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Cloning and Sequencing of the Genes Involved in the Conversion of 5-Substituted Hydantoins to the Corresponding L-Amino Acids from the Native Plasmid of *Pseudomonas* sp. Strain NS671

KEN WATABE,* TAKAHIRO ISHIKAWA, YUKUO MUKOHARA, AND HIROAKI NAKAMURA

Odawara Research Center, Nippon Soda Co., Ltd., 345 Takada, Odawara, Kanagawa 250-02, Japan

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***Pseudomonas* sp. strain NS671, which produces L-amino acids asymmetrically from the corresponding racemic 5-substituted hydantoins, harbored a plasmid of 172 kb. Curing experiments suggest that this plasmid, designated pHN671, is responsible for the conversion of 5-substituted hydantoins to their corresponding L-amino acids by strain NS671. DNA fragments containing the genes involved in this conversion were cloned from pHN671 in *Escherichia coli* by using pUC18 as a cloning vector. The smallest recombinant plasmid, designated pHPB12, contained a 7.5-kb insert DNA. The nucleotide sequence of the insert DNA was determined, and three closely spaced open reading frames predicted to encode peptides with molecular masses of 75.6, 64.9, and 45.7 kDa were found. These open reading frames were designated *hyuA*, *hyuB*, and *hyuC*, respectively. Cell extracts from *E. coli* carrying deletion derivatives of pHPB12 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gene products of *hyuA*, *hyuB*, and *hyuC* were identified. The functions of these gene products were also examined with the deletion derivatives. The results indicate that both *hyuA* and *hyuB* are involved in the conversions of D- and L-5-substituted hydantoins to corresponding *N*-carbamyl-D- and *N*-carbamyl-L-amino acids, respectively, and that *hyuC* is involved in the conversion of *N*-carbamyl-L-amino acids to L-amino acids.**

Racemic 5-substituted hydantoins are intermediates in the conventional chemical synthesis of the corresponding racemic amino acids. It has been reported that some bacteria belonging to the genera *Bacillus* (33, 35, 37), *Flavobacterium* (22, 27), and *Arthrobacter* (30), etc., can convert racemic 5-substituted hydantoins to the corresponding L-amino acids. The bacterial conversion consists of the following three successive steps (22, 30, 31, 35, 36, 39). The first step is either spontaneous or the enzymatic racemization of 5-substituted hydantoins. The second step is a ring-opening hydrolysis of 5-substituted hydantoins with an enzyme called hydantoinase, and corresponding *N*-carbamyl-amino acids are produced. This step is thought to be either L-isomer specific or nonspecific but reversible. In the third step, *N*-carbamyl-L-amino acids are hydrolyzed to L-amino acids with an L-isomer-specific enzyme. As a result, the whole racemic 5-substituted hydantoins are converted to corresponding L-amino acids.

Pseudomonas sp. strain NS671, which produces L-amino acids asymmetrically from corresponding racemic 5-substituted hydantoins, is one of the bacteria isolated in our laboratory from soil (14). T o investigate the molecular mechanisms of the reactions catalyzed by strain NS671, we attempted to clone the genes involved in the conversion of 5-substituted hydantoins to corresponding L-amino acids. In this paper, we describe the cloning and sequencing of the genes and examine the functions of these gene products.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strain NS671 (FERM P-9543, Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan), which is able to convert racemic 5-substituted hydantoins to corresponding L-amino acids, was isolated from soil and identified as a *Pseudomonas* sp. (14). Plasmid DNA, designated pHN671, was isolated from strain NS671 by the alkaline lysis method (4), purified by CsCl-ethidium bromide density gradient centrifugation (17), and used as a gene source. The vector for cloning was pUC18, and those for sequencing were M13mp18 and M13mp19 (38). *Escherichia coli* JM103 (19) was used as a host for recombinant plasmids.

Media, culture conditions, and chemicals. Strain NS671 was grown in LB medium (17) at 30°C. *E. coli* JM103 carrying recombinant plasmids were grown in LB medium supplemented with 50 μg of ampicillin per ml at 30°C. The agar plate of M9 medium (17), containing 0.1% DL-5-(2-methylthioethyl)hydantoin as the sole nitrogen source instead of NH_4Cl and supplemented with 10 μg of $MnSO_4$ per ml, 1 μg of $FeSO_4$ per ml, 1 μg of thiamine per ml, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), was used as a selective plate for gene cloning. Optically active 5-(2-methylthioethyl)hydantoin and *N*-carbamylmethionine were prepared from D- or L-methionine (28) in our laboratory.

Plasmid curing. Test tubes containing 1.5 ml of LB medium and various concentrations of acridine orange or sodium dodecyl sulfate (SDS) were inoculated with strain NS671 and shaken at 30°C overnight. Cells from the tube containing the highest concentration of the potential curing reagent that just allowed growth were spread on an LB agar plate and incubated at 30°C overnight. Colonies were tested for their ability to convert 5-substituted hydantoins to corresponding amino acids as follows. The cells from each colony were inoculated into M9 medium containing 0.5% DL-5-(2-methylthioethyl)hydantoin instead of NH_4Cl and supplemented with 10 μg of $MnSO_4$ per ml, 1 μg of $FeSO_4$ per ml, 1 μg of thiamine per ml, and 0.01% yeast extract. After incubation at 30°C with shaking overnight, the culture medium was analyzed by thin-layer chromatography on

* Corresponding author.

silica gel 60 F-254 (Merck) with a solvent system of 1-butanol–acetic acid–water (9:1:1, by volume), and the products were detected by the iodoplatinate method (34).

Cloning of DNA. The plasmid pHN671 was partially digested with *Mbo*I, and the DNA fragments were ligated into the *Bam*HI site of pUC18. Competent cells of *E. coli* JM103 were transformed by using the ligated DNA. The transformants were incubated in LB medium supplemented with 1 mM IPTG at 30°C for 1.5 h, spread on the selective plate described above, and incubated at 30°C for about 2 weeks. Recombinant clones having the ability to utilize 5-(2-methylthioethyl)hydantoin as a nitrogen source are expected to form colonies.

DNA sequencing. Overlapping DNA fragments from the cloned DNA were generated by using appropriate restriction endonucleases and cloned in both orientations into M13mp18 and/or M13mp19. Progressive unidirectional deletions of the insert DNAs were created by the method of Henikoff (10) by using a deletion kit (Takara Shuzo, Kyoto, Japan). These recombinants were transfected into *E. coli* JM103 to produce single-stranded templates. Nucleotide sequences of the clones were determined by the dideoxy chain termination method of Sanger et al. (26) by using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequence analysis was totally carried out for both strands. For computer analysis, SDC-Genetyx software (Software Development Co., Tokyo, Japan) was used.

Identification of gene products. *E. coli* JM103 carrying a deletion derivative of pHPB12 was grown in 1.5 ml of LB medium with shaking at 30°C. IPTG (7.5 μl of a 200 mM solution) was added to the culture at an A_{600} of about 0.2, and incubation was continued for another 2 h. The cells were harvested by centrifugation (3,000 × *g*, 5 min, 4°C), suspended in 600 μl of 25 mM Tris-HCl (pH 8.0) containing 50 mM glucose and 10 mM EDTA, and disrupted by sonication on ice for 30 s with an ultrasonic homogenizer UR-20P (Tomy Seiko Co., Tokyo, Japan). The cell debris was removed by centrifugation (18,000 × *g*, 10 min, 4°C). The supernatant was heated in the sample buffer containing 5% (vol/vol) β-mercaptoethanol for 2 min over boiling water and analyzed with a 12% polyacrylamide gel by the method of Laemmli (16). After electrophoresis, the gel was stained with Coomassie brilliant blue. Protein standards used for molecular mass estimation were phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), and carbonic anhydrase (30.0 kDa) (electrophoresis calibration kit; Pharmacia LKB, Uppsala, Sweden).

Whole-cell reactions. *E. coli* JM103 carrying a deletion derivative of pHPB12 was grown in 1.5 ml of LB medium with shaking at 30°C. IPTG (7.5 μl of a 200 mM solution) and optically active 5-(2-methylthioethyl)hydantoin or *N*-carbamylmethionine (150 μl of a 2% solution) were added to the culture at an A_{600} of about 0.2. The incubation was continued for 16 h for 5-(2-methylthioethyl)hydantoin or for 3 days for *N*-carbamylmethionine. The culture medium was analyzed by the thin-layer chromatography described above.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to DDBJ, EMBL, and GenBank and assigned the accession number D90469.

RESULTS

Isolation and characterization of the plasmid pHN671. A DNA band corresponding to covalently closed circular DNA was detected by the CsCl-ethidium bromide density gradient centrifugation of crude DNA from strain NS671. This DNA was analyzed by agarose gel electrophoresis, and a single plasmid, designated pHN671, was found. pHN671 was digested with restriction endonucleases and analyzed by agarose gel electrophoresis. The sizes of the fragments generated with *Bgl*II, *Kpn*I, and *Pfl*MI added up to 170, 175, and 170 kb, respectively (data not shown). Thus, the total length of pHN671 was estimated to be 172 kb.

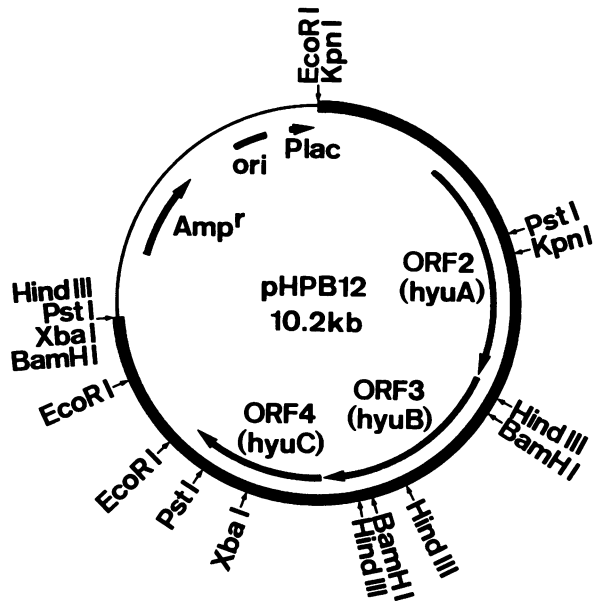

FIG. 1. Structure of pHPB12. The thick line represents the fragment from pHN671. The thin line represents the pUC18 vector. The regions of the complete ORFs found in the insert are indicated by arrows.

To determine whether pHN671 is related to the conversion of 5-substituted hydantoins to corresponding L-amino acids, we attempted to cure strain NS671 with acridine orange (10 μg/ml) or SDS (0.005%). Thirty-two colonies were tested for their conversion ability after treatment. Two strains from the acridine orange treatment and one strain

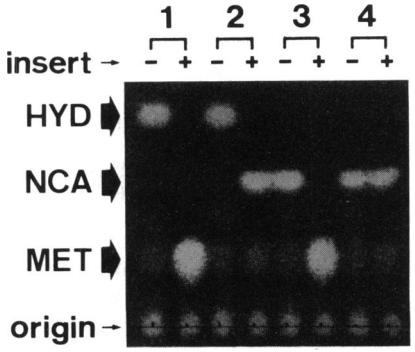

FIG. 2. Conversions of 5-(2-methylthioethyl)hydantoin and *N*-carbamylmethionine by *E. coli* carrying pHPB12. *E. coli* carrying either pUC18 (insert, −) or pHPB12 (insert, +) was incubated with 5-(2-methylthioethyl)hydantoin (lanes 1 and 2) or *N*-carbamylmethionine (lanes 3 and 4), and the resultant culture media were analyzed by thin-layer chromatography. Lanes 1 and 3 are the L-isomer and lanes 2 and 4 are the D-isomer of each substrate. HYD, 5-(2-methylthioethyl)hydantoin; NCA, *N*-carbamylmethionine; MET, methionine.

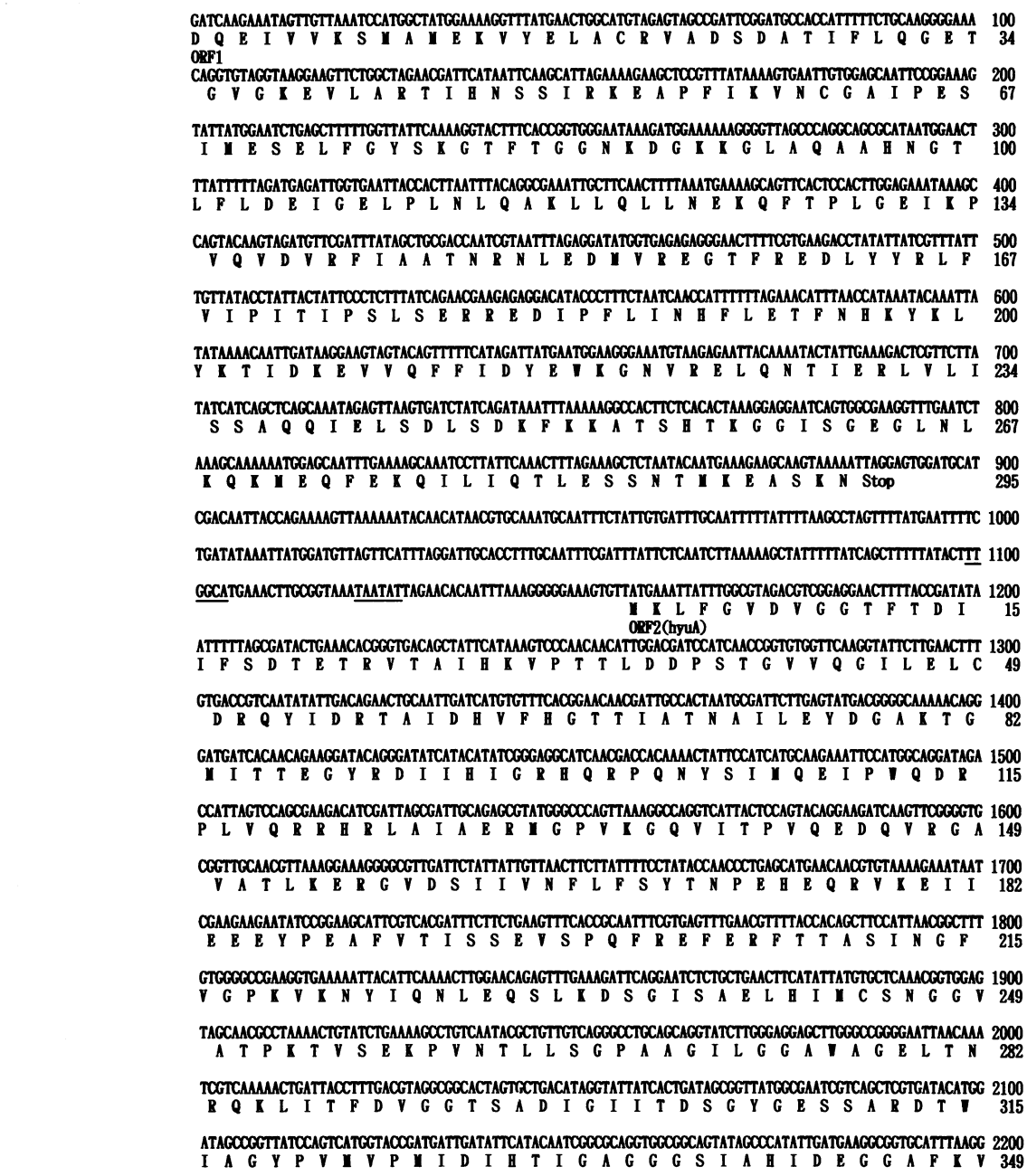

FIG. 3. Nucleotide sequence of the insert DNA of pHPB12 and deduced amino acid sequences of ORFs. Nucleotides are numbered at the right of the sequence lines, with nucleotide 1 corresponding to the first nucleotide of the insert DNA. Amino acids are numbered at the right of the sequence lines, with amino acid 1 corresponding to the first amino acid of each ORF. Sequences similar to the −10 and −35 consensus sequences for *E. coli* promoters are underlined.

from the SDS treatment lost the conversion ability and simultaneously lost the plasmid. It was later found that pHN671 is spontaneously eliminated from the host if the bacterium is stored for a long time, and the resulting plasmidless mutants lost the conversion ability. These results suggest that pHN671 contains genes responsible for the conversion of 5-substituted hydantoins to corresponding L-amino acids by strain NS671.

Cloning of the genes involved in the conversion of 5-substituted hydantoins to the corresponding L-amino acids. The experimental approach used for the cloning of the genes involved in the conversion of 5-substituted hydantoins to corresponding L-amino acids was to select the clones of the transformed *E. coli* cells with the ability to utilize 5-(2-methylthioethyl)-hydantoin as a nitrogen source. Because *E. coli* does not grow on a medium containing 5-(2-methylthioethyl)hydantoin as the sole nitrogen source, it was a feasible host for this experimental approach. A partial *Mbo*I library of pHN671 was constructed by insertions within the *Bam*HI site of pUC18. Approximately 20,000 recombinant clones were screened for their ability to form a colony on a selective plate containing 5-(2-methylthioethyl)hydantoin as the sole nitrogen source. Four clones formed colonies, and they were ampicillin resistant. Plasmid DNAs were prepared from

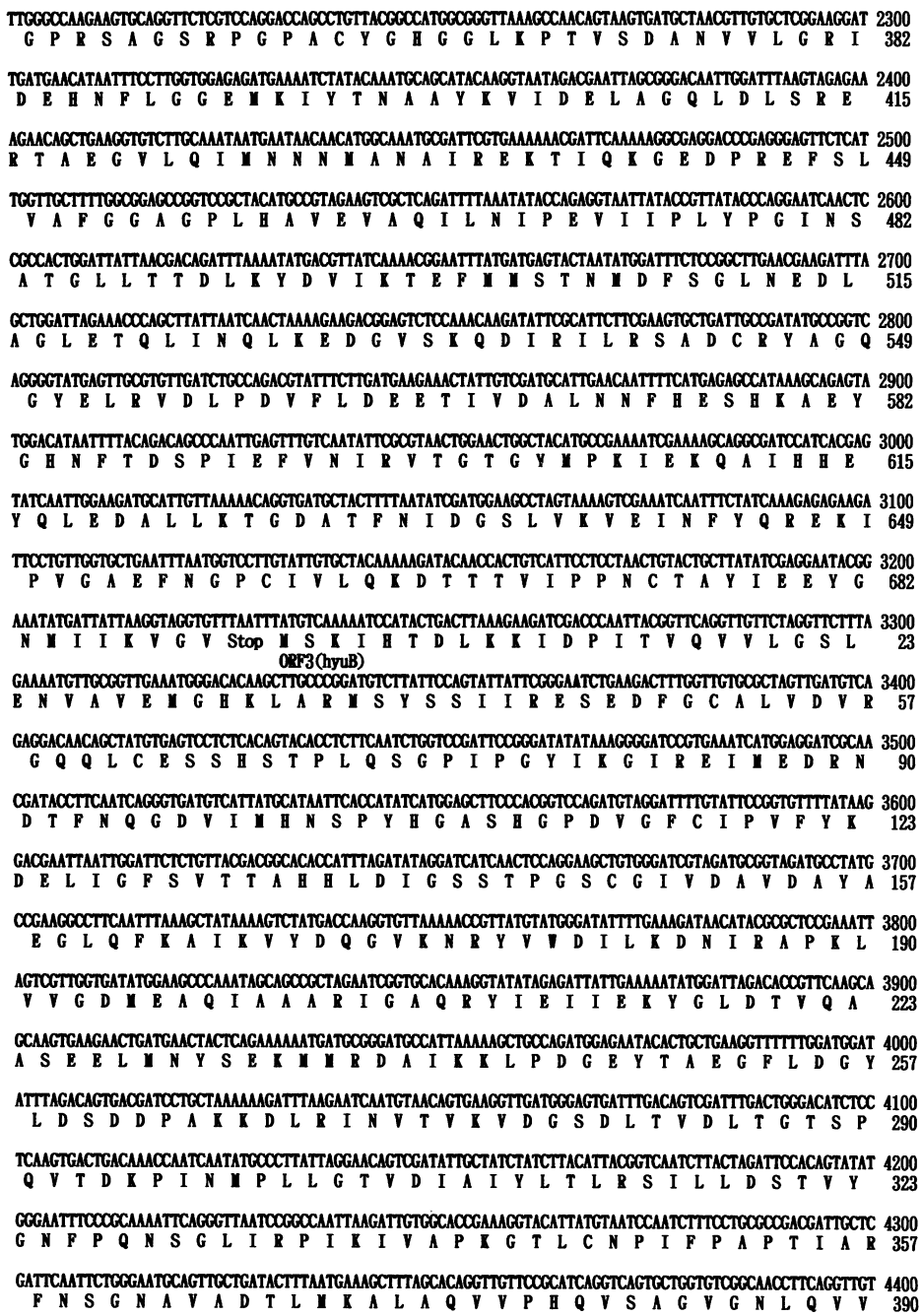

FIG. 3—*Continued.*

these clones and analyzed with *Pst*I. Each of them consisted of two common *Pst*I fragments of 4.6 and 4.1 kb and an additional *Pst*I fragment of either 1.5 (two clones), 2.3, or 6.8 kb. This result suggests that the inserts of the recombinant plasmids are derived from the identical portion in pHN671 and oriented in the same direction, but the terminals of the inserts opposite from the *lac* promoter are different. Detailed restriction analysis was performed on the smallest plasmid, designated pHPB12, which contains an insert of 7.5 kb, and the restriction map is shown in Fig. 1. Retransformation of *E. coli* with pHPB12 yielded transformants that could grow on an agar plate containing either 5-(2-methylthioethyl)hydantoin or *N*-carbamylmethionine as the sole nitrogen source.

The conversions of 5-(2-methylthioethyl)hydantoin and *N*-carbamylmethionine by *E. coli* carrying pHPB12 were examined, and the results are shown in Fig. 2. Both L-5-(2-methylthioethyl)hydantoin and *N*-carbamyl-L-methionine were converted to methionine, which was found to be the L-isomer by thin-layer chromatography by using a Chiral plate (Macherey-Nagel). D-5-(2-Methylthioethyl)hydantoin was converted to *N*-carbamylmethionine but not to methionine. This *N*-carbamylmethionine was thought to be the D-isomer since *N*-carbamyl-D-methionine was not converted

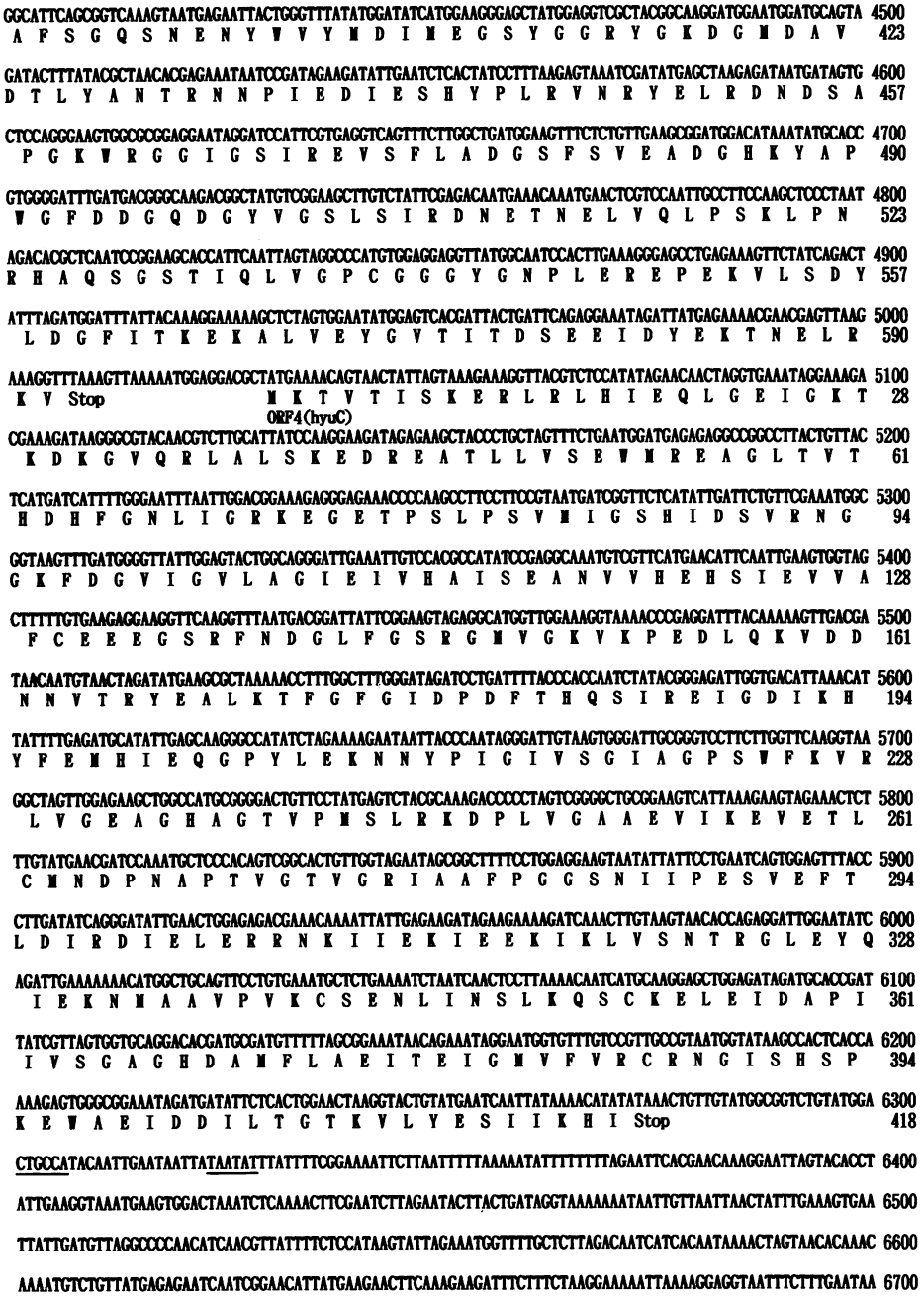

FIG. 3—*Continued.*

by *E. coli* carrying pHPB12 (Fig. 2, lane 4). To determine the configuration of the *N*-carbamylmethionine produced from D-5-(2-methylthioethyl)hydantoin, we converted it chemically to methionine by the sodium nitrite method (36), and it was found to be the D-isomer judging from the configuration of this methionine. Thus, *E. coli* carrying pHPB12 is able to convert D- and L-5-(2-methylthioethyl)hydantoin to *N*-carbamyl-D- and *N*-carbamyl-L-methionine, respectively, and to convert *N*-carbamyl-L-methionine to L-methionine.

Nucleotide sequence analysis. The sequence of the 7.5-kb insert DNA of pHPB12 is shown in Fig. 3. Analysis of the sequence revealed five open reading frames (ORFs), designated ORF1, ORF2, ORF3, ORF4, and ORF5. All of these ORFs were in the same orientation. ORF1 and ORF5 were truncated, and only the 3′ portion of ORF1 (295 amino acids) and the 5′ portion of ORF5 (245 amino acids) were present in the insert. ORF2, ORF3, and ORF4 were closely spaced, suggesting that these ORFs may be translationally coupled (23). ORF2 and ORF3 were preceded by possible ribosome-binding sites (Shine-Dalgarno sequences) and were predicted to encode peptides of 690 and 592 amino acids, with calculated molecular masses of 75.6 and 64.9 kDa, respectively. ORF4 started with two adjacent ATG triplets (positions 5019 and 5031), but a possible ribosome-binding site (GGAGGA)

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CGTTAATGAAAAGGGGATTAGTTTAAAGGGAAAAGACATCATGCCGACAGTGAACAAGGAAAGAAATATGTCCTCAATAGCGTATTTTATTTTATGGGTT 6800
                                    M  P  T  V  N  K  E  R  N  M  S  S  I  A  Y  F  I  L  W  V   20
                                   ORF5
GGGATAGCTGTACAACTAGTAACCCCCATAACAGCGGCTCAGCTTTATCCAGCATTATCACCCCTGCAAATCATCATAGCTTGTATCCTTGGTAATCTAA 6900
G  I  A  V  Q  L  V  T  P  I  T  A  A  Q  L  Y  P  A  L  S  P  L  Q  I  I  I  A  C  I  L  G  N  L  I   54

TTGTTGCTATTCTTTTAACATTATTAGGGGATATTGGGGTGAGATATGGAATTCCATATGCGGTTTATATTAGGGCGAGCTTCGGTTATTTAGGAGCCCA 7000
 V  A  I  L  L  T  L  L  G  D  I  G  V  R  Y  G  I  P  Y  A  V  Y  I  R  A  S  F  G  Y  L  G  A  H   87

TATACCAGGTATTGTACGTGCCGTTCCAGCGGTATTCTGGTTTGGTTTTCAAACATGGATGGGTGCATATGCCTTGGATGCAATCATGGAAATGTTAACG 7100
 I  P  G  I  V  R  A  V  P  A  V  F  W  F  G  F  Q  T  W  M  G  A  Y  A  L  D  A  I  M  E  M  L  T  120

GGTTACTCGAACCTTACATTGTTAATTATTTTATTTGGTGTAGTGCAAATCATTAACACTGCCATGGGAATGGAAGCTATTACAAAATTTGAATGGCTGG 7200
G  Y  S  N  L  T  L  L  I  I  L  F  G  V  V  Q  I  I  N  T  A  M  G  M  E  A  I  T  K  F  E  W  L  A  154

CTTCCCCTAGTATTTTAATTATAGGTATTATTTTACAAGTATATATTATGAAACAGCATCATTTAACATTCAGTGAGATTTTCTCCCATGGGGGTGACGG 7300
 S  P  S  I  L  I  I  G  I  I  L  Q  V  Y  I  M  K  Q  H  H  L  T  F  S  E  I  F  S  H  G  G  D  G  187

TGGTGTTTCAATGGGCTATGCTGTCGTCGTAATGATGGGAACATATATTACGATGGCTTTAAATGCCCCCGGATTTTACCCGGTTCTTAAAGACAAAAACT 7400
 G  V  S  M  G  Y  A  V  V  V  M  M  G  T  Y  I  T  M  A  L  N  A  P  D  F  T  R  F  L  K  T  K  T  220

GGTAGTAGTGAGCCTAATTGGTGGAAAGTCAATAAAGGAAGTTTTTGGGCACATACATTTGGTTTGATAGGATCC                    7475
G  S  S  E  P  N  W  W  K  V  N  K  G  S  F  W  A  H  T  F  G  L  I  G  S                      245
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FIG. 3—*Continued.*

was present between these triplets. Therefore, the proper translational initiation codon would be the second triplet, and ORF4 was predicted to encode a peptide of 414 amino acids, with a calculated molecular mass of 45.7 kDa.

The nucleotide compositions of the intergenic regions between ORF1 and ORF2 and between ORF4 and ORF5 were A+T rich (72% for both) compared with those of ORF2, ORF3, and ORF4 (58% on the average). Upstream of ORF2 and ORF5, sequences similar to the −10 and −35 consensus sequences for *E. coli* promoters were found. These sequences are TTGGCAN_{16}TAATAT (positions 1099 to 1126) and CTGCCAN_{16}TAATAT (positions 6301 to 6328). Downstream of ORF4, two palindromic sequences (positions 6280 to 6311 and 6504 to 6526) were found. The latter sequence was followed by a run of T residues, which is typical of the ρ-independent transcriptional termination signals (25).

Construction of deletion derivatives of pHPB12 and identification of the gene products of the ORFs. To identify the gene products of the ORFs, we constructed some deletion derivatives of pHPB12 as follows. pHPB12 was digested independently with *Bam*HI, *Xba*I, and *Kpn*I, and the fragments were self-ligated. The desired recombinant plasmids were verified by restriction analysis and designated pDBA35, pDXB51, and pDKP46, respectively. pDKP46 was digested with *Xba*I, and the fragment was self-ligated to yield pDKX35. pHPB12 was double digested with *Sma*I-*Stu*I and *Pfl*MI-*Stu*I independently, and the latter was treated with mung bean nuclease to produce blunt ends. These fragments were self-ligated, and the resulting recombinant plasmids were designated pDST38 and pDSP73, respectively. The deletion derivatives obtained above are summarized in Fig. 4.

The cell extracts from *E. coli* carrying the deletion derivatives of pHPB12 were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). *E. coli* carrying pHPB12, as compared with that carrying pUC18, exhibited three additional peptide bands with apparent molecular masses of 76, 66, and 45 kDa. The 76-kDa peptide appeared only in the case where the deletion derivatives contained intact ORF2, and the apparent molecular mass was in good agreement with the calculated molecular mass of the ORF2 product (75.6 kDa). Similarly, the 66- and 45-kDa peptides appeared only in the cases where the deletion derivatives contained intact ORF3 and intact ORF4, respectively, and the apparent molecular masses were in good agreement with the calculated molecular masses of the ORF3 product (64.9 kDa) and ORF4

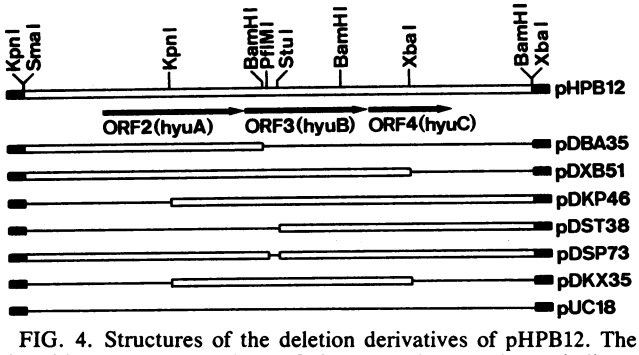

FIG. 4. Structures of the deletion derivatives of pHPB12. The closed boxes represent the pUC18 vector, the open boxes indicate insert DNAs, and the thin lines indicate deleted regions. Restriction endonucleases used to construct the deletion derivatives are shown at the top of the figure. The regions of the complete ORFs found in the insert of pHPB12 are indicated by arrows.

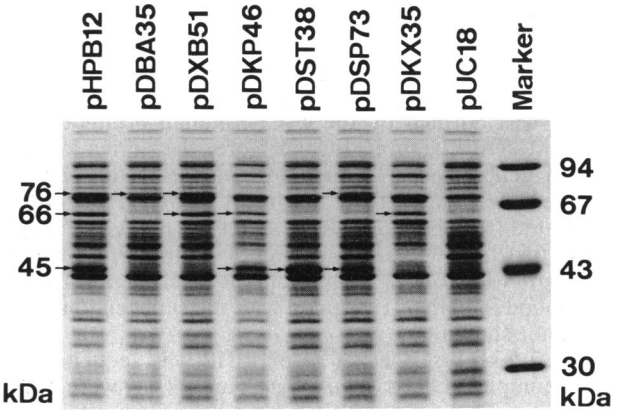

FIG. 5. Identification of the gene products of the ORFs found in the insert of pHPB12. The cell extracts of *E. coli* carrying the deletion derivatives of pHPB12 defined in Fig. 4 were analyzed by SDS-polyacrylamide gel electrophoresis. Peptide bands not seen in the extract of the cells carrying pUC18 are indicated by arrows.

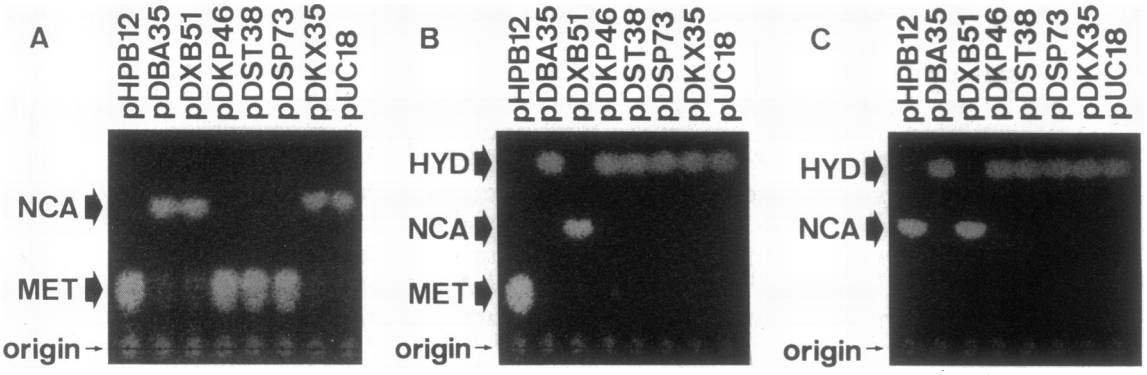

FIG. 6. Conversions of 5-(2-methylthioethyl)hydantoin and *N*-carbamylmethionine by *E. coli* carrying the deletion derivatives of pHPB12. *E. coli* carrying the deletion derivatives of pHPB12 shown in Fig. 4 were incubated with *N*-carbamyl-L-methionine (A), L-5-(2-methylthioethyl)hydantoin (B), or D-5-(2-methylthioethyl)hydantoin (C), and the resultant culture media were analyzed by thin-layer chromatography. HYD, 5-(2-methylthioethyl)hydantoin; NCA, *N*-carbamylmethionine; MET, methionine.

product (45.7 kDa), respectively. These results indicate that the 76-, 66-, and 45-kDa peptides are the gene products of ORF2, ORF3, and ORF4, respectively.

Functions of the gene products of the ORFs. To determine the functions of the gene products of the ORFs, we examined the conversions of 5-(2-methylthioethyl)hydantoin and *N*-carbamylmethionine by *E. coli* carrying the deletion derivatives of pHPB12. *N*-carbamyl-L-methionine was converted to L-methionine only by the cells containing intact ORF4 (Fig. 6A). D- and L-5-(2-Methylthioethyl)hydantoin were converted only by the cells containing both intact ORF2 and intact ORF3 (Fig. 6B and C). When using pDXB51, deficient in ORF4, *N*-carbamyl-L-methionine was produced from L-5-(2-methylthioethyl)hydantoin (Fig. 6B). The *N*-carbamylmethionine produced from D-5-(2-methylthioethyl)hydantoin was the D-isomer so that it was not converted to methionine by the cells carrying pHPB12 (Fig. 6C). These results indicate that both ORF2 and ORF3 are involved in the conversions of D- and L-5-(2-methylthioethyl)hydantoin to *N*-carbamyl-D- and *N*-carbamyl-L-methionine, respectively, and that ORF4 is involved in the conversion of *N*-carbamyl-L-methionine to L-methionine. Thus, ORF2, ORF3, and ORF4 were renamed *hyuA*, *hyuB*, and *hyuC* (hydantoin utilization), respectively.

DISCUSSION

The results of curing experiments suggest that pHN671 is responsible for the conversion of 5-substituted hydantoins to corresponding L-amino acids by strain NS671. In fact, the genes involved in the conversion were cloned from pHN671 which was purified by the CsCl-ethidium bromide density gradient centrifugation. Furthermore, the derivation of the cloned genes was confirmed by comparing the restriction fragments generated from the insert of pHPB12 with those generated from pHN671. Restriction fragments corresponding to the *Ban*II (5.45-kb), *Eco*NI (4.44-kb), *Hpa*I (5.45-kb), and *Pst*I (4.06-kb) fragments from the insert of pHPB12 were also found in pHN671 digested with the same endonucleases (data not shown).

The 7.5-kb insert DNA of pHPB12 was sequenced, and three complete ORFs (*hyuA*, *hyuB*, and *hyuC*) were found in this insert. The potential initiation codons for *hyuA* and *hyuB* are ATG triplets at positions 1156 and 3232, respectively, considering the apparent molecular masses of the gene products (Fig. 5). For *hyuC*, there are two potential initiation codons at positions 5019 and 5031. The gene product of *hyuC* was purified from *E. coli* carrying pDST38, containing complete *hyuC*. Its N-terminal amino acid sequence was analyzed and found to be M-K-T-V-T-I-S-K-E-X-L (where X indicates unidentified residue). Therefore, the translational start of *hyuC* is thought to be the ATG triplet at position 5031, which is reasonable considering the position of the potential Shine-Dalgarno sequence (positions 5021 to 5026).

Nucleotide sequence identities were found between *hyuA* and *hyuC* (43%) and between *hyuB* and *hyuC* (46%), suggesting that these genes have evolved from a common ancestor by gene duplications. In contrast with the nucleotide sequence identities, an amino acid sequence identity was found only between C-terminal regions of the deduced sequences of *hyuA* and *hyuB* (15% in 126 amino acids).

The deduced amino acid sequences of the ORFs found in the insert of pHPB12, except that of ORF1, had no significant similarity to the known sequences in the NBRF (release 26) and SWISS (release 15) protein data bases. The deduced amino acid sequence of ORF1 was found to share strong homology with the gene products of *nifA* (40 to 46% in 237 to 287 amino acids) from different species (2, 6, 9, 18, 21, 32), *ntrC* (42 to 44% in 248 to 264 amino acids) from *Klebsiella pneumoniae* (6) and *E. coli* (20), *dctD* (42% in 231 amino acids) from *Rhizobium leguminosarum* (24) and *Rhizobium meliloti* (15), *xylR* (40% in 229 amino acids) from *Pseudomonas putida* (13), *tyrR* (40% in 229 amino acids) from *E. coli* (7), and *hydG* (37% in 290 amino acids) from *E. coli* (29). All of these are regulatory proteins which function at a transcriptional level. It is of interest that, upstream of *hyuA* and ORF5, sequences similar to the consensus sequence (CTGGYAYRN$_4$TTGCA) for nitrogen-regulated or nitrogen fixation promoters (Ntr or Nif promoters) (1, 3) were found. These sequences are TTGGCATGN$_4$TTGCG (positions 1099 to 1115) and CTGCCATAN$_3$TTGAA (positions 6301 to 6316), overlapping the sequences similar to the consensus sequences for *E. coli* σ^{70} promoters (Fig. 3). The Ntr or Nif promoters are recognized by the σ^{54} RNA polymerase (11, 12), which requires the transcriptional activator *ntrC* or *nifA* product (5, 8). Future efforts to establish the function of the ORF1 product should yield information concerning the regulation of the expression of the genes involved in the conversion of 5-substituted hydantoins to L-amino acids.

E. coli carrying pHPB12 converted D-5-(2-methylthioethyl)hydantoin only to *N*-carbamyl-D-methionine, while the original bacterium strain NS671 converted it to L-methio-

nine. This fact indicates that strain NS671 can racemize 5-(2-methylthioethyl)hydantoin. The search for the additional gene responsible for the reaction is now in progress.

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