4, 0.1% sodium pyrophosphate, 5× Denhardt solution, 0.1% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml. For hybridization, 1×10^6 to 5×10^6 cpm of ^{32}P-labeled probes was used. Genomic DNA was isolated by the phenol extraction procedure of Maniatis et al. (19).

Materials. Unless otherwise mentioned, all biochemicals were obtained from Sigma Chemical Co. Oligonucleotide linkers and restriction enzymes were purchased from either New England BioLabs, Inc., or International Biotechnologies, Inc. Plasmid pPL-lambda and *E. coli* N4830 were purchased from Pharmacia, Inc.

RESULTS

Transcriptional orientation of the *hut* genes. Consevage et al. (7) first studied the arrangement of *hut* genes from *P. putida* by analyzing their expression in *E. coli* strains containing cosmids derived from pMC1. They observed that pLH1 expressed urocanase and FIGLUase from *hutU* and *hutF* genes, respectively; pLH2 expressed IPAase (*hutI*) and FGase (*hutG*), whereas pMC4 expressed histidase (*hutH*) and urocanase. These data led to the conclusion that the *hut* genes are arranged in the order (*hutG*, *hutI*)-*hutH*-*hutU*-*hutF* (Fig. 1). In addition, the *hutC* gene encoding the repressor was found to be located between *hutU* and *hutF*, spanning the *Bam*HI site on the right side of pMC1 as represented in Fig. 1. Using Tn*1000* insertion mutagenesis, Consevage et al. (7) also determined that *hutU* and *hutH* are located in the same transcriptional unit, with *hutU* transcription preceding *hutH* transcription. However, the transcription direction of the remaining genes, the number of transcriptional units, and the regulatory regions of the *hut* genes were not established in that study.

To determine the direction of transcription for *hutI* and *hutG*, pLH2 was mutagenized by Tn*1000* insertion. Expression of these genes in pLH2 is probably from a promoter on the pB'ℭ322 vector (7). The transconjugants with Tn*1000* insertions were first assayed for the loss of IPAase or FGase activity, and the plasmids with insertions in these genes were then isolated and mapped for the locations of the insertions (Fig. 3). Transconjugants 49 and 79, corresponding to insertions approximately 2 kb from the *Bam* HI site, showed a complete loss of the FGase activity but a normal IPAase activity (specific activity, approximately 0.060). On the other hand, the insertions in transconjugants 35, 81, 82, and 87, located 3.2 to 5.0 kb from the *Bam*HI site, eliminated the IPAase activity and reduced but did not completely eliminate the FGase activity (specific activity, 0.004 to 0.006). The results indicated that *hutG* is located adjacent to *hutI* which is itself next to *hutH*. Since the loss of FGase activity did not affect IPAase activity but the loss of IPAase activity reduced FGase activity, it is concluded that *hutI* and *hutG* share a transcriptional unit, with *hutI* preceding *hutG*. The remaining low FGase activity in transconjugants 35, 81, 82, and 87 is apparently due to a weak promoter in front of *hutG*. Further evidence to support this conclusion will be presented shortly.

To determine the transcription direction of *hutF*, *hutG*, *hutH*, and *hutC*, individual genes were cloned into pPL-lambda next to the p_L promoter (Fig. 2). The resulting

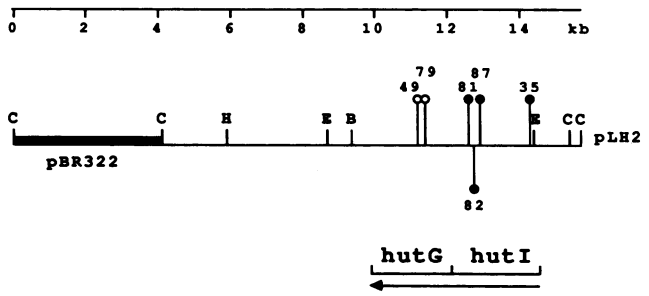

FIG. 3. Tn*1000* insertions in pLH2. The 11.2-kb *Cla*I fragment from pMC1 was cloned into the *Cla*I site of pBR322. The vertical lines mark the position of Tn*1000* inserts in pLH2, as determined by restriction mapping. The vertical lines above and below the horizontal line represent the Tn*1000* inserts in different orientations. The numbers above the vertical lines indicate the mutants with the insertion. The abbreviations of the restriction sites are the same as in Fig. 1. The deduced transcriptional orientation of *hutI* and *hutG* is indicated with the arrow at the bottom. Symbols: ○, no FGase activity but normal IPAase activity; ●, no IPAase activity but low levels of FGase activity. The normal specific activity (micromoles of product formed per minute per milligram of protein) was 0.060 for FGase and 0.065 for IPAase expressed from the pLH2 clone. A specific activity of 0.004 to 0.006 is considered a low activity. Restriction enzyme sites are indicated as follows; C, *Cla*I; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI.

plasmids were transformed into *E. coli* N4830, a lambda lysogen containing a temperature-sensitive *c*I repressor. At higher temperatures at which the *c*I repressor is inactivated, the expression of a gene should be enhanced when it is properly oriented downstream of the p_L promoter but should be inhibited by the strong antisense transcription when the gene is transcribed in the direction opposite to this promoter. Clones containing DNA fragments for expected genes were tested for the activities of the corresponding enzymes (Table 1). Plasmids pLH22 and pLH23 contained the 1.9-kb *Sma*I fragment from pLH1 (Fig. 1) inserted in opposite orientations in pPL-lambda. The bacteria containing each of these two plasmids were assayed for FIGLUase activity. At 28°C, FIGLUase was expressed in both clones, regardless of the insertion orientation. After a temperature shift to 42°C for 2 h, the enzyme activity was enhanced approximately 40-fold for pLH22 and abolished completely for pLH23. Similarly, the 2.9-kb *Xho*I fragment from pLH2 was inserted in both orientations to construct pLH20 and pLH21. The clones were assayed for the expression of *hutG*. FGase was expressed at 28°C independent of orientation, whereas at 42°C, the activity of FGase was enhanced for one orientation (pLH20) but inhibited for the other (pLH21). Therefore the transcriptional orientation of *hutF* is opposite to that of *hutG* (Fig. 2). The orientation of *hutG* determined here is consistent with that determined by Tn*1000* insertion (Fig. 3).

TABLE 1. Expression of the *hut* genes in pPL-lambda derivatives[a]

Plasmid	Inducer	Enzyme	Specific activity at [b]:	
			28°C	42°C
pLH10		Histidase	0.000	0.000
pLH11		Histidase	0.000	1.100
pLH20		FGase	0.043	1.440
pLH21		FGase	0.048	0.021
pLH22		FIGLUase	0.042	1.700
pLH23		FIGLUase	0.041	0.000
pLH24		FGase	0.013	ND[c]
pLH24	Urocanate	FGase	0.040	ND
pLH24	FG	FGase	0.034	ND

[a] *E. coli* N4830 containing pLH24 was grown on 0.2% glucose in minimal A medium plus 15 mM urocanate or FG; when containing other plasmids, this host was grown in LB medium.

[b] Specific activities are given in micromoles of product formed per minute per milligram of protein in the crude extracts. The temperatures refer to growth conditions; assays were all conducted at 30°C. Cultures were grown aerobically at 28°C to an A_{600} of 0.4 to 0.6 and then either continued at this temperature or shifted to 42°C for an additional 2 h prior to harvest. No activities of these enzymes were detected in N4830 without plasmids.

[c] ND, Not done.

To determine the transcription direction of *hutC*, a 1.7-kb *Eco*RI-*Sal*I fragment containing *hutC* (Fig. 2) was inserted into the *Hpa*I site of pPL-lambda in different orientations. The resulting plasmids pLH18 and pLH19 were transformed into *E. coli* N4830. At 28°C, no repressor activity was detected in the strains containing either pLH18 or pLH19, whereas the activity was enhanced in the clone containing pLH18 but not pLH19 at 42°C. Therefore, *hutC* was found to be expressed in the direction opposite to *hutF*, and thus in the same direction as the remaining *hut* genes. The details of the construction of *hutC* clones and an assay of repressor DNA-binding activity will be described in a subsequent paper (L. Hu et al., manuscript in preparation).

Transcriptional units of the *hut* genes. The genes *hutU* and *hutH* were previously shown to be in the same transcriptional unit by Tn*1000* insertion mutagenesis in pMC4 (7). It was also suggested that *hutI* and *hutG* share a transcriptional unit, with *hutI* preceding *hutG*; but there is evidence that *hutG* may have its own promoter, based on Tn*1000* mutagenesis in pLH2 (see above) and on the uncoordinated synthesis of FGase and other Hut enzymes (14). The total number of transcriptional units involved in the *hut* system of *P. putida* is not known, however.

To determine whether expression of *hutI* and *hutG* is related to the *hutUH* transcriptional unit, *P. putida* BJA53a and BJA54 were isolated from PRS1 by Tn*5* insertion mutagenesis. The insertion was localized to the *hutUH* region for each mutant by Southern blot analysis and was found to abolish histidase activity but did not affect the level of urocanase (data not shown). These two mutants were assayed for the activities of IPAase and FGase (Table 2). As a control, PRS1 expressed no detectable IPAase activity and a basal level of FGase activity in the absence of the inducers; the activities of these two enzymes were inducible in the presence of histidine plus urocanate. FGase activity was also induced by FG, although to a lower level. For the *hutH* insertion mutants, the IPAase activity was not detectable in the presence or absence of the inducers, indicating that *hutI* is in the same transcriptional unit as *hutUH*. The FGase activity in these mutants when induced by histidine plus urocanate was approximately 25% of wild-type activity; this indicates clearly that *hutG* can be separately induced, and it

TABLE 2. FGase and IPAase activities in different bacterial strains[a]

Bacterium	Strain	Inducer[b]	Specific activity[c]	
			IPAase	FGase
P. putida	PRS1		0.000	0.005
P. putida	PRS1	His + Uro	0.075	0.060
P. putida	PRS1	FG	0.000	0.030
P. putida	BJA53a		0.000	0.005
P. putida	BJA53a	His + Uro	0.000	0.016
P. putida	BJA53a	FG	0.000	0.029
P. putida	BJA54		0.000	0.005
P. putida	BJA54	His + Uro	0.000	0.015
P. putida	BJA54	FG	0.000	0.028
E. coli	RDP145(pLH4)		0.252	0.154
E. coli	RDP145(pLH4a)		0.000	0.015
E. coli	RDP145		0.000	0.000

[a] The *P. putida* strains were grown in 0.4% succinate medium with the indicated inducers at 30°C. The *E. coli* strains were grown in LB medium at 39°C.

[b] Inducers were added at 15 mM each. Uro, Urocanate.

[c] Specific activities are expressed as described in Table 1, footnote b.

is likely that FGase levels could be even higher were it not for the generally poor ability of urocanate to serve as an inducer (14). However, the 3-fold induction of FGase by urocanate in these mutants versus the 10-fold induction in the wild-type strain (data not shown) suggests that much of the FGase activity in the wild type is contributed by the transcription of the *hutUHI* unit, although the ratio of the transcription from each unit cannot be simply estimated from this result only. In these two mutants the level of FG-induced activity remained unchanged compared with the activity in the wild-type strain. Hence, *hutG* also can be said to have its own transcriptional unit whose expression is controlled by either FG or urocanate.

The transcriptional units of *hutI* and *hutG* were determined by another approach. Plasmids pLH4 and pLH4a contained the 9.6-kb *Cla*I-*Hin*dIII fragment corresponding to *hutI* and *hutG* from pLH2 inserted downstream of the *bla* promoter in pBEU1 (14). The transcriptional orientation of these two genes was consistent with expression from the *bla* promoter in pLH4 but opposite to that of the promoter in pLH4a. Plasmids derived from pBEU1 have a high copy number at a temperature of 39°C (28). As a negative control, the host strain RDP145 itself did not express these two enzymes. High levels of activities for both IPAase and FGase were detected (Table 2) when the genes were under the control of the vector promoter (possibly the *bla* promoter) at 39°C (in pLH4), while no activity of IPAase and only a low level of FGase were detected when the genes were opposite to the *bla* promoter (in pLH4a). These results further indicate that *hutI* and *hutG* share a transcriptional unit whose promoter is not present in this cloned 9.6-kb *Cla*I-*Hin*dIII fragment and that *hutG* also possesses its own weak promoter.

To determine whether expression of *hutC*, *hutF*, and *hutU* is correlated, pLH1, which contained these three genes, was mutagenized by Tn*1000* insertion. The location of each insertion was mapped by *Bam*HI and *Cla*I digestions, and enzyme assays were conducted on the mutants (Fig. 4). The inducibility of the enzyme activities was detected by addition of the gratuitous inducer imidazole propionate, since the product of urocanase would accumulate and is known to be inhibitory to urocanase (20). Three groups of insertions (represented in Fig. 4 by the closed circles, the triangles, and the open circles) eliminated the activities of urocanase, the *hut* repressor (leading to a constitutive expression of the other two enzymes), and FIGLUase, respectively. The locations of the insertions defined the approximate sizes of these three genes. A fourth group, indicated in Fig. 4 by the open squares, did not affect the activities of these three proteins. All but one (insertion 28) are located on the right of pLH1, indicating that this region encoded no *hut* genes.

Inactivation of urocanase by Tn*1000* insertions did not affect FIGLUase, and vice versa. Inactivation of either one of these two enzymes also did not affect the inducibility of the other, indicating that the *hut* repressor activity was not affected. Inactivation of the repressor by insertion in the *hutC* region resulted in the constitutive expression of urocanase and FIGLUase. In other words, inactivation of any one of the three proteins did not alter the activities of the other two. Therefore, the three genes, *hutU*, *hutC*, and *hutF*, must have different transcriptional units for their own expression. The inducible expression of *hutU* and *hutF* also indicates

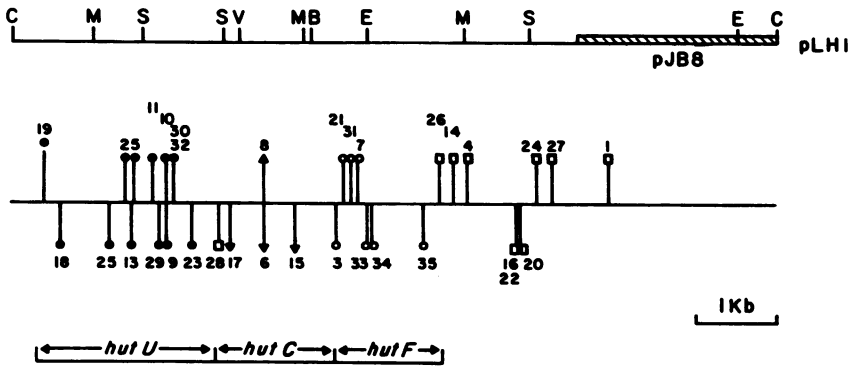

FIG. 4. Tn*1000* insertions in pLH1. The restriction map of pLH1 is shown at the top of the figure. The positions of individual Tn*1000* insertions (designated by the numbers) are shown in the middle by the vertical lines. The lines above and below the horizontal line represent Tn*1000* insertions in opposite orientations. The effect of each individual Tn*1000* insertion is shown by the following symbols: ●, no urocanase activity but normal inducible FIGLUase activity; ▲, normal constitutive activities for both enzymes; ○, no FIGLUase activity but normal inducible urocanase activity; and □, normal inducible activities for both enzymes. The normal activity refers to a specific activity of 0.040 to 0.070 for FIGLUase and 0.020 to 0.040 for urocanase. Constitutive activity is normal activity in the presence or absence of the inducer, imidazole propionate, and inducible activity is normal activity reduced two- to three-fold in the absence of the inducer. Restriction enzyme sites are indicated as follows: C, *Cla*I; M, *Sma*I; S, *Sal*I; V, *Eco*RV; B, *Bam*HI; E, *Eco*RI.

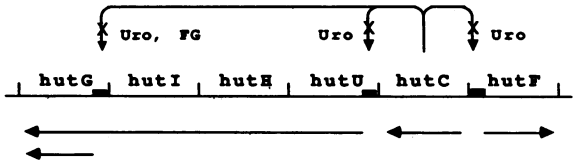

FIG. 5. Organization and regulation pattern of the *hut* genes. The transcriptional units and their expression directions are indicated with the horizontal arrows. The negative regulation of the transcription units for the structural genes by *hutC* is indicated with the vertical arrows. Derepression (induction) by the inducers, urocanate (Uro) and formylglutamate (FG), is shown with an X. The postulated regulatory regions are shown by the small black boxes.

that the transcriptional units are controlled by their own promoter-operator regions.

Identification of multiple regulatory regions controlling *hut* gene expression. Taken together, the data presented thus far indicate the existence of four transcriptional units for the *hut* genes, representing the grouping of *hutUHIG*, *hutG*, *hutF*, and *hutC*. The data also indicate that the expression of the first three transcriptional units (excluding *hutC*) is controlled by the *hutC* product, the repressor.

To establish the presence of promoter-operator regions for the *hut* structural genes, DNA fragments containing each of the expected regulatory regions and its following genes were cloned into the same plasmid with the *hutC* gene, and the inducibility of the enzyme activities were examined. First, pLH24 was constructed with a 1.7-kb *hutC*-containing *Eco*RI-*Sal*I fragment from pLH1 inserted downstream of but opposite to the p_L promoter in pPL-lambda to form pLH19 and a 2.9-kb *hutG*-containing *Xho*I fragment from pLH2 inserted into the *Sal*I site of pLH19 in the same direction as *hutC* (Fig. 2). At 28°C (at which the p_L promoter did not function), *E. coli* containing this plasmid expressed FGase activity that was induced two- to threefold by urocanate or by FG (Table 1). Second, pLH17 was constructed with the 3.7-kb *Sal*I fragment from pLH1 containing *hutF* and *hutC* genes inserted into pBR322 (Fig. 1). *E. coli* RDP145 containing the plasmid expressed FIGLUase activity that was induced fourfold by urocanate and threefold by imidazole propionate. Third, pLH6 was constructed and contained the 4.1-kb *Eco*RI-*Cla*I fragment from pLH1 encoding urocanase and the *hut* repressor inserted into pBEU1 (Fig. 1; see Materials and Methods for details of the construction). *E. coli* RDP145 containing pLH6 expressed urocanase activity when induced by urocanate but showed no detectable urocanase in the absence of inducer (imidazole propionate was not tested). These results indicate the existence of a promoter-operator region for each of the three transcriptional units. More direct evidence for these will be offered in a study of the *hutC* gene product and its recognition requirements (Hu et al., manuscript in preparation). The organization and regulation of the *hut* genes are summarized in Fig. 5. There currently is no indication of control of *hutC* by its product, despite evidence for *hutC* expression independent of the other genes.

Comparison of restriction patterns of pMC1 and PRS1 genomic DNA. Except for the Tn*5* insertion mutagenesis studies, all experiments described above for the study of *hut* gene arrangements were performed with *E. coli* clones and assumed that *hut* genes had not been rearranged during the cloning by cosmid ligation and in vitro packaging of lambda phage (7). To prove this, pMC1 and genomic DNA of *P. putida* PRS1 were isolated, digested with restriction enzymes, and subjected to Southern blot analysis.

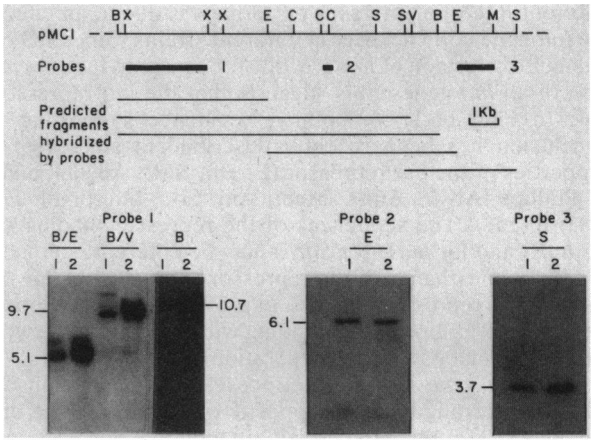

FIG. 6. Comparison of the restriction patterns of pMC1 and PRS1 DNA. DNA of PRS1 (lanes 1) and pMC1 (lanes 2) was isolated, digested with the restriction enzymes indicated above each gel panel, and hybridized with one of the probes in Southern blot analysis. The left set of six lanes was hybridized against probe 1, the middle set of two lanes against probe 2, and the right set of two lanes against probe 3. The size (in kilobases) of each hybridized band is indicated. A restriction map of pMC1 is included to indicate the locations of the probes and the predicted restriction fragments. Restriction enzymes are indicated as follows: B, *Bam*HI; X, *Xho*I; E, *Eco*RI; C, *Cla*I; S, *Sal*I; V, *Eco*RV; M, *Sma*I. The restriction maps for *Xho*I, *Sal*I, and *Sma*I are not complete on pMC1.

Probe 1 (2.9 kb), corresponding to the *hutG* region, hybridized to a 5.1-kb *Bam*HI-*Eco*RI fragment, a 9.7-kb *Bam*HI-*Eco*RV fragment, and a 10.7-kb *Bam*HI fragment, all from pMC1. Probe 2 (0.3 kb) from the *hutH* region and probe 3 (1.2 kb) from the *hutF* region hybridized to a 6.1-kb *Eco*RI fragment and 3.7-kb *Sal*I fragment of pMC1, respectively. By using the same probes, these five fragments were also observed in PRS1 DNA digested with the same enzymes (Fig. 6). Therefore the 13.3-kb-long *hut* gene region in pMC1 between the *Bam*HI and the *Sal*I sites shares the same restriction patterns with PRS1 DNA, indicating that there is no obvious rearrangement in this region during and after cloning.

DISCUSSION

The *hut* genes in *P. putida* are arranged in the order *G-I-H-U-C-F*. There are four transcriptional units for *hut* gene expression: *hutUHIG*, *hutF*, *hutC*, and *hutG*, although the last one may not be a major unit. The direction of *hutF* expression is opposite to that of the other *hut* genes. Expression of all *hut* structural genes (except perhaps *hutC*) is induced by urocanate, whereas that of *hutG* is induced by FG as well; this behavior was detected both in *E. coli* clones and in *P. putida*.

The regulation of *hutG* expression is more complicated than that of the other *hut* genes. It can be transcribed from its own promoter in addition to the promoter preceding *hutU*. The expression of *hutG* in both *P. putida* and the *E. coli hutG-hutC* clone is elevated by addition of urocanate or FG, indicating that there is a repressor-binding region close to the promoter of *hutG*. A low constitutive level of FGase activity in *P. putida* PRS1 (Table 2) suggests that the *hutG* promoter-operator may not be repressed completely in the absence of any inducer. It also appears that the level of the FGase activity varies from *E. coli* clones to *P. putida*, which is probably due to efficiency of promoter recognition by

different RNA polymerases or perhaps is due to an effect of the *hut* gene copy number in different strains. Since FG only induces expression of *hutG*, while urocanate induces expression of all *hut* genes, this suggests that the *hut* repressor is able to bind both compounds. Evidence supporting this conclusion has been recently described in studies of the properties of the *hut* repressor (L. Hu, S. L. Allison, and A. T. Phillips, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H83, p. 158). The sequences of the repressor binding sites for *hutG* and for *hutU* (or *hutF*) may be different, so that the conformation change of the repressor upon FG binding only releases the repression of *hutG* expression. A more thorough study of repressor-inducer binding will be detailed elsewhere (Hu et al., manuscript in preparation).

The *hutF* gene in *P. putida* possesses its own transcriptional unit arranged in the opposite orientation to the other *hut* genes. Coote and Hassall (9) pointed out that in *P. testosteroni*, FIGLUase activity could be induced by urocanate as well as by its substrate, formiminoglutamate (FIGLU). In contrast, *hutF* expression in *P. putida* is only induced by urocanate or its analog, imidazole propionate; similar results were obtained in *E. coli* clones. If the original observation is indeed correct, this would seem to be a species difference in the recognition specificity for the repressor protein.

In *S. typhimurium* (25) and *K. aerogenes* (12), two transcriptional units are expressed in the same direction, and the *hutC* gene shares one of them with two structural genes. It therefore appears that expression of the *hutC* gene is autoregulated and induced by urocanate in *S. typhimurium* and *K. aerogenes*. The regulation of *hutC* in *P. putida* is not clear, although it appears to be expressed as a separate transcriptional unit. Further conclusions on the regulation of *hutC* expression await DNA sequence information.

As revealed by studies on histidine dissimilation, the number of enzymes involved in the Hut pathway varies among organisms, the arrangement of *hut* genes is not uniform, and the regulation of *hut* gene expression is diverse. These observations have raised a very interesting question about how the Hut pathway evolved in *Pseudomonas* species. In *P. putida*, two enzymes, FIGLUase and FGase, instead of one, catalyze the conversion of FIGLU to glutamate. The two genes encoding these enzymes are located at each end of the *hut* gene cluster, and the expression of at least one of these two genes is regulated differently from the other *hut* genes. It is also clear that there is considerable diversity among genera as to the route taken from FIGLU to glutamate. One speculation is that evolution led to modification of the primordial gene(s) used for this process and as a result, several solutions arose. In the case of *Klebsiella* and related genera, we now see a single gene encoding an enzyme which catalyzes the conversion of FIGLU to glutamate plus formamide. Mammalian systems appear to have followed a related path to glutamate, although in these systems the formimino group of FIGLU is transferred to tetrahydrofolate rather than to water. *Pseudomonas* species followed a different evolutionary path to arrive at a two-enzyme system. It would seem most reasonable to propose that a duplication of part of the *hut* gene region encoding FIGLU-utilizing activity generated *hutF* and a regulatory region which was similar to that for *hutU* expression. Since the *hutF* gene product, FIGLUase, only converts FIGLU into FG plus 1 mol of ammonia, the bacteria were forced to recruit the gene for another hydrolytic activity, now seen as FGase, to degrade FG to glutamate. The ancestor of *hutF* was later deleted when the five-enzyme system was established. Some of the properties of FGase are consistent with its recruitment as an *N*-acetylglutamate hydrolase capable of acting on FG (14).

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