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The 46-Kilodalton-Hemolysin Gene from *Treponema denticola* Encodes a Novel Hemolysin Homologous to Aminotransferases

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The 46-kDa hemolysin produced by *Treponema denticola* may be involved in the etiology of periodontitis. In order to initiate a genetic analysis of the role of this protein in disease, its gene has been cloned. Synthetic oligonucleotides, designed on the basis of the previously reported amino-terminal amino acid sequence of the 45-kDa hemolysin, were used as primers in a PCR to amplify part of the hemolysin (*hly*) gene. This PCR product was then used to clone the entire *hly* gene from libraries of *T. denticola* genomic DNA. Constructs containing the entire cloned region on plasmids in *Escherichia coli* produced both hemolysis and hemoxidation activities either on sheep blood agar plates or in liquid assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis revealed that the constructs synthesized a protein with molecular size of about 46 kDa which was reactive with anti-*T. denticola* hemolysin. Nucleotide sequence analysis indicated that the largest open reading frame could encode a protein with a calculated molecular size of 46.2 kDa. The first 31 amino acids encoded by this open reading frame were identical to the experimentally determined amino-terminal sequence of the 45-kDa hemolysin. These results indicate that the entire *hly* gene has been cloned. The deduced amino acid sequence of the *T. denticola hly* gene is homologous (23 to 37% identity) to those of proteins that are members of a family of pyridoxal-phosphate-dependent aminotransferases. This suggests that the 46-kDa hemolysin may be related to an aminotransferase and have a novel mechanism of hemolysis. However, the functional aspects of this relationship remain to be investigated.

Oral treponemes have been implicated in the etiology of periodontal disease (13, 43, 44). They are often located at diseased sites and in subgingival plaque, and they are positively associated with increases in plaque index, gingival inflammation, connective tissue attachment loss, and bleeding in periodontal disease progression (19, 34, 41). To date, only three oral spirochetes have been cultured: *Treponema pectinovorum*, *Treponema socranskii*, and *Treponema denticola. T. denticola* appears to be a major member of the pathogenic periodontal microbiota. It is a putative pathogenic agent in adult periodontitis, acute necrotizing ulcerative gingivitis, and juvenile and human immunodeficiency virus-related periodontal diseases (1, 18, 20, 26, 28, 35, 39). From in vitro studies, *T. denticola* is known to produce a number of virulence factors that could contribute to disease progression. For example, the organism synthesizes a variety of proteases which may contribute to host tissue destruction (12, 25, 29, 32, 39, 46).

A critical component of the pathogenic potential of an organism is its ability to acquire iron from the host. *T. denticola* has several mechanisms for iron acquisition, including the binding of lactoferrin (but not transferrin) and the binding of hemin (7, 38, 42). In addition, *T. denticola* produces at least two molecules associated with hemoxidation and hemolysis (6). These proteins could be involved in lysing erythrocytes in vivo in order for *T. denticola* to acquire iron and other factors essential for its growth. Chu et al. (5, 6) demonstrated that one of the hemolysins, with a molecular size of ≤1 kDa (i.e., a tripeptide), is secreted into the growth medium. They showed that the second hemolysin was a 45-kDa cell-associated protein that also had hemoxidation activity. The activity of the larger hemolysin was cysteine dependent but was not inhibited by cholesterol, as are those of other cysteine-dependent bacterial hemolysins (14, 31). The amino-terminal amino acid sequence of the 45-kDa hemolysin from *T. denticola* did not have significant identities with those of any other hemolysins. These results suggest that the 45-kDa hemolysin might be unique. In order to extend this conclusion and to begin to understand the mechanism of action of this toxin, we have cloned and sequenced the gene for the 45-kDa hemolysin from *T. denticola*. This report presents these results, which suggest that this hemolysin uses a previously undescribed mechanism.

MATERIALS AND METHODS

Bacterial strains and plasmids. *T. denticola* ATCC 33404 (TD-4), obtained from the American Type Culture Collection (Rockville, Md.), was used as the source of genomic DNA. The cells were grown in GM-1 medium (50). Plasmids pUC18 and pUC19 were used as vectors for the cloning, sequencing, and expression of the *T. denticola* DNA. Recombinant constructs were propagated in *Escherichia coli* TB-1 after transformation by the $CaCl_2$ procedure or by electroporation (2).

DNA isolation and hybridizations. A miniprep method involving alkaline lysis and boiling (2) was used for the isolation of plasmid DNA from *E. coli*. For the construction of particular subclones, DNA fragments were recovered from agarose gels by electroelution (51) and then cloned into the appropriate vector by standard techniques. *T. denticola* chromosomal DNA was isolated by a detergent-proteinase K lysis procedure that included treatment with cetyltrimethylammonium bromide to remove polysaccharides and cell wall debris (2). DNA samples for Southern blot hybridizations were transferred from 0.75% agarose gels to nitrocellulose filters. For hybridizations with oligonucleotide probes, the filters were first hybridized in 10× Denhardt's solution with 100 μg of denatured salmon sperm DNA per ml and 0.1% sodium dodecyl sulfate (SDS) at 42°C (15). The filters were then hybridized overnight in a hybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10× Denhardt's solution, 0.1% SDS, 0.1 mg of denatured salmon sperm DNA per ml) containing oligonucleotides labelled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP. Finally, the filters were washed three times for 10 min each in 6× SSC with 0.02% SDS at 42°C.

When double-stranded DNA fragments were used as hybridization probes, they were labelled with [α-^{32}P]dATP by using a nick translation system from Life Technologies (Gaithersburg, Md.). With these probes, prehybridizations and hybridizations were done at 60°C in 10× Denhardt's solution–50 mM Tris (pH

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7.5)–4× SSC containing 0.1 mg of denatured salmon sperm DNA per ml and 0.1% SDS. Filters were washed three times at 60°C in 4× SSC.

PCR amplification. Primers Tdent7 (5′-ATGATITAYGAYTTYACIACIAA-3′) and Tdent8 (5′-CCIACYTCIGGRTTYTGISDYTACAT-3′) (I, inosine; Y, adenine or guanine; D, adenine or thymine; R, cytosine or thymine; S, cytosine or guanine) were used in PCRs with *T. denticola* chromosomal DNA as the substrate. *Taq* DNA polymerase and the reagents from a PCR kit (Promega) were used in these reactions. An initial denaturation step (7 min, 93°C) was followed by 40 cycles of amplification (2 min, 93°C; 1 min, 45°C; and 1 min, 72°C) in a model PTC-100 thermocycler from M.J. Research. Primers Tdent9 (5′-CAAGAAAAAACTTAGGT-3′), Tdent10 (5′-CATTAAATCCCACTTAAG-3′), and the pUC forward and reverse sequencing primers were used in PCRs with DNAs from appropriate ligation mixtures. A PCR extender kit (Stratagene) was used in these reactions, and the DNA was amplified by 20 cycles of amplification consisting of 7 min at 93°C, 1 min at 45°C, and 5 min at 72°C. PCR fragments were isolated for sequencing or cloning by electroelution from agarose gels (51) or by extraction from 6% acrylamide gels (21) for fragments of <300 bp.

Construction and screening of limited libraries of *T. denticola* genomic DNA. *T. denticola* genomic DNA (40 μg) was digested with the restriction endonuclease(s) of choice and electrophoresed on a 0.75% agarose gel. A pool of DNA fragments from a region of the gel that encompassed the size range of the hybridizing DNA fragment, as determined by a preliminary Southern blot, was isolated by electroelution (51). For some experiments, the DNA fragments of a limited size range were isolated from sucrose gradients (2). After extraction, each pool of DNA fragments was ligated into either pUC18 or pUC19, which had been linearized with the appropriate restriction endonuclease(s), and transformed into *E. coli*. Colonies of these limited libraries were replica plated onto duplicate nitrocellulose filters and screened for the correct insert by colony blot hybridization (14). The hybridization and washing conditions were the same as the ones described above for Southern blot analysis.

DNA sequencing and computer analysis. The nucleotide sequences of portions of various clones were determined by the Sanger dideoxy-chain termination method (40) with double-stranded DNA templates and a Sequenase sequencing kit (United States Biochemical). Both strands were sequenced independently, and appropriate oligonucleotides were used as primers at approximately 300-bp intervals. The deduced amino acid sequence of the *T. denticola* hemolysin was compared with the Swiss-Protein and Protein-Identification-Resource databases by using the FastDB program (Intelligenetics).

Hemolysis and hemoxidation assays. The hemolytic activities of transformants were determined with sheep erythrocytes as described previously (5). Bacterial cells to be tested for hemolysis and hemoxidation activities were grown to stationary growth phase in 5 ml of Luria-Bertani broth containing 50 μg of ampicillin per ml. The cells were harvested by centrifugation at 10,000 × *g*, and the concentration of the protein in the whole cells was determined with a protein assay kit (Bio-Rad). The cells were suspended in 5 mM sodium citrate–0.15 M NaCl (pH 6.8) containing 2 mM TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) and phenylmethylsulfonyl fluoride and frozen-thawed (−20°C and room temperature) three times prior to the assay. Hemoxidation activity was assayed by the procedure of Leahy and Smith (17). Enriched tryptic soy agar with 5% (vol/vol) sheep blood plates, with or without 12 mM cysteine, was used to measure cysteine-dependent hemolysis activity. Two-microliter portions of the above-described cultures for each sample were dropped onto the plates, inoculated at 37°C for 16 h, and then placed at 4°C for 4 days.

SDS-PAGE and Western blot (immunoblot) analysis. The discontinuous gel system of Laemmli (16) was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel consisted of 10% acrylamide in 0.125 M Tris-glycine (pH 8.8), and the stacking gel contained 4% acrylamide in 0.125 M Tris (pH 6.8). The gels were either stained with 0.025% Coomassie brilliant blue R-250 after electrophoresis or transferred to nitrocellulose (0.45-μm pore size) for Western blotting (7). The purified 45-kDa hemolysin from *T. denticola* was prepared for the analysis as described by Chu and Holt (5). The *E. coli* cells of various transformants were grown in LB broth containing 50 μg of ampicillin per ml at 30°C overnight. The cells (0.5 ml) were harvested by centrifugation at 10,000 × *g* and resuspended in 0.125 ml of 0.15 M NaCl–0.125 ml of 2× SDS treatment buffer.

RESULTS

Cloning and sequencing the gene for the 45-kDa hemolysin from *T. denticola*. An oligonucleotide approach was used to clone the gene encoding the 45-kDa hemolysin from *T. denticola*, since the sequence of the first 31 amino acids in the protein had been determined previously (5). Two degenerate oligonucleotides, Tdent4 and Tdent5, which should hybridize to sequences encoding amino acids 1 to 10 and 17 to 26, respectively, were used independently as hybridization probes of Southern blots of *T. denticola* DNA. Both probes hybridized to a 6.6-kb *Hin*dIII fragment and to a 5.5-kb *Bgl*II fragment (data not shown), suggesting that these genomic DNA fragments contained at least part of the hemolysin gene. Limited genomic libraries were constructed from *T. denticola Hin*dIII or *Bgl*II DNA fragments in these size ranges, and the libraries were screened with oligonucleotide Tdent4. No clones carrying the correct sequences were found despite the screening of thousands of insert-containing colonies. Apparently the hemolysin gene was not represented in either of the libraries. One possible explanation for the inability to find the hemolysin gene in the limited libraries is that an adjacent sequence inhibits the growth of *E. coli*.

As an alternate approach, PCR was used to clone a more limited region of the gene. In the first step, two primers, Tdent7 and Tdent8, were used in a PCR with total *T. denticola* genomic DNA as a substrate. Tdent7 is a degenerate oligonucleotide containing sense sequences for amino acids 1 to 8 of the hemolysin, and Tdent8 is a degenerate oligonucleotide containing antisense sequences for amino acids 22 to 31. When used as a primer pair, they should generate a 92-bp PCR product that encompasses the nucleotides encoding amino acids 9 to 21 of the 45-kDa hemolysin. Indeed, a 92-bp PCR product was generated, and when this product was subcloned into pUC18 and sequenced, the deduced amino acid sequence was identical to the sequence from the protein (data not shown).

To clone the remainder of the coding region of the hemolysin gene, oligonucleotide Tdent9 was used as one primer in a PCR with either the pUC forward or reverse sequencing primer as the other primer. The substrate for the reaction was the ligation mixture used as described above to generate the *Hin*dIII limited library. Since Tdent9 is an exact match for the sense strand encoding amino acids 10 to 15, it should amplify the rest of the hemolysin-coding region as well as the downstream sequences up to a *Hin*dIII recognition site. Both primer pairs gave a PCR product that was approximately 1.3 kb in size (Fig. 1). Each PCR product was cloned into pUC18, and then the insert DNA was sequenced at the cloning junctions. In each case, one of the junction sequences could encode a peptide that was identical in sequence to amino acids 9 to 31 of the 45-kDa hemolysin, proving that the correct gene had been cloned. Therefore, the sequences of both inserts were determined; a large open reading frame that included the known hemolysin sequence was found in both sequences. However, the two sequences differed at 3 bases, and those differences changed the deduced amino acids at three positions. Since these two clones were derived from the same DNA template, the changes are presumed to be due to mutations introduced during the PCR amplification.

Because the PCR approach gave somewhat ambiguous sequence results and because the amino terminus and promoter of the gene had not yet been cloned, genomic clones were isolated from new limited libraries. The majority of the coding region was cloned from a limited library containing 0.8- to 2.0-kb *Hin*dIII-*Pst*I *T. denticola* DNA fragments, and the rest of the coding region with its 5′ flanking sequences was cloned from a limited library of *Pst*I fragments. The hybridization probes used were the 1.0-kb and the 0.3-kb fragments, respectively, generated by a *Hin*dIII-*Pst*I digestion of the 1.3-kb PCR product described above (Fig. 1). Several clones were identified in this screen, and the inserts from two of them, pLC44 and pLC61, were sequenced (Fig. 2). The largest open reading frame found could encode a protein whose first 31 amino acids were identical to those of the 45-kDa *T. denticola* hemolysin (5). Thus, we had cloned the hemolysin gene (called *hly*).

The open reading frame identified in the cloned DNA could encode a protein with a calculated molecular size of 46,256 Da. This is in excellent agreement with the size of the hemolysin

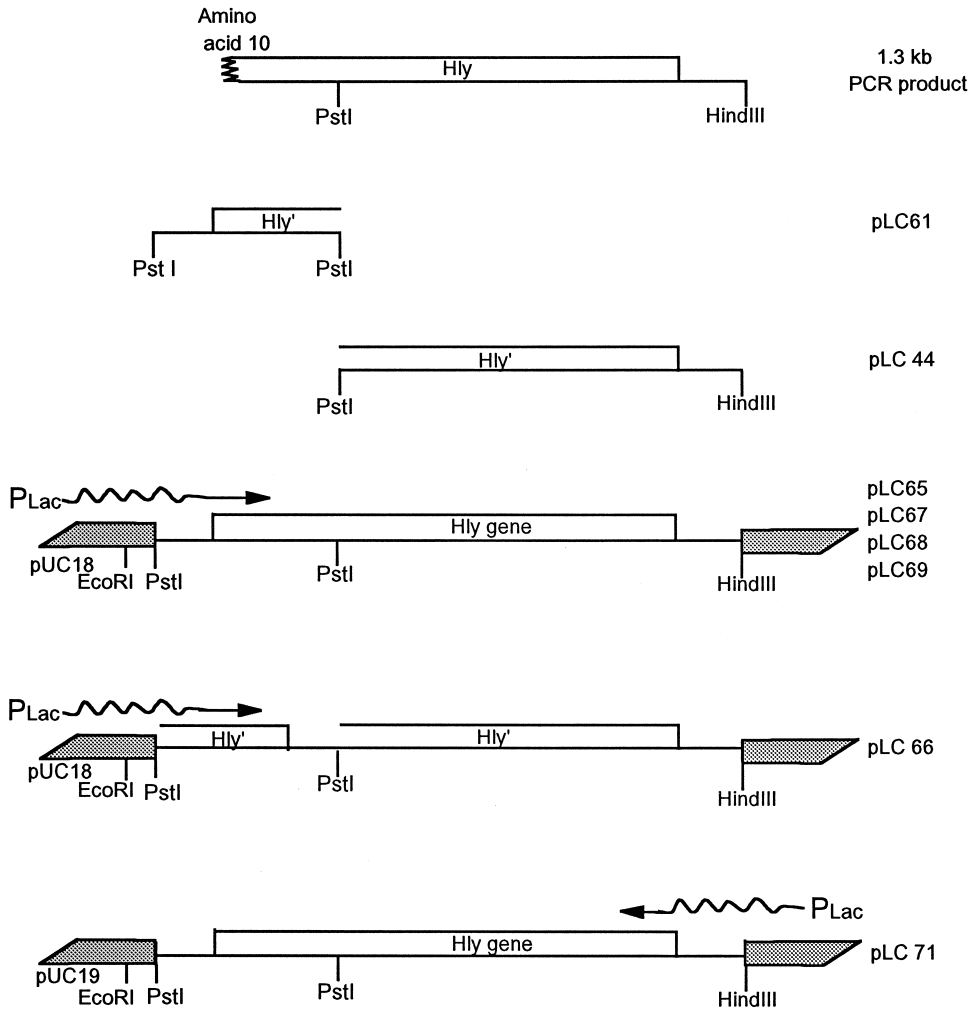

FIG. 1. Restriction map of PCR products and genomic clones containing the *T. denticola hly* gene. The shaded boxes represent vector (pUC18 or pUC19) sequences. The wavy lines indicate the direction of transcription from the vector promoter P_{lac}.

isolated from *T. denticola*, i.e., 45 kDa (5). The deduced protein has a calculated pI of 6.1 and does not contain any long stretches of hydrophobic amino acids, suggesting that it is a fairly typical cytosolic protein. There are six cysteines in the protein, some of which are presumably important since the hemolytic activity of the protein increases under reducing conditions (5, 6). The number of methionines found, 13, is 5 more than expected for an average protein of this size (9), but the significance of this is unknown. Finally, just upstream of the deduced translation initiation site, there is a good match (five of seven positions) to the Shine-Dalgarno sequence for a ribosome binding site in *E. coli* (11).

Properties of the hemolysin protein expressed in *E. coli*. In order to prove that the entire hemolysin gene had been cloned, the hemolysis and hemoxidation activities of several recombinant constructs were tested. In plasmids pLC65, -67, -68, and -69, the 0.38-kb *Pst*I fragment from pLC61 was ligated into the *Pst*I site of pLC44 to create what should be a full-length *hly* gene (Fig. 1). As a negative control, the same 0.38-kb *Pst*I fragment was ligated into the *Pst*I site of pLC44 in the reverse orientation to create plasmid pLC66 (Fig. 1); this would disrupt the reading frame identified as the *hly* gene. Each plasmid was transformed into *E. coli*, and the transformants were tested for hemolysis activity on sheep blood agar plates (Fig. 3A). Rings of hemolysis were found only around cells containing plasmids with the full-length *hly* gene. Cells with the vector alone or with plasmid pLC66, which has the beginning of the gene in the incorrect orientation, did not show hemolysis. The hemolysis shown by cells with plasmids pLC65, -67, -68, and -69 was enhanced by the addition of cysteine to the medium (Fig. 3B). Cysteine-enhanced hemolysis is a property demonstrated by the 45-kDa hemolysin isolated directly from *T. denticola* (5, 6). To quantify these results and to check for hemoxidation activity, another property of the *T. denticola* protein, hemolysis and hemoxidation assays were performed with extracts of *E. coli* cells containing the various plasmids. The results clearly show that cells containing plasmids pLC65 and -67 with the full-length *hly* gene have significantly more hemolysis and hemoxidation activities than cells with control plasmid pUC18 or pLC66 (Table 1).

One formal possibility is that the activity expressed in *E. coli* is an *E. coli*-encoded activity that is somehow increased by the presence of the cloned *T. denticola* gene. To help rule this out, extracts from *E. coli* cells containing various plasmids were examined by SDS-PAGE. Extracts from cells containing plasmids with the full-length *hly* gene (Fig. 4A, lane 2) had a

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-78              5' CTG CAG TTA TGG AAG CAA ATA AAA GGC AAA TTA TCG AAG ATG CCT TAC TTG CTG AAA CTA AAG AAT AGG AGA AAA AAA

  1 ATG ATT TAC GAT TTT ACA ACA AAA ATT TCA AGA AAA AAC TTA GGT TCA CTT AAG TGG GAC TTA ATG TAT TCA CAA AAT CCT GAG GTT GGA
    Met Ile Tyr Asp Phe Thr Thr Lys Ile Ser Arg Lys Asn Leu Gly Ser Leu Lys Trp Asp Leu Met Tyr Ser Gln Asn Pro Glu Val Gly

 91 AAT GAG GTG GTT CCT CTT TCG GTA GCA GAC ATG GAA TTT AAA AAT CCG CCG GAA CTT ATT GAA GGT TTA AAA AAA TAT CTT GAC GAA ACA
    Asn Glu Val Val Pro Leu Ser Val Ala Asp Met Glu Phe Lys Asn Pro Pro Glu Leu Ile Glu Gly Leu Lys Lys Tyr Leu Asp Glu Thr

181 GTG TTA GGA TAT ACA GGA CCT ACT GAA GAG TAT AAA AAA ACC GTA AAA AAA TGG ATG AAG GAT AGA CAT CAA TGG GAT ATT GAA ACT GAC
    Val Leu Gly Tyr Thr Gly Pro Thr Glu Glu Tyr Lys Lys Thr Val Lys Lys Trp Met Lys Asp Arg His Gln Trp Asp Ile Glu Thr Asp

271 TGG ATA ATA AAT ACT GCC GGA GTC GTT CCT GCA GTG TTC AAT GCC GTT AGG GAA TTT ACA AAA CCT GGA GAC GGA GTC ATT ATC ATC ACC
    Trp Ile Ile Asn Thr Ala Gly Val Val Pro Ala Val Phe Asn Ala Val Arg Glu Phe Thr Lys Pro Gly Asp Gly Val Ile Ile Ile Thr

361 CCC GTT TAT TAT CCT TTC TTT ATG GCA ATA AAA AAT CAA GAG CGT AAA ATC ATA GAA TGT GAA TTA TTG GAA AAA GAC GGA TAT TAT ACA
    Pro Val Tyr Tyr Pro Phe Phe Met Ala Ile Lys Asn Gln Glu Arg Lys Ile Ile Glu Cys Glu Leu Leu Glu Lys Asp Gly Tyr Tyr Thr

451 ATC GAT TTT GAA AAA CTA GAA AAA TTA TCC AAG GAT AAA AAC AAT AAG GCT TTG CTC TTT TGC TCG CCG CAC AAT CCT GTA GGC AGG GTT
    Ile Asp Phe Glu Lys Leu Glu Lys Leu Ser Lys Asp Lys Asn Asn Lys Ala Leu Leu Phe Cys Ser Pro His Asn Pro Val Gly Arg Val

541 TGG AAA AAA GAT GAA CTT CAA AAA ATA AAG GAT ATA GTT TTA AAA TCC GAT CTT ATG CTA TGG TCA GAT GAA ATC CAC TTT GAC TTA ATT
    Trp Lys Lys Asp Glu Leu Gln Lys Ile Lys Asp Ile Val Leu Lys Ser Asp Leu Met Leu Trp Ser Asp Glu Ile His Phe Asp Leu Ile

631 ATG CCC GGT TAT GAG CAT ACA GTG TTT CAA TCT ATT GAT GAG CAG CTT GCC GAT AAA ACG ATT ACT TTT ACT GCT CCG TCT AAA ACA TTT
    Met Pro Gly Tyr Glu His Thr Val Phe Gln Ser Ile Asp Glu Gln Leu Ala Asp Lys Thr Ile Thr Phe Thr Ala Pro Ser Lys Thr Phe

721 AAT ATT GCA GGA ATG GGC ATG AGC AAT ATA ATA ATT AAA AAT CCG GAT ATC AGG GAA AGG TTT ACA AAG TCT CGG GAT ATT ACA AGC GGA
    Asn Ile Ala Gly Met Gly Met Ser Asn Ile Ile Ile Lys Asn Pro Asp Ile Arg Glu Arg Phe Thr Lys Ser Arg Asp Ile Thr Ser Gly

811 ATG CCA TTT ACT ACA CTC GGA TAT AAG GCT TGC GAA ATT TGC TAT AAA GAA TGC GGA AAA TGG CTG GAT GGC TGC ATA AAG GTA ATA GAC
    Met Pro Phe Thr Thr Leu Gly Tyr Lys Ala Cys Glu Ile Cys Tyr Lys Glu Cys Gly Lys Trp Leu Asp Gly Cys Ile Lys Val Ile Asp

901 AAA AAT CAA AGA ATT GTA AAA GAT TTT TTT GAA GTA AAT CAC CCC GAA ATA AAG GCT CCT CTT ATT GAA GGC ACC TAT TTA CAA TGG ATA
    Lys Asn Gln Arg Ile Val Lys Asp Phe Phe Glu Val Asn His Pro Glu Ile Lys Ala Pro Leu Ile Glu Gly Thr Tyr Leu Gln Trp Ile

991 GAT TTT AGA GCT TTA AAA ATG GAT CAT AAG GCT ATG GAA GAA TTT ATG ATT CAC AAA GCT CAA ATA TTT TTT GAT GAA GGC TAT ATT TTC
    Asp Phe Arg Ala Leu Lys Met Asp His Lys Ala Met Glu Glu Phe Met Ile His Lys Ala Gln Ile Phe Phe Asp Glu Gly Tyr Ile Phe

1081 GGA GAC GGA GGT ATA GGA TTT GAA AGA ATC AAC TTG GCA GCT CCA TCA TCT GTT ATT CAA GAA AGT TTA GAA AGA CTA AAT AAG GCC TTA
     Gly Asp Gly Gly Ile Gly Phe Glu Arg Ile Asn Leu Ala Ala Pro Ser Ser Val Ile Gln Glu Ser Leu Glu Arg Leu Asn Lys Ala Leu

1171 AAA GAT CTT AAA AAC CGG CAT TTA AAA TAA AAT TTA CTG CCG GTA AAA TTC CTC CTC ATA TTT AAT ATA TAA AAT GTG GGG AGG TCT TTT
     Lys Asp Leu Lys Asn Arg His Leu Lys xxx

1261 ATA AAG GCT TAT TTT TGC TGT TAT TTT TTT CGA GAA TAT CGG TAT AAG CTT GGC 3'
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FIG. 2. DNA sequence of the *T. denticola hly* gene. The complete nucleotide sequence determined is presented. The deduced amino acid sequence of the *hly* gene product is also shown. The sequence that matches the *E. coli* Shine-Dalgarno translation consensus sequence at 5 of 7 bases is underlined.

46-kDa protein that was not present in extracts from cells with the control plasmids (Fig. 4A, lanes 4 and 5). This protein was identical in size to the purified *T. denticola* hemolysin protein (Fig. 4A, lane 1). Western blot analysis of proteins from the same extracts showed that polyclonal antisera against the purified *T. denticola* hemolysin reacted only with the 46-kDa protein expressed in the extracts from *E. coli* cells containing the plasmid with the full-length *hly* gene (Fig. 4B). These results indicate that the 46-kDa *T. denticola* protein is expressed in *E. coli* and, in combination with the results described above, they confirm that the open reading frame identified as the *hly* gene is indeed correct and prove that the entire gene has been cloned.

Since all of the plasmids with the full-length *hly* gene expressed reasonable amounts of hemolysin protein in *E. coli*, we wanted to determine if the expression was dependent upon a promoter in the vector or if the mRNA for the protein might be initiating from *T. denticola* sequences. This could not be deduced from the fact that pLC65, -67, -68, and -69 expressed the protein, since the P_{lac} promoter in the pUC18 vector could be driving the synthesis of *hly* in these clones (Fig. 1). Therefore, the insert from pLC67 was isolated as a *Hin*dIII-*Eco*RI DNA fragment and cloned into pUC19. In this new plasmid, pLC71, the P_{lac} promoter will be transcribing in a direction opposite to the sense orientation of the cloned *hly* gene (Fig. 1). *E. coli* containing plasmid pLC71 was hemolytic and expressed hemoxidation activity, although at only about 50% of the level with pLC67 (Table 1). In addition, cells with plasmid pLC71 contained the 46-kDa cross-reactive protein but at a lower level than pLC67-containing cells (data not shown). This strongly suggests, but does not prove, that the transcription of the *hly* gene in *E. coli* is initiating in the *T. denticola* sequences that are upstream of the *hly* gene. This result was somewhat surprising, since there is only 72 bp of *T. denticola* DNA in front of the *hly* gene and there are no good matches to the −10 and −35 consensus sequences for typical *E. coli* promoters within that sequence. However, more-definitive proof that the *hly* RNA is initiating at *T. denticola* sequences in *E. coli* will require mapping of the *hly* RNA start site.

The *T. denticola* hemolysin is homologous to aminotransferases. When the hemolysin protein sequence was used to search several protein databases, there were no sequences for previously reported hemolysins among the top 60 scores. However, 18 of the top 20 scores were for proteins that are mem-

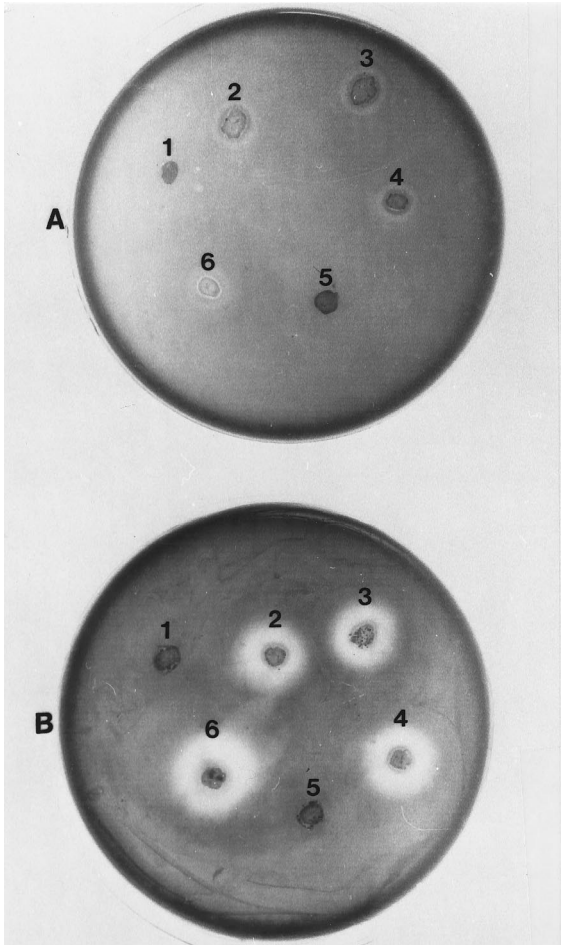

FIG. 3. Hemolytic activity of the cloned hemolysin (in *E. coli* cells) on enriched tryptic soy agar plates without cysteine (A) and with cysteine (B). Colonies 1 to 6 have plasmids pLC66, pLC67, pLC68, pLC69, pUC18 alone, and pLC65, respectively. pLC65, -67, -68, and -69 have the entire *hly* gene; pLC66 has the first part of the *hly* gene in the opposite orientation.

bers of the pyridoxal-phosphate-dependent aminotransferase family of proteins (22). The five proteins with the most significant identity (greater than four standard deviations above the mean) to the *T. denticola* hemolysin were the PatB protein from *Bacillus subtilis* (47) (37% identity), the MalY protein from *E. coli* (33) (29% identity), the C-S lyase from *Corynebacterium glutamicum* (37) (29% identity), the aspartate aminotransferase from a *Bacillus* sp. (45) (25% identity), and the rat tyrosine aminotransferase (23) (24% identity). The enzymatic activities of the first two proteins are unknown, but they both have been suggested to be aminotransferases on the basis of their homologies to known aminotransferases. When the sequences of all five of these proteins were aligned with the deduced sequence of the *T. denticola* hemolysin, 13 residues were found to be invariant in all six sequences (Fig. 5). Four of these residues (Fig. 5, closed circles) are also invariant when various pyridoxal-phosphate-dependent aminotransferases are aligned (23). Included in this group of four residues is the lysine to which pyridoxal-phosphate attaches. The fact that five of the other nine residues that are invariant in this alignment are also commonly found in aminotransferases (Fig. 5, open circles) strongly suggests that the *T. denticola* hemolysin catalyzes changes at the α-carbon of amino acids. This possibility is

TABLE 1. Hemolysis and hemoxidation activities in different recombinant constructs and controls

Source[a]	Activity (U/mg of protein)[b]	
	Hemolysis	Hemoxidation
Control solution	0.84 ± 0.15	3.4 ± 0.21
pLC65	195.2 ± 16.5	724.6 ± 92.5
pLC67	188.5 ± 27.7	658.9 ± 82.4
pLC71	75.7 ± 13.5	257.4 ± 22.5
pLC66	9.8 ± 7.5	22.8 ± 14.5
pUC19	12.5 ± 6.7	40.5 ± 8.5
pUC18	7.6 ± 5.4	15.6 ± 6.7
Purified 46-kDa *T. denticola* protein	1,532.5 ± 134.2	4,058.8 ± 248.5

[a] Plasmids were in whole *E. coli* cells. Plasmids pLC65 and -67 in *E. coli* have the entire *hly* gene in pUC18. pLC71 has the entire *hly* gene in pUC19. pLC66 in pUC18 has the first part of the *hly* gene in the opposite orientation. Plasmids pUC18 and -19 alone in *E. coli* were negative controls. The positive control, purified 46-kDa protein, was from the soluble cell fraction of *T. denticola.*

[b] Results are means ± standard deviations from three experiments.

being tested. These results indicate that the *T. denticola* hemolysin is a novel hemolysin that has not been described previously.

DISCUSSION

In this study, the hemolysin (*hly*) gene from *T. denticola* TD-4 was cloned, sequenced, and shown to have hemolysis and hemoxidation activities when expressed in *E. coli*. The first 31 amino acids of the protein sequence deduced from the cloned gene were identical to those in the amino-terminal sequence derived from the hemolysin protein purified from *T. denticola*. In addition, the cloned gene expressed a protein in *E. coli* that comigrated on SDS-PAGE with the 45-kDa protein from *T. denticola* and which also reacted with anti-*T. denticola* hemolysin antibody. These results demonstrate that the entire *T. denticola hly* gene has been cloned.

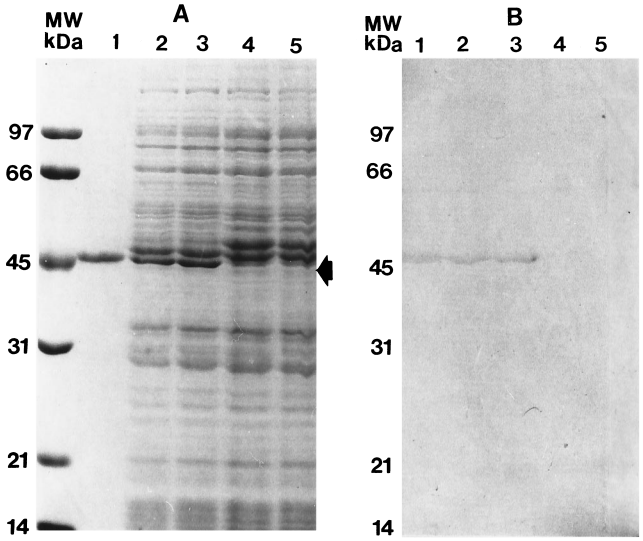

FIG. 4. (A) SDS-PAGE (10% acrylamide) of purified *T. denticola* hemolysin and cloned hemolysin (in *E. coli* cells). The gel was stained with Coomassie brilliant blue R-250. (B) Western blot analysis with anti-45-kDa-hemolysin antiserum. Lanes: 1, purified *T. denticola* 46-kDa hemolysin; 2, pLC67; 3, pLC69; 4, pLC66; 5, pUC18 alone. The arrow indicates the position of the hemolysin protein.

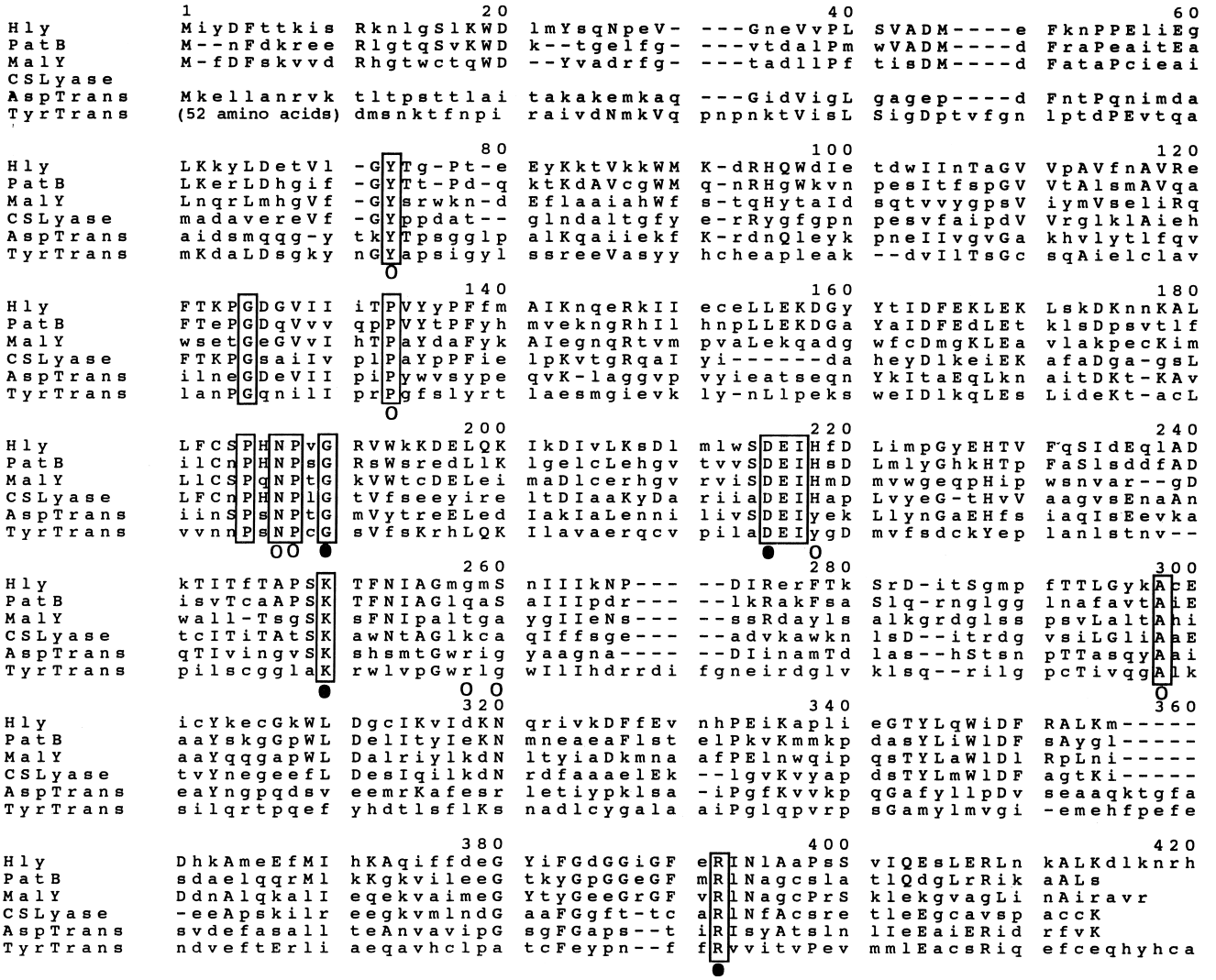

FIG. 5. Alignment of the 46-kDa hemolysin with related proteins. The amino acids positioned identically in all six sequences are boxed. The four residues previously reported to be invariant in aminotransferases (22, 23) are marked by closed circles, and the other eight positions that are found in most aminotransferases are marked by open circles. The capital letters indicate positions with amino acid identity to the 45-kDa *T. denticola* hemolysin. Hly, 46-kDa hemolysin from *T. denticola* (the last 2 amino acids are not shown); PatB, PatB protein from *B. subtilis* (47); MalY, MalY protein from *E. coli* (33); CSLyase, C-S lyase from *C. glutamicum* (37); AspTrans, aspartate aminotransferase from a thermophilic bacillus (45); TyrTrans, tyrosine aminotransferase from rat (23) (the first 52 and last 9 amino acids are not shown).

The amino acid sequence deduced from the *hly* gene was not homologous to the sequences of any other hemolysins in the protein databases. For example, thiol-activated hemolysins from a number of organisms (*Streptococcus suis*, *Clostridium perfringens*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus alveoli*, and *Streptococcus pneumoniae*) have similar amino acid sequences, but none are homologous to *T. denticola* Hly (8, 14, 49). In fact, some of the predicted physical properties of *T. denticola* Hly are quite different from those of other classes of hemolysins. Whereas most thiol-activated hemolysins have one (ceriolysin) or two (alveolysin, perfringolysin O, streptolysin O, listeriolysin, and pneumolysin) cysteine residues per molecule (8, 10, 12), the *T. denticola* hemolysin has six. *T. denticola* Hly is less hydrophobic than hemolysins that function by binding to the erythrocyte membrane (4, 24, 27). Most of these pore-forming hemolysins have several clusters of hydrophobic amino acids, but the hemolysin from *T. denticola* is predicted to have only one short stretch of hydrophobic residues. These predicted differences in physical properties are consistent with the hypothesis that the *T. denticola* 46-kDa hemolysin functions by a unique mechanism. This postulate is supported by the fact that the predicted amino acid sequence of the Hly protein is homologous to those of proteins from the family of pyridoxal-phosphate-dependent aminotransferases.

The homology of the *T. denticola* Hly protein to pyridoxal-phosphate-dependent aminotransferases raises the question of the physiological role of the Hly protein. One possibility is that Hly functions in vivo as a hemolysin by a novel mechanism. Another possibility, suggested by the homology to the aminotransferases, is that *T. denticola* Hly is involved in amino acid degradation in the cell and that the hemolytic activity is a fortuitous side reaction found under the laboratory conditions used. Of course, it is also possible that both functions are important for *T. denticola*. Finally, one interesting possibility is suggested by the homology of *T. denticola* Hly to the C-S lyase of corynebacteria: perhaps the primary in vivo function of *T. denticola* Hly is to produce H_2S. The fact that cysteine and glutathione, but not dithiothreitol, enhance *T. denticola* Hly

activity (5, 6) is consistent with their use as substrates for Hly in the production of H_2S. This gas is toxic to host cells (3, 48) and thus may be involved in virulence. In fact, the levels of H_2S have been reported to be higher in deep periodontal pockets than in subgingival crevices from periodontally healthy subjects (30, 36). The enzymatic activities of the *T. denticola* Hly protein are being investigated in order to address these possibilities. The availability of the cloned gene makes it possible to design genetic experiments to help determine the function of Hly in vivo.

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