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Cloning and Expression of the Aspartate Carbamoyltransferase Gene from *Treponema denticola*

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Treponema denticola **seems to play a central role in the etiology of human periodontal disease. We have cloned an antigenic protein-coding sequence from *T. denticola* ATCC 33520. The protein-coding region was found to be a 3-kbp *Hin*dIII-*Hin*dIII fragment. The open reading frame consists of 1,426 bp and codes for a protein with an M_r of 54,919. The deduced amino acid sequence showed 33.8% homology with that of the aspartate carbamoyltransferase of *Escherichia coli*. The gene products showed aspartate carbamoyltransferase activity.**

The mechanisms responsible for periodontal disease have not been clarified, but subgingival bacterial infection by organisms such as gram-negative rods and spirochetes is a major etiological factor in the disease process. Elevated numbers of oral spirochetes including *Treponema denticola* have been demonstrated in the subgingival plaque of patients with various forms of periodontitis (1, 17, 18, 35). Moreover, the invasion of spirochetes into the gingival tissue has been observed in patients with periodontitis (17). Many studies have demonstrated that *T. denticola* inhibits proliferation of fibroblasts (4) and lymphocytes (32), neutrophil degranulation (3), and superoxide production (31). This microorganism also has proteolytic enzymes (10, 25) and hemolytic activity (9). These properties may have significant roles in the development of periodontal disease.

We have been attempting to clone and sequence several antigenic proteins of *T. denticola*. In these studies, we detected a gene product with a deduced amino acid sequence showing 33.8% homology with an aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) of *Escherichia coli*. This enzyme catalyzes the first committed step of de novo pyrimidine nucleotide biosynthesis. This enzyme from *E. coli* is known to be allosteric. In this study, we examined the ATCase activity of the gene product from *T. denticola* and compared it with that of the enzyme from *E. coli*.

T. denticola ATCC 33520 was maintained and grown at 37°C in an anaerobic chamber in TYGVS medium (25). Chromosomal DNA was prepared by the method of Marmur (21), partially digested with *Hin*dIII, and ligated to the *Hin*dIII site of the phage vector λL47.1 (19). The chimeric DNAs were packaged into a phage. *E. coli* Q358 (20) was infected with phage particles and plated onto Luria-Bertani medium with top agar. Immunoscreening with rabbit antiserum against whole cells of *T. denticola* was carried out by the method of Miyamoto et al. (24). A total of 18 positive clones screened with rabbit antiserum were picked from more than 10,000 plaques. One clone which reacted strongly, designated λTDS12, was the subject of this study. Three *Hin*dIII-*Hin*dIII fragments of λTDS12 were subcloned into plasmid vector pACYC184 (6) and transformed into *E. coli* HB101 (5). One subclone was found to contain a 3-kbp *Hin*dII-*Hin*dIII fragment of *T. denticola*. These plasmid and bacterial clones were designated pTDS12 and TD12, respectively. Sonicated extracts of bacterial clone TD12 were subjected to electrophoresis on a 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel by the method of Laemmli (15) and blotted by the method of Towbin et al. (36) by using a Transblot cell (Bio-Rad, Richmond, Calif.). For the immunological detection of recombinant protein, rabbit antiserum against *T. denticola* 33520 was used as the first antibody, and goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad) was used as the second antibody. Protein bands reacted were developed by the method of Miyamoto et al. (24). This clone produced an approximately 57.5-kDa protein (data not shown). Analysis of the Southern blot clarified that this gene exists in *T. denticola* ATCC 33521, ATCC 35404, and ATCC 35405 (Fig. 1), but no reaction was observed in other oral treponemes (data not shown). The restriction map of the 3-kbp fragment determined by restriction endonuclease analysis is shown in Fig. 2. The fragment contains two *Sac*I sites, a *Hin*cII site, and a *Cla*I site. For nucleotide sequence determination, restriction fragments from pTDS12 were subcloned into pBluescript SK and KS (Stratagene, San Diego, Calif.) with *E. coli* MV1184 (37). The single-stranded DNA templates were isolated by the procedure of Messing (22). The double-stranded DNA template of pTDS12 was purified by the alkali lysis method (20) and CsCl-ethidium bromide density gradient centrifugation. DNA sequencing was performed by the dideoxy chain termination method described by Sanger et al. (29) with an Applied Biosystems model 373A automated DNA sequencer. Oligonucleotide primers used for sequencing double-stranded DNA were prepared by an Applied Biosystems model 391 DNA synthesizer on the basis of sequence data for pTDS12. From the resultant DNA sequence data, only one open reading frame capable of coding for the 57.5-kDa protein could be identified on the 2.5-kbp *Sac*I-*Hin*dIII fragment (Fig. 2).

These sequence data for ATCase and the deduced amino acid sequence are shown in Fig. 3. The open reading frame begins with an ATG codon (position 1) and terminates with a TAG codon (position 1426). A Shine-Dalgarno sequence was observed 7 to 11 bp upstream from this ATG codon. Potential promoter elements homologous to the −10 and −35 consensus sequences (7) could be identified. However, transcription start site determination will be necessary to accurately identify the promoter region for this gene. This protein contains 486 amino acids and has a calculated M_r of 54,919.

Searches for DNA and protein homologies were performed by using the National Institutes of Health, GenBank

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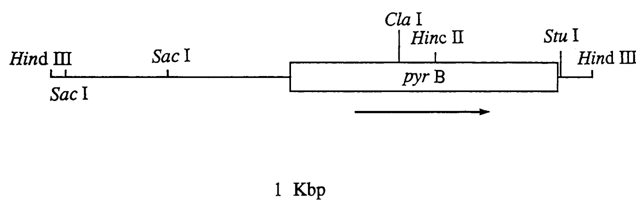

FIG. 1. Restriction map of the 3-kbp fragment from *T. denticola* genomic DNA. *pyr*B, open reading frame encoding aspartate carbamoyltransferase.

(June 1991 version), and Protein Identification Resource (March 1991 version) data bases. In the DNA sequence, no significant homology was identified, but the deduced amino acid sequence showed 33.8% homology with that of the catalytic subunit of ATCase of *E. coli* (30) (Fig. 4) and about 30% homology with the sequences of ATCase in *Salmonella typhimurium* (23) and the CAD protein of golden hamster (34). The *Bacillus subtilis* ATCase (14) showed 24.3% homology with this sequence. Unlike the *E. coli* enzyme, a sequence-coding regulatory subunit was not found downstream of the sequence. This resembled the *B. subtilis* ATCase, which lacks a regulatory subunit (16, 26). The ATCases of *T. denticola* and *E. coli* differ in size (475 and 310 amino acid residues, respectively). Of the 475 residues in the *T. denticola* enzyme, 93 are identical to those in *E. coli* ATCase and 104 are replaced in *E. coli* by homologous residues. This homology is located in the center of the sequence (33.8% identity in residues 50 to 306 of *E. coli* ATCase, which correspond to residues 60 to 336 of *T. denticola* ATCase). Three-dimensional structure determination of the enzyme with *N*-(phosphoacetyl)-L-aspartate (14) or carbamyl phosphate plus succinate (8) provides information concerning the specific groups on the enzyme which interact with carbamoyl phosphate. The residues that interact with carbamoyl phosphate include Ser-52, Thr-53, Arg-54, Thr-55, Arg-105, and Lys-134; Gln-137 from one catalytic chain; and Ser-80 and Lys-84 from the adjacent catalytic chain. Of these residues, Thr-55, Arg-105, and His-134 act

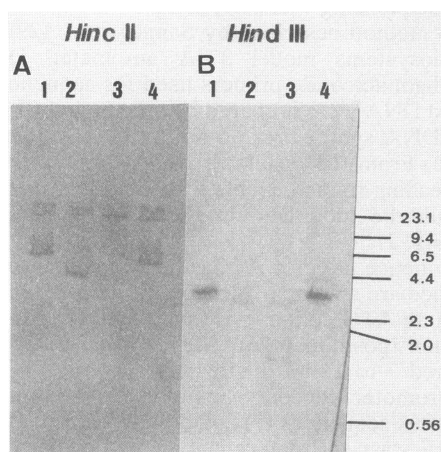

FIG. 2. Southern blot analysis of *Hin*cII-digested (A) and *Hin*dIII-digested (B) chromosomal DNAs from *T. denticola*. The biotinylated *Hin*dIII fragment of pTDS12 was used as a probe. Lanes: 1, ATCC 33520; 2, ATCC 335521; 3, ATCC 35404; 4, ATCC 35405. Numbers on the *right* indicate molecular masses in kilodaltons.

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-100 AATTACTAGA AGCTGCAAAT TTTGCCACCC TCGTTTATTT TGATAACATC
                           -35                     -10

 -50 GGTTTTGCCG ATGTTTTTTT TTGCATTTTT GTTTTTAACA GGAGTTTAAA
                                                SD

   1 ATG GAA AAC AAA TTT ATG GGG CGT TCA TTG ACG GTT ATC GAT GAC TTA TCG ATC
   1  M   E   N   K   F   M   G   R   S   L   T   V   I   D   D   L   S   I

  55 GAT GAA AGA AAA TAC CTT TTT GAC AAG ACA AAG CGC TTA AAA AAA GCC ATT CAA
  19  D   E   R   K   Y   L   F   D   K   T   K   R   L   K   K   A   I   Q

 109 GAA GAT GAT CAA AAG GTT ATG GAT GAA TTT AGA ATT AAC GAC AAA GAT TTC GGT
  37  E   D   D   Q   K   V   M   D   E   F   R   I   N   D   K   D   F   G

 163 ATT TAT GAG GTT TTT TTG GAA CCC AGT ACC CGC ACA AAG GAA TCA TTT AGG AAT
  55  I   Y   E   V   F   L   E   P   S   T   R   T   K   E   S   F   R   N

 217 GCA GCC AAA TTT CAT CAA GTA AAG TTA AGT GAT TTA GCC GCT GAA TCT TCC TCT
  73  A   A   K   F   H   Q   V   K   L   S   D   L   A   A   E   S   S   S

 271 TTT AAC AAG GGA GAA AGC TAT GCC GAC ACC TTT AAT ACT CTT GCA GGT TAT CAA
  91  F   N   K   G   E   S   Y   A   D   T   F   N   T   L   A   G   Y   Q

 325 AAC AGC ATT TTT ATT GTA CGC AGC GAG GTA GAA GGG GTA TGC CGC TGG CTT GAA
 109  N   S   I   F   I   V   R   S   E   V   E   G   V   C   R   W   L   E

 379 GAT GAG GCT CAA GCC TTT TAT CAA AGA AAT AAC TTA AAA AGA AAG CCT GCC TTT
 127  D   E   A   Q   A   F   Y   Q   R   N   N   L   K   R   K   P   A   F

 433 ATA AAC GCC GGA GAC GGA AAG CAT GAG CAC CCT ACT CAG GAG CTG CTT GAC GAA
 145  I   N   A   G   D   G   K   H   E   H   P   T   Q   E   L   L   D   E

 487 TTT ACC TTT ATC GAA GAT AAT AAC TGG TCA TTC GAT AAG ATT CAC ATA GCC TTA
 163  F   T   F   I   E   D   N   N   W   S   F   D   K   I   H   I   A   L

 541 GTA GGC GAC TTG TAC CAC GGA AGA ACT GTA CAC TCA AAG GCC GAC GGT CTT AAA
 181  V   G   D   L   Y   H   G   R   T   V   H   S   K   A   D   G   L   K

 595 ATC TTT AAG TCC GTA AAG GTT GAC CTT ATA GCT CCT GCC GAA CTT GCT ATG CCT
 199  I   F   K   S   V   K   V   D   L   I   A   P   A   E   L   A   M   P

 649 GAA TAT TAC AAG GTC AGG ATG CAG GAA AAC GGC TTT ACC GTA AGG GAA TTT TCT
 217  E   Y   Y   K   V   R   M   Q   E   N   G   F   T   V   R   E   F   S

 703 TCG ATA GAG GAA TAT CTG AGA CAA GCC GAT GTC GCT CTT ATT TGG TAC TTT ACC
 235  S   I   E   E   Y   L   R   Q   A   D   V   A   L   I   W   Y   F   T

 757 CGC CCC CAG CTT GAA AGA ATG GGT GAG CAG GTT TTA AAA AAG CAA GAC GAG CTC
 253  R   P   Q   L   E   R   M   G   E   Q   V   L   K   K   Q   D   E   L,

 818 CGC CGC TCC ATA ACT TTC CGA AAA GAA TTT ATC GAA AAA CTT CCC GAA AAT ACC
 271  R   R   S   I   T   F   R   K   E   F   I   E   K   L   P   E   N   T

 865 CGC TTC TAT CAT CCC CTC CCA AGA CAC CGC GTA CAT CCG ACT ATT CCC ACT TTT
 289  R   F   Y   H   P   L   P   R   H   R   V   H   P   T   I   P   T   F

 919 TTG GAT GCA ACC CCC CTT AAC GGT TGG GAA AGA CAG TCC ATA AAC GGA ATG TAC
 307  L   D   A   T   P   L   N   G   W   E   R   Q   S   I   N   G   M   Y

 973 GTA AGA ATG GTT CTT CTT TCG ATG ATT GCA GGT AAA ATA GGT GAT GAC TAT AAG
 325  V   R   M   V   L   L   S   M   I   A   G   K   I   G   D   D   Y   K

1027 GGG CCT GAA CCT AAA TCT TGT GAA CGT GTC GAA GAT GAA GAC TAC ATA GTT GAA
 343  G   P   E   P   K   S   C   E   R   V   E   D   E   D   Y   I   V   E

1081 GTT CCC ATA AAT AAT TCC AAA GAA AGC AAG GTT GAA ACC TTT TCC GAA GGT GTA
 361  V   P   I   N   N   S   K   E   S   K   V   E   T   F   S   E   G   V

1135 CGC CCC ATA CAA AAC GGA ATA GTT ATT GAC CAT ATT TGC CGA GGA GAC AAG CCC
 379  R   P   I   Q   N   G   I   V   I   D   H   I   C   R   G   D   K   P

1189 TCA GTA ATA AGA CAT CAC ATG TCC AAG ATT ATA AAT GTT ATG GGA CTT GAA GAA
 397  S   V   I   R   H   H   M   S   K   I   I   N   V   M   G   L   E   E

1243 GGC AAG GGC GGC GAA TGG GTA TCC ACT TCT ACA AAA GAT AAG GGA ACC TTT AAA
 415  G   K   G   G   E   W   V   S   T   S   T   K   D   K   G   T   F   K

1297 GGT ATA ATT TTC CGT CCG GGA GAG TAT AAG TTT TCA AGG GCA GAT TTA AAG AGG
 433  G   I   I   F   R   P   G   E   Y   K   F   S   R   A   D   L   K   R

1351 CTG TCT GCC GTT GCT TCG AGC TGT ACC CTT AAC CTA ATA AAA GAC GGA AAA AGA
 433  L   S   A   V   A   S   S   C   T   L   N   L   I   K   D   G   K   R

1405 CGG AAA AAT TCA GTC AAA GTA TAG
 451  R   K   N   S   V   K   V   *

1429 GACGCACCTT CCTCCGCGTA TTTATAATTT TGAAGACTTA ATTTGCAAGA ACGAGGCCTG

1489 TATTTCACAT CCGGCACAAT CCGAAGCGTC CCCGCCATTT TTTACCGCAC TATAGACAAC

1549 AGATATGCCT GCCAATATTG CGGAACAATC CATACCTTTA AAGAAATATG GGGCGAAAAA

1609 AAGAACTAAA ATCTATATCC GTTCCTATCG TTTTGAAGCT T  3'
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FIG. 3. Nucleotide sequence of the *T. denticola* ATCase gene. Potential −10 and −35 promoter sequences and a potential Shine-Dalgarno (SD) sequence are underlined.

with the carbonyl group of carbamyl phosphate and are conserved in the aspartate and ornithine carbamoyltransferases that have been sequenced (2, 11, 16, 34, 38). The present findings indicate that these residues may be essential for carbamyl phosphate binding. In the ATCase of *T. denticola*, these residues were identical with those in the ATCase of *E. coli*. This result suggests that this protein binds to carbamyl phosphate.

FIG. 4. Comparison of amino acid sequences of *T. denticola* and *E. coli* ATCases. Gaps have been introduced for maximum alignment. :, identical residue; ., homologous residue.

E. coli ME8359 (*thyA pyrB*::Tn*5*), which lacked ATCase (kindly provided by National Institute of Genetics, Mishima, Japan), was transformed with pTDS12. Ultrasonicated materials from recombinants were screened for ATCase activity. Cells were repeatedly ultrasonicated at 100 W for 5 min on ice, and the supernatant was collected by centrifugation at 10,000 $\times$ *g* for 30 min. The carbamyl aspartate-producing reaction was performed under the conditions of Sheperdson and Pardee (33). Produced carbamyl aspartic acid was measured by the method of Prescott and Jones (27). The ATCase activity of the sonicated extract of our clone is shown in Table 1. *E. coli* ME8359 bearing pTDS12 showed apparent ATCase activity (2.5×10^{-3} U/mg of protein); the ATP case activity of *E. coli* ME8359 bearing pACYC was less than 1×10^{-5} U/mg of protein. CTP did not suppress this reaction. This result indicated that carbamyl aspartic acid was not produced by *E. coli* ATCase but was produced by *T. denticola* ATCase. We attempted to purify the enzyme from *E. coli* HB101. Harvested cells of *E. coli* HB101 were repeatedly ultrasonicated for 5 min on ice, and the supernatant was collected by ultracentrifugation at 105,000 $\times$ *g* for 2 h. Ammonium sulfate was added to the supernatant to make a final concentration at 60% saturation. The precipitate was collected, suspended in 50 mM buffer (pH 7.0), and dialyzed against the buffer. The dialyzed sample was applied to an ion-exchange column for chromatography with an S Sepharose Fast Flow (2.6 by 20 cm; Pharmacia, Uppsala, Sweden). The column was extensively washed and eluted with a linear gradient of 0 to 0.5 M NaCl at a flow rate of 5 ml/min. Fractions (5 ml) were collected and monitored for A_{280}. The recombinant protein bound to the column and could be eluted at 0.15 M NaCl (Fig. 5). Appropriate fractions were pooled and concentrated by membrane ultrafiltration (UP-20; Advantec, Tokyo, Japan). The SDS-polyacrylamide gel electrophoresis (PAGE) pattern of partially purified ATCase demonstrated a main band with a molecular mass of approximately 57 kDa (Fig. 6A). This fraction of ATCase possessed 0.462 U of ATCase activity per mg of protein. The enzymatic activity was not inhibited by CTP. The result revealed that the enzyme derived from the *T. denticola* gene. The partially purified ATCase reacted strongly with antiserum against whole cells of *T. denticola* ATCC 33520 (Fig. 6B), indicating

TABLE 1. ATCase activity in *E. coli* ME8359 (*pyrB*::Tn*5*) cells bearing recombinant plasmids[a]

E. coli ME8359 and treatment	ATCase activity (U/mg of protein)[b]
Bearing pTDS12	2.5×10^{-3}
Bearing pTDS12 + 0.2 mM CTP	3.0×10^{-3}
Bearing pACYC 184	$<10^{-5}$
Alone	$<10^{-5}$

[a] Crude extracts of each clone were prepared and assayed for ATCase activity as described in the text. Each value is derived from the activities determined with five different dilutions of each extract.

[b] Specific activities in 1 U of crude preparations were defined as production of 1 μmol of carbamoyl aspartate per min.

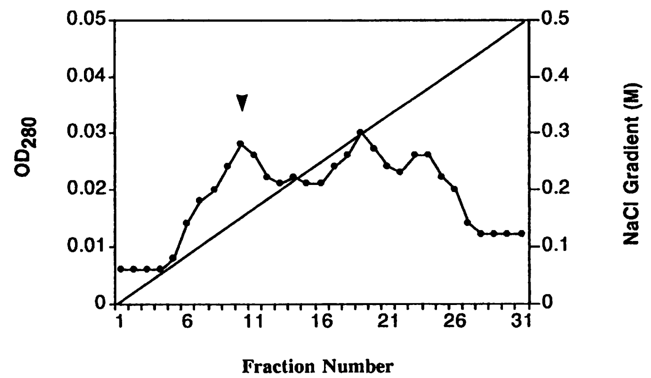

FIG. 5. S Sepharose Fast Flow chromatography of TDS12 gene products. Symbols: ●, protein A_{280} (OD_{280}); ——, NaCl gradient.

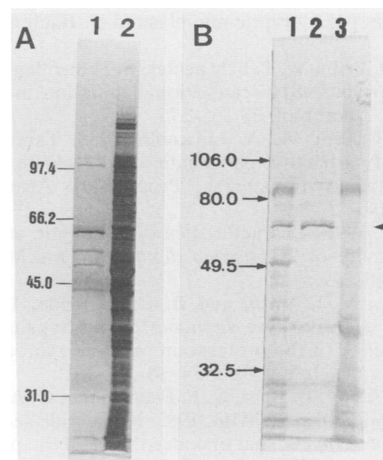

FIG. 6. SDS-PAGE analysis of the TDS12 gene products from *E. coli* HB101 containing pACYC184. (A) Silver stain of polyacrylamide gel. Lanes: 1, partially purified ATCase from TDS12; 2, sonicate from pTDS12. (B) Western blot (immunoblot) analysis with antiserum against whole cells of *T. denticola* ATCC 33520. Lanes: 1, sonicate from TDS12; 2, partially purified ATCase from TDS12; 3, TDS12 containing pACYC184. Numbers at the left of each panel indicate molecular masses in kilodaltons.

that the ATCase is a potent antigenic protein of the microorganism. In spirochetes, chymotrypsinlike proteases of *T. denticola* (28) and nucleic acid metabolism of *Leptospira* spp. (12, 13) were analyzed. The present study was initiated to clone the potent antigen of *T. denticola* strains. Our cloned ATCase is the dominant antigen. It is possible that the ATCase is a unique enzyme in which a few amino acid sequences possess strong antigenicity. Further investigation concerning the treponemal biosynthesis of pyrimidine remains to be done. The cloned gene encoding for ATCase was specific to *T. denticola*. These results indicate that the cloned ATCase gene is a useful DNA probe for identification of *T. denticola* and is applicable for clinical examinations.

Nucleotide sequence accession number. These data have been submitted to GenBank and have been assigned accession number D10052.

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