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Purification of Histidase from *Streptomyces griseus* and Nucleotide Sequence of the *hutH* Structural Gene

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Histidine ammonia-lyase (histidase) was purified to homogeneity from vegetative mycelia of *Streptomyces griseus*. The enzyme was specific for L-histidine and showed no activity against the substrate analog, D-histidine. Histidinol phosphate was a potent competitive inhibitor. Histidase displayed saturation kinetics with no detectable sigmoidal response. Neither thiol reagents nor a variety of divalent cations had any effect on the activity of the purified enzyme. High concentrations of potassium cyanide inactivated histidase in the absence of its substrate or histidinol phosphate, suggesting that, as in other histidases, dehydroalanine plays an important role in catalysis. The N-terminal amino acid sequence of histidase was used to construct a mixed oligonucleotide probe to identify and clone the histidase structural gene, *hutH*, from genomic DNA of the wild-type strain of *S. griseus*. The cloned DNA restored the ability of a histidase structural gene mutant to grow on L-histidine as the sole nitrogen source. The deduced amino acid sequence of *hutH* shows significant relatedness with histidase from bacteria and a mammal as well as phenylalanine ammonia-lyase from plants and fungi.

Histidine ammonia-lyase (EC 4.3.1.3; histidase) is the enzyme that converts L-histidine to urocanate and ammonia as the first step in the degradation of histidine. Histidase has been purified from pseudomonads (9, 10, 23), *Bacillus subtilis* (29, 32), and rat liver (7, 44), and the structural genes have been cloned. In bacteria the synthesis of histidase is regulated by induction (8, 11, 28, 32, 33, 39), carbon catabolite repression (8, 31), and nitrogen regulation (36). The histidase structural gene (*hutH*) is located within an operon that also contains the structural gene encoding urocanase (*hutU*). In *Pseudomonas putida* (17) and enteric bacteria (5, 6), *hutU* is the first gene of the operon; in *B. subtilis*, *hutH* lies upstream of *hutU* and is separated from the promoter by a regulatory locus, *hutP* (33). Although some investigators (24, 30) have suggested the participation of less active, disulfide-linked oligomers in the inactivation of pseudomonad histidase in vitro, there is no evidence that histidase activity in these bacteria is modulated by aggregation or any other type of posttranslational regulation in vivo.

Histidase from streptomycetes appears to be regulated differently. Neither nitrogen regulation nor carbon catabolite repression seems to govern histidase synthesis in streptomycetes (22, 25). Enzymological evidence suggests that histidase is synthesized constitutively in these bacteria but is maintained in an inactive form unless histidine or urocanate is present in the medium (26) and that posttranslational control may play a major role in regulating histidase activity (25, 26). Streptomycete histidase shows nonlinear reaction kinetics: in crude extracts prepared from *Streptomyces griseus* (25) and *S. coelicolor* (20), the reaction rate increases during a 10-min incubation. In contrast, the synthesis of urocanase, the enzyme that catalyzes of the second step in histidine degradation, appears to be inducible. The lack of coordinate regulation of histidase and urocanase in *S. griseus* implies that these two proteins are encoded in different transcription units. To begin to determine the molecular reasons for the novel kinetic and regulatory characteristics of streptomycete histidase, we purified and characterized histidase from *S. griseus* and determined the nucleotide sequence of the corresponding structural gene.

MATERIALS AND METHODS

Enzyme purification. Histidase was purified from a culture of SKK821 (wild-type; NRRL B-2682) (21) grown in 10 liters of 2×YTH medium (26) in a 14-liter fermentor (New Brunswick Scientific Co., Edison, N.J.). The culture was aerated at a flow rate of 6 liters of air (liter of medium)$^{-1}$ min^{-1} and was agitated mechanically by impellers at a rate of 400 rpm. Mazu DF-60P organic antifoaming agent (Mazer Chemicals, Inc., Gurnee, Ill.) was added as needed to prevent excessive foaming. An activated spore suspension (ca. 5×10^9 CFU) (21) was the inoculum. The culture was incubated at 30°C for 20 h, at which time the exponentially growing mycelia were harvested. From this point, all steps were conducted at 4°C unless noted otherwise. Chilled cells were concentrated in a Pellicon horizontal-flow filtration device (0.45-μm-pore-size filter; 1.5 square feet [ca. 0.139 m^2] of total filter area; Millipore Corp., Bedford, Mass.) and then collected by centrifugation at 27,000 × *g* for 15 min. The cells were washed twice with 1 M KCl, divided into 50-g (wet weight) portions, and frozen at −20°C until use.

Mycelia (approximately 50 g [wet weight]) were suspended in 400 ml of 50 mM Tris hydrochloride–5 mM EDTA (disodium salt)–1 mM dithiothreitol (pH 8.0) and disrupted by passage twice through a French pressure cell operated at 12,000 lb in^{-2} (25). The protein concentration of the cleared extract was adjusted to 5 to 10 mg ml^{-1} with extraction buffer. The extract was heated rapidly with stirring to 60°C and maintained at this temperature for 10 min before rapid cooling to 4°C in an ice bath. The cooled extract was combined with one-eighth volume of chloroform, mixed by inversion, and centrifuged at 27,000 × *g* for 15 min.

Potassium chloride was added to the cleared aqueous phase to a concentration of 0.25 M, and the solution was

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allowed to equilibrate for 15 min. Polyethyleneimine (Polymin P; Bethesda Research Laboratories, Gaithersburg, Md.) was added slowly to a final concentration of 0.25% (vol/vol) from a stock solution of 10% (vol/vol) in water. After 3 h of equilibration with stirring, additional KCl was added to adjust the concentration to 0.5 M. This solution was equilibrated for 12 h, and then the particulate material was removed by centrifugation at 27,000 × *g* for 15 min. Ammonium sulfate (ICN Biomedicals, Costa Mesa, Calif.) was added to the supernatant to a concentration of 1.2 M and equilibrated for 3 h. The precipitate was removed by centrifugation at 27,000 × *g* for 15 min. Additional ammonium sulfate was added to the supernatant to bring the concentration to 2.0 M, the mixture was equilibrated for 3 h, and the particulate material was recovered by centrifugation as described above. The pellet was dissolved in 50 mM Tris hydrochloride (pH 8.0) containing 0.1 M KCl, 5 mM EDTA, and 1 mM dithiothreitol. This solution was passed at a flow rate of 15 ml h^{-1} through a gel filtration column (2.5 by 55 cm, Bio-Gel A-0.5 m; Bio-Rad, Richmond, Calif.) equilibrated with the same buffer. Fractions of 3 ml were collected and assayed for histidase activity. The active fractions were pooled and applied to a column (1.5 by 12.5 cm) containing DEAE-Sephadex (Sigma Chemical Co., St. Louis, Mo.) that had been equilibrated with 50 mM Tris hydrochloride (pH 8.0)–0.1 M KCl–10% (vol/vol) glycerol. After unbound protein was removed by washing with 8 column volumes of loading buffer, the bound protein was eluted with a linear gradient of 200 ml of 0.1 to 0.4 M KCl in 50 mM Tris hydrochloride (pH 8.0)–10% (vol/vol) glycerol. Fractions of 2 ml were collected and assayed for histidase and protein; the active fractions (eluting at ca. 0.2 M KCl) were pooled.

The pooled sample from the DEAE-Sephadex column was loaded at a flow rate of 1 ml min^{-1} onto a MonoQ HR5/5 anion-exchange column (Pharmacia LKB, Piscataway, N.J.) that had been equilibrated with 50 mM Tris hydrochloride (pH 8.0)–0.2 M KCl–10% (vol/vol) glycerol. The column was washed at the same flow rate with 20 ml of loading buffer before applying a KCl concentration gradient increasing at a rate of 20 mM min^{-1}. After 5 min (0.3 M KCl), the rate was lowered to 5 mM min^{-1}, and 1-ml fractions were collected for 70 min. Fractions containing active histidase (eluting at approximately 0.35 M KCl) were pooled and adjusted to 0.2 M KCl and 50% (vol/vol) glycerol. Aliquots of the pooled fractions were stored at −20°C.

Enzyme activity was determined by using a spectrophotometric assay procedure that measures the rate of formation of urocanate (25, 37). One unit of activity is the amount of histidase that produces 1 μmol of urocanate per min. Protein concentration was measured by using the UV absorption method of Ehresmann et al. (13). Kinetic studies were carried out with a Kontron Uvikon 930 thermoregulated recording spectrophotometer operated at 30°C except when the temperature optimum was measured. Enzyme purity was monitored by polyacrylamide gel electrophoresis under denaturing conditions (27). Proteins were detected on gels by using the silver stain method of Gerton and Millette (14) or by staining with Coomassie blue R250. The molecular weight of the native enzyme was determined by chromatography on a Superose 6 column (Pharmacia) with standards ranging in M_r from 100,000 to 440,000.

For N-terminal amino acid analysis, 2 μg (36 pmol) of purified histidase was electrophoretically transferred to an Immobilon membrane (Millipore) after denaturing polyacrylamide gel electrophoresis. Sequencing was carried out by using Edman degradation of the protein with an Applied Biosystems (Foster City, Calif.) model 470A protein sequencer.

Hybridization analysis. A mixture of oligonucleotides was prepared on the basis of the N-terminal amino acid sequence of histidase. The mixture was not biased to favor codons used most frequently in high-G+C DNA. The oligonucleotide mixture was end labeled with polynucleotide kinase and [γ-^{32}P]ATP (38) and purified from unincorporated nucleotides by using a spun column (38). The end-labeled probe was used at a concentration of 9 pmol ml^{-1} in the hybridizations. Either genomic DNA from the wild-type strain of *S. griseus* or DNA from a library of *S. griseus* genomic DNA prepared in λEMBL4 was hybridized overnight at 37°C with the radiolabeled probe. The membrane was washed four times for 10 min each in 6× SSC (1× SSC is 0.15 M sodium citrate plus 0.015 M sodium citrate)–0.05% sodium pyrophosphate at room temperature and then in the same buffer at 42°C for 30 min and subjected to autoradiography for 20 h at −70°C.

For Southern hybridization analysis (41), genomic DNA from *S. griseus* SKK821 and *S. coelicolor* M145 was digested with restriction enzymes and separated on a 1% agarose gel. After capillary transfer of the alkali-denatured DNA to a nylon membrane (Zeta-Probe; Bio-Rad), the membrane was dried under vacuum and hybridized to *Eco*RI-digested pKK540 that had been labeled by the random primer (Promega; Madison, Wis.) method. The membrane was washed at increasing temperatures (55 to 75°C) in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and 0.2× SSC–0.1% SDS before autoradiography.

DNA manipulations. Restriction endonuclease digestions and ligations were performed as described previously (2). For nucleotide sequence determination, nested deletions of pKK501, pKK538, and pKK540 were made by using exonuclease III-digested DNA (15) that had been purified twice through a cesium chloride-ethidium bromide gradient.

Plasmids were constructed according to the following scheme. Where appropriate, fragments were isolated from agarose gels by using Prep-a-Gene (Bio-Rad). Plasmids pKK500 and pKK501 were made by ligating the 8-kb *Eco*RI-*Bgl*II fragment from λKK500 and the 2.5-kb *Eco*RI-*Bam*HI fragment from λKK501, respectively, into pGEM-4Z (Promega) that had been digested with *Eco*RI and *Bam*HI. Plasmid pKK535 was constructed by digesting pKK500 with *Mlu*I and *Hin*dIII and ligating the resulting 1.6-kb fragment into similarly digested pKK501. This ligation resulted in the reconstruction of the 1.5-kb *hutH* structural gene joined to approximately 0.8 kb of upstream genomic DNA and 0.5 kb of downstream genomic DNA. For unidirectional digestion with exonuclease III (15), the resulting fragment was excised from pKK535 with *Eco*RI and *Hin*dIII, and the ends were filled in with Klenow fragment (38) before ligation to *Sma*I-digested pUC18, generating pKK538 and pKK540; these two plasmids contained the *hutH* gene and adjacent DNA in both orientations. Complementation of the Hut$^-$ defect in mutant strain SKK896 (26) was assessed after transformation with plasmid pKK593; this plasmid was constructed by ligating the 2.4-kb *Bam*HI fragment of pKK540 (containing the *hutH* structural gene and approximately 600 bp of upstream sequence) to *Bgl*II-digested pIJ702 (19). Transformation of *S. griseus* was conducted as described previously (2).

DNA sequencing. Nucleotide sequencing was carried out with plasmid minipreparations essentially as described by Babcock and Kendrick (3), but with the following modifications. The replacement of dGTP with 7-deaza-dGTP in the nucleotide mixtures as suggested by the manufacturer (Se-

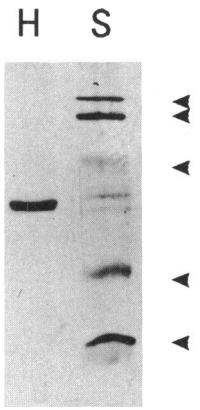

FIG. 1. Analysis of the purification of histidase by SDS-polyacrylamide gel electrophoresis. Purified histidase (200 ng) was subjected to electrophoresis through a 9% polyacrylamide gel under reducing and denaturing conditions (27). The gel was stained with silver nitrate (14). Lanes: H, purified histidase (MonoQ pooled fractions); S, molecular weight standards (M_rs of 116,000, 97,400, 66,000, 45,000, and 29,000).

quenase version 2.0; U.S. Biochemical Corp., Cleveland, Ohio) resolved most artifacts caused by compressions or other secondary structures. The incorporation of 10% dimethyl sulfoxide in the annealing reaction reduced background interference significantly. Use of T7 DNA polymerase at 0.4 U μl^{-1} and incubation of the termination reactions at 45°C rather than at 37°C also reduced compression artifacts. Deoxycytidine 5′-[α-^{35}S]thiotriphosphate was used to label the newly synthesized strand. The Wisconsin Genetics Computer Group (Madison, Wis.) program (12) was used for analysis of the nucleotide and amino acid sequences.

Determination of the direction of transcription. Plasmid pKK501 was linearized with either *Eco*RI or *Hin*dIII. Runoff transcripts were generated in vitro by incubation with T7 RNA polymerase or SP6 RNA polymerase, respectively. Samples of these reactions were separated by agarose-formaldehyde gel electrophoresis in 3-(*N*-morpholino)propanesulfonic acid-acetate-EDTA buffer (38) and transferred to a nitrocellulose membrane by capillary action (38). The RNA on the membrane was hybridized to an end-labeled oligonucleotide probe overnight at 37°C as described above.

Nucleotide sequence accession number. The *hutH* sequence has been assigned the GenBank accession number M77841.

RESULTS

Purification of histidase. *S. griseus* histidase was purified approximately 3,000-fold by differential precipitation, gel filtration, and anion-exchange chromatography. A typical purification from 50 g of vegetative mycelium yielded 0.6 mg (12.72 U) of protein. The enzyme preparation was estimated to be >98% pure by densitometric scanning of SDS-polyacrylamide gels stained with Coomassie brilliant blue or silver nitrate (Fig. 1). Heating of the crude extract at 60°C for 10 min before purification resulted in a twofold increase in specific activity. This procedure ensured that the starting material contained only fully active histidase.

Histidase activity was maximal in Tris hydrochloride buffer at pH 8.5 and at a temperature of 50°C. The purified enzyme displayed typical saturation kinetics in this buffer.

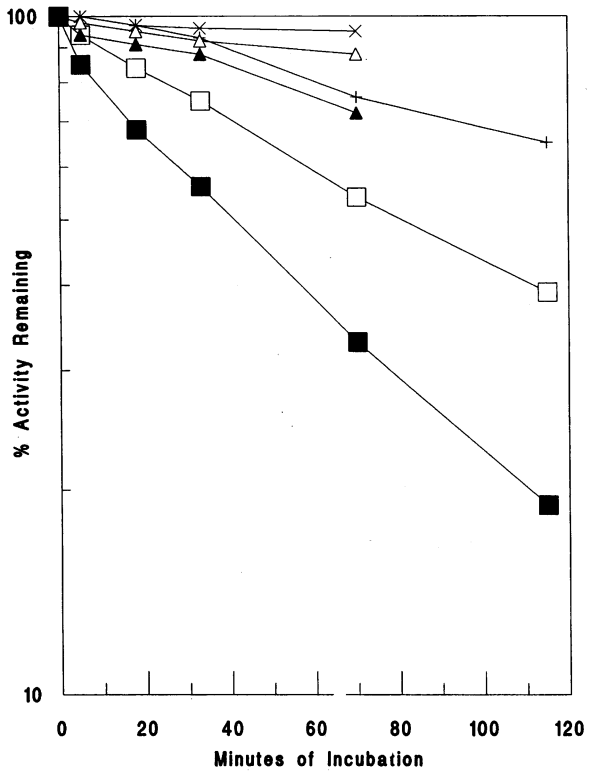

FIG. 2. Cyanide inactivation of histidase activity. Purified histidase (12 mU) was combined with potassium cyanide at the indicated concentrations in 100 mM Tris hydrochloride buffer (pH 8.8) in a total volume of 100 μl. At intervals during incubation of the mixture at 25°C, samples (20 μl) were removed and assayed for activity. In two cases, histidine or histidinol phosphate was combined with histidase before the addition of potassium cyanide. x, 0 mM KCN; +, 50 mM KCN; □, 100 mM KCN; ■, 150 mM KCN; △, 100 mM KCN and 5 mM histidinol-phosphate; ▲, 100 mM KCN and 10 mM L-histidine.

The reaction rate was proportional to enzyme concentration and linear for at least 10 min. This latter result contrasts significantly with the results obtained with unheated crude extracts, in which the rate increased over the course of a 10-min reaction (20, 25).

The enzymatic activity was unaffected by the chloride salts of the divalent cations of Mn, Ca, Mg, Co, or Zn; likewise, neither 10 mM EDTA, 1 mM dithiothreitol, nor 5 mM β-mercaptoethanol had any effect. The apparent M_r of the histidase subunit was 55,000 (Fig. 1). The elution profile of histidase upon gel filtration through Superose 6 suggested that the M_r of the active protein was 230,000 to 240,000. The N-terminal amino acid sequence was determined to be Met-Asp-Met-His-Thr-Val-Val-Val-Gly-Thr-Ser-Gly.

Purified histidase showed a K_m for L-histidine of 0.6 mM and was competitively inhibited by D-histidine (K_i of 11.3 mM). Histidinol phosphate was a potent competitive inhibitor of histidase, with a K_i of 0.27 mM. Histidase had a half-life of approximately 80 min when incubated with 100 mM potassium cyanide. Both 5 mM histidinol phosphate and 10 mM L-histidine afforded protection from inactivation by cyanide (Fig. 2).

Cloning the *hutH* gene. We designed a mixture of oligonucleotides that would encode the N-terminal 7 amino acids of purified histidase. Before using this mixture to identify

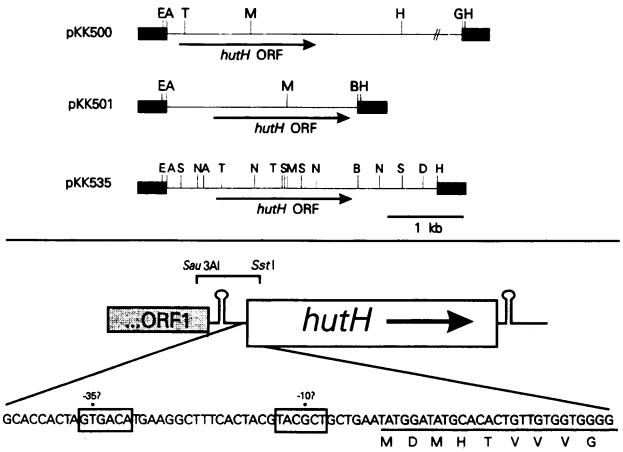

FIG. 3. Maps of the cloned DNA. The top part of the figure is a restriction map of the *hutH* region, showing the location and direction of the *hutH* coding sequence. Dark boxes indicate the plasmid vector sequences. The double shills (//) in pKK500 indicate a region of *S. griseus* genomic DNA that has been foreshortened in the drawing. Abbreviations: A, *Sau*3AI; B, *Bam*HI; D, *Nde*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; M, *Mlu*I; N, *Nco*I; S, *Sal*I; T, *Sst*I. Features of the cloned *hutH* gene are shown at the bottom of the figure. The location of the *hutH* gene downstream of a partial open reading frame is indicated, as are the region surrounding the *hutH* translation initiation site and a putative *hutH* promoter (boxed). The *Sau*3AI-*Sst*I restriction fragment used in the promoter-probe analysis and the deduced N-terminal amino acid sequence are shown.

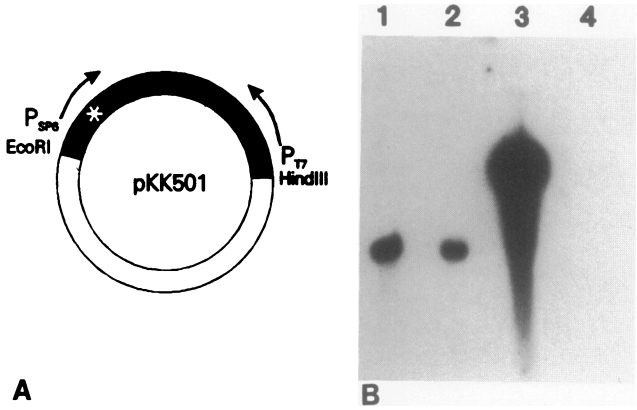

FIG. 4. Determination of the direction of transcription of *hutH*. (A) Schematic diagram of pKK501. The asterisk indicates the region of pKK501 that hybridized with the oligonucleotide probe. (B) Plasmid pKK501 linearized by digestion with *Eco*RI or *Hin*dIII and incubated with T7 RNA polymerase or SP6 RNA polymerase, respectively, to obtain transcripts in vitro. Reactions were carried out according to the instructions of the pGEM-4Z supplier (Promega). Transcripts were separated by agarose gel electrophoresis and hybridized to radiolabeled N terminus-specific oligonucleotide (38). Lanes: 1, 2.6-kb molecular weight standard; 2, 2.4-kb transcript transcribed from the T7 promoter (*Eco*RI-cut template); 3, 8-kb molecular weight standard; 4, transcript made from the SP6 promoter (*Hin*dIII-cut template).

hutH-containing clones in a λEMBL4 library, we confirmed that genomic DNA from *S. griseus* SKK821 contained a 4.75-kb *Bam*HI fragment, a 7.6-kb *Mlu*I fragment, a 0.9-kb *Nco*I fragment, and a 1.54-kb *Sal*I fragment, each of which hybridized with the probe. Subsequently we identified three λ phage isolates that hybridized strongly with the probe. All three contained overlapping DNA fragments with a 16-kb *Sau*3AI fragment in common. DNA located at the end of the inserts proximal to *cosR* hybridized with the oligonucleotide mixture. Portions of two of these inserts (λKK500 and λKK501) were ligated to pGEM-4Z for further analysis.

Restriction enzyme mapping of the subcloned inserts in the transcription vector pGEM-4Z and hybridization to the end-labeled oligonucleotide mixture showed that the N terminus of the histidase structural gene mapped near the *Eco*RI site (Fig. 3). It was therefore unclear whether we had cloned only the N-terminal coding portion of *hutH* and adjacent upstream DNA or the entire *hutH* structural gene. To resolve this question, we identified the coding strand by using runoff transcription assays and hybridization to the N terminus-specific oligonucleotide mixture. If the *hutH* gene were oriented in the clockwise direction as drawn in Fig. 4, then we would expect the radiolabeled oligonucleotide to hybridize only to the in vitro transcript generated from the T7 promoter. In contrast, orientation in the opposite direction would permit hybridization only to the P_{SP6}-generated transcript. The results (Fig. 4) showed that the *hutH* structural gene is transcribed in the direction from the *Eco*RI site to the *Bam*HI site as drawn in Fig. 3. On the basis of the length of the insert in pKK500 (8 kb) and the histidase subunit M_r (55,000), we concluded that we had cloned the entire *hutH* gene.

Features of the nucleotide sequence. The nucleotide sequence of the 2.9-kb *Eco*RI-*Hin*dIII fragment of pKK540 is shown in Fig. 5. The key features of the sequenced DNA are depicted in Fig. 3. The N-terminal coding sequence of *hutH* was identified by its strict correspondence to the N-terminal amino acid sequence of the purified enzyme. Computer analysis of the gene product indicated that *hutH* encodes a protein of M_r 53,000, in agreement with the electrophoretically determined subunit size. Alignment of streptomycete histidase with other histidases (Fig. 6) shows regions of highly conserved amino acid sequence located primarily within the central portion of the primary structure. Construction of a phylogenetic tree on the basis of maximum parsimony (43), using the Wisconsin Genetics Computer Group program PILEUP to align the amino acid sequences (12), revealed that streptomycete histidase is distantly related to the other bacterial and mammalian histidases (Fig. 7). The histidase amino acid sequence also is significantly similar to sequences of phenylalanine ammonia-lyase (EC 4.3.1.5) from plants and fungi (44).

Inspection of the nucleotide sequence immediately upstream of the N-terminal coding sequence of *hutH* revealed a region with a high degree of homology with streptomycete promoters that are active during vegetative growth: five of six nucleotides match the consensus sequences of the −35 and −10 regions (TTGACa/g and TAg/cg/a/cg/aT, respectively, where the lowercase letters separated by shills indicate equally common bases [42]), which are separated by a 17-nucleotide spacer (Fig. 3) (16, 42). Preliminary analysis with the promoter-probe pIJ487 (45) in *S. lividans* suggested that the approximately 400-bp *Sau*3AI-*Sst*I fragment (highlighted in Fig. 3) that contains the N-terminal coding sequence of *hutH* and extends approximately 300 bp upstream has promoter activity.

Further upstream of *hutH* is the C-terminal portion of an open reading frame that probably encodes a polypeptide in *S. griseus* (4). Computer searches have revealed no significant similarities with any sequenced gene products. Between the upstream open reading frame and the *hutH* structural-

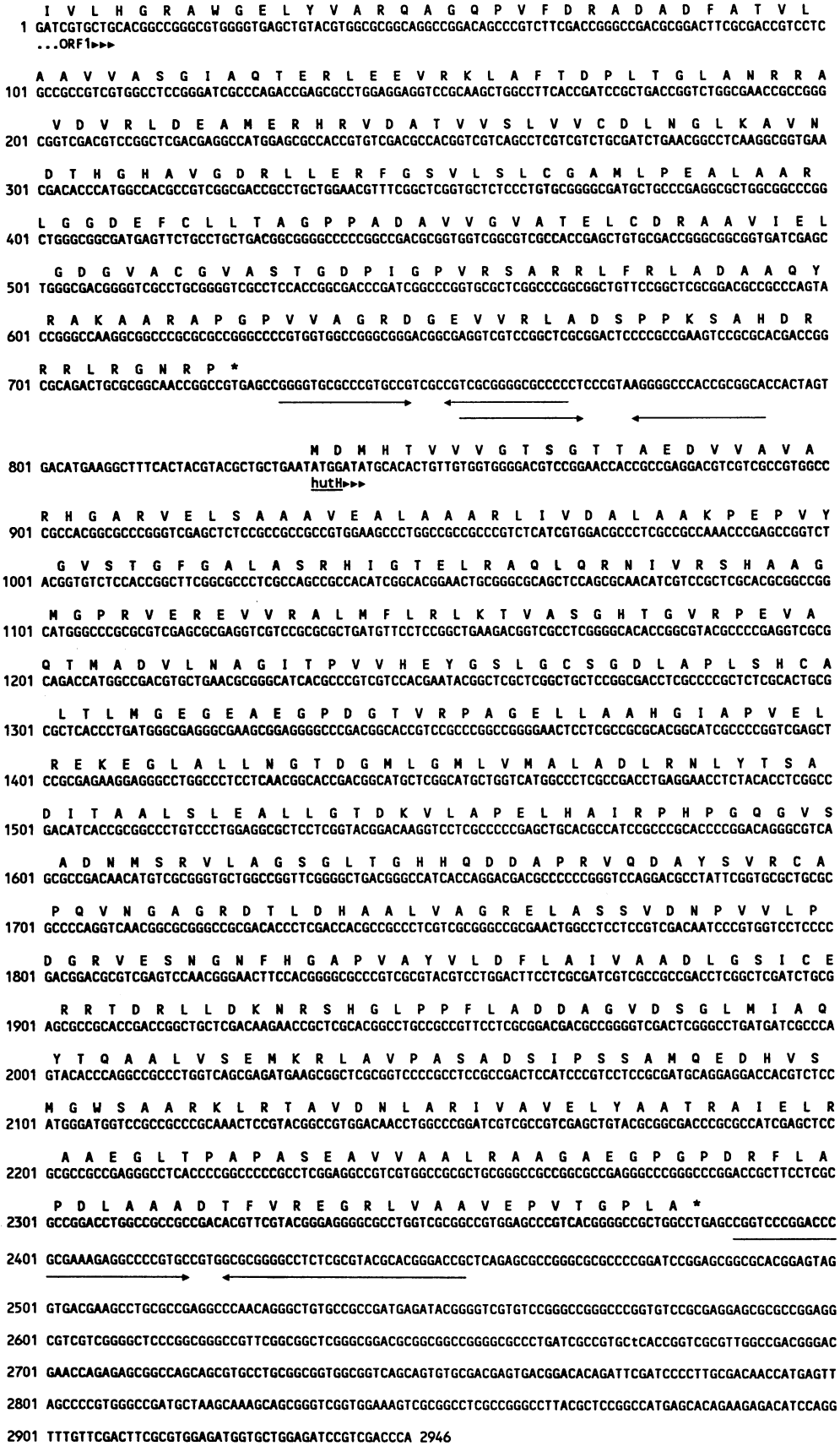

FIG. 5. Sequence of the *hutH* gene and adjacent DNA. ORF1 ►►► indicates the partial open reading frame upstream of the *hutH* structural gene. Arrows indicate inverted repeats.

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            1                                                                                  80
S. griseus  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
P. putida   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B. subtilis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Rat         MPRYTVHVRG EWLAVPCQDG KLSVGWLGRE AVRRYMKNKP DNGGFTSVDE VRFLVRRCKG LGLLDNEDLL EVALEDNEFV

            81                                                                                160
S. griseus  ---------- ---------- --------MD MHTVVVGTSG TTAEDVVAVA RHGARVELSA AAVEALAAAR LIVDALAAKP
P. putida   ---------- ---------- ---------- -TE.T.KPGT L.LAQ.R..H AAPV..Q.D. S.AP..D.SV AC..Q.I.ED
B. subtilis ---------- ---------- ---------- --M.T.DG.S L.TA..AR.L FDFEEAAA.E ES..R.KKS. AA..R.VRDE
Rat         EVVIEGDVMS PDFIPSQPEG VFLYSKYREP EKY.A.DGDS L.T....N.. .GHY..K.TS I.EKK.QQS. E...S.IKER

            161                                                                               240
S. griseus  EPVYGVSTGF GALASRHIGT ELRAQLQRNI VRSHAAGMGP RVEREVVRAL MFLRLKTVAS GHTGVRPEVA QTMADVLNAG
P. putida   RTA...N... .L...TR... HDLE....S. .L.......A ...D....L. .V...N..SR .F....RK.I DA.IA....E
B. subtilis KT....N... .KFSDVL.QK .DS.A..L.. .L...C...D .FPEC.S... .L..ANA.LK .F....A..I EQ.LAF..KR
Rat         TV........ .KF.RTV.PA NKLQE..V.. .R..SS...K ..SP.RC.M. .A...NV..K .Y...SL.TL KQ.I..F..S

            241                                                                               320
S. griseus  ITPVVHEYGS LGCSGDLAPL SHCALTLMGE GEAEGPDGTV RPAGELLAAH GIAPVELREK EGLALLNGTD GMLGMLVMAL
P. putida   .Y.H.PLK.. ..A....... ATMS.V.... .K.RY-K.QW LS.T.A..VA ..E..T.AA. .........Q .ST.YA.RG.
B. subtilis .H...PQQ.. ..A....... ..L..A...Q ..VFF-E.ER M..MTG.KKA ..Q..T.TS. .........Q ..T..G...Y
Rat         CLSY.P.K.. ..A....... ..L..G.... .KMWS.KSGW AD.KY..E.. ..K..V..P. .........Q M.TS.GCE..

            321                                                                               400
S. griseus  ADLRNLYTSA DITAALSLEA LLGTDKVLAP ELHAIRPHPG QGVSADNMSR VLAGSGLT-- -GHHQDDAPR VQDAYSVRCA
P. putida   FYAED..AA. IAC....... ....RSPFDA R..EA.GQ.. .ID..ACFRD ...---D.SE VSLSHKNCD. ...P.....Q
B. subtilis I.AEK.AYQT .RI.S..... .Q.IIDAFDE ...LA.GYQE .IDV..R.RF Y.SD....TS Q.EL-----. .........I
Rat         ERASA.ARQ. ..V......V .K..T.AFDT ........R. .IEV.FRFRS ..DSDHHPSE I.ESHRFCD. .........C

            401                                                                               480
S. griseus  PQVNGAGRDT LDHAALVAGR ELASSVDNPV VLPD-GRVES NGNFHGAPVA YVLDFLAIVA ADLGSICERR TDRLLDKNRS
P. putida   ...M..CLTQ .RQ..E.L.I .ANAVS.... .FAAE.D.I. G....AE... MAA.N...AI ......S... ISL....HM.
B. subtilis ...H..TWQ. .GYVKEKLEI ..NAAT.... .FN.GD..I. G.....Q... .A....K.AI S..AN.A... I....NP.LN
Rat         ...H.VVN.. .AFVKD.ITT ..N.AT.... .FASR.ETI. G.....EYP. KA......GV H..AA.S... I...CNPSL.

            481                                                                               560
S. griseus  HGLPPFLADD AGVDSGLMIA QYTQAALVSE MKRLAVPASA DSIPSSAMQE DHVSMGWSAA RKLRTAVDNL ARIVAVELYA
P. putida   Q-.....V.N ...N..F... .V.A...A.. N.A.SH.H.V .......N.. .....APA.. ...WEMA..T RG.P...WL.
B. subtilis D-.....SPH P..Q..A..M ..AA.S.... N.T..H...V .......N.. ......TI.. .HAYQV.A.T R......AIC
Rat         E-..A..VA. ...N..F... HC.A...... S.A.CH.S.V ...S...AT. ......GW.. ..ALRV..H. EQ......L.

            561                                                                               640
S. griseus  ATRAIELRAA EGLTPAPASE AVVAALRAAG AEGPGPDRFL APDLAAADTF VREGRLVAAV EPVTGPLA*- ----------
P. putida   .CQ.....-- K..KTSAKL. KARQ...SEV .-HYDR...F ....EK.VEL .AK.S.T.L. PAGVL.SL*- ----------
B. subtilis .LQ...Y.-- GIEHA.SY.K Q.FQE..KVV P-SIQQ..VF SY..ERLTD. ..KES..PDH QNKELRGMNI *---------
Rat         .CQ...FL-- RP.KTTTPL. K.YDL..SVV R-PWIK.... ....E..HRL .L.Q..WEVA A.YIEKYRME HIPESRPLSP

            641                    662
S. griseus  ---------- ---------- --
P. putida   ---------- ---------- --
B. subtilis ---------- ---------- --
Rat         TAFSLESLRK NSATIPESDD L*
```

	S. griseus	P. putida	B. subtilis	Rat
S. griseus	100/100	56/38	52/38	52/37
P. putida		100/100	57/42	58/42
B. subtilis			100/100	56/42
Rat				100/100

FIG. 6. Alignment of histidase primary structures. The amino acid sequence of *S. griseus* histidase is shown in entirety. Only nonidentical residues in the other histidases (10, 34, 44) are specified; identical residues are indicated by dots. Gaps are indicated by dashes. Amino acids were classified into the following similarity groups: M, V, I, and L; E and D; N and Q; A and G; S and T; F, Y, and W; K and R; C; P; and H. The alignment was constructed by using the Wisconsin Genetics Computer Group program PILEUP. The percent similarity (numerator) and percent identity (denominator) in pairwise comparisons among the sequenced histidases are tabulated in a matrix at the end of the aligned sequences. These values were calculated using the Wisconsin Genetics Computer Group program GAP with a gap penalty of 5 and a gap length penalty of 0.3.

gene are two regions containing potential secondary structures (Fig. 5) that may function in transcription termination.

Southern analysis of *hutH* in genomic DNA prepared from the wild-type strain of *S. griseus* indicated that no major rearrangement occurred during the cloning of the *hutH* gene, nor is there evidence for a second histidase structural gene that is similar to the one we have cloned (Fig. 8). A related gene is present in *S. coelicolor* (Fig. 8), consistent with enzymological and genetic data (20, 22). Introduction of the cloned *hutH* gene into a putative *hutH* structural gene mutant of *S. griseus* (26) resulted in a high level of histidase

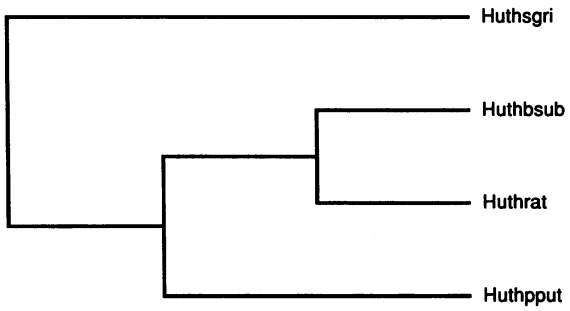

FIG. 7. Cladogram of the relatedness of amino acid sequences of histidine ammonia-lyases. The tree was generated by using maximum parsimony analysis according to the computer program PAUP (43). The *S. griseus* HutH sequence (HuthSgri) diverged from the other HutH primary structures at a confidence level of 95%. Sequences of histidases from rat (Huthrat), *B. subtilis* (HuthBsub), and *P. putida* (HuthPput) were obtained from the SwissProt data base.

activity and restored the ability of this mutant to utilize L-histidine as a nitrogen source.

DISCUSSION

We purified histidase from *S. griseus* and noted that divalent metal cations, sulfhydryl reagents, or a chelating agent had no effect on the activity of the purified enzyme. Our earlier results (25, 26) indicated that these reagents also did not affect histidase in crude extracts from *S. griseus*. The nonlinear rate observed in crude extracts (25) is therefore likely to be a consequence of extract components other than histidase itself or an irreversible change in conformation of histidase that occurs during purification. We favor the former possibility because we have obtained preliminary evidence that histidase can be activated enzymatically (46). These observations supported our analysis of histidase activity in crude extracts of *S. griseus* (25) and suggest that there are distinct differences between streptomycete histidase and that from pseudomonads, which requires Mn^{2+} and

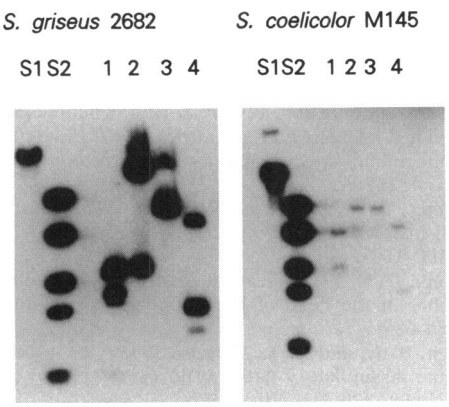

FIG. 8. Southern hybridization analysis of *hutH* in genomic DNA from *S. griseus* and *S. coelicolor*. Genomic DNA from *S. griseus* NRRL B-2682 and *S. coelicolor* M145 was digested with *Sal*I (lanes 1), *Mlu*I (lanes 2), *Bam*HI (lanes 3), or *Nco*I (lanes 4) and hybridized with radiolabeled pKK540, which contains the *hutH* coding sequence. Molecular weight standards in kilobases: S1, 26.3; S2, 6.1, 3.2, 1.5, 1.0, and 0.2.

reducing agent for maximal activity (9, 37). Because we have found no evidence that divalent cations or thiol reagents modulate the activity of histidase from *S. griseus*, in contrast to their effects on pseudomonad histidase (9, 23, 24, 37), it appears that the requirements for maximal activity of the *S. griseus* histidase are different from those of the *P. putida* histidase.

Despite the significant kinetic differences from pseudomonad histidase, streptomycete histidase shares some important properties with other bacterial histidases. The apparent molecular mass of histidase from *S. griseus* is similar to the M_r (53,000 to 55,000) of histidase from *B. subtilis* (29, 33) and pseudomonads (9, 37); it is likely that histidase is active as a tetramer, as has been determined for histidase from other bacteria (9, 23, 29). Cyanide, which is thought to inactivate histidase by reacting with dehydroalanine in the active site (9), also inactivates streptomycete histidase. The lowest concentration of cyanide that inactivated streptomycete histidase, however, was significantly higher than that required for inactivation of histidase from *P. putida*: the half-life of *P. putida* histidase activity in the presence of 4 mM KCN was 30 min (9), whereas *S. griseus* histidase required exposure to more than 150 mM KCN to acquire a similar half-life (Fig. 2). The conditions we used for cyanide inactivation were slightly different from those employed by Consevage and Phillips, however (9): we measured the susceptibility of histidase to potassium cyanide in Tris hydrochloride buffer (pH 8.8) rather than diethanolamine buffer (pH 9.5), because under the latter conditions the inactivation curves for *S. griseus* histidase were multiphasic. Protection from inactivation was afforded by the substrate L-histidine. Unlike the results of Consevage and Phillips (9), however, no divalent cation was required for substrate-mediated protection of the streptomycete histidase. Histidinol phosphate also protected the streptomycete histidase from inactivation by cyanide. Consistent with the protection from targeted inactivation by cyanide, kinetic evidence indicated that histidinol phosphate is a potent competitive inhibitor of L-histidine. Because both phosphorylated and carboxylated substrates are known to bind to arginine in active sites (34), these results suggest that an arginyl residue in *S. griseus* histidase interacts with both histidinol phosphate and L-histidine. To our knowledge, *S. griseus* histidase is the first example for which histidinol phosphate has been identified as an inhibitor. Preliminary experiments indicate that *Klebsiella aerogenes* histidase is not significantly inhibited by histidinol phosphate (46). It may be that the lower sensitivity to cyanide and the competitive inhibition by histidinol phosphate are characteristics of a kinetically distinct histidase, exemplified by that found in streptomycetes. The cladogram presented as Fig. 7 is consistent with this possibility.

Although the overall composition of the *hutH* structural gene (74% G+C) is comparable to those of other streptomycete coding sequences, the G+C content at the N terminus of *hutH* is relatively low. Indeed, we were fortunate not to have biased our oligonucleotide mixture in favor of codons that contain G or C in the third position, because such a mixture probably would not have permitted us to detect the proper clones.

Immediately downstream of the *hutH* coding sequence is an inverted repeat (Fig. 5) that may play a role in transcription termination. There is no evidence that the 0.5 kb of DNA extending downstream of *hutH* encodes either a protein or a tRNA. We do not know whether the *S. griseus hutH* gene is expressed as a monocistronic transcript. The adja-

cent inverted repeat and the absence of an open reading frame downstream of *hutH* support the hypothesis that transcription terminates just beyond the *hutH* coding sequence. Although an open reading frame has been identified upstream of *hutH*, the deduced amino acid sequence of the partial gene product (M_r of >25,400) is distinct from those of HutP of *B. subtilis* (33), HutU of *P. putida* (35), and HutC of either *P. putida* (1) or *K. aerogenes* (40). Thus, either the streptomycete *hutH* gene is part of a *hut* operon containing a different organization of the *hut* genes, or *hutH* does not lie within a *hut* operon in the *S. griseus* chromosome.

There is no obvious ribosome binding site immediately upstream of the *hutH* translation initiation site. The absence of a ribosome binding site is a key trait of genes in which transcription and translation initiate at the same nucleotide. Recent studies have established the efficient translation of leaderless transcripts of *Streptomyces* genes (18). The nucleotide sequence of the region immediately upstream of the *hutH* coding sequence (nucleotides 799 through 827) has characteristics of a relatively strong streptomycete promoter (42) that would be expected to be active during vegetative growth, and evidence from promoter-probe analysis is consistent with the localization of a promoter within the 300 nucleotides preceding the *hutH* translation initiation site. Thus it is possible that the ATG that marks the amino terminus of *hutH* also marks the transcription start site. We are undertaking more detailed studies to determine precisely the transcription start point of *hutH*. These studies will also confirm whether the *hutH* gene in *S. griseus* is expressed constitutively, as our physiological data suggest.

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