Cloning and nucleotide sequence of the carboxynorspermidine decarboxylase gene from *Vibrio alginolyticus*

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The gene (*nspC*) encoding carboxynorspermidine decarboxylase (CANS DC), the last enzyme in norspermidine biosynthesis, in *Vibrio alginolyticus* was isolated by immuno-screening and its complete nucleotide sequence was determined. Sequence analysis of the subcloned fragment (2.0 kb) revealed an ORF of 1131 bp encoding a protein of 377 amino acids with a calculated molecular mass of 42 008 Da. The sequence of 20 N-terminal amino acids of purified CANS DC was found to be identical to that predicted from the *nspC* gene. A putative ribosome binding sequence was observed 8 bp upstream from the translation start site (ATG), and promoter- and terminator-like sequences were detected upstream and downstream of the ORF, respectively. Database searches identified no similar proteins, but the deduced amino acid sequence contained a putative pyridoxal 5'-phosphate binding region similar to those of the bacterial meso-2,6-diaminopimelate decarboxylases and eukaryotic ornithine decarboxylases. Another full ORF was found on the opposite strand downstream from the *nspC* gene. It encoded a protein of 69 amino acids with a calculated molecular mass of 7441 Da, which exhibited some weak similarity to ScrR, a repressor protein of *V. alginolyticus*, in the helix-turn-helix DNA binding domain, but did not appear to be expressed in the host cells.

Keywords: *Vibrio alginolyticus*, carboxynorspermidine, decarboxylase gene, polyamines

INTRODUCTION

In addition to the three prototypical polyamines, putrescine, spermidine and spermine, a wide variety of unusual polyamines, most homologues of the prototypes, such as norspermidine (NSPD) and homospermidine, have been identified particularly in prokaryotes, and recently polyamine analysis has proved to be a valuable tool for chemotaxonomic classification of some bacterial species (Hamana & Matsuzaki, 1992). However, the amounts of unusual polyamines found in most bacteria seem too low to suggest their functional roles in producer strains. In contrast, members of the genus *Vibrio*, some of which are important pathogens for humans and animals (Janda *et al.*, 1988), are characterized by possessing NSPD as a main polyamine species instead of spermidine

(Yamamoto *et al.*, 1990). This is quite a unique type of polyamine occurrence in mesophilic bacteria. In addition, the novel biosynthetic pathways for NSPD in some *Vibrio* species have been delineated (Fig. 1) (Yamamoto *et al.*, 1986) and the three enzymes responsible have been purified from *V. alginolyticus* (Nakao *et al.*, 1989, 1990, 1991).

The genes involved in biosynthesis of the usual polyamines, putrescine and spermidine, have been cloned from *Escherichia coli* and extensively characterized (Boyle *et al.*, 1984; Tabor & Tabor, 1987; Szumanski & Boyle, 1990; Moore & Boyle, 1990; biosynthetic ornithine decarboxylase, GenBank accession no. M33766). However, to our knowledge, no genetic study regarding the biosynthetic enzymes for unusual polyamines has been reported. From a viewpoint of the novelty of NSPD biosynthesis in vibrios, it would be of great interest to elucidate the structure, organization and regulation of the genes encoding these enzymes. In addition, isolation and characterization of the genes responsible may provide the means to develop specific DNA probes or PCR primers

Abbreviations: CANS DC, carboxynorspermidine decarboxylase; NSPD, norspermidine; PLP, pyridoxal 5'-phosphate; DAP DC, meso-2,6-diaminopimelate decarboxylase; ODC, ornithine decarboxylase.

The GenBank accession number for the nucleotide sequence data reported in this paper is D31783.

Fig. 1. Biosynthetic pathway for norspermidine in *Vibrio* species. Enzymes: A, L-2,4-diaminobutyrate decarboxylase; B, carboxynorspermidine synthase; C, CANS DC. ASA, aspartic β -semialdehyde; DABA, L-2,4-diaminobutyrate.

for detection and identification of *Vibrio* species. Thus, we started to clone the genes involved in NSPD biosynthesis from *V. alginolyticus*.

In this study, we have isolated the gene, named *nspC*, encoding the pyridoxal 5'-phosphate (PLP)-dependent carboxynorspermidine decarboxylase (CANS DC), catalysing the last step of NSPD biosynthesis, and completely sequenced this gene. The deduced amino acid sequence was compared with those from other bacterial decarboxylases, and Southern hybridization with part of the cloned DNA as a probe was performed to find homologous sequences in other *Vibrio* species.

METHODS

Bacterial strains and plasmids. *V. alginolyticus* type strain ATCC 17749 was used as a source of the chromosomal DNA. *E. coli* HB101 (Boyer & Roulland-Dussoix, 1969) was used as a host for cloning and sequencing experiments. The plasmids pUC18 and 19 (Norrander *et al.*, 1983) were used as cloning and subcloning vectors, and pUC19 and pBluescript II SK(+) (Stratagene) as vectors in preparation of unidirectional deletion plasmids for sequencing.

Purification of CANS DC and determination of N-terminal amino acid sequence. CANS DC was purified from *V. alginolyticus* as described by Nakao *et al.* (1990). The N-terminal amino acid sequence was determined by automated sequential Edman degradation with subsequent identification and quantification of phenylthiohydantoin-labelled amino acids by reversed-phase HPLC.

Antibody preparation. Polyclonal antibodies against purified CANS DC were raised in a rabbit as previously described (Yamamoto *et al.*, 1992). The antiserum was absorbed with sonicated HB101 cells grown in LB broth (Sambrook *et al.*, 1989).

Western blotting and assay of CANS DC activity. *E. coli* transformants carrying the recombinant plasmid DNA were cultured in LB broth supplemented with ampicillin at 37 °C for 8 h. Cells from 200 ml of the culture were harvested by centrifugation and were suspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol and 0.1 mM EDTA. The cell

suspension was disrupted by sonication and the cell debris removed by centrifugation (40000 g, 30 min at 4 °C). The supernatant was used for Western blot analysis and CANS DC assay (Nakao *et al.*, 1990). Western blotting was carried out as follows. A sample of the supernatant (approx. 50 µg protein) was separated by SDS-PAGE by the method of Laemmli (1970). The gels were equilibrated in a transfer buffer (48 mM Tris, 30 mM glycerol and 20%, v/v, methanol) and then transferred electrophoretically (15 V, 30 min) onto nitrocellulose membranes (Hybond-C, Amersham), using a Trans-Blot SD electrophoretic blotting cell (Bio-Rad). The membranes were blocked for 1 h in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) plus 3% (w/v) skimmed milk (Difco), and incubated with rabbit antibodies against CANS DC (1:500 dilution) for 1 h. Goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2000 dilution; Boehringer Mannheim) was bound to the membrane in TBST. After washing with TBST, the membrane was visualized by the addition of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate colour development substrates.

Genomic and plasmid DNA isolation. *V. alginolyticus* ATCC 17749 was grown in the modified marine fermentation oxidation medium (Yamamoto *et al.*, 1979) containing 2% (w/v) NaCl for 8 h at 37 °C in a reciprocal waterbath shaker. Cultures were harvested by centrifugation (9800 g, 10 min at 4 °C) during exponential growth ($\text{OD}_{660} = 0.8$). Genomic DNA was prepared by the procedure of Murray & Thompson (1980) using hexadecyltrimethylammonium bromide. *E. coli* strains harbouring a plasmid were cultivated in LB broth containing 100 µg ampicillin ml⁻¹ at 37 °C. Plasmid DNA was isolated by the alkaline extraction procedure of Birnboim & Doly (1979).

Cloning of the CANS DC (*nspC*) gene. Genomic DNA from *V. alginolyticus* was partially digested with *Sau3A*, and the fragments (4–10 kb) collected from an agarose gel were ligated with the DNA ligation kit (Takara Shuzo) into *Bam*HI-digested pUC19 previously treated with calf intestine alkaline phosphatase (Toyobo) for 30 min at 37 °C. The ligation mixture was used to transform competent cells of *E. coli* HB101. Ampicillin-resistant transformants were immobilized on nitrocellulose membranes according to the procedure of Helfman *et al.* (1983), and then CANS DC protein-containing colonies were immunologically detected as described for Western blotting. Enzymic manipulation and subcloning of DNA were carried out using standard methods (Sambrook *et al.*, 1989). Restriction enzymes were purchased from Nippon Gene.

Nucleotide sequencing. Unidirectional deletion subclones were prepared by exonuclease III/mung bean nuclease digestion (Kilo-Sequence Deletion kit, Takara Shuzo) with appropriate sets of restriction enzymes according to the manufacturer's recommendations. To determine the span of the CANS DC structural gene, the reactivities to the antiserum of each colony of *E. coli* HB101 transformed with a deleted plasmid and its crude lysate were monitored by dot blotting and Western blotting, respectively. The plasmid DNA was denatured by treatment with 2 M NaOH/1 mM Na₂EDTA, and the nucleotide sequence of both strands of the cloned DNA was determined by the method of Sanger *et al.* (1977) using [α -³⁵S]CTP and the *Bca*BEST Dideoxy Sequencing kit (Takara Shuzo). Approximately 300 bases were read from each sample.

DNA hybridization. Total cellular DNAs from *V. alginolyticus* and other pathogenic *Vibrio* species were digested to completion with *Xba*I and separated by electrophoresis. After denaturation by soaking the gel with 0.5 M NaOH/1 M NaCl for 30 min at room temperature, the DNA was transferred onto a nylon membrane (Pall) (Southern, 1975) and hybridized with plasmid

pCDC14-3 (see Fig. 3) as a probe containing the major part of the structural gene of CANS DC. Hybridizations were conducted overnight at 42 °C in a solution of 5 × SSC containing 0.1% sodium *N*-lauroylsarcosine, 3% (w/v) casein, 0.02% SDS and 50% (v/v) formamide, and the bands hybridized were visualized by the alkaline phosphatase-conjugated universal probe (Nakagami *et al.*, 1991) (Wakunaga Pharmaceuticals) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

N-Terminal sequence of CANS DC

The N-terminal sequence of the CANS DC protein was determined by Edman degradation as MQQNELKTPY-FMINEDKLIE.

Cloning of *nspC* in *E. coli* HB101

Prior to screening the pUC19 library, the specificity and titre of the antiserum against CANS DC were determined by immuno-dot blots of both *E. coli* HB101 and *V. alginolyticus* cells. A 1:500–1000 dilution was determined to give the best positive signal over the *E. coli* background. Of several thousand ampicillin-resistant *E. coli* transformants tested, one was found to strongly react with the antiserum. Western blots of proteins from this transformant separated by SDS-PAGE identified a band as reacting with the antibodies (Fig. 2). A corresponding band of homology was present in an extract of *V. alginolyticus* but not in that of *E. coli* HB101 containing pUC19. The crude extract of *E. coli* HB101 harbouring this clone showed CANS DC activity ($1.4 \mu\text{mol mg}^{-1} \text{h}^{-1}$) approximately threefold higher than that of *V. alginolyticus* ATCC 17749. However, activities of L-2,4-diamino-

butyrate decarboxylase and NSPD synthase, two other enzymes responsible for NSPD biosynthesis, were not detected in the same extract, when assayed by the methods of Nakao *et al.* (1989, 1991). The plasmid recovered from this clone was designated pCDC14, which contained a 4 kb insert, and was used for further subcloning.

Physical mapping and subcloning for the cloned gene

Various restriction enzymes were used to generate a physical map of the insert of pCDC14 (Fig. 3). Single restriction sites were found for *EcoRI*, *PvuII* and *SacI*; two sites each were found for *SalI* and *XbaI*. No restriction sites were found for *ApaI*, *BamHI*, *DraII*, *KpnI*, *NotI*, *PstI*, *SmaI*, *SpeI*, *SphI* and *XhoI*. This information was used to verify the accuracy of the determined sequence. Subcloning of the 3.6 kb *SacI*–*PstI* (located within pUC19) fragment of the pCDC14 into pUC18, which was previously digested with the same restriction enzymes, in an inverse orientation with relation to the *lac* promoter (*P_{lac}*) (pCDC14 versus pCDC41) resulted in nearly equal levels of enzyme activity (Fig. 3), indicating that transcription of the *nspC* gene is initiated at its own promoter.

To localize the *nspC* gene, three deletion subclones were prepared on the basis of the restriction map and were used to transform *E. coli* HB101. The results indicated that the *nspC* structural gene and its expected promoter were located in the 2.0 kb *EcoRI*–*Sau3A* region containing two *XbaI* sites (Fig. 3), and this clone was named pCDC14-2. Furthermore, to confirm that the cloned DNA fragment was derived from *V. alginolyticus*, the plasmid, pCDC14-3, containing the 1.2 kb *XbaI* fragment from pCDC14 was used to probe the genomic DNA of *V. alginolyticus* by Southern blotting. The probe showed strong hybridization with the *XbaI*-digested plasmid pCDC14, which was used as a positive control. Only an identical 1.2 kb band was visible in the genomic *XbaI* digest from *V. alginolyticus*, and no cross-hybridization with the *XbaI*-digested *E. coli* genomic DNA was detected (data not shown), indicating that the *nspC* gene is present in *V. alginolyticus* as a single copy.

Nucleotide and deduced amino acid sequence of the *nspC* gene

The *EcoRI*–*PstI* (vector) fragment of pCDC14-2 was recloned into pBluescript II SK(+) to construct pCDC14-2 SK, which was also used to prepare the unidirectional deletion plasmids for sequencing in a reverse direction. A series of nested deletion plasmids were generated from both the *SalI* and *EcoRI* sites in pCDC14-2 and pCDC14-2 SK, respectively. Approximately 500 bp deletions from the *EcoRI* site resulted in complete disappearance of the protein cross-reactive with the antiserum against CANS DC, suggesting that the possible start site of the *nspC* gene is located near the *EcoRI* site. Hence the *nspC* gene is inserted into the pUC19 vector in an opposite orientation with respect to the *P_{lac}*.

Nucleotide sequence analysis indicated two ORFs, ORF1 (1131 bp) encoding 377 amino acids, and ORF2 (207 bp)

Fig. 2. Western blot analysis of the CANS DC proteins cross-reactive with anti-CANS DC antiserum. Lanes 1 and 3 represent the crude extract of *E. coli* HB101 and the partially purified CANS DC from *V. alginolyticus*, respectively. Lane 2 is a cross-reactive protein in the crude extract from *E. coli* HB101 harbouring pCDC14. The arrow indicates the position of the *V. alginolyticus* CANS DC (43.5 kDa). The sizes of the molecular mass markers are indicated in kDa on the left.

Fig. 3. Restriction map, subclones and deletion derivatives of the plasmid pCDC14. The *Bam*HI site of pUC19 was eliminated by cloning. The thin and bold lines represent pUC19 DNA and *V. alginolyticus* DNA, respectively. CANS DC activity is indicated for each construct. The dotted arrow indicates the position and the direction of the *nspC* gene.

encoding 69 amino acids (Fig. 4). ORF1 contained a stretch of 20 amino acids, beginning with Met, that corresponded perfectly to the N-terminal part of mature CANS DC as determined by N-terminal sequencing of the protein. This clearly demonstrates that the procedure has led to the cloning of the CANS DC structural gene, and shows that there is no posttranslational modification of the enzyme in the N-terminal part. The ATG start codon was not preceded by a classical ribosome binding site (RBS) (Shine & Dalgarno, 1974), but the AAGG sequence identical to the RBS in the *V. alginolyticus* *scrK* gene encoding fructokinase (Blatch *et al.*, 1990) was recognized 8 bp upstream from the start codon. A promoter-like sequence (Harley & Reynolds, 1987) is located upstream from the start codon with a -35 sequence of TTGCGT followed by a 16 bp space and a -10 region of TATAGT. Interestingly, an inverted repeat with a calculated ΔG value of $-23.4 \text{ kcal mol}^{-1}$ ($-97.9 \text{ kJ mol}^{-1}$) spans the -35 region. The positioning of this stem-loop structure would function in gene regulation by blocking access to RNA polymerase. This may be supported by the fact that CANS DC activity in *E. coli* HB101 harbouring a subcloned plasmid was low compared with levels in *V. alginolyticus*, in spite of the high copy number of the pUC19 vector. Alternatively, it could also act as a terminator site for an undefined upstream ORF. Immediately downstream from the stop codon TAA of the *nspC* gene, there is another region of dyad symmetry followed by a TTATT sequence which resembles a *p*-independent transcription termination signal (Platt, 1986). The ΔG value for the formation of this putative terminator structure was calculated to be $-21.5 \text{ kcal mol}^{-1}$ ($-89.9 \text{ kJ mol}^{-1}$), within the range typically observed for *p*-independent terminators.

It was calculated from the deduced amino acid sequence that the exact M_r and pI of the CANS C subunit were 42008 Da and 4.62 respectively. These values are in reasonable agreement with those reported for CANS DC (M_r 43500 and pI 4.25) purified from *V. alginolyticus* (Nakao *et al.*, 1990).

The mol% G+C content of the coding region was 47.0%, consistent with the 45–47% overall G+C content in the genomic DNA of *V. alginolyticus* (Baumann & Schubert, 1984). The codon usage of the *nspC* gene was compared with the mean usage calculated from the *V. alginolyticus* collagenase gene (Takeuchi *et al.*, 1992) as well as those of 16 other *V. alginolyticus* genes (Blatch & Woods, 1991) (data not shown). The percentage synonymous use of each codon in the *nspC* gene reflected the bias essentially similar to other *V. alginolyticus* genes. The codons for leucine (CUA), serine (UCA) and threonine (ACA and ACG), which each have been reported to be rare in *E. coli* genes (Andersson & Kurland, 1990), were used preferentially in the *nspC* gene. In contrast, the preferred codon reported for *E. coli* for serine (UCC) was not used in the *nspC* gene.

Comparison of CANS DC protein sequence with the sequences of other decarboxylases

The *nspC* nucleotide and deduced amino acid sequences were not significantly homologous with any of the sequences of bacterial decarboxylases and other proteins in the current versions of GenBank, EMBL or PIR, suggesting that putative homologous genes in other organisms may not yet have been isolated. In addition, despite the absolute requirement of PLP for CANS DC activity, the deduced amino acid sequence did not show the usual features of a PLP-binding domain, S(N,T)-X-H-K, found in many bacterial α -amino acid decarboxylases such as arginine, glutamate, histidine, lysine and ornithine decarboxylases (Smith *et al.*, 1991). Recently, in aligning the amino acid sequences among prokaryotic *meso*-2,6-diaminopimelate decarboxylases (DAP DCs) and eukaryotic ornithine decarboxylases (ODCs), none of which contain the H-K sequence, Mills & Flickinger (1993) identified seven conserved regions and proposed that the consensus lysine residue in the region 1 sequence, Y(F)-A-S(Q,V)-K-A or C(S)-F(N,C) or N(H), is responsible for Schiff-base formation with PLP in all these decarboxylases. When the deduced amino acid sequence of the *nspC* gene product was compared directly with those of the above enzymes, two regions (aa 38–43, L-A-L-K-C-F, and 219–224, L-A-L-K-A-F) having significant similarity to the proposed PLP-binding segment were detected (Table 1). Considering the relative position, Lys-40 is more likely to be involved in Schiff-base formation. However, the possibility that Lys-222 is involved in PLP-binding cannot be excluded, until more is known about the binding of PLP to this enzyme. Regardless, the occurrence of these conserved segments allowed us to confirm that the *nspC* gene encodes one of the structural features typical of a decarboxylase. No similarity to the other regions proposed as catalytic and substrate-binding sites by Mills & Flickinger (1993), however, could be identified in CANS DC; probably because of differences in structure between CANS and DAP or ornithine substrates. However, eight histidine and seven cysteine residues are present in CANS DC, one of which in each may be associated with the catalytic site. The requirement of dithiothreitol for CANS DC activity (Nakao *et al.*,

-101 TTTAGCGACTC
 -35 -10 RBS
 -90 TTATTTGTACGTTTAAAGCCGATGCACTTGCCTCGGCTTTGTTTCGATTTTATAGTGTCTTTGCTAAGGCGAAGGGATCGTAAGGTATCAAA
 1 ATGCAACAAAACGAACATAAAAACGCCATACTTCAGTATCAATGAAGACAAGCTGATTGAGAACCTAGAGAAAGCAAAACAGCTAAAAGAC
 1 M Q Q N E L K T P Y F M I N E D K L I E N L E K A K Q L K D
 ↳ORF1(NspC)
 91 ATCTCTGGTGTGAAGCTTGTATTAGCACTGAAGTGCTTCTCAACATGGGGTGATTTCGACATCATCAAGCCTTACCTAGACGGAACAACA
 31 I S G V K L V L A L K C F S T W G V F D I I K P Y L D G T T
 181 AGTTCTGGCCCGTTGAAGTGAAGCTTGGCTACGAAACGTTTCGGTGGTGAGACACAGCATACAGTGTGGGTACAGCGAAGATGACGTT
 61 S S G P F F E A V K L G Y E T F G G E T H A Y S V G Y S E D D V
 271 CGCGATGTGGCGGACATCTGTGACAAGATGATCTTTAACTCTCAGTCACAGCTAGCGGCTTATCGTCATATTGTGCAAGGCAAAGCATCG
 91 R D V A D I C D K M I F N S Q S Q L A A Y R H I V E G K A S
 361 ATTGGCTTGCGCCTAAACCCAGGTGTGAGCTATGCAGGCAAGACTTAGCAAACCCAGCGCTCAATTCTCTCGTCTTGGTGTTTCAGGCT
 121 I G L C R L N P G V S Y A G Q D L A N P A R Q F S R L G V Q A
 451 GATCATCAACCCGAAATTTTGTGATGTTGATGGCGTAATGTTCCACATGAACRTGTGAGAACAAGACGTTGGATGCGTTTATCGGC
 151 D H I K P E I F D G I D G V M F H M N C E N K D V D A F I G
 541 TTA CT CGATGCTATTTTCAGCTCAGTTTGGCGAGTACCTAGATAAGCTGGACTGGGTAAGCATGGGCGGCGGCGTGTCTTCACATGGCCG
 181 L L D A I S A Q F G E Y L D K L D W V S M G G G V F F T W P
 631 GGTTATGACATCGAAAAGCTTGGCCTAGCGCTGAAAGCGTTTGCTGAAAAACACGGCGTGCAAATGTACCTAGAACC GGCGGAACGGATC
 211 G Y D I E K L G L A L K A F A E K H G V Q M Y L E P G E R I
 721 ATTACCAAGACGACTGACCTAGTGGTCACTGTGGTTGATATCGTTGAGAACGTAAGAAAGACGGCGGATGTGCGATTGAGCCACAGAAAGCT
 241 I T K T T D L V V T V D I V E N V K K T A I V D S A T E A
 811 CACCGCTTAGATACGTTGATCTACAATGAGCCAGCGTCAATCTTGAAGCATCTGAAAATGGTGAACACGAGTATGTGATCGGCTCGTGC
 271 H R L D T L I Y N E P A S I L E A S E N G E H E Y V I G S C
 XbaI XbaI
 901 TCTTGTGGTGGGACGAGTCTGCGTGGCGAACTTCGAACAGCCTCTAGAGATAGGACAGCGTCTGCATATTCTAGATAGTGCCGGT
 301 S C L A G D Q F C V A N F E Q P L E I G Q R L H I L D S A A G
 991 TACACCATGGTGAAACTGAACTGGTTTAAACGGTCTACGTATGCCGTGGTCTACTGTGACGCTCGAATGGTGACATTCAAAAAGTGAAT
 331 Y T M V K L N W F N G L R M P S V Y C T E R S N G D I Q K L N
 PvuII
 1081 GAATTTGACTACTCTGATTTCAAACGTTCACTGTCTCAGTGGTCAGTGATATAAAACGTTATTGAGTATTAGAACGCAAAAGACGAGCTGA
 361 E F D Y S D F K R S L S Q W S V I ***
 1171 ATTGCTGCTCTTTTATTGGGGAAGCGTTAAAGGTATGCACTTACGTTTCTTACAATTACACGAGTGTCTTCACTCAGAGATTCTAACTCA
 69 *** S E S I V R T D E S L S E L E
 1261 TTGGCTATGCTCTTCAATTTCTAGCTCGCTAGGTAGTTTTATCGCCATTTTAGCGCTGAATAAGCTAGAGCTTACCCCTCCGCTCTGTGA
 54 N A I D E I E L E S P L K I A M K A S F L S S S V G G G A A
 RBS
 1351 ATGAATACACGGTGACAATCCATATCTAAAATTGAGATACCTTGATTATCGAGAACGTTGAGTAATTTCACTTCACAATACCCGCTCTATC
 24 I F V R Q C D M D L I S I G Q N D L V N L K M
 -10 -35 SalI ORF2
 1441 GTTGGCATCTAAACGAAGTGAAGATAGTGTCTTCACGATATGAGTGAGGCTCAGAGTCGACGAACCTAACAAACAAGAGTCTGACAAGAAG
 1531 AGAACGCTTGTGTTAAACGATGTGGGCAATTTCTCTTGGAAAGCTCTACTTTGATGACACCTGCAACTTGGTCTTCGATAAAGTTTACTTTGC

Fig. 4. Nucleotide sequence of the *V. alginolyticus* CANS DC gene, deduced amino acid sequence of the enzyme, and sequences of the 5'- and 3'-flanking regions. The start and the direction of the ORFs are indicated by horizontal arrows. The Shine-Dalgarno ribosome binding sites (RBS) and probable -35 and -10 promoter sequences are underlined. The translation termination codons and a potential transcription termination signal are marked with asterisks and facing arrows, respectively. Relevant restriction sites are indicated above their corresponding recognition sequences. The amino acid sequence as revealed by N-terminal sequencing of the protein is double underlined.

1990) would be accounted for by the involvement of a cysteine residue in the active site.

Nucleotide and deduced amino acid sequences of ORF2

Sequence analysis also revealed the presence of ORF2 on the opposite strand immediately downstream from the *uspC* gene, which is terminated at the putative terminator common with the *uspC* gene (Fig. 4). The sequence showed a putative RBS, GGGT, a promoter-like sequence (GTGAAG-N₁₈-TTAGAT) upstream from the ATG start codon, and the TAA stop codon followed by a

terminator sequence. The translated product of ORF2 would be predicted to have a molecular mass of 7441 Da. Part of the deduced amino acid sequence (aa 21–40) displayed some weak similarity to ScrR, a repressor protein of *V. alginolyticus* (Blatch & Woods, 1991) (data not shown), which has a helix-turn-helix motif typical of prokaryotic DNA-binding proteins (Pabo & Sauer, 1984). Eleven out of the 20 amino acid residues are identical or belong to the same family, and two of three highly conserved residues are identical. However, the peptide band corresponding to the presumptive product of this gene was not detected by SDS-PAGE in crude lysates of *E. coli* HB101 harbouring pCDC14-2. This may be that

Table 1. Similarity of a putative PLP-binding domain of the *V. alginolyticus* CANS DC to those of bacterial DAP DCs and eukaryotic ODCs, all of which lack the conserved amino acid sequence, -S(N,T)-X-H-K, for PLP-binding

Identical amino acid residues are marked with asterisks. The PLP-binding domain for eukaryotic ODCs is summarized from the data of Mills & Flickinger (1993).

Organism	Enzyme	PLP-binding domain	GenBank accession number
<i>V. alginolyticus</i>	CANS DC	38 L-A-L-K-C-F * * *	This study
		219 L-A-L-K-A-F * * *	
<i>Bacillus subtilis</i>	DAP DC	65 Y-A-S-K-A-F * * *	D90189
<i>B. methanolicus</i>	DAP DC	63 Y-A-S-K-A-F * * *	L188790
<i>Corynebacterium glutamicus</i>	DAP DC	72 Y-A-S-K-A-F * * *	X07563
<i>Pseudomonas aeruginosa</i>	DAP DC	57 F-A-V-K-A-N * * *	M23174
<i>E. coli</i>	DAP DC	51 F-A-Q-K-A-C * *	J01614
Eukaryotes	ODCs	Y (F) -A-S-K-C (S) -N (H)	

ORF2 is not effectively transcribed or translated in an *E. coli* background, as is seen with some genes in the plasmid pJM1 of *V. anguillarum* (Farrell *et al.*, 1990).

Genomic hybridization with other *Vibrio* species

Genomic DNAs of other *Vibrio* species completely digested with *Xba*I were probed with the pCDC14-3 containing a majority of the *nspC* structural gene to investigate whether homologous sequences could be found. All the strains tested showed a single hybridization band, but surprisingly most of the hybridized fragments were much larger than that of *V. alginolyticus* (1.2 kb) (Table 2). This, however, at least indicates that the homologous *nspC* genes are widely distributed in *Vibrio* species, presumably reflecting divergence from a common ancestral gene. In accordance with these results, CANS DC activity (Table 2) and a protein with a similar M_r to CANS DC of *V. alginolyticus*, which reacted intensely with the anti-CANS DC antiserum (data not shown), were also found in each cell lysate of the strains, thereby confirming that NSPD in vibrios is indeed synthesized through a novel pathway (Yamamoto *et al.*, 1986). No positive signal was detected for the *Xba*I-digested genomic DNA from *Paracoccus denitrificans* NCIB 8944, which produces spermidine from carboxyspermidine through the action of a carboxyspermidine decarboxylase functionally very similar to CANS DC (Tait, 1976).

Conclusions

The gene encoding CANS DC from *V. alginolyticus* has been cloned and sequenced. Isolation and characterization of the *nspC* gene as well as its distribution in other *Vibrio*

Table 2. Occurrence of the *nspC* gene in other *Vibrio* species and their CANS DC activities

Genomic DNA was completely digested with *Xba*I, separated by electrophoresis, and transferred onto a nylon membrane. Plasmid pCDC14-3 was used as a probe, and the hybridized band was visualized with the alkaline phosphatase-conjugated universal probe (Nakagami *et al.*, 1991). CANS DC activity of the 50–70% ammonium sulphate precipitate from each strain was determined according to the method of Nakao *et al.* (1990).

Strain	Fragment hybridized (kb)	CANS DC activity (nmol NSPD formed mg ⁻¹ h ⁻¹)
<i>V. anguillarum</i> NCMB 17749	10.2	540
<i>V. cholerae</i> Non O1 NCTC 8042	7.2	485
<i>V. cholerae</i> Non O1 NCTC 4716	7.2	948
<i>V. fluvialis</i> ATCC 33809	9.7	655
<i>V. furnissii</i> ATCC 35016	12.0	271
<i>V. hollliae</i> ATCC 33564	3.2	179
<i>V. mimicus</i> ATCC 33653	9.7	449
<i>V. parahaemolyticus</i> AQ 3354	7.2	569
<i>V. parahaemolyticus</i> ATCC 17802	7.2	799
<i>V. vulnificus</i> CDC A6546	7.0	371

species demonstrates the existence of the novel biosynthetic route for NSPD. The *nspC* gene appeared to be in a separate transcriptional unit, and activities of the two other enzymes, L-2,4-diaminobutyrate decarboxylase and

CANS synthase, responsible for NSPD biosynthesis were not detected even in the longest clone, pCDC14. Therefore, the organization of NSPD biosynthetic genes differs from that in *E. coli* where the genes essential for spermidine biosynthesis, *speE* encoding spermidine synthase and *speD* encoding *S*-adenosylmethionine decarboxylase, constitute an operon (Tabor & Tabor, 1987). However, since nothing is known about the location of two other genes, whether these genes are located in an operon or closely clustered in the region downstream from the *nspC* gene requires further investigation.

A presumptive PLP-binding domain similar to those of DAP DCs from several bacterial sources and of eukaryotic ODCs was identified in the deduced amino acid sequence, but alignments of the nucleotide and deduced amino acid sequences of the *nspC* gene with those of these decarboxylase genes revealed no significant homology. In addition, the apparent ubiquity of homologous *nspC* genes among *Vibrio* species was indicated by Southern blot hybridizations. Thus, it is likely that CANS DC may be one of a new family of decarboxylases which have evolved uniquely among *Vibrio* species. It will be interesting, therefore, to use the *nspC* gene as a probe for specific detection of *Vibrio* species.

In addition to ORF1, ORF2 encoding a possible repressor-like peptide was detected on the opposite strand immediately downstream from ORF1. However, at present there is no evidence that an ORF2 product would be involved in the regulation of ORF1 gene expression.

ACKNOWLEDGEMENTS

We thank Drs H. Nakao and K. Inagaki for determining the N-terminal amino acid sequence, and Dr T. Moritani for her help in the homology search and data analyses. We thank the Okayama University Gene Research Center for allowing us to use the Geneline apparatus. This work was supported by a General Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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Received 20 May 1994; revised 18 July 1994; accepted 21 July 1994.