JOURNAL OF BACTERIOLOGY, May 1990, p. 2224–2229
0021-9193/90/052224-06$02.00/0

Vol. 172, No. 5

Sequence Analysis of the *hutH* Gene Encoding Histidine Ammonia-Lyase in *Pseudomonas putida*

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Received 21 September 1989/Accepted 27 January 1990

The complete nucleotide sequence of the *hutH* gene, encoding histidine ammonia-lyase (histidase), in *Pseudomonas putida* ATCC 12633 has been determined from the appropriate portions of the *hut* region that had been cloned into *Escherichia coli*. The resulting DNA sequence revealed an open reading frame of 1,530 base pairs, corresponding to a protein subunit of approximate molecular weight 53,600, in the location previously identified for the histidase gene by Tn*1000* mutagenesis. Translation began at a GTG codon, but direct protein sequencing revealed that the initiating amino acid was removed posttranslationally to provide an N-terminal threonine; 11 additional residues completely agreed with the predicted amino acid sequence. This sequence excluded the possibility that a dehydroalanine unit, the postulated coenzyme for histidase, is attached at the N terminus of histidase subunits. Comparison of the *P. putida* histidase gene sequence with that of a *Bacillus subtilis* region encoding histidase revealed 42% identity at the protein level. Although the *hutU* (urocanase) and *hutH* (histidase) genes are induced by urocanate and normally are transcribed as a unit beginning with *hutU*, analysis of the region immediately upstream of the histidase gene revealed a potential weak promoter that may possibly be used to maintain a basal level of histidase for the generation of inducer (urocanate) when histidine levels are elevated.

Histidine ammonia-lyase (histidase; EC 4.3.1.3) from *Pseudomonas putida* possesses an essential electrophilic center whose properties are consistent with its tentative identification as a dehydroalanine (DHA) unit (5). This conclusion is based on the chromatographic detection of [^{3}H]alanine from acid hydrolysates of histidase that had been inactivated by reduction with NaB^3H_4 and a similar identification of [^{14}C]aspartate from acid-hydrolyzed enzyme that had been treated with $Na^{14}CN$. Analogous findings have been obtained for the histidase from *Pseudomonas acidovorans* ATCC 11299b (9, 31). Little is known regarding the possible mechanistic involvement of DHA in the action of the enzyme or the nature of its binding to the protein.

We have previously shown (5) that *P. putida* histidase is a tetramer with identical subunits of molecular weight approximately 55,000 and 4 mol of DHA per mol of tetrameric protein, although total activity is lost upon covalent modification of one of the DHA units. It was also found that DHA residues are present in the native unpurified enzyme, thereby indicating that they do not arise by β elimination of a carbohydrate or similar moiety during the purification process (5). Furthermore, Givot and Abeles inactivated rat liver histidase in vivo by modification of its electrophilic center with nitromethane and demonstrated that the products formed were the same as those found with the *P. acidovorans* enzyme (8).

The structural gene for histidase, along with genes for the other enzymes and major control elements of histidine utilization (*hut*) in *P. putida*, has been cloned into *Escherichia coli* on a cosmid vector, and some details are available concerning the expression and relative location of the various structural genes on the cloned element (6, 12). A portion of the original *hut* DNA was subcloned to generate a plasmid expressing both histidase and urocanase (the first and second enzymes, respectively, in the pathway) as well as the *hut* repressor gene product; this plasmid, pMC4, was used by Consevage et al. (6) in Tn*1000* insertional mutagenesis experiments to locate the approximate positions of the histidase and urocanase genes and to establish the direction of transcription. It was concluded that these were generally expressed as a single transcriptional unit from a promoter preceding the urocanase gene (6, 12). This start site has recently been located by S1 mapping just upstream of the *hutU* gene, encoding urocanase, and adjacent to a repressor-binding site (S. L. Allison and A. T. Phillips, manuscript in preparation). To study further the structures of histidase and its associated coenzyme component, we used the information available concerning pMC4 to prepare new plasmids, which were then sequenced in the regions corresponding to the histidase gene. Our report describes this nucleotide sequence and analyzes the predicted protein sequence for its properties.

MATERIALS AND METHODS

Strains. Strain RDP210 (6) is a derivative of *E. coli* C600 and has the genotype F$^-$ *lacY1 leuB6 thi-1 hsdR hsdM rpsL supE44*. *E. coli* JM103 (19) has the genotype F128 *lacI*^{q} *traD36*/Δ(*lac-pro*) *supE thi rpsL endA sbcB15 hsdR4*.

Cell growth and plasmid manipulations. All cultures were grown with shaking at 37°C in LB medium (20) supplemented with antibiotics as appropriate (ampicillin, [50 μg/ml] and chloramphenicol [10 μg/ml]). For plasmid preparations, cells were grown to 100 Klett units (no. 42 filter) in LB medium plus ampicillin and then treated with chloramphenicol (170 μg/ml) for 16 h. When cells contained pBR325 (23) or its derivatives, an equivalent amount of spectinomycin replaced chloramphenicol. Plasmid isolations were conducted as described by Maniatis et al. (17).

Plasmid pMC4, which contains genes for histidase and urocanase and the *hut* repressor from *P. putida* ATCC 12633, was described in an earlier paper (6). Appropriate regions of pMC4 were inserted into either pBR325 or pUC8 (29) to construct pMC5 and pMC6, as indicated. The resulting plasmids were transformed into *E. coli* RDP210 or JM103

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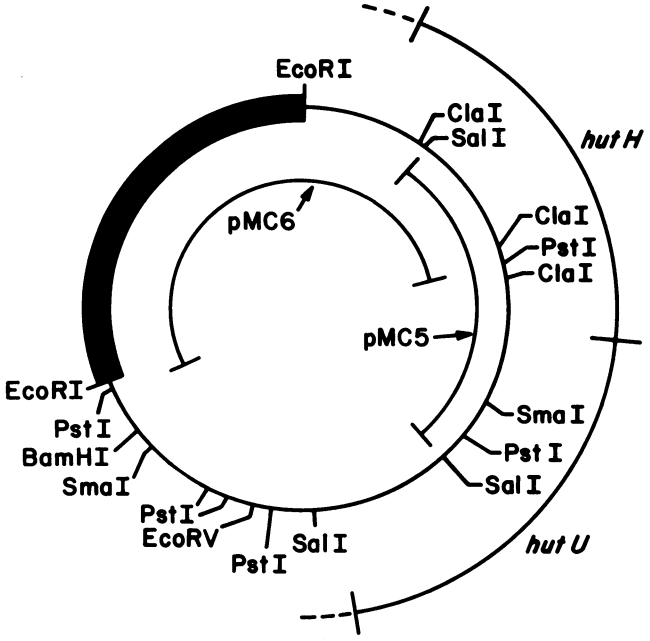

FIG. 1. Restriction map of pMC4, showing the portions used for construction of pMC5 and pMC6. Plasmid pMC4 is composed of a 6.1-kbp *Eco*RI fragment containing *hutH*, *hutU*, and *hutC* genes from *P. putida* inserted into pUC8.

by the $CaCl_2$-RbCl method of Kushner (15). Restriction enzyme digestions and ligations were conducted as recommended by Maniatis et al. (17).

Labeling of fragments and DNA sequencing. For 5′ labeling of fragments to be sequenced, the procedure was that of Maxam and Gilbert (18) with T4 kinase and [γ-^{32}P]ATP, followed by agarose gel electrophoresis, electroelution, and purification on DEAE-Sephacel (17). For 3′ labeling, reactions contained 25 pmol of potentially labeled sites, 50 pmol of the appropriate α-^{32}P-labeled deoxynucleoside triphosphate, and 8 U of avian myeloblastosis virus reverse transcriptase in 50 μl of 30 mM Tris hydrochloride (pH 8.3) plus 6 mM $MgCl_2$ and 40 mM KCl. After incubation at 37°C for 6 h, the labeled fragments were isolated as outlined above.

Nucleotide-specific cleavages were performed by the procedures outlined by Maxam and Gilbert (18). Electrophoresis was performed on 8% polyacrylamide gels at 1,600 V and 55°C. Fragments less than 60 nucleotides long were analyzed on 20% gels at 2,000 V. Gels were autoradiographed at −70°C for 24 to 48 h on Kodak XAR-5 film.

The sequence of *hutH* reported here was submitted to GenBank and assigned the accession number M28873.

Edman sequencing of the histidase N terminus. Histidase was purified to greater than 95% homogeneity from *P. putida* by our published procedure (5). This material was sequenced directly in a Beckman 890C Sequenator.

RESULTS

Construction and sequencing of pMC5 and pMC6. As part of the strategy developed for sequencing of the region containing the histidase gene, two smaller plasmids were constructed from portions of pMC4. Our earlier results (6) from insertion mutagenesis established that over half of the histidase-coding region lay within a 2.3-kilobase-pair (kbp) *Sal*I fragment in pMC4 (Fig. 1). This segment was isolated by ligating a *Sal*I digest of pMC4 into the corresponding site of pBR325 and then transforming the digest into RDP210 and selecting for resistance to ampicillin and chloramphenicol. The resulting plasmid having the expected size was designated pMC5. The remaining portion of the histidase gene was contained in the adjacent 0.9-kbp segment of pMC4 located between *Sal*I and *Eco*RI. To have some degree of overlap between this region and the 2.3-kbp *Sal*I fragment of pMC5, a second plasmid was constructed by digestion of pMC4 with *Pst*I and religation. Transformation into *E. coli* JM103 and screening for ampicillin resistance provided a plasmid of the expected size. This plasmid, termed pMC6, contained the pUC8 vector region of pMC4 and the *Eco*RI-to-*Pst*I sequence overlapping a portion of pMC5 (Fig. 1). Detailed restriction digestions were performed with both pMC5 and pMC6 and are presented in Fig. 2, along with indications of the specific fragments sequenced.

The DNA sequence extending over much of the cloned regions of pMC5 and pMC6 was determined, and the portions located between two *Nco*I sites, corresponding to 2,249 nucleotide pairs, are shown in Fig. 3; both strands were completely sequenced in this region. We deduced that the coding region for histidase began at a GTG codon positioned 8 nucleotides downstream from an acceptable Shine-Dalgarno sequence (GGAG) and was followed by an extended open reading frame containing 1,530 nucleotides; this represents a sequence of 510 amino acids (including the initiator amino acid). This translational start site was confirmed by sequencing of the N-terminal region of histidase (see below).

Protein sequencing by Edman degradation. Protein sequence determination on the intact histidase molecule revealed the sequence Thr-Glu-Leu-Thr-Leu-Lys-Pro-Gly-Thr-Leu-Thr-Leu in 12 cycles, which corresponded completely with the predicted N-terminal sequence if the initiating formylmethionine was removed through posttranslational modification. Also important was the observation that the terminal position (Thr) was not blocked. In explaining their findings on the existence of DHA in histidase, Givot et al. (9) suggested that attachment of DHA through its carboxyl group to the protein would permit the amino function of DHA to participate in a Schiff base linkage with some carbonyl group located elsewhere on the protein. If this suggestion is still appropriate, then DHA should be attached at a side chain position (e.g., the ε-amino group of lysine) rather than at the N terminus. Other possibilities, such as DHA being part of a larger nonprotein structure bound to the enzyme, cannot be eliminated at this time.

Codon frequencies in the *hutH* gene. The codon usage pattern for the *hutH* gene is presented in Table 1. The relatively high G+C content (62%) of *P. putida* forces a bias similar to that observed for other *Pseudomonas* sequences (7, 30). This bias is reflected in a clear dominance, usually around 85%, of G and C at the wobble positions, with the most obvious exception being the GAA codon used for glutamate. Beach and Rodwell (1) recently reported that use of the GAA codon for glutamate in the *mvaA* gene of *P. mevalonii* was 70%, whereas its occurrence in other *Pseudomonas* genes (mainly from *P. aeruginosa*) did not exceed 50%. We found that this glutamate codon was used 75% of the time in the histidase gene (compared with 25% for GAG), but furthermore, we noted several other examples of genes from *P. putida* (2) and *P. fluorescens* (27) in which GAA was the dominant (70% or more) codon for glutamate. Thus, a predominance of GAA over GAG codons for glutamate appears not to be unusual for many *Pseudomonas* species.

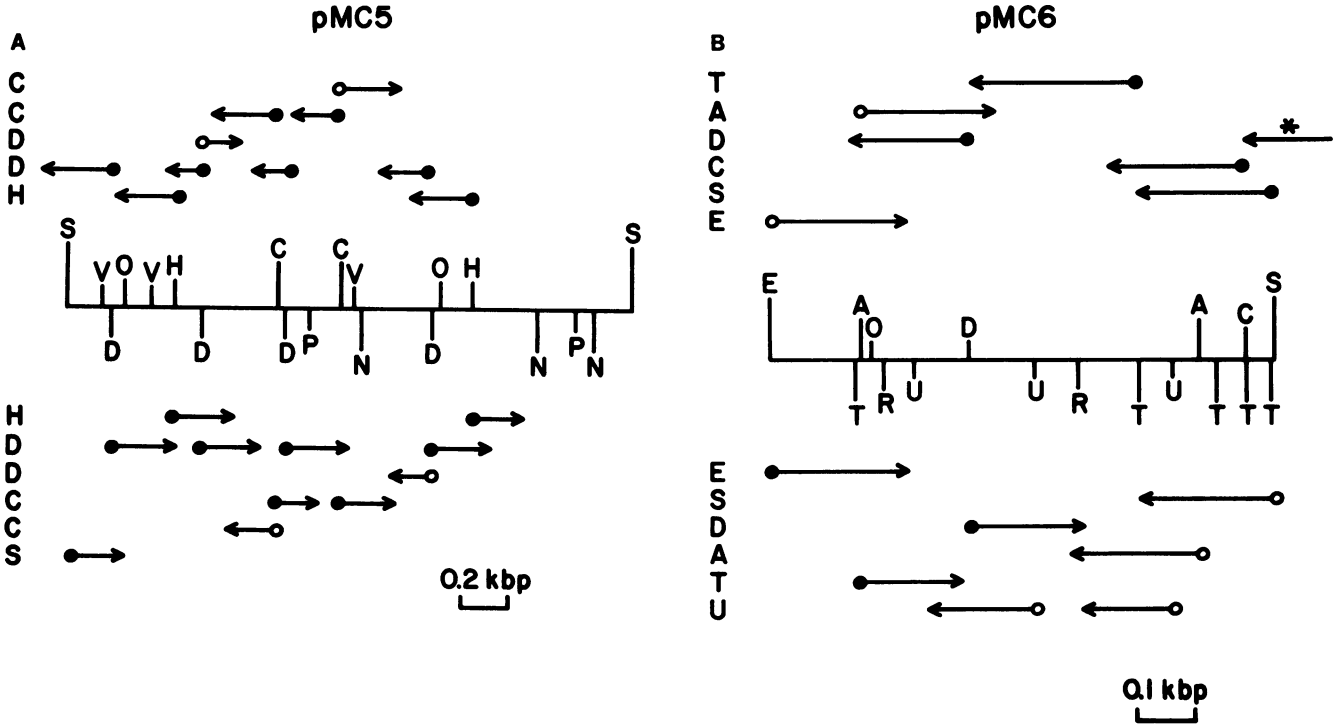

FIG. 2. Restriction maps and sequencing strategy for two plasmids containing portions of the *hutH* gene. (A) Plasmid pMC5, which contains a 2.3-kbp *SalI* section of pMC4 inserted into pBR325 (not shown). (B) A 0.9-kbp portion of plasmid pMC6 that extends from *SalI* to the nearest *Eco*RI site; the region that overlaps part of pMC5 (*PstI* to *SalI*) is not shown. Arrows depict regions cut with the indicated restriction enzymes, labeled, and sequenced. Those indicated with an open symbol were labeled at the 5′ end; those indicated with a closed symbol were labeled at the 3′ end. Enzyme abbreviations: A, *Ava*II; C, *Cla*I; D, *Dde*I; E, *Eco*RI; H, *Hin*fI; N, *Nci*I; O, *Nco*I; P, *Pst*I; R, *Rsa*I; S, *Sal*I; T, *Taq*I; U, *Alu*I; V, *Pvu*II. The *Dde*I fragment marked with an asterisk extends across the region covered by both pMC5 and pMC6. *Alu*I, *Ava*II, *Rsa*I, and *Taq*I sites in pMC5 are not illustrated, nor are *Nci*I sites shown for pMC6.

Aside from this difference, the overall codon use patterns were fairly similar to those tabulated for *P. aeruginosa* (30).

DISCUSSION

Predicted characteristics of histidase. From sequence data, the histidase molecule was calculated to have a subunit molecular weight of 53,574, excluding the initial formylmethionine, which is apparently removed during translation. The predicted composition of the protein agreed quite well with direct amino acid analysis results reported by us earlier (5) if corrections were made for a slight molecular weight difference. The only serious discrepancies were found with half-cystine (4 reported, 7 predicted) and serine (31 reported, 39 predicted), both of which are often lost on hydrolysis. A Kyte-Doolittle hydrophobicity profile (16) exhibited no evidence for a membrane-spanning region. Chou-Fasman predictions for major secondary structure (4) revealed 10 helices and four regions of β structure that each contained a minimum of 10 residues.

Earlier studies on histidases from *P. acidovorans* ATCC 11299b by Klee and Gladner (14) and *P. testosteroni* NCIB 10808 by Hassall and Soutar (11) reported that the native tetramer form of these proteins could be modified by iodo[^{14}C]acetate, from which a labeled tryptic peptide could be isolated and characterized. Whereas only the composition was reported by Klee and Gladner (14), Hassall and Soutar (11) determined its sequence to be Gly-Leu-Leu-Asp-Gly-Ser-Ala-Ile-Asn-Pro-Ser-His-Pro-Asn-CMCys-Gly-Arg. The composition of the peptide isolated by Klee and Gladner (14) was quite similar, also having 17 residues and differing only in three amino acids. Because the carboxymethylated form of the enzymes retained weak activity, it was concluded that this reactive cysteinyl residue is not essential for the reaction mechanism but might instead be involved in a polymerization that is commonly noted and results in less active higher-molecular-weight forms.

Consevage and Phillips (5) found that the *P. putida* histidase did not readily undergo a similar polymerization, and we were therefore interested in determining whether it contained a homologous peptide. Examination of the predicted protein sequence revealed one region having a cysteinyl residue in a somewhat similar environment. At positions 258 to 275 (nucleotides 775 to 828), there is present an 18-residue tryptic sequence, Asp-Leu-Leu-Gly-Asp-Ser-Ser-Glu-Val-Ser-Leu-Ser-His-Lys-Asn-Cys-Asp-Lys, that bears a reasonable resemblance to the *P. testosteroni* tryptic peptide, especially in the C-terminal portion. If these sequences are indeed analogous, then this fact would suggest that subtle changes in the sequence of the *P. putida* peptide have resulted in a decreased tendency to participate in the polymerization process or, alternatively, that this peptide alone is not directly responsible for the polymerization behavior.

Comparisons between *P. putida* and *Bacillus subtilis* histidases. Recently, Oda et al. (22) described the cloning and sequencing of a region of the *B. subtilis* genome that corresponded to the *hutH* gene. They found an open reading frame coding for 508 amino acids (molecular weight, 55,676) and concluded that this was the structural gene for histidase

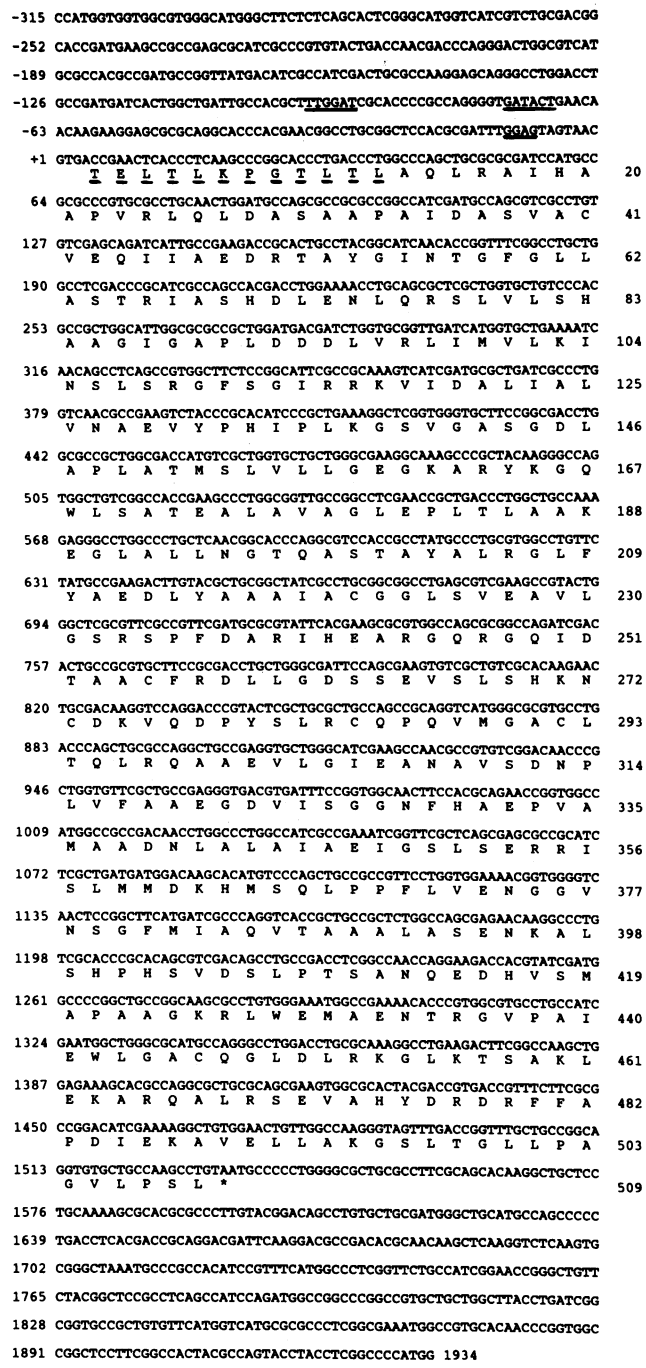

FIG. 3. Complete nucleotide sequence for the *hutH* gene from *P. putida* and the predicted amino acid sequence for histidase. A potential weak promoter sequence located between −69 and −97 is indicated, as is the likely ribosome-binding site at −10. Amino acids underlined have been confirmed by direct sequencing of the protein.

because a restriction fragment containing it had *hutH*$^+$ transforming activity. We compared the predicted sequence of amino acids for *B. subtilis* histidase with that of *P. putida* histidase and found a 42% identity between the two sequences if an internal deletion of two amino acids was assumed in the *B. subtilis* sequence (Fig. 4). Similarity was more evident in the interior regions than in the terminal portions, but there seemed to be no greater similarity in the N-terminal half of each than in the C-terminal half, or vice versa.

Although the various *Pseudomonas* histidases appear to retain a cysteine-containing peptide that may have some role in catalytic activity, there is no corresponding cysteine in an equivalent location in the *B. subtilis* histidase. This is not surprising, since Chasin and Magasanik (3) found that the *B. subtilis* histidase activity was unaffected by sulfhydryl-modifying reagents, including iodoacetate. It has been suggested by Klee (13) that in *Pseudomonas* histidase, this reactive sulfhydryl group is involved in the binding of histidine to the enzyme via a metal bridge complex. In any event, its modification does not completely eliminate activity, and it may simply be that carboxymethylation, like disulfide bond-related polymerization, brings about an unfavorable conformational change.

The 42% identity between the *B. subtilis* and *P. putida* histidases is relatively high for such distantly related genera, although not quite equal to the 56% identity observed for the highly conserved *trpB* gene products from *P. aeruginosa* and *B. subtilis* (10). Even so, the relatedness between these histidases is impressive when considered in relation to the glutamine-binding subunit of anthranilate synthase from *P. putida*, which is 62% identical to that from *Acinetobacter calcoaceticus* (7) and 43% identical to the same protein from *Serratia marcescens* (28). We conclude from these comparisons that histidases may turn out to be structurally well conserved.

Immunological relatedness studies of histidases from different *Pseudomonas* species (25) have also indicated that a close relationship exists for the proteins from the fluorescent group of pseudomonads (e.g., *P. putida* and *P. aeruginosa*) but not when these proteins are compared with those from other groups, including such members of the acidovorans family as *P. testosteroni* and *P. acidovorans*. Because the limited sequence information available on a reactive cysteine-containing peptide of histidase from these latter organisms suggests some degree of homology to the *P. putida* histidase, further structural comparisons of these histidases should be helpful in identifying catalytically important domains.

Potential modes for attachment of DHA to histidase in *P. putida*. Givot et al. (9) recognized that DHA with an unsubstituted amino group would readily undergo tautomerization and hydrolysis to pyruvate if not stabilized against this by formation of a Schiff base with a donor carbonyl group or some equivalent blocking process. This reasoning is supported by studies of Recsei and Snell (24) on the pyruvate-containing histidine decarboxylase of *Lactobacillus* spp. In this enzyme, an inactive proenzyme form containing an internal Ser-Ser sequence becomes dehydrated to generate a Ser-DHA sequence, which is rapidly cleaved to produce one subunit with a C-terminal seryl residue and another subunit with a (transient) DHA residue at the N-terminal position. This latter unstable DHA structure spontaneously converts to an N-terminal pyruvoyl residue plus ammonia. A similar senario for histidase could be envisioned, but one in which DHA residues were somehow stabilized against hydrolysis to pyruvate. Alternatively, one might propose that a Ser-DHA sequence formed as described above could remain as the result of this conversion, thereby placing the DHA at an interior position.

The primary sequence of histidase from *P. putida* does not support a proenzyme process for generating DHA, at least not at an N-terminal position. The histidase molecule is virtually identical in size to that predicted from the DNA

TABLE 1. Codon usage analysis for the histidase gene[a]

Codon and amino acid	No. of times used	Codon and amino acid	No. of times used	Codon and amino acid	No. of times used	Codon and amino acid	No. of times used
TTT, Phe	0	TCT, Ser	0	TAT, Tyr	2	TGT, Cys	1
TTC, Phe	11	TCC, Ser	8	TAC, Tyr	6	TGC, Cys	6
TTA, Leu	0	TCA, Ser	0	TAA, —[b]	1	TGA, —	0
TTG, Leu	5	TCG, Ser	17	TAG, —	0	TGG, Trp	3
CTT, Leu	0	CCT, Pro	1	CAT, His	1	CGT, Arg	8
CTC, Leu	6	CCC, Pro	2	CAC, His	11	CGC, Arg	18
CTA, Leu	0	CCA, Pro	1	CAA, Gln	1	CGA, Arg	0
CTG, Leu	59	CCG, Pro	18	CAG, Gln	17	CGG, Arg	1
ATT, Ile	5	ACT, Thr	3	AAT, Asn	0	AGT, Ser	1
ATC, Ile	19	ACC, Thr	16	AAC, Asn	15	AGC, Ser	13
ATA, Ile	0	ACA, Thr	0	AAA, Lys	7	AGA, Arg	0
ATG, Met	10	ACG, Thr	0	AAG, Lys	11	AGG, Arg	0
GTT, Val	1	GCT, Ala	11	GAT, Asp	7	GGT, Gly	9
GTC, Val	11	GCC, Ala	47	GAC, Asp	19	GGC, Gly	31
GTA, Val	2	GCA, Ala	4	GAA, Glu	22	GGA, Gly	0
GTG, Val	17	GCG, Ala	17	GAG, Glu	7	GGG, Gly	1

[a] The GTG initiation codon is excluded from this compilation.
[b] —, Termination codon.

sequence, excluding the existence of a much larger proenzyme precursor. Moreover, the finding of an N-terminal threonine immediately following the initiator amino acid eliminates the possibility that DHA would be in a terminal position but leaves open the possibility that it is present as part of the main peptide chain or is somehow attached at a side chain position (e.g., the ε-amino group of lysine). There is a Ser-Ser dipeptide sequence predicted for positions 263 to 264, but it is not known whether this actually is a stable Ser-DHA sequence. In the *B. subtilis* sequence there are two Ser-Ser sequences predicted, but neither is conserved at a corresponding position in the *P. putida* histidase.

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TELTLKPGTLTLAQLRAIHAAPVRLQLDASAAPAIDASVACVEQIIAEDR   50
 MVTLDGSSLTTADVARVLFDFEEAAASEESMERVKKSRAAVERIVRDEK   49

TAYGINTGFGLLASTRIASHDLENLQRSLVLSHAAGIGAPLDDDLVRLIM  100
TIYGINTGFGKFSDVLIQKEDSAALQLNLILSHACGVGDPFPECVSRAML   99

VLKINSLSRGFSGIRRKVIDALIALVNAEVYPHIPLKGSVGASGDLAPLA  150
LLRANALLKGFSGVRAELIEQLLAFLNKRVHPVIPQQGSLGASGDLAPLS  149

TMSLVLLGEGKARYKGQWLSATEALAVAGLEPLTLAAKEGLALLNGTQAS  200
HLALALIGQGEVFFEGERMPAMTGLKKAGIQPVTLTSKEGLALINGTQAM  199

TAYALRGLFYAEDLYAAAIACGGLSVEAVLGSRSPFDARIHEARGQRGQI  250
TAMGVVAYIEAEKLAYQTERIASLTIEGLQGIIDAFDEDIHLARGYQEQI  249

DTAACFRDLLGDSSEVSLSHKNCDKVQDPYSLRCQPQVMGACLTQLRQAA  300
DVAERIRFYLSDSGLT--TSQGELRVQDAYSLRCIPQVHGATWQTLGYVK  297

EVLGIEANAVSDNPLVFAAEGDVISGGNFHAEPVAMAADNLALAIAEIGS  350
EKLEIEMNAATDNPLIFNDGDKVISGGNFHGQPIAFAMDFLKIAISELAN  347

LSERRISLMMDKHMSQLPPFLVENGGVNSGFMIAQVTAAALASENKALSH  400
IAERRIERLVNPQLNDLPPFLSPHPGLQSGAMIMQYAAASLVSENKTLAH  397

PHSVDSLPTSANQEDHVSMAPAAGKRLWEMAENTRGVPAIEWLGACQGLD  450
PASVDSIPSSANQEDHVSMGTIAARHAYQVIANTRRVIAIEAICALQAVE  447

LRKGLKTSAKLEKARQALRSEVAHYDRDRFFAPDIEKAVELLAKGSLTGL  500
YRGIEHAASYTKQLFQEMRKVVPSIQQDRVFSYDIERLTDWLKKESLIPD  497

LPAGVLPSL*                                           509
HQNKELRGMNI*                                         508
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FIG. 4. Comparison of protein sequences for histidases from *P. putida* (upper sequence) and *B. subtilis* (lower sequence). Vertical bars connect identical residues.

Identification of a promoterlike region near the *hutH* gene. Although transcription of the *hutH* gene in *P. putida* appears to be predominantly initiated from a promoter located upstream of *hutU* (12), a promoterlike sequence was observed in the *hutU-hutH* intergenic region. A good match for the *E. coli* RNA polymerase consensus in the Pribnow box −10 region was found (Fig. 3), having the sequence GGTGAT ACTGA centered at −72 and predicting an mRNA start at −61. This 11-nucleotide sequence is identical to that seen for the lambda p_L promoter Pribnow box (26). The similarity to the P_L promoter in the polymerase recognition region (−35 region) is much less impressive, having the sequence CTT TGGATCGC centered at −94; this contrasts with the corresponding sequence TGTTGACATAA noted for the p_L promoter −35 region but still retains the highly conserved TTG in the proper position. The spacing between the two regions is also a significant factor in expression, at least in the case of *E. coli* RNA polymerase, with an optimum of 17 nucleotides separating the consensus hexamers (21). Taking as the hexamers those nucleotides underlined in Fig. 3 around −94 and −71, the observed spacing is 17 nucleotides. Whether this would function even weakly as a promoter in *P. putida* is less clear, and direct proof remains to be established.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant DK13198 from the National Institutes of Health and by the Pennsylvania Agricultural Experiment Station (project 2984).

We acknowledge the assistance of Nels Pederson with the secondary-structure analysis, Steve Allison in sequence comparisons, and David Speicher (Wistar Institute, Philadelphia, Pa.) for protein sequencing.

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