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Identification of Multiple Repressor Recognition Sites in the *hut* System of *Pseudomonas putida*

LAN HU,† STEVEN L. ALLISON, AND ALLEN T. PHILLIPS*

Department of Molecular and Cell Biology, Althouse Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802

Received 21 October 1988/Accepted 2 May 1989

The *hutC* gene in *Pseudomonas putida* encodes a repressor protein that negatively regulates the expression of all *hut* genes. We have overexpressed this cloned *hutC* gene in *Escherichia coli* to identify *P. putida hut* regions that could specifically bind the repressor. Ten restriction fragments, some of which were partially overlapping and spanned the coding portions of the *P. putida hut* region, were labeled and tested for their ability to recognize repressor in a filter binding assay. This procedure identified three binding sites, thus supporting previous indications that there were multiple operons. A 1.0-kilobase-pair *Sal*I restriction fragment contained the operator region for the *hutUHIG* operon, whereas a 1.9-kilobase-pair *Sma*I fragment contained the *hutF* operator. A 2.9-kilobase-pair *Xho*I segment appeared to contain the third operator, corresponding to a separate and perhaps little used control region for *hutG* expression only. The addition of urocanate, the normal inducer, caused dissociation of all operator-repressor complexes, whereas *N*-formylglutamate, capable of specifically inducing expression of the *hutG* gene, inhibited binding only of repressor to fragments containing that gene. Formylglutamate did not affect the action of urocanate on the repressor-*hutUHIG* operator complex, indicating that it binds to a site separate from urocanate on the repressor. DNA footprinting and gel retardation analyses were used to locate more precisely the operator for the *hutUHIG* operon. A roughly 40-base-pair portion was identified which contained a 16-base-pair region of dyad symmetry located near the transcription initiation site for this operon.

Expression of the histidine utilization (*hut*) genes in *Pseudomonas* species is predominantly under negative control by a single repressor, the *hutC* gene product (10); similar negative regulation exists in *Klebsiella aerogenes* and *Salmonella typhimurium* (5, 11, 18), whereas only positive regulation of *hut* gene expression is believed to occur in *Bacillus subtilis* (15). We have recently shown (7) that the *hut* genes of *Pseudomonas putida* are organized into three major transcriptional units, *hutUHIG*, *hutC*, and *hutF*, for which the corresponding gene products are urocanase (*hutU*), histidase (*hutH*), imidazolone propionate hydrolase (*hutI*), formylglutamate amidohydrolase (*hutG*), and formiminoglutamate iminohydrolase (*hutF*). However, expression of the *hutG* gene can be induced by either urocanate or *N*-formylglutamate (FG), whereas the other four *hut* genes are induced only by urocanate (6). Thus it appears that the *hutG* gene, in addition to being expressed as part of the *hutUHIG* operon, may have its own promoter and operator region. To explain this dual transcriptional mechanism, we proposed that the *hutC* gene product in *P. putida* is a bifunctional repressor molecule that can bind to either of these inducers (7). Furthermore, for FG to inhibit binding of repressor to the *hutG* operator only, and thus selectively allow *hutG* expression, the interaction of the repressor with the *hutG* operator site should be different from its interaction with the *hutUHIG* and *hutF* operators.

Consevage et al. (4) previously cloned all of the genes necessary for histidine utilization by *P. putida* onto a cosmid vector and expressed these in *Escherichia coli*. In the present work we overexpressed the *hutC* gene of *P. putida* in *E. coli* to obtain sufficient quantities of repressor protein for its partial purification, and we studied its binding to potential operator-containing restriction fragments and the influence of inducers on this binding. In addition to identification of three binding regions for the repressor, we also obtained detailed information about the structure of the *hutUHIG* operator by gel retardation and DNase I footprint analysis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and bacterial hosts referred to in this study have been described previously (4, 7). Figure 1 illustrates the relationships of the cloned portions of each plasmid to the overall *hut* gene arrangement. Vector pPL-lambda and its host, *E. coli* N4830, were purchased from Pharmacia-LKB Biotechnology, Inc. Plasmid pLH18 contained the *hutC* gene in a 1.7-kilobase-pair (kbp) *Eco*RI-*Sal*I fragment that had been filled to produce blunt ends and cloned into the *Hpa*I site on pPL-lambda. Plasmid pLH19 contained the same fragment but in the opposite orientation with respect to the p_L promoter, as shown by restriction patterns obtained upon *Bam*HI digestion.

Transformants were grown at 28°C in either Luria broth (LB) or 0.2% glucose–salts A minimal medium (14) supplemented with histidine, leucine, and valine at 40 μg/ml each. To overexpress the *hutC* gene, the culture was grown at 28°C to an A_{600} of 0.4 and then shifted to 42°C for 2 h.

Repressor-DNA binding assay. Repressor-DNA binding was conducted by the method of Johnson et al. (8) with minor modifications. A 20-μl volume of *hut* repressor protein, appropriately diluted in TEDG buffer (10 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA, 0.1 mM dithiothreitol, 100 mM NaCl, 10% glycerol), was added to 0.3 ml of assay buffer (10 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA, 10 mM $MgCl_2$, 50 mM KCl, 100 μg of bovine serum

* Corresponding author.
† Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA 02115.

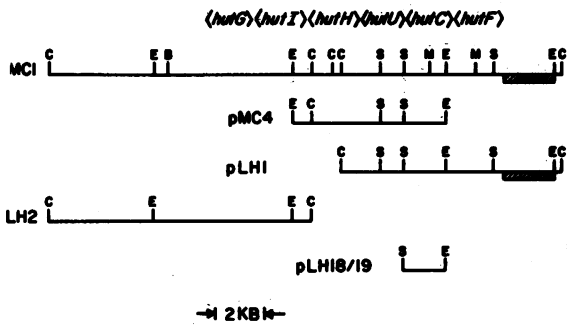

FIG. 1. Partial restriction maps of the principal plasmids used in this study and their relationship to the *hut* region from *P. putida*. The vector portion (▨) is illustrated only for pMC1 and pLH1. The actual coding region sizes are known only for the histidase and urocanase genes, and thus the gene locations are approximate. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; M, *Sma*I; S, *Sal*I. Not all sites that have been identified are shown.

albumin per ml, 5% dimethyl sulfoxide, 50 μg of DNA from *E. coli* [Sigma Chemical Co.; type VIII] per ml, 3 × to 10^5 to 7 × 10^5 cpm of labeled DNA prepared by nick translation with [α-^{32}P]dCTP per ml) (13). When urocanate or other inducers were required, they were added in a volume of 10 μl, and the amount of TEDG buffer was correspondingly reduced. Unless stated otherwise, the urocanate concentration was 5 mM. The total mixture was incubated at room temperature for 20 min and then loaded onto a nitrocellulose filter (Millipore Corp.; HA filter, 0.45-μm pore size) that had been presoaked in wash buffer (identical to assay buffer but without radiolabeled DNA, bovine serum albumin, and *E. coli* DNA). The solution was slowly drawn through the filter with an aspirator, and the filter was rinsed with 0.5 ml of wash buffer before scintillation counting in 10 ml of Liquiscint solution (National Diagnostics Corp.). Under these conditions, less than 0.5% of the labeled DNA was retained on the filter in the absence of repressor.

The 1.0-kbp *Sal*I fragment derived from pMC4 (Fig. 1) was purified by electrophoresis (2) and used routinely for the binding assay. It was normally labeled to a level of 5 × 10^7 cpm/μg of DNA, and thus each assay contained 5 ng of this fragment. The specific activity of the repressor was defined as nanograms of this DNA bound per milligram of protein under the assay conditions. Protein was determined by the method of Bradford (3) with ovalbumin as the standard.

Preparation of crude extracts. Cultures (5 ml) were grown at 28°C in LB medium containing 50 μg of ampicillin per ml. At an A_{600} of 0.4, the temperature was raised to 42°C and held there for 2 h. Cells were then centrifuged rapidly and suspended in 0.5 ml of cold TEDG buffer. Disruption was by sonic treatment (Branson model 450 sonifier) with a microprobe operated at one-third power and 40% duty cycle for four 30-s treatments at 6°C. Cell debris was removed by centrifugation at 13,000 × *g* for 10 min, and the supernatant portion was kept on ice until assays were performed.

For isolation of repressor on a larger scale, cells were grown as above in a 100-liter fermenter, the temperature was shifted for 2 h, and cells were frozen after harvest. A 40-g quantity of cells was thawed in 80 ml of TEDG buffer and sonicated at 40% of full power and 50% duty cycle with a standard probe for four 4-min intervals at 6°C.

Gel retardation analysis. Various amounts of partially purified repressor (0 to 1 μg of protein) were mixed with 5 ng of 3′-labeled 380-bp *Bss*HII-*Sau*3A DNA fragment plus 20 μl of a solution containing 10 mM Tris hydrochloride (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 50 μg of bovine serum albumin per ml, and 10 μg of *E. coli* DNA. When required, urocanate was added to a concentration of 5 mM. The final volume was 30 μl. After samples were incubated at room temperature for 20 min, 1 μl of gel loading buffer (30% sucrose, 0.2% bromophenol blue) was added to the mixture, and electrophoretic analysis was carried out on a 4% polyacrylamide gel (25:1, acrylamide-bisacrylamide). A buffer containing 0.1 M Tris hydrochloride [pH 8.3], 0.1 M boric acid, and 2 mM EDTA was used in the gel and as the running buffer. After pre-electrophoresis for 10 min at 20 V/cm, the samples were loaded onto the gel and subjected to electrophoresis for 10 min at 20 V/cm and then 10 V/cm for 1 to 3 h, depending on the size of the DNA fragment (9, 12). The gel was subsequently dried and autoradiographed by exposure on Kodak XAR-5 film at −70°C. DNA labeling was achieved with DNA polymerase I (Klenow fragment) and [α-^{32}P]dCTP (13).

Footprint analysis. DNase I footprint analysis was performed with a procedure modified from that of Schmitz and Galas (17). The binding mixture consisted of 0 to 4 μg of repressor protein purified through the DNA-cellulose step, 5 ng of 3′-labeled DNA (10,000 cpm), and 50 μl of a solution containing 10 mM Tris hydrochloride (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, and 10 μg of *E. coli* DNA per ml; the final reaction volume was 60 μl. After incubation at room temperature for 20 min, 10 ng of DNase I (Bethesda Research Laboratories, Inc.) was added from a stock solution containing 0.5 μg of DNase I per ml in 10 mM Tris hydrochloride (pH 7.9) and 50 mM $CaCl_2$. The DNase I digestion was allowed to continue for 60 s, and the reaction was terminated by adding 50 μl of a stop solution composed of 1% sodium dodecyl sulfate, 20 mM EDTA, 0.2 M NaCl, and 200 μg of tRNA per ml. The DNA was further purified by extraction with an equal volume of phenol-chloroform and precipitated with 0.3 M sodium acetate (pH 5.2) and 3 volumes of absolute ethanol at −70°C for 30 min. The DNA was recovered by centrifugation for 10 min in a microcentrifuge, washed with 75% ethanol, and dried in a vacuum concentrator. The pellet was dissolved in 2 μl of loading buffer and subjected to electrophoresis at 1,600 V on an 8% polyacrylamide–urea sequencing gel (13).

Materials. Unless otherwise specified, chemicals were purchased from Sigma. FG and imidazole propionate were synthesized by methods previously described (16, 19). Electrophoresis reagents were obtained from Bio-Rad Laboratories. Restriction enzymes, DNA ligase, and DNA polymerase I (Klenow fragment) were purchased from either New England BioLabs or International Biotechnologies, Inc. Labeled materials were purchased from DuPont, NEN Research Products. LB medium was obtained from GIBCO Laboratories.

RESULTS

Expression of *hutC* subclones. Insertion mutagenesis with Tn*1000* has established the approximate location of the *hutC* gene as being between the genes for urocanase (*hutU*) and formiminoglutamate iminohydrolase (*hutF*) (7). By using this information, pLH18 and pLH19 were constructed to place the *hutC* gene under control of the lambda p_L promoter. To determine whether *E. coli* N4830 containing pLH18 or pLH19 expressed the *hutC* gene from this promoter, both clones were grown at 28°C and then shifted to 42°C for 1, 2, and 4 h. Crude extracts from the cells were assayed for

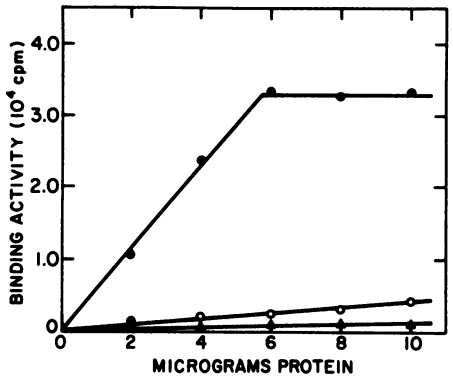

FIG. 2. DNA-binding activity in extracts of cells containing the cloned *hutC* gene. Cultures of N4830 containing either pLH18 or pLH19 were grown as described in the text and heat treated for 2 h to permit expression from the p_L promoter, and extracts were prepared. Filter binding assays were conducted with the amount of protein as indicated and 10^5 cpm of the 1.0-kbp *Sal*I fragment from pMC4 (4) as labeled DNA. Other details of the assays are included in Materials and Methods. Symbols: ●, N4830(pLH18) with no urocanate; ○, N4830(pLH18) with 5 mM urocanate; ▲, N4830 (pLH19) with no urocanate.

DNA-binding activity by the nitrocellulose filter method. The binding assay used a 1.0-kbp *Sal*I fragment (Fig. 1) that contained the N-terminal portion of *hutU* and the upstream flanking region believed to contain the regulatory region for *hutU* expression (7; Allison and Phillips, manuscript in preparation).

Neither N4830(pLH18) nor N4830(pLH19) extracts prepared from cells kept at 28°C showed any specific DNA-binding activity. For cells shifted to 42°C for 2 h, binding activity was observed with N4830(pLH18) extracts and increased to a maximum when roughly one-third of the input radioactivity was bound (Fig. 2). Activity at 1 and 4 h was somewhat reduced compared with that at 2 h of heat induction. In addition, this binding activity was abolished when the inducer, urocanate, was added in the binding assay. No binding activity was detected in N4830(pLH19) extracts prepared from cells after a 2-h temperature induction (Fig. 2). These results indicate that pLH18 encodes a protein that exhibits the specific DNA-binding properties expected for the *hut* repressor, and that the orientation of the insert in pLH18 allows the gene to be expressed in a temperature-inducible manner from the p_L promoter. This finding is consistent with earlier results of Hu and Phillips (7), which indicated that the *hutC* gene is transcribed in the opposite direction of *hutF* but in the same direction as the remaining *hut* structural genes.

The repressor was partially purified from extracts of heat-induced N4830(pLH18) by using the standard DNA-binding filter assay to monitor activity in the presence and absence of urocanate. Cells harvested after heat induction were disrupted by sonication, followed by centrifugation at 13,000 × *g* for 2 h. The repressor was purified from the supernatant by precipitation with 0.6% polyethylenimine and subsequent elution from the pellet with 0.6 M KCl, followed by gel filtration on a 400-ml column of Sephacryl S-200 equilibrated with TEDG buffer and chromatography on a 50-ml column of calf thymus DNA-cellulose (Sigma), eluting with a 300-ml linear gradient from 0.1 to 1.0 M NaCl in TEDG buffer. Although these steps enriched the preparation 78-fold with a recovery of over 80%, the resulting material was not homogeneous when analyzed by sodium dodecyl sulfate-gel electrophoresis; three distinct components of molecular weights 70,000, 67,000, and 27,000 were present, and their separation has not yet been achieved. Although we have not established conclusively that any of these components corresponds to the *hutC* product, evidence from DNA sequence analysis of the *hutC* region points to a likely value of 27,000 for the subunit molecular weight of the repressor (S. L. Allison and A. T. Phillips, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H84, p. 159). Details of the purification and properties of the repressor protein will be described later when a complete purification has been accomplished. All of the following experiments utilized this partially purified repressor preparation.

Identification of repressor-binding regions. Earlier studies on the organization and regulation of the *hut* genes in *P. putida* (7) indicated, on the basis of insertional inactivation and subcloning, that there existed three, and possibly four, regulatory regions for *hut* gene expression; these correspond to transcriptional units for *hutF*, *hutC*, and *hutUHIG* and a secondary one for *hutG* located near the gene. To identify these regions directly, 10 restriction fragments, some of which contained overlapping regions, were isolated from previously constructed plasmids pLH1, pLH2, and pMC4 (4). The locations of these fragments on the map of pMC1 are shown in Fig. 3. Each fragment was labeled by nick translation and assayed for repressor-binding activity. The repressor was able to bind five of the 10 fragments tested (Fig. 3). The binding activity to these fragments was significantly reduced upon the inclusion of 5 mM urocanate in the assay, indicating specificity in the recognition. Alignment of the fragments along the map of pMC1 permitted localization of distinct repressor-binding regions preceding *hutF* (and *hutC*), *hutU*, and *hutG*, in support of our earlier findings (7). We did not find restriction fragments that could distinguish between the possibility of one or two repressor-binding sites located between the *hutC* and *hutF* genes. In fact, since these are apparently transcribed in opposite directions, it is probable that a single repressor-binding site located between the two genes can control transcription of both.

To compare the repressor-binding affinities of the different operator regions, the binding activity values were normalized to counts per minute per kilobase of DNA. As revealed by the binding curves, the repressor showed similar affinity for the *hutUHIG* operator (the 1.0-kbp *Sal*I fragment) and the *hutF-hutC* operator (the 1.7-kbp *Sal*I-*Eco*RI and 1.9-kbp *Sma*I fragments), but the normalized binding activities for the 5.6-kbp *Eco*RI and 2.9-kbp *Xho*I fragments were only 5 and 2.5%, respectively, of values obtained for *hutUHIG*, suggesting that the *hutG* operator might be a weaker binding site. The binding activities to the *hutF-hutC* and *hutUHIG* operators were abolished by urocanate, whereas that to *hutG* was only reduced to 50%.

Inhibition of repressor-operator binding by inducers. Since repressor-operator binding activity was affected by the addition of urocanate, several other potential inducers were tested for their ability to alter the repressor-operator binding equilibrium. In these experiments one of three different operator-containing DNA fragments was incubated with repressor and various amounts (0.02 to 20 mM) of the compound to be tested. The concentrations of DNA and repressor were held constant, with the amount of repressor present being less than saturating in each case. The equilibrium amount of repressor-operator complex was then measured by the filter assay, and the relative binding activity (ratio of complex detected in the absence and presence of

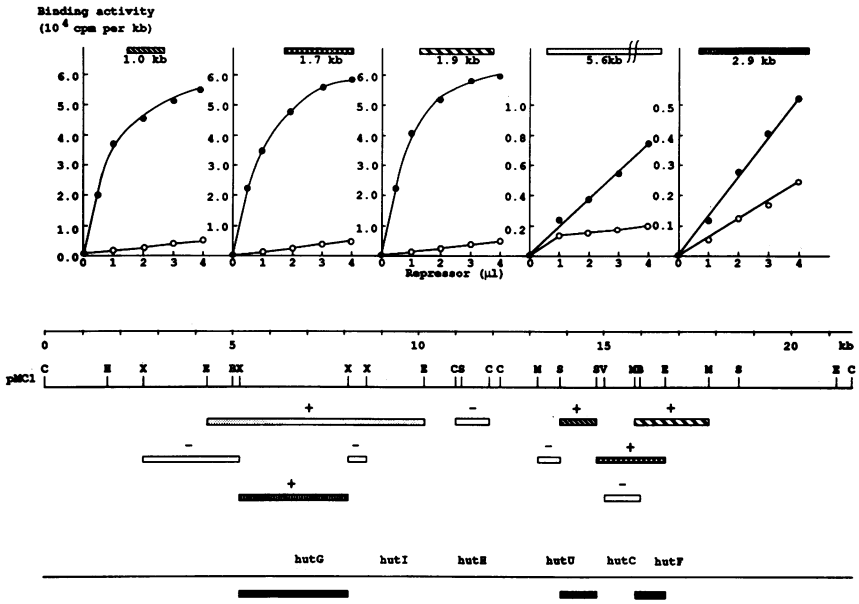

FIG. 3. Repressor binding to various restriction fragments. The DNA fragments were labeled by nick translation, and binding activity was tested by using the same molar amount for each (0.03 nM) and repressor as indicated; the protein (repressor) preparation used was 50 μg/ml, whereas other conditions were as in the standard assay. Symbols: ●, assayed without urocanate; ○, assayed with 5 mM urocanate. A partial restriction map of pMC1 containing all *hut* genes is shown, and the locations of the fragments assayed are indicated. Fragments: +, detectable binding activity (greater than 10× background); −, without activity. Quantitative results are shown only for those fragments exhibiting binding. Binding curves used, from left to right, the 1.0-kbp *Sal*I, 1.7-kbp *Sal*I-*Eco*RI, 1.9-kbp *Sma*I, 5.6-kbp *Eco*RI, and 2.9-kbp *Xho*I fragments. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Sma*I; S, *Sal*I; V, *Eco*RV; X, *Xho*I. All sites for *Xho*I, *Sal*I, and *Sma*I are not indicated on pMC1. The deduced repressor-binding regions are indicated on the bottom as the heavy lines below the gene locations.

inducer) was plotted against the concentration of inducer (Fig. 4).

Among six potential inducers tested, histidine, formiminoglutamate, and glutamate showed no effect on repressor-operator equilibrium. On the other hand, the addition of urocanate and imidazole propionate (a nonmetabolizable urocanate analog) caused dissociation of all three repressor-operator complexes, whereas FG inhibited binding to the *hutG* operator only. Binding to the *hutUHIG* and *hutF-hutC* operators could be reduced 50% by 30 μM urocanate or 40 to 50 μM imidazole propionate, whereas up to 20 mM FG had no effect (Fig. 4).

In the case of repressor binding to the *hutG* operator fragment, FG was as effective as urocanate or imidazole propionate in reducing the binding of repressor to this fragment, although the concentrations required in this experiment for 50% reduction in binding were all between 2 and 4 mM. Because the actual amount required for half reduction in binding is dependent on many factors internal to the experiment (e.g., absolute repressor or fragment concentration, ionic strength, and pH), it cannot be taken as necessarily indicative of the true binding constant for the inducer to free repressor or repressor-operator complex (1). Within an experiment with a given operator fragment, however, such assays provide a reasonable index of inducer potency. When pure repressor becomes available, direct equilibrium binding measurements should provide a better indication of the relative binding efficiency of FG and urocanate to the repressor and various operator-repressor complexes.

Urocanate but not FG inhibited binding of the repressor to the *hutUHIG* operator, but both compounds could affect to some extent the binding of repressor to the *hutG* operator. FG should therefore affect the inhibition of repressor binding to the *hutUHIG* operator by urocanate if these two inducers bind at the same site on the repressor. To test this, a competition assay was conducted with the repressor and the *hutUHIG* operator (1.0-kbp *Sal*I fragment) incubated with 30 μM urocanate in the presence of various amounts of FG. It was found that the ability of 30 μM urocanate to reduce repressor-operator binding by 50% was not altered by addition of FG up to 10 mM. Therefore, it can be concluded that FG and urocanate do not bind at the same site on the repressor. An alternative explanation, namely, that some or all of the binding to the *hutG* operator and competition by FG is due to a second and different binding protein in the extracts tested, cannot be dismissed at this time. Possibly arguing against this are the observations that genetic evidence indicates a single *hut* repressor (7) and the lack of binding of *E. coli* N4830 extracts (minus pLH18) to the various operators, but the matter awaits the availability of pure repressor before definitive resolution.

Identification of the operator site preceding *hutU*. To define more closely the repressor binding site preceding *hutU*, several experiments were conducted. First, a set of overlapping restriction fragments was generated, corresponding to a 1.4-kbp region containing the 1.0-kbp *Sal*I fragment used in the standard assay. These fragments were purified, labeled by nick translation, and assayed for repressor-binding activity (Fig. 5). A 128-bp region was identified that was common to all of the fragments demonstrating repressor-binding ability. This region has been sequenced along with the entire *hutU* gene and found to contain a region of dyad symmetry composed of 16 bp located near the −35 site relative to the transcriptional initiation site for *hutU* (Allison and Phillips, in preparation).

Second, gel retardation analysis was performed with a

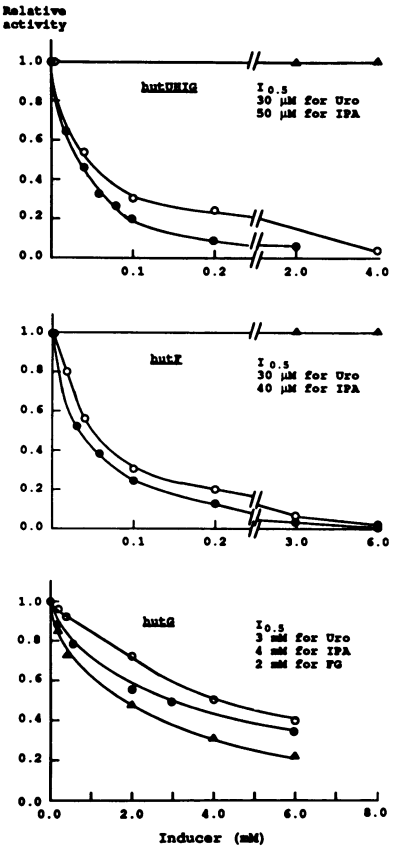

FIG. 4. Effect of inducers on the repressor-operator binding equilibrium. Constant amounts of repressor protein and operator DNA were incubated in the presence of various levels of inducers. Relative binding activity refers to the ratio of activity exhibited in the absence of inducer to that found in its presence. The repressor-binding fragments used were *hutUHIG* (1.0-kbp *Sal*I fragment), *hutF-hutC* (1.9-kbp *Sma*I fragment), and *hutG* (5.6-kbp *Eco*RI fragment). Symbols for inducers: ●, urocanate (Uro); ○, imidazole propionate (IPA); ▲, formylglutamate (FG). $I_{0.5}$ is the amount of inducer needed to reduce the binding of repressor to a given operator fragment by 50%.

380-bp DNA fragment containing the 16-bp symmetry region. Addition of the repressor to the DNA caused a mobility shift of the end-labeled fragment, whereas urocanate abolished this shift (Fig. 6), indicating that this fragment contained the operator region. To test whether the symmetrical segment, which contains an *Rsa*I site, was in the repressor recognition site, the 380-bp fragment used in the gel retardation analysis was digested by *Rsa*I in the presence or absence of the repressor. The repressor specifically protected the *Rsa*I site that was in the −35 region from cleavage by *Rsa*I, but another *Rsa*I site located 230 nucleotides upstream in the same DNA fragment was not protected.

Footprint analysis was employed to confirm the location of the repressor-binding site (Fig. 7). The same 380-bp DNA fragment was labeled and incubated with the repressor before DNase I digestion. The samples were then subjected to polyacrylamide gel electrophoresis. A DNase I-protected region was identified which covered an approximately 40-bp portion of the probe (from positions −10 to −50 relative to the transcriptional initiation site). As expected, the 16-bp symmetrical region was near the middle of the protected segment.

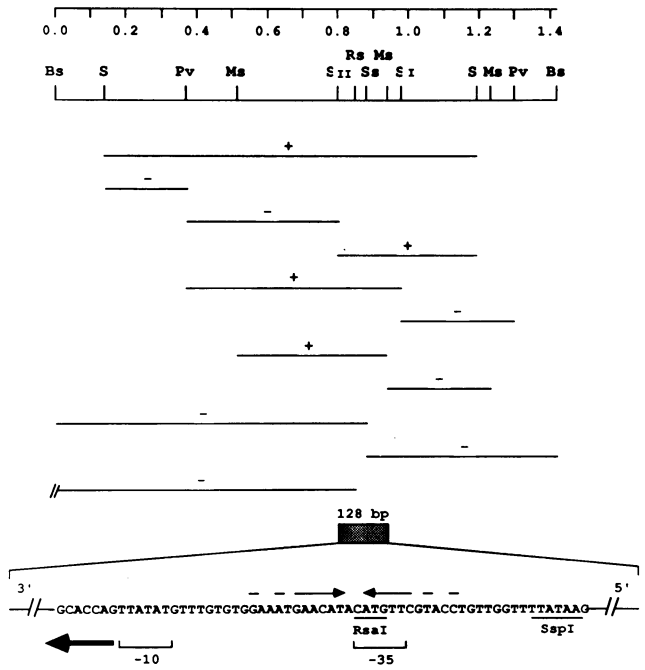

FIG. 5. Detailed mapping of the repressor-binding site preceding *hutU*. A 1.4-kbp *Bst*EII fragment, containing the 1.0-kbp *Sal*I fragment used in the standard assay, was digested to generate a set of overlapping oligonucleotides. These were labeled by nick translation and assayed for repressor-binding activity as follows: +, positive binding; −, no binding activity (less than twofold over background value). The partial sequence of the region so revealed is shown at the bottom. The −35 and −10 regions are relative to the transcription initiation site for this operon (Allison and Phillips, in preparation). Restriction sites: Bs, *Bst*EII; Ms, *Msp*I; Pv, *Pvu*II; Rs, *Rsa*I; S, *Sal*I; SI, *Sac*I; SII, *Sac*II; Ss, *Ssp*I.

DISCUSSION

The *hutC* gene of *P. putida* was overexpressed from the lambda p_L promoter in *E. coli*, and this allowed a partial purification of the repressor. The repressor specifically bound to certain *hut* region DNA fragments; as a result, three potential operator sites were localized. These regions were found just upstream of the transcriptionally related units previously indicated on the basis of insertional inactivation studies (7). Urocanate and its analog imidazole propionate inhibited repressor binding to all three operators, whereas FG affected binding to the *hutG* operator only. This agrees with the earlier result that synthesis of all Hut enzymes is induced by urocanate but FGase can be induced by its substrate as well (6).

Urocanate inhibits the binding of the repressor to each of the operator-containing fragments, whereas histidine itself has no effect; this provides direct evidence that urocanate and not histidine is the real physiological inducer. It is therefore apparent that these organisms will synthesize Hut enzymes only when a level of histidine in the cells is sufficiently high to allow some conversion to urocanate. In the uninduced state urocanate would accumulate until sufficient degradative capacity was generated, whereupon induction would be initiated and continue as long as there was a high level of histidine for conversion to urocanate. This rationale, however, requires a mechanism for maintaining some low level of histidase production even in the absence of urocanate. We suggest this can be most easily achieved by

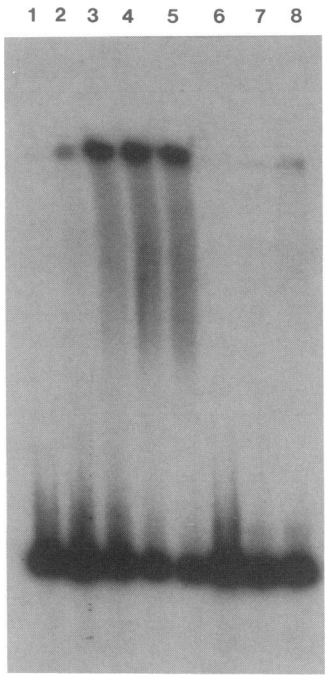

FIG. 6. Binding of the repressor to the *hutUHIG* operator region. A 380-bp *Bss*HII-*Sau*3A fragment containing the 16-bp symmetrical region was used in this gel retardation experiment. Details of the binding mixtures are provided in Materials and Methods. Samples on the gel contained the following amounts of repressor (lanes): 1, no repressor; 2, 0.1 μg; 3 and 6, 0.2 μg; 4 and 7, 0.4 μg; 5 and 8, 1 μg. Samples in lanes 6 through 8 also contained 20 mM urocanate.

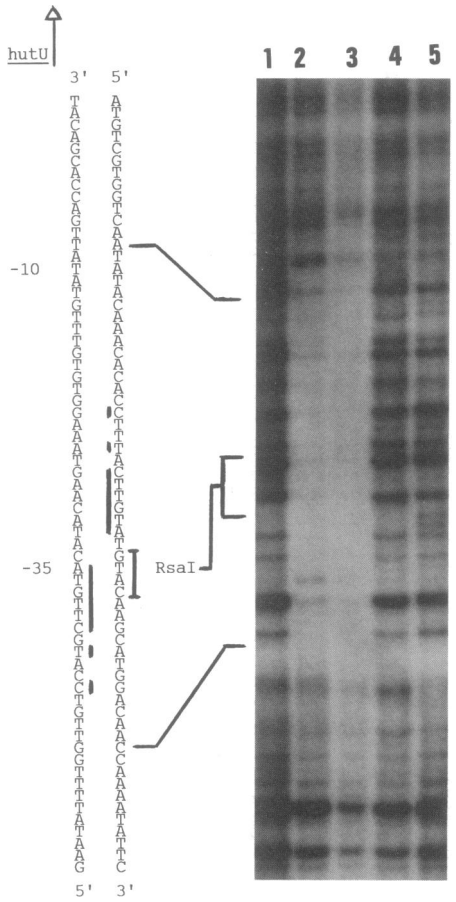

FIG. 7. DNase I footprint analysis. Reactions were carried out as described in Materials and Methods. The 380-bp *Bss*HII-*Sau*3A probe was employed in this experiment. Lanes: 1 and 5, no repressor; 2 and 3, 2 and 4 μg of repressor, respectively; 4, 2 μg of repressor plus 20 mM urocanate. The location of the footprint on a portion of the sequence of the fragment is illustrated.

having a nonregulated, inefficient promoter immediately preceding the *hutH* gene; this would permit a minimal level of transcription and subsequent translation to ensure that some histidase will always be present to convert excess histidine to urocanate for purposes of *hut* operon induction. DNA sequencing studies of the *hutH* and *hutU* genes should allow this proposal to be evaluated.

The *hutG* gene can be expressed from a promoter preceding *hutU* and from its own weaker promoter as well (6). This conclusion is supported by the results presented here that both of these regions can be recognized by the repressor and that binding to either region was inhibited by urocanate, whereas FG only inhibited binding to the *hutG* fragment. This would account for the noncoordinate synthesis of FGase from that of other Hut enzymes (6). However, the binding of the repressor to the *hutG* operator appears to be less favorable than that to the *hutUHIG* or *hutF-hutC* operators, and higher concentrations of inducers were required to inhibit repressor binding to *hutG* operator-containing fragments compared with those required for binding to *hutUHIG* or *hutF-hutC* fragments. Hence this *hutG* regulatory region is less likely to be part of the normal control of *hutG* expression than is the region preceding *hutU*, and thus a polycistronic *hutUHIG* mRNA model would be required. It is not obvious what purpose would be served by retaining a separate promoter-operator locus for *hutG*, and it is possible that this region is a vestige of the evolutionary events that may have led to the introduction of a fifth enzyme into the Hut pathway (7).

The repressor has similar binding activity for both *hut UHIG* and *hutF-hutC* operators, and the same amount of urocanate was required for equivalent inhibition of binding. It has recently been found that the nucleotide sequence of the *hutF-hutC* operator site is nearly identical to that of the *hutUHIG* operator site (Allison and Phillips, in preparation), indicating that the DNA-repressor interactions are probably identical at these two loci. The *hutG* operator may be different in sequence and structure from the *hutUHIG* and *hutF* operators, and thus the repressor would bind it with a different efficiency. Since the binding sites for FG and urocanate on the repressor are probably separate, the conformational change produced in the repressor by FG binding would affect only the interaction between the repressor and the *hutG* operator.

The interaction between the *hutUHIG* operator and the repressor sheds light on the mechanism of the regulation of *hut* gene expression and negative control by the repressor. The repressor binds a roughly 40-bp region from positions −10 to −50 relative to the transcription initiation site for *hutU*, with a section of dyad symmetry around position −35 probably serving as a recognition sequence. Therefore the repression of *hutU* transcription is most likely due to steric hindrance by the repressor at the RNA polymerase-binding site.

Studies are planned that will utilize pure repressor, when available, in more direct binding experiments with charac-

terized fragments containing the various operator regions so as to confirm the results presented here, especially concerning the unusual binding characteristics of repressor preparations with the *hutG* operator region. These studies should distinguish among the several possibilities discussed here, including potential aberrant properties of the repressor due to its nonpure state or the use of a very large *hutG* operator-containing fragment for binding measurements.

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