

Cloning and Expression in *Escherichia coli* of Histidine Utilization Genes from *Pseudomonas putida*[†]

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Received 21 August 1984/Accepted 20 January 1985

A library of the *Pseudomonas putida* chromosome, prepared through the use of the cosmid pJB8 ligated to a partial *Sau3A* digest of bacterial DNA, followed by in vitro packaging into bacteriophage lambda particles, was used to construct a strain of *Escherichia coli* which contained the genes for histidine utilization. This isolate produced a repressor product and all five enzymes required in *Pseudomonas* spp. for histidine dissimilation, whereas none of these could be detected in the nontransduced parent *E. coli* strain. When this transductant was grown on various media containing histidine or urocanate as the inducer, it was observed that production of the cloned histidine degradative enzymes was influenced somewhat by the choice of nitrogen source used but not by the carbon source. The recombinant cosmid was isolated and found to consist of 21.1 kilobase pairs of DNA, with approximately 16 kilobase pairs derived from *Pseudomonas* DNA and the remainder being from the pJB8 vector. Digestion of this insert DNA with *EcoRI* provided a 6.1-kilobase-pair fragment which, upon ligation in pUC8 and transformation into an *E. coli* host, was found to encode histidine ammonia-lyase and urocanase. The inducible nature of this production indicated that the *hut* repressor gene also was present on this fragment. Insertional inactivation of the histidine ammonia-lyase and urocanase genes by the gamma-delta transposon has permitted location of these structural genes and has provided evidence that transcription proceeds from urocanase through histidine ammonia-lyase. Mapping of the 16-kilobase-pair *Pseudomonas* DNA segment with restriction enzymes and subcloning of additional portions, one of which contained the gene for formiminoglutamate hydrolase and another that could constitutively express activities for both imidazolone propionate hydrolase and formylglutamate hydrolase, has provided evidence for the organization of all *hut* genes.

Several of the histidine utilization enzymes from *Pseudomonas* species have novel coenzymes or display unusual coenzymatic features. Histidine ammonia-lyase, the initial enzyme in the pathway, contains dehydroalanine which functions in catalysis by an α,β -elimination mechanism which is not well understood (14). Urocanase, the second enzyme, contains one residue of tightly bound NAD whose role in catalysis appears to involve covalent attachment to the urocanate molecule rather than intermediate conversion to NADH (23). As part of our effort in studying these enzymes from *Pseudomonas putida*, we wished to examine their gene structure and control at the DNA level to obtain further information on the structural properties of both enzymes.

In *Pseudomonas* spp., unlike many other species of bacteria, the pathway leading to glutamate from histidine requires five catalytic steps instead of four (21). After the formation of urocanate and ammonia from histidine by histidine ammonia-lyase (histidase), urocanase action on urocanate produces the unstable intermediate imidazolone propionate (IPA) which is converted by a hydrolase to formiminoglutamate (FIGLU). FIGLU in most organisms is converted into glutamate plus a 1-carbon unit (either formiminotetrahydrofolate or simply formamide), but in *Pseudomonas* spp., FIGLU is instead hydrolytically deaminated by FIGLU hydrolase to produce formylglutamate (FG). FG is then hydrolyzed by FG hydrolase to produce glutamate and

formate. Initial mapping of the *P. putida* histidine utilization (*hut*) genes by Leidigh and Wheelis (20) established the existence of a clustered set of genes controlled by a single repressor locus (*hutC*). Recent data with *P. putida* have suggested a likely arrangement of the *hut* genes into at least two transcriptional units, with genes for urocanase (*hutU*) and histidase (*hutH*) being coordinately controlled, whereas the FG hydrolase gene (*hutG*) can be expressed separately. Control of expression of genes for FIGLU hydrolase (*hutF*) and IPA hydrolase (*hutI*) was not explicitly established. All units can be induced by urocanate, but FG hydrolase also is specifically induced by its substrate (A. T. Phillips and L. M. Mulfinger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K27, p. 141). Similar results have been reported for *Pseudomonas testosteroni* (6).

The *hut* genes from *Salmonella typhimurium* and *Klebsiella aerogenes* have previously been isolated on F' episomes and can be expressed in *Escherichia coli* as well as in each other (9, 10, 26); *E. coli* itself apparently contains no *hut* genes (37). Urocanate has been found to be the actual inducer for the *hut* genes in *S. typhimurium* (13), *K. aerogenes* (34), and *Pseudomonas aeruginosa* (21). Furthermore, growth under limiting nitrogen conditions or under conditions which would promote high intracellular levels of cyclic AMP derepressed the *hut* genes of *K. aerogenes* (29). Mapping studies with *S. typhimurium* (35) and *K. aerogenes* (2, 3, 11) revealed that the *hut* genes are organized into two transcriptional units with separate promoters. In contrast, the *hut* genes in *Bacillus subtilis* were found to function as a single transcriptional unit (18).

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[†] Paper no. 7015 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

This report describes the cloning of the *hut* genes from *P. putida* into *E. coli* and presents data on the regulation of expression of these genes under various growth conditions. The results of subcloning experiments and mapping of some of the genes by $\gamma\delta$ -insertional mutagenesis clarify the organization of the *P. putida hut* operon and permit a comparison with that found in *S. typhimurium* and *K. aerogenes*.

MATERIALS AND METHODS

Genotypes of principal *E. coli* strains used. Strain RDP210 is a derivative of strain C600 and has the genotype F⁻ *lacY1 leuB6 thi-1 hsdR hsdM rpsL supE44*. Strain RDP186 is F42 *lac* Δ (*lac-pro*) *thi-1 recA1 rpsE*. Strain AB1157 is F⁻ *lacY1 galK2 xyl-5 mtl-1 ara-14 proA2 argE3 his-4 leuB6 thr-1 tsx-33 rpsL31 supE44 rpsL*. Strain JM103 is F128 *lacI^r traD36* Δ (*lac-pro*) *supE thi rpsL endA sbcB15 hsdR4*. Strain MWC1 consists of the recombinant cosmid pMC1 in strain RDP210; its construction is described below.

Restriction enzyme digestions and ligations. All restriction enzymes were purchased from New England BioLabs, Inc. Restriction digestions were conducted at 37°C with the appropriate buffers described by Maniatis et al. (22). The digested DNA was analyzed by agarose gel electrophoresis, and the size of fragments was determined by comparison to lambda DNA digested with either *Pst*I or *Hind*III. Ligations were performed as described by Maniatis et al. (22).

Isolation of plasmid DNA. Cultures of *E. coli* were grown in Luria broth (LB [24]) containing the appropriate antibiotic, plasmid synthesis was amplified with chloramphenicol, and DNA was isolated by the procedures of Ish-Horowitz and Burke (16) and Maniatis et al. (22). Large-scale preparations usually were purified by CsCl gradient centrifugation at 44,000 rpm for 40 h in a Beckman 70.1 Ti rotor.

Isolation of high-molecular-weight DNA from *P. putida*. A 2-liter culture of *P. putida* ATCC 12633 was grown at 30°C in LB to a final A₆₆₀ of 0.6. The cells were harvested and treated with 60 ml of 50 mM Tris-hydrochloride (pH 8.0) containing 25% (wt/vol) sucrose and 1.5 mg of lysozyme per ml. After 20 min at 25°C, 20 ml of 0.25 M EDTA (pH 8.0) was added, and incubation was continued for an additional 20 min. To obtain complete lysis, 0.2 ml of a 25% (wt/vol) sodium dodecyl sulfate solution was added, followed by a 5-min incubation at 57°C. The lysed culture was treated with 8 mg of RNase for 60 min at 37°C, followed by a similar treatment with 0.1 mg of proteinase K, and then the DNA was purified by centrifugation in a 58% (wt/wt) CsCl gradient. DNA isolated in this manner was judged to be at least 60 kilobase pairs (kbp) when compared with lambda DNA on 0.5% agarose gel electrophoresis.

Preparation of a cosmid library of DNA from *P. putida*. Cosmid pJB8 was prepared by the method of Maniatis et al. (22). High-molecular-weight *P. putida* DNA was digested to various extents with *Sau*3A, and the digested products were combined and sized on a 5 to 40% sucrose gradient. Fractions from the gradient which contained DNA of approximately 30 to 40 kbp were pooled, ethanol precipitated, and ligated to the *Bam*HI site of pJB8 by the method of Ish-Horowitz and Burke (16). This ligated mixture was used for in vitro packaging by means of extracts prepared from lambda-defective mutants BHB2688 and BHB2690 (7). The packaged cosmids were transduced into the recipient *E. coli* RDP210 and plated on LB-ampicillin plates (50 μ g/ml). Ampicillin-resistant transductants were isolated and tested for their ability to utilize histidine as the sole carbon source.

Subcloning of pMC1. pUC8, pBR322, or pBR325 was used as the recipient vector for subcloning experiments. pMC1

was digested with enzymes indicated in the text, and the resulting fragments were ligated into the appropriate sites of the above vectors. When pUC8 was used, strain JM103 served as the host for transformation. After transformation, cells were plated on LB-ampicillin plates with 2.5 ml of 0.6% plating agar containing 0.1 ml of 2% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside and 40 μ l of 100 mM isopropyl-1-thio- β -D-galactopyranoside. White colonies were isolated and analyzed for the desired fragments. Strain RDP210 was used for subcloning experiments with pBR322 and pBR325. All transformations were performed by the CaCl₂-RbCl method of Kushner (19).

Gamma-delta insertion mutagenesis. Insertion mutagenesis with $\gamma\delta$ was performed by the method of Guyer (12) with several modifications. Plasmids containing the fragment of interest were first placed in *E. coli* RDP186 by transformation. RDP186 is a Str^s *recA* strain containing F42 *lac*. The F42 *lac* episome carries a functional $\gamma\delta$ which allows it to conduct other compatible plasmids to a conjugational recipient strain as transient $\gamma\delta$ insertion cointegrates. Cultures of the Str^r recipient strain (AB1157) and the plasmid-containing derivatives of strain RDP186 were grown to about 10⁸ cells per ml in LB at 37°C, and equal volumes of the two cultures were mixed for mating. After 2 h of mating at 37°C, streptomycin was added to a final concentration of 100 μ g/ml, and the mixed culture was incubated at 37°C for another 2 h to allow the killing of the Str^s donor cells. The cells then were pelleted and washed twice with minimal salts to remove any β -lactamase released by the donor cells. Cells resuspended in one-half of the original volume of minimal salts then were plated on LB plates containing 100 μ g of streptomycin per ml and 500 μ g of ampicillin per ml. This selected for cells of the recipient strain that received the Ap^r plasmid from the donor strain by F42 *lac*-mediated conduction. Transconjugants from each mating were tested for the loss of expression of enzymes encoded for by the original cloned DNA fragment. Only one $\gamma\delta$ insertion isolate was kept from each mating.

Enzyme and protein assays. Cells were grown either in LB medium or in minimal medium 56 consisting of 0.26% KH₂PO₄, 0.43% Na₂HPO₄, 0.01% MgSO₄, and 2.5 μ g of thiamin per ml, to which carbon and nitrogen sources were added to a final concentration of 0.5 and 0.2%, respectively. Amino acid supplements were added at a final concentration of 50 μ g/ml as required. Antibiotics were added as needed at the following final concentration (micrograms per milliliter): ampicillin, 50; tetracycline, 10; and chloramphenicol, 10. Cultures were grown at 30°C to a Klett reading of 150 (42 filter).

Cell extracts were prepared from 150 ml of late-log-phase cultures by sonification in 1 ml of 50 mM potassium phosphate (pH 7.3). The cellular debris was pelleted by centrifugation, and the supernatant was retained for assays. Histidase was assayed spectrophotometrically at 277 nm by the method of Rechler and Tabor (30). Urocanase was assayed essentially as described by George and Phillips (8), except the assay was conducted in a final volume of 1.0 ml. The FIGLU hydrolase assay contained 0.2 M Tris-hydrochloride (pH 8.5), 10 mM reduced glutathione, 10 μ M MnCl₂, and crude extract in a total volume of 0.1 ml. The mixture was preincubated at 30°C for 10 min, followed by initiation of the assay by the addition of 0.7 μ mol of Na FIGLU in a volume of 0.1 ml. After 30 min at 30°C, the reaction was terminated by the addition of 0.8 ml of saturated sodium tetraborate, and the amount of FIGLU remaining was determined colorimetrically by the method of Tabor and Rabinowitz (36). FG

TABLE 1. Inducibility of the various *hut* clones^a

| Plasmid | Carbon source | Inducer ^b | Sp act ^c | | | | |
|-----------------------|---------------|----------------------|---------------------|-----------|----------|-------|--------|
| | | | Histidase | Urocanase | FIGLUase | FGase | IPAase |
| pMC1 | LB | - | 0.021 | 0.012 | 0.051 | 0.003 | ND |
| | LB | + | 0.036 | 0.018 | 0.073 | 0.010 | ND |
| | Glucose | - | 0.042 | 0.018 | 0.087 | 0.006 | ND |
| | Glucose | + | 0.095 | 0.040 | 0.137 | 0.020 | 0.060 |
| | Histidine | + | 0.107 | 0.060 | 0.171 | 0.023 | ND |
| pMC2 | Glucose | - | 0.000 | 0.000 | 0.140 | 0.000 | ND |
| | Glucose | + | 0.000 | 0.000 | 0.140 | 0.000 | 0.000 |
| pMC3 | Glucose | - | ND | ND | ND | 0.033 | ND |
| | Glucose | + | 0.083 | 0.037 | 0.000 | 0.035 | 0.043 |
| pMC4 | Glucose | - | 0.026 | 0.013 | 0.000 | 0.000 | 0.000 |
| | Glucose | + | 0.058 | 0.042 | 0.000 | 0.000 | 0.000 |
| pLH1 | Glucose | - | 0.000 | 0.000 | 0.034 | 0.000 | 0.000 |
| | Glucose | + | 0.000 | 0.000 | 0.044 | 0.000 | 0.000 |
| pLH2 | Glucose | - | 0.000 | 0.000 | 0.000 | 0.086 | 0.201 |
| | Glucose | + | 0.000 | 0.000 | 0.000 | 0.086 | 0.210 |
| Host strain only | Glucose | + | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| <i>P. putida</i> only | Glucose | - | 0.004 | 0.002 | 0.011 | 0.000 | ND |
| | Glucose | + | 0.158 | 0.062 | 0.184 | 0.030 | ND |

^a The host strain used for plasmids was RDP210. Enzyme assays were conducted as described in the text. Medium was either LB or minimal with (NH₄)₂SO₄ as the nitrogen source plus leucine at 50 µg/ml for strain RDP210; leucine was omitted for experiments involving *P. putida*.

^b The inducer was histidine (0.2%) plus urocanate (0.2%).

^c Specific activities are given in terms of micromoles of product formed or substrate degraded per minute per milligram. The sensitivity limit on all assays was 0.5 to 2.0 nmol min⁻¹ mg⁻¹. Enzymes: FIGLUase, FIGLU hydrolase; FGase, FG hydrolase; IPAase, IPA hydrolase. ND, Not determined.

hydrolase was assayed radioisotopically by measuring the [¹⁴C]glutamate formed from formyl-[¹⁴C]glutamate; details of this assay will be reported elsewhere (L. Hu and A. T. Phillips, manuscript in preparation). IPA hydrolase was assayed spectrophotometrically by following the disappearance of IPA at 260 nm, as described by Kimhi and Magasanik (18). Protein was determined by the method of Bradford (1) with ovalbumin as the standard.

Rapid screening of subclones for expression of *hut* genes. Four of the five *hut* gene products could be assayed with 1 ml of an overnight culture grown in LB medium containing either 0.5% histidine or urocanate and the appropriate antibiotic. Cells from approximately 1 ml of culture were resuspended in 100 µl of the appropriate assay buffer (described above) containing the desired substrate and 0.025% cetyltrimethylammonium bromide. After 2 to 4 h at room temperature, the cells were removed by centrifugation, and the supernatant was retained. A positive test for histidase or urocanase was the appearance or disappearance, respectively, of UV-absorbing urocanate when 5 µl of the supernatant was spotted on filter paper and viewed under short-wave UV. The presence of FIGLU hydrolase was indicated by the disappearance of the brown color due to FIGLU when 40 µl of the supernatant was assayed for FIGLU (36). FG hydrolase activity was indicated by a significant radioactivity remaining after washing a small circle of cation-exchange resin paper onto which 60 µl of the supernatant from a FG hydrolase assay mixture had been previously spotted.

Protein blotting. The protein blotting procedure used was that described by Renart and Sandoval (31). Antihistidase antibodies from rabbit were produced and purified as reported by Consevage and Phillips (5).

RESULTS

Expression of the *hut* region in *E. coli*. The *hut* genes of *P. putida* were selected from a cosmid library of *P. putida* DNA that had been packaged into lambda particles in vitro. The library was screened by transduction into *E. coli* RDP210. Since *E. coli* normally is unable to utilize histidine as a carbon source, presumably due to the absence of the *hut* enzymes, identification of cells containing the *hut* genes linked to pJB8 was based on selecting an ampicillin-resistant transductant capable of growth on histidine as carbon source. A transductant which had the desired phenotype was isolated and designated strain MWC1. Growth of this organism improved rapidly upon subculturing with histidine as carbon source; evidence to be presented later suggests that this growth improvement may have been due to deletion of a nonessential portion of the recombinant cosmid. Assays for the *hut* enzymes were conducted on strain MWC1 as well as on the parental strain RDP210. All enzymes were detected in strain MWC1 containing the cosmid pMC1, whereas none could be found in the nontransduced parent (Table 1).

The specific activities of most of the histidine-utilizing enzymes in strain MWC1 were approximately half of those found in *P. putida* under nearly identical growth conditions and inducer concentrations (Table 1). Strain MWC1 grew relatively well on histidine and (NH₄)₂SO₄ minimal medium with a doubling time of 150 min. This is in accord with the 80- to 90-min generation time for *P. putida* under similar conditions. The recombinant plasmid pMC1 was isolated from this histidine-utilizing clone and was found to be 21.1 kbp, with approximately 16 kbp of *Pseudomonas* DNA. A restriction map of pMC1 was constructed for sites cleaved

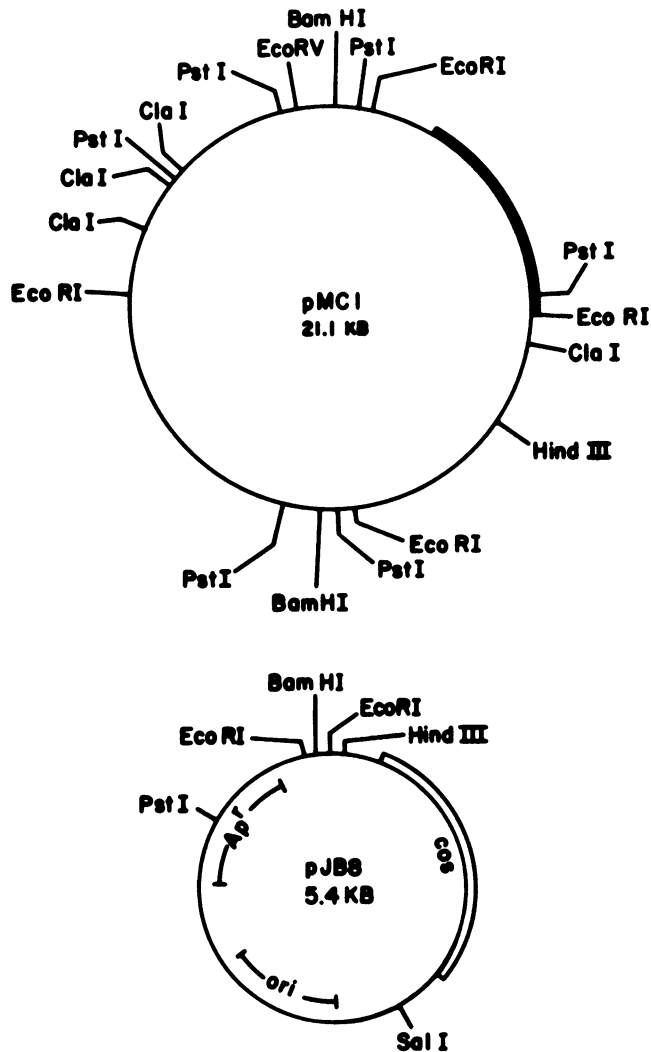


FIG. 1. Restriction maps of pMC1 and pJB8. The top restriction map is of the cosmid which contains the *P. putida hut* genes. The thick line represents that portion of DNA derived from the vector pJB8. The thin line designates approximately 16.1 kbp of *Pseudomonas* DNA. The bottom map shows major restriction sites and other features of the cosmid vector pJB8 (16). KB, Kilobase pairs.

by *Bam*HI, *Pst*I, *Eco*RI, *Cla*I, *Eco*RV, and *Hind*III. *Bam*HI digestion split the cosmid into two essentially equal parts of 10.5 and 10.6 kbp, whereas an *Eco*RI digest yielded four fragments between 4.8 and 6.1 kbp (Fig. 1). A *Cla*I digest cleaved the cosmid into two large fragments of approximately 10 kbp each and two smaller fragments.

Properties of plasmids derived from pMC1. To determine the portion of pMC1 which corresponded to pJB8 and to map the relative position of the various *hut* genes, several derivatives of pMC1 were constructed. Since the vector pJB8 has two *Eco*RI sites surrounding the *Bam*HI cloning site (22), we attempted to recover from pMC1 a self-replicating *Eco*RI fragment which conferred ampicillin resistance. This resulted in the isolation of a 5-kbp plasmid, designated pMC7, which contained single *Eco*RI, *Sal*I, and *Pst*I sites (Fig. 2). A comparison of pMC7 with pJB8 showed that pMC7 was 400 base pairs smaller and lacked not only the *Bam*HI cloning site but also in the *Hind*III site of pJB8

which is located near one of the two *Eco*RI sites (see Fig. 1 for sites on pJB8). This difference indicates that part of pJB8 had been deleted in forming pMC1. Since pMC7 conferred ampicillin resistance, the β -lactamase gene would have to be intact and would include the *Pst*I restriction site. Furthermore, the *cos* region, located near the *Sal*I restriction site, was still present in a functional form since pMC1 could be mobilized, presumably as a dimer, by helper phage transduction. The region shown to the left of the *cos* site, which normally would contain the *Hind*III site of pJB8, must contain the junction of pJB8 and *Pseudomonas* DNA created by a deletion event. Thus, it would appear that the 21-kbp pMC1 resulted from a deletion event which occurred on an originally larger cosmid; this deletion placed one *Eco*RI site located within *Pseudomonas* DNA near the position expected for the original pair of *Eco*RI sites on pJB8. Similar deletions have been seen to occur with *P. aeruginosa* DNA in cosmids transduced into *E. coli* (4).

A *Bam*HI or *Cla*I digestion of pMC1 cleaved the plasmid into several large segments, each of which represents approximately 45 to 50% of pMC1 (Fig. 1). Thus, subclones of the four major segments could be of significant value in determining the position of the *hut* genes. Furthermore, the entire DNA region which gave rise to pMC7 is also contained within one of the *Bam*HI fragments and one of the large *Cla*I fragments. Therefore, it should be possible to isolate these two pieces as self-replicating subclones. Some of the other nonreplicating fragments could be isolated by ligating a *Cla*I or *Bam*HI digest of pMC1 into the appropriate sites of either pBR322 or pBR325.

To isolate the self-replicating subclones, pMC1 was digested with either *Cla*I or *Bam*HI, religated under dilute DNA conditions, and placed into strain RDP210 by transformation. This permitted the isolation of two types of ampicillin-resistant transformants. The first type, derived from the *Bam*HI digest, contained a 10.6-kbp plasmid (designated pMC2) with a single *Bam*HI and *Hind*III site; the second type, obtained from the *Cla*I religation, contained a 9.6-kbp plasmid (pLH1) with the unique *Eco*RV site of pMC1. Restriction maps of both plasmids were constructed and compared (Fig. 2). Assays of cells containing either plasmid for the enzymatic activities of the *hut* gene products revealed only FIGLU hydrolase activity (Table 1). Therefore, this gene would obviously have to be located somewhere in the 5.7-kbp region of DNA common to both plasmids. The length of DNA to the right of the β -lactamase gene in pLH1 is less than 500 nucleotides and would not contain enough sequence to code for the FIGLU hydrolase gene. As a result, this gene must be located in the region between the left of *cos* and the *Bam*HI site on pMC2 or pLH1 (Fig. 2). Preliminary $\gamma\delta$ insertion mutagenesis experiments (data not presented) also indicate that the FIGLU hydrolase gene resides in this area.

The other *Bam*HI fragment was isolated by cloning a *Bam*HI digest of pMC1 into the *Bam*HI site of pBR325. After ligation, transformation of strain RDP210 was performed, and chloramphenicol-resistant, ampicillin-resistant, tetracycline-sensitive transformants were isolated. These transformants were found to contain a 10.5-kbp insert. The plasmid was designated pMC3 and coded for histidase, urocanase, IPA hydrolase, and FG hydrolase activities but not FIGLU hydrolase activity (Table 1). A restriction analysis of pMC3 was performed (Fig. 2).

The other large *Cla*I fragment containing the single *Hind*III site was inserted into the *Cla*I site of pBR322. Assays conducted on strain RDP210 containing this recombinant

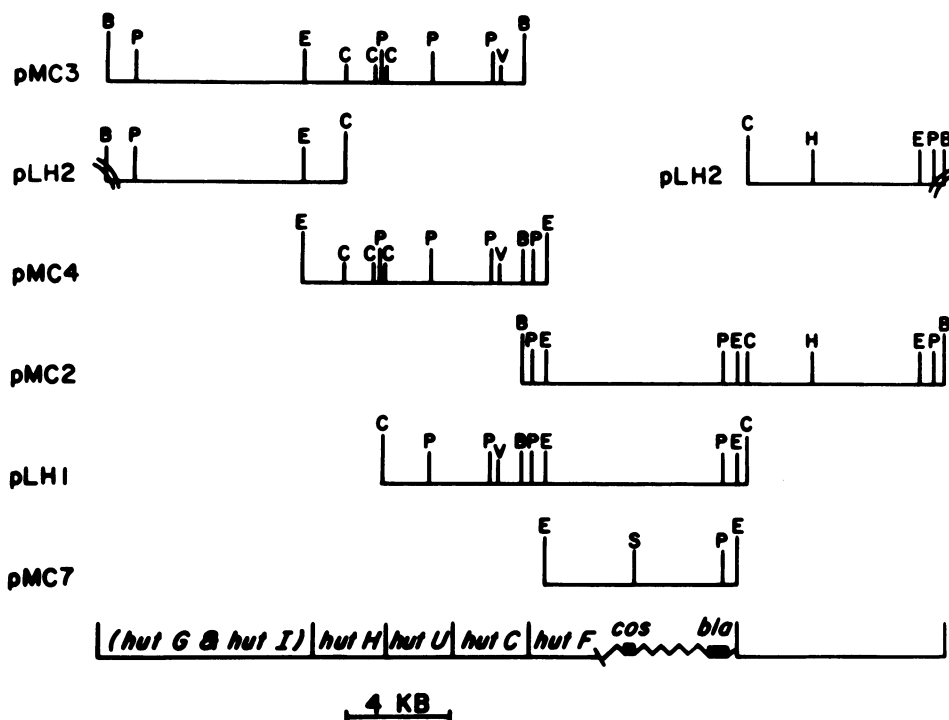


FIG. 2. Derivatives of pMC1. Restriction maps of several derivatives of pMC1 and the regions corresponding to the various *hut* genes are shown. The subclones are arranged to reveal their approximate position with respect to pMC1. Vectors used in subcloning (pUC8, pBR322, or pBR325) are not shown. Abbreviations: B, *Bam*HI; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; V, *Eco*RV; *hut*C, repressor protein; *hut*F, FIGLU hydrolase; *hut*G, FG hydrolase; *hut*H, histidase; *hut*I, IPA hydrolase; *hut*U, urocanase; *bla*, β -lactamase. The wavy line shown at the bottom refers to the location of DNA derived from pJB8. The slash to the left of *cos* represents the junction of pJB8 and *Pseudomonas* DNA. The order of the *hut*G and *hut*I genes is not known and could be reversed. The structure of pLH2 is shown divided at its *Bam*HI site to depict its relationship to pMC2 and pMC3. Kb, Kilobase pairs.

plasmid (pLH2) revealed the presence of IPA hydrolase and FG hydrolase activities. Since pMC3 also coded for these two activities, the genes would have to be located in the region between the *Cl*aI and *Bam*HI sites which are common to both plasmids (Fig. 2).

The combined data from pMC3 and pLH2 indicated that the histidase and urocanase genes probably were located between the regions identified as containing the IPA-FG hydrolase genes and FIGLU hydrolase gene. To support this conclusion, an *Eco*RI digest of pMC1 was ligated into the *Eco*RI site of pUC8 and placed into strain JM103 to isolate a strain which contained histidase or urocanase or both activities. A 6.1-kbp insert in pUC8 was isolated which produced both histidase and urocanase. This plasmid, pMC4 (Fig. 2), contained the *Eco*RI fragment which divided between pMC2 and pMC3. This confirmed that these genes were contained in that part of the *Eco*RI fragment found entirely in pMC3. A detailed restriction map of pMC4 was constructed (Fig. 3A), and the exact location of these genes was mapped through $\gamma\delta$ insertional mutagenesis (see below).

Physical mapping of histidase and urocanase genes in pMC4. The regions which code for the histidase and urocanase genes in pMC4 were mapped by using $\gamma\delta$ insertion mutagenesis. Plasmid pMC4 was transformed into strain RDP186 which was streptomycin sensitive and which contained an F42 *lac* episome. Mating between this strain and a streptomycin-resistant, ampicillin-sensitive recipient (strain AB1157) allowed the isolation of transconjugants which were resistant to both antibiotics. After screening 120 of these, 13 were found to be deficient in histidase activity due to $\gamma\delta$ insertion. Further enzymatic analysis revealed that

three classes of histidase-negative strains existed. The first class had normal levels of urocanase activity but contained no detectable histidase. The second class exhibited neither activity, whereas the third class consisted of those that had no urocanase but had slightly above background levels of histidase. Plasmid DNA was isolated from all three classes, and the position of the $\gamma\delta$ element was mapped by analyzing *Bam*HI and *Eco*RI digestion patterns (Fig. 3B). The mapping and enzymatic data were indicative of a single transcriptional unit consisting of urocanase and histidase. In this unit the gene for histidase would be located downstream and the promoter would be located upstream with respect to urocanase. The slight histidase activity seen in the third class of insertions probably was the result of insertion into urocanase at sites far from histidase, with some secondary promoter sites being used to provide expression of histidase.

Based on the location of the $\gamma\delta$ inserts and subunit molecular weights of 55,000 for urocanase (8) and 56,000 for histidase (5), the region which codes for these two enzymes was identified. We estimated that 1.6 kbp of DNA was needed to code for each enzyme. The NH₂-terminus region of urocanase was assumed to lie just beyond the first insert that was of the class 2 type (no detectable urocanase activity), whereas the COOH terminus of histidase was assumed to be located outside of the class 1 insert most distant from urocanase. Based on these assumptions, the maximum range for these two genes could be determined (Fig. 3B). A 0.3-kbp uncertainty exists for the dividing point between the two genes due to the ambiguity in location of the urocanase NH₂-terminus region and the COOH terminus for histidase.

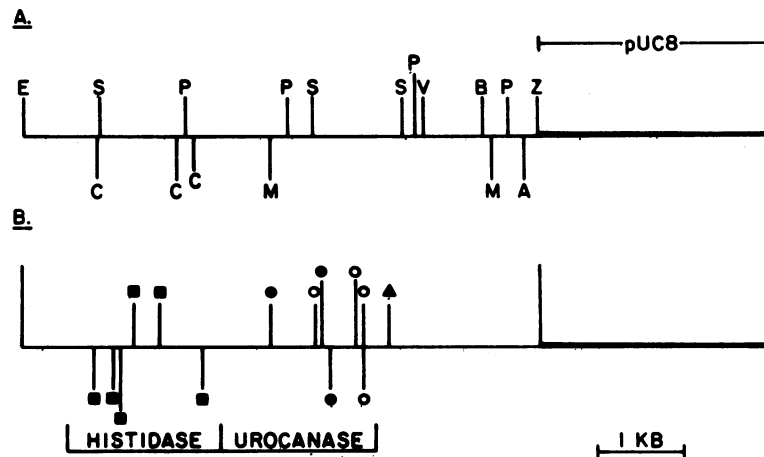


FIG. 3. (A) Restriction map of pMC4. This 6.1-kbp *EcoRI* fragment from the 16.1-kbp *Pseudomonas* insert was cloned into the *EcoRI* site of pUC8. This fragment codes for histidase and urocanase. Abbreviations: A, *AvaI*; B, *BamHI*; C, *ClaI*; E, *EcoRI*; M, *SmaI*; P, *PstI*; S, *SalI*; V, *EcoRV*; Z, *EcoRI* site plus the rest of the *lacZ* region of pUC8. (B) Mapped position of $\gamma\delta$ insertions. The lines mark the position of $\gamma\delta$ inserts into pMC4. Lines above the horizontal represent $\gamma\delta$ inserts in one orientation, whereas lines below designate the opposite orientation. Specific activity (SA) is presented in terms of micromoles per minute per milligram as described in the text. Symbols: ■, inserts with no histidase activity (SA, 0.000) and normal urocanase activity (SA, 0.019 to 0.030); ●, inserts with no histidase activity (SA, 0.000) and no urocanase activity (SA, 0.000); ○, inserts with background histidase levels (SA, 0.002 to 0.006) and no urocanase activity (SA, 0.000); ▲, insert with both histidase (SA, 0.040) and urocanase activity (SA, 0.032). KB, Kilobase pairs.

Demonstration of the origin of cloned histidase. To provide evidence that the products of *hut* expression were derived from *Pseudomonas* genes, protein blots for histidase were performed. The *E. coli* parental strain, RDP210, showed no detectable bands when examined on sodium dodecyl sulfate gels with antibody directed towards histidase purified from *P. putida*. The *E. coli* histidine utilization-positive transductant, strain MWC1, produced a single band when exposed to the same antihistidase antibodies, and this corresponded to the major component present in a sample of crude extract of induced *P. putida* (Fig. 4). The presence of this immunologically detectable material in strain MWC1 confirmed the expression of the *Pseudomonas* histidase gene in *E. coli*. Control experiments to evaluate the specificity of the antihistidase antibody revealed that this material was able to neutralize *Pseudomonas cepacia* 249 histidase 90% relative to *P. putida* histidase, whereas histidase from *K. pneumoniae* ATCC 13773 was 19% neutralized, and histidase from *S. typhimurium* ATCC 14028 was unaffected by the antibody.

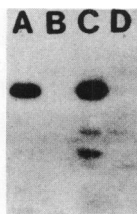


FIG. 4. Western blot of histidase. An autoradiogram of the protein blot which was probed with antibody directed towards histidase purified from *P. putida* is shown. The columns are designated: A, strain RDP210 containing pMC1; B, strain RDP210; C, *P. putida* which was induced for the production of the *hut* genes; D, noninduced *P. putida*. Crude extracts tested were prepared from cells grown on LB plus 0.2% urocanate except for the noninduced *P. putida* culture which instead was grown on a succinate minimal medium with $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source.

Effect of various media on expression of the cloned genes. Previous reports of poor expression of *Pseudomonas* genes cloned into an *E. coli* background have been attributed to the poor recognition of a *Pseudomonas* promoter (33). However, these genes could be expressed at a higher efficiency if they were placed behind an *E. coli* promoter. Since the expression of the cloned *hut* genes gave rise to enzyme levels which were often within a factor of 2 of the normal *P. putida* levels, either as a result of efficient expression or high plasmid copy number, several experiments were conducted to determine whether these genes were being expressed from a plasmid promoter. Induction of the *hut* operon in *P. putida* occurs maximally during growth on limiting nitrogen, e.g., with cytidine as nitrogen source, and with the addition of the inducer, urocanate, or histidine (15). In contrast to *hut* operons from other sources, the *P. putida* enzyme levels were not subject to a glucose-linked catabolite repression but instead were affected by a cyclic AMP-independent repression when succinate served as carbon source (28). Therefore, the levels of the cloned gene products were monitored under a variety of growth conditions to determine whether they responded in a typical *Pseudomonas* fashion.

To produce various degrees of catabolite repression, strain RDP210 containing pMC1 was grown on either glucose or glycerol (27). When the levels of the *hut* enzymes were determined, no effect was noted due to changes in these conditions (Table 2); control experiments demonstrated that β -galactosidase production in strain RDP210 was affected by catabolite repression (data not presented). In addition, the expression of the genes was not altered when the carbon source was changed to succinate. However, the levels of enzymes were affected to a limited extent by the type of nitrogen source provided. Specific activities increased when the nitrogen source was changed from ammonium to a poorer source, represented by proline or nitrate in *E. coli*. It was interesting to note that cytidine, an extremely poor nitrogen source in *P. putida* that results in increased expression of *hut* genes, had no effect on *hut* expression in *E. coli*, whereas proline, a relatively good nitrogen source for *P.*

putida, increased the level of *hut* enzymes. Nitrate, a poor source of nitrogen in both species, resulted in a twofold elevation of the *hut* enzymes in *E. coli*. As expected, strain MWC1 grew slowly and *hut* enzyme induction was greatest when urocanate was used as sole nitrogen source; this is probably due to the poor uptake of urocanate which creates a nitrogen-deficient condition.

To substantiate these findings further, the levels of enzymes were measured under induced and noninduced conditions in strain RDP210 containing derivatives of pMC1. Induction was clearly evident for histidase and urocanase in strain RDP210 containing pMC4, whereas one or more hydrolase genes were found to be expressed constitutively in strain RDP210 harboring pMC2, pMC3, and pLH2 (Table 1). However, the FIGLU hydrolase gene was slightly but reproducibly inducible in pLH1. This suggests the presence of *hut* repressor gene in pLH1 and indicates that the repressor gene is located within the region common to pLH1 and pMC4 (Fig. 2). Furthermore, the combined $\gamma\delta$ insertional mutagenesis data and induction studies indicate that the expression of the *hut* genes in the case of pMC1 and pMC4 was due to the recognition of a *Pseudomonas* promoter and operator rather than a plasmid promoter.

DISCUSSION

The *Pseudomonas* genes for histidine degradation were cloned and expressed in *E. coli* at a relatively high level. When *E. coli* RDP210 containing pMC1 was grown on various carbon and nitrogen sources, the levels of enzymes indicated that the *hut* genes in an *E. coli* background were being regulated in a fashion consistent with control by nitrogen availability but not by carbon-linked catabolite repression. Pahel and co-workers (25) and Rothman et al. (32) have documented that the *hut* operon of *K. aerogenes* is under the control of the positive regulatory elements of the *gln* operon in both *K. aerogenes* and *E. coli*. Previous data by Janssen et al. showed that histidase levels in *Pseudomonas aeruginosa* paralleled the expression of glutamine synthetase and urease (17). These findings suggest that the *hut* genes from *P. putida* might be controlled by some of the same heterologous elements which regulate the *gln* operon. However, what constitutes a poor nitrogen source in *P. putida*, and thereby results in *gln* and *hut* activation, can be different from that in *E. coli*. The present data do indicate that specificity for control of the cloned *hut* genes by nitrogen source was determined by the *E. coli* host, although the extent of regulation was not large under the conditions examined.

Urocanate was able to induce all the *hut* enzymes coded for by pMC1, an indication that the gene for the *hut* repressor as well as the five structural genes required for histidine catabolism were contained in this cosmid. The efficiency of *hut* regulation by the repressor was considerably less for the cloned gene than for *P. putida* (Table 1). We are currently unable to state whether this difference is a reflection of a higher level of endogenous inducer or whether expression of the repressor gene is reduced. Induction experiments with the plasmids derived from pMC1 demonstrated that the gene products of pMC4 and pLH1 were inducible, whereas the FIGLU hydrolase gene of pMC2 and FG hydrolase and IPA hydrolase genes of pLH2 were expressed constitutively. These data indicated that the repressor protein was coded for somewhere on the 6.1-kbp insert in pMC4. Although the position of the repressor was not mapped, it is unlikely to overlap the 3-kbp area mapped

TABLE 2. Enzymatic levels of histidase and FIGLU hydrolase in strain MWC1 grown on various carbon and nitrogen sources^a

| Source ^b | | Sp act ^c | | Generation time (hr) |
|---------------------|---|---------------------|-----------------|----------------------|
| Carbon | Nitrogen | Histidase | FIGLU hydrolase | |
| Glycerol | (NH ₄) ₂ SO ₄ | 0.10 | 0.13 | 2.0 |
| | Glutamine | 0.10 | 0.13 | 2.0 |
| | Cytidine | 0.12 | 0.10 | 2.5 |
| | Proline | 0.14 | 0.15 | 3.0 |
| | NaNO ₃ | 0.23 | 0.20 | 8.0 |
| | Urocanate only | 0.30 | 0.21 | 9.2 |
| Glucose | (NH ₄) ₂ SO ₄ | 0.10 | 0.13 | 1.8 |
| | Glutamine | 0.10 | 0.13 | 1.8 |
| | Cytidine | 0.13 | 0.13 | 2.2 |
| | Proline | 0.18 | 0.14 | 4.0 |
| | NaNO ₃ | 0.23 | 0.19 | 7.8 |
| | Urocanate only | 0.29 | 0.20 | 9.1 |
| Succinate | (NH ₄) ₂ SO ₄ | 0.11 | 0.09 | 2.0 |
| | Glutamine | 0.11 | 0.09 | 2.0 |
| | Cytidine | 0.12 | 0.10 | 2.1 |
| | Proline | 0.15 | 0.15 | 3.7 |
| | NaNO ₃ | 0.29 | 0.19 | 9.0 |
| | Urocanate only | 0.30 | 0.20 | 9.1 |

^a Strain MWC1 containing the *hut* genes on pMC1 was grown on minimal salts plus 50 μ g of both leucine and ampicillin per ml as described in the text.

^b Final carbon and nitrogen concentrations were 0.5 and 0.2%, respectively. Urocanate, 0.2%, was added to all cultures.

^c Specific activity was measured in terms of micromoles per minute per milligram of protein.

for histidase and urocanase. Therefore, the repressor would most likely be located in the 2-kbp region upstream from the urocanase gene which is in common with pLH1 (Fig. 2).

The $\gamma\delta$ insertion mutagenesis studies showed that histidase and urocanase genes were transcribed as a unit in pMC4. Insertion upstream of this unit did not affect the levels of activity for the enzymes produced from these two genes, although the question of whether the repressor gene function was altered by insertion has not been addressed. These results indicated that the promoter and operator regions were likely contained in this fragment next to the urocanase gene and suggest that these *hut* genes were being transcribed from *Pseudomonas* promoters.

Several groups have shown two transcriptional units for the *hut* genes in *S. typhimurium* (35) and in *K. aerogenes* (3, 11). Since *Pseudomonas* spp. differ from these two organisms in the presence of an additional catalytic step in this pathway, the gene arrangement might be somewhat different. Enzymatic data for *P. putida* as well as *P. testosteroni* (3) suggested two to three transcriptional units that may be similar to the ones described for *K. aerogenes* (3, 11). The limited mapping data confirm these results and showed that urocanase and histidase genes were transcribed as a unit in right-to-left orientation (Fig. 3), whereas the genes for FIGLU hydrolase and repressor gene were located 2 to 6 kbp upstream. Since the histidase-urocanase unit functions by itself without the hydrolase gene and vice versa, these likely constitute two separate transcriptional units. The first unit would probably consist of FIGLU hydrolase plus repressor, whereas the second unit would contain genes for urocanase and histidase. Attempts to define the direction of transcription in the first unit are currently in progress. Assignment of the repressor gene (*hutC*) to the FIGLU hydrolase transcriptional unit is quite tentative and is based only on the observation that $\gamma\delta$

insertions located upstream of the urocanase gene (*hutU*) had no adverse effect on expression of *hutU* and *hutH*. This must be confirmed by further studies.

Expression of IPA hydrolase and FG hydrolase genes in pLH2 points to their location downstream from the histidase gene, but it should be noted that the level of this expression was abnormally high, raising the possibility that in this plasmid these genes were being expressed from a non-*Pseudomonas* promoter (the *tet* promoter from pBR322 is one possibility). Thus these data cannot be used in support of a transcriptional unit for *hutI* and *hutG* independent of that encompassing histidase and urocanase genes. However, as mentioned earlier, there is evidence that the FG hydrolase gene (*hutG*) may be independently regulated in *P. putida*. Synthesis of this enzyme is not fully coordinated with that of histidase or urocanase, and it can be independently induced by formylglutamate. This would require a third transcriptional unit but one which is limited to the gene for FG hydrolase. These initial findings imply that the *P. putida* genes are arranged slightly differently from those in *S. typhimurium* and *K. aerogenes*; in these organisms the genes for IPA hydrolase and FIGLU hydrolase make up one transcriptional unit, with genes for histidase and urocanase comprising another (3). Additional experiments are in progress to map the exact location of all three hydrolase genes and the regulatory regions of the various transcriptional units.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AM-13198 from the National Institutes of Health and by project 2684 from the Pennsylvania Agricultural Experiment Station.

The assistance of Lan Hu in plasmid construction and enzyme assays and Kathy Rockey in manuscript preparation is gratefully acknowledged.

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