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Cloning and Expression of a Neutral Phosphatase Gene from *Treponema denticola*

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We have isolated and characterized a neutral phosphatase gene, *phoN*, from *Treponema denticola* ATCC 35405. The gene was isolated from a *T. denticola* clone bank constructed in the medium-copy-number plasmid vector pMCL19. Subcloning and nucleotide sequencing of the DNA insert from one phosphatase clone, pTph14, revealed that the activity corresponded to an open reading frame consisting of 1,027 bp coding for a 37.9-kDa protein. Hydrophobicity analysis indicated that the protein exhibits some hydrophobic regions. Indeed, partial purification of the phosphatase suggested that the enzyme was membrane associated both in *T. denticola* and in the *Escherichia coli* clone. The pH optimum of the enzyme, approximately pH 6.4, indicated that it corresponded to a neutral phosphatase activity from *T. denticola*. An examination of possible natural substrates for the enzyme suggested that this enzyme hydrolyzes nucleoside di- and triphosphates. Northern (RNA) blot analysis revealed that this phosphatase gene is not likely to be present in an operon structure.

Numerous species of bacteria have been isolated from the human oral cavity, and several of these species, including spirochetes, have been implicated in periodontal diseases (20, 21). However, many of these spirochetes have not been identified yet. The oral spirochete *Treponema denticola* is one of the species associated with chronic periodontal diseases (1). However, only recently have potential virulence factors been identified for these microorganisms (8, 33, 40). Among these, several proteases such as a chymotrypsin-like protease (40), lymphocyte suppression factors (33), and a fibronectin-binding protein (8) have been identified and suggested to play a role in virulence. In addition, the effects of the outer membrane of *T. denticola* on bone resorption were recently reported (11).

Phosphatases may play a role in mineralization (9) as well as in dental plaque formation. High levels of phosphatase activity can be detected in exudates from inflamed periodontal tissues (4). Such activity derived from the host or subgingival plaque bacteria could play a role in bone resorption characteristic of severe periodontitis (10). Therefore, the elaboration of phosphatase activity by these microorganisms could serve as potential virulence factors. However, only limited information is currently available regarding the phosphatase activities in oral microorganisms. For this reason, we have initiated an investigation into the phosphatase activities of *T. denticola*. In the present report, we describe the isolation and preliminary characterization of a neutral phosphatase gene, *phoN*, from *T. denticola.*

MATERIALS AND METHODS

Media. Luria-Bertani broth and Luria-Bertani agar were previously described by Miller (25). Ampicillin (60 μg/ml) and chloramphenicol (30 μg/ml) were added to Luria-Bertani broth and agar where indicated. To screen for expression of phosphatase activity by bacterial colonies, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, St. Louis, Mo.) and potassium phosphate were added to agar plates at final concentrations of 200 μg/ml and 20 mM, respectively (XP plates). Recombinant clones harboring genes from *T. denticola* were incubated at 30°C. *T. denticola* ATCC 35405 (7) was maintained and grown at 37°C in a Coy anaerobic (80% N_2, 10% CO_2, 10% H_2) chamber in TYGVS medium (29).

Extraction of genomic DNA from *T. denticola*. *T. denticola* chromosomal DNA was isolated from *T. denticola* cells grown in 500 ml of TYGVS medium. Cells were harvested after growth in culture for 2 days, washed with TE (Tris-EDTA [pH 8.0]) buffer, and resuspended in 5 ml of TE buffer. Then 250 μl of 10% sodium dodecyl sulfate (SDS; Sigma) was added to this suspension, and the mixture was incubated at 65°C for 10 min. Proteinase K (Boehringer Mannheim, Indianapolis, Ind.) was added to a final concentration of 100 μg/ml, and incubation was continued for an additional 60 min at 37°C. The suspension was then extracted with phenol and chloroform. After precipitation with ethanol, the crude DNA preparation was treated with 50 μg of RNase (Sigma) per ml at 37°C for 60 min. Finally, DNA was extracted with phenol and chloroform and reprecipitated with ethanol.

Recombinant DNA methods. Standard procedures for recombinant DNA manipulation were carried out as described by Sambrook et al. (31). For construction of the *T. denticola* clone bank, chromosomal DNA from strain ATCC 35405 was partially digested with *Sau*3AI and ligated to the moderate-copy-number plasmid pMCL19 (27). Ligated DNA was then transformed into *Escherichia coli* HB101 (5) by electroporation (Bio-Rad, Richmond, Calif.). After a 1-h incubation in SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 20 mM glucose) at 30°C, transformants were inoculated onto XP plates and incubated at 30°C overnight. Phosphatase-positive clones were identified by their blue color on XP plates.

Southern blot analysis. For Southern blot analysis, chromosomal DNA from *T. denticola* was digested with *Sau*3AI or with *Hin*cII and *Pst*I. After electrophoresis on 1.0% agarose gels, the DNA fragments were transferred to Nytran (Amersham, Arlington Heights, Ill.) by using the PosiBlot pressure-blotting system (Stratagene, La Jolla, Calif.). The enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham) was used to label the probe (476-bp *Hin*cII-*Hin*dIII DNA fragment) (see Fig. 1). Hybridization was performed at 42°C overnight, and hybridized membranes were washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 6 M urea and 0.4% SDS at 42°C and at room temperature as specified by the supplier.

Northern (RNA) blot analysis. Total RNA from *T. denticola* was extracted by the boiling method (35) and treated with RNase-free DNase (1 U/ml; Promega, Madison, Wis.). The isolated RNA was separated on 0.66 M formaldehyde–1.0% agarose gels and transferred to a Nytran filter by capillary transfer (38). The ECL direct nucleic acid labeling and detection system was used to label the probe and detect the hybridized band as outlined for Southern blot analysis.

DNA sequencing. DNA fragments from chimeric plasmid pTph14 were subcloned into pBluescript KS+ and pBluescript KS− (Stratagene) in *E. coli* MV1184 (41). Single-stranded template DNA was isolated by the procedure of Messing (24). For sequencing of unstable fragments, the low-copy-number plasmid pGD103 (12), containing treponemal DNA fragments, was used as a template. DNA was sequenced with double-stranded plasmid DNA or single-stranded DNA as template by the dideoxynucleotide chain termination method (32) with a Sequenase II kit (United State Biochemical, Cleveland, Ohio) together with M13 or synthetic oligonucleotide primers and ^{35}S-dATP.

Fractionation of crude extracts of *T. denticola*. A 3-day culture of *T. denticola*

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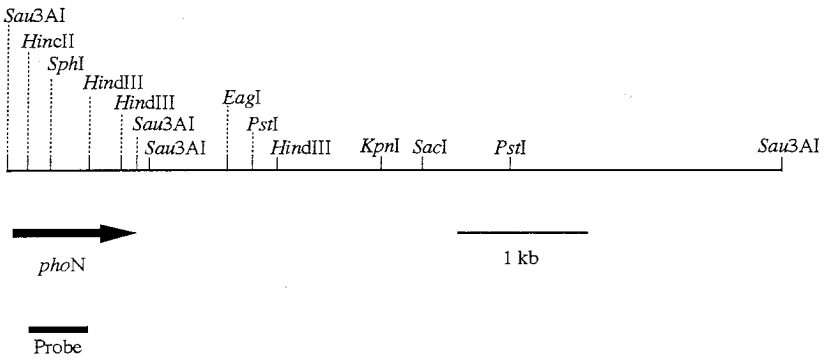


FIG. 1. Restriction map of the *T. denticola* fragments in plasmid pTph14. The locations of the *phoN* gene and the DNA fragment used for subsequent Southern and Northern blot analysis are also indicated.

was harvested by centrifugation at 8,000 × *g* and 4°C for 20 min. The cells were washed twice with 20 mM Tris-HCl buffer (pH 8.0) (Tris buffer) and resuspended in the same buffer. The cells were then disrupted at 100 W for 1 min in a model 450 sonifier (Branson, Danbury, Conn.), and sonication was repeated five times at 1-min intervals. Unbroken cells were removed by centrifugation (8,000 × *g* for 20 min) in a Sorvall SS34 rotor (Dupont, Wilmington, Del.), and the supernatant fluid was ultracentrifuged at 105,000 × *g* for 2 h in a 50 Ti rotor (Beckman, Fullerton, Calif.). The sedimented material was resuspended with Tris buffer, and the supernatant and precipitate were used as soluble and membrane fractions, respectively. The activities in equivalent volumes of buffer were compared, and the corresponding specific activities were calculated.

Purification of the phosphatase. *E. coli* HB101 containing recombinant plasmid pTph14 was grown for 14 h at 30°C, and the cells were harvested and washed twice with Tris buffer. The cells were disrupted by sonication as described above, and the unbroken cells were discarded following centrifugation at 8,000 × *g* for 20 min. The supernatant fluid was applied to an anion-exchange Econopack MonoQ cartridge (Bio-Rad) which was equilibrated with Tris buffer. The flowthrough fraction was solubilized by the modified method of Maeshima and Yoshida (22). The flowthrough fraction was diluted in Tris buffer containing 0.1% Triton X-100, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 20% glycerol, 1 mM dithiothreitol, and 2 mM $MgCl_2$ (Tris-GDEM buffer). Lysophosphatidylcholine (Sigma) was then added to a final concentration of 0.4%, and the solution was incubated at 4°C for 10 min. Triton X-100 was added to a final concentration of 1%, and the insoluble material was removed by centrifugation at 6,000 × *g* for 20 min. The resultant soluble fraction was applied to a cation-exchange Econopack MonoS cartridge (Bio-Rad) which was equilibrated with Tris-GDEM buffer and eluted with a 0 to 1 M NaCl gradient. Protein concentrations were determined by the method of Bradford (6) following precipitation with 10% trichloroacetic acid. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (18).

Enzyme assays. Standard phosphatase activity was assayed at 37°C with 5 mM *p*-nitrophenylphosphate (*p*-NPP) as a substrate at pH 6.4 in 0.025 M cacodylate buffer. One unit of enzyme activity corresponded to 1 μmol of substrate hydrolyzed per min at 37°C. The effect of various cations on enzyme activity was examined by using 0.1 M cacodylate buffer (pH 6.4) containing 5 mM *p*-NPP with 5 mM KCl, $MgCl_2$, $MnCl_2$, NaCl, $ZnCl_2$, or $CaCl_2$. The substrate specificity of the enzyme was examined by using the following esters: *p*-NPP, β-glycerophosphate, D-glucose 6-phosphate, ATP, GTP, ADP, GDP, AMP, GMP, PP_1, tyrosine-*o*-phosphate, and UTP (each at 5 mM). The P_i produced was detected with a P_i quantitative kit (Sigma).

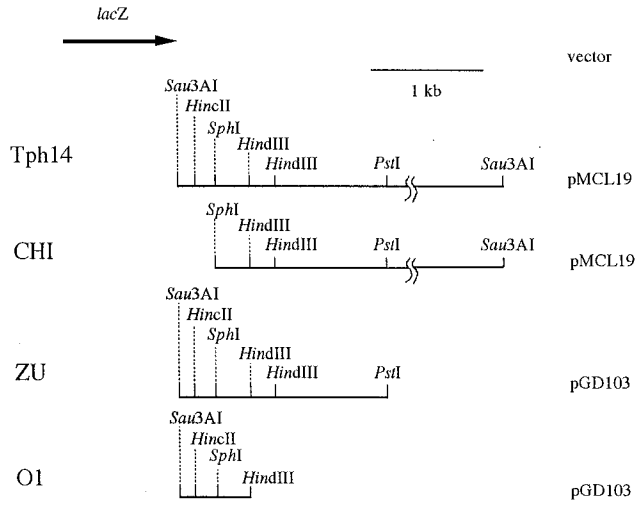
Nucleotide sequence accession number. The nucleotide sequence of the *phoN* gene has been deposited in the GenBank database and assigned accession number L25421.

RESULTS

Phosphatase activities of *T. denticola*. Recent results have shown the presence of *T. denticola* phosphatase activity exhibiting two pH optima at pH 4.8 and 6.2 (28). This suggested the presence of both acidic and neutral phosphatases in cell extracts. The phosphatase activity exhibiting a neutral pH optimum was designated as a neutral phosphatase. The phosphatase activity of *T. denticola* ATCC 35405 was determined by using a pH 6.4 assay buffer with the synthetic substrate *p*-NPP. This activity was not detected in the culture supernatant fluid but was expressed in treponemal cell extracts. After sonication and ultracentrifugation of the cells, the activity was detected exclusively in the membrane-associated sedimented material (5.26×10^{-3} U/mg) and was not detected in soluble fractions from *T. denticola*. The substrate specificity of this preparation showed strong nucleoside triphosphatase activity and very weak glucose-6-phosphatase activity (data not shown). These data suggested that the neutral phosphatase activity of *T. denticola* is associated with the membrane fraction and hydrolyzes primarily nucleoside phosphates.

Isolation of the neutral phosphatase gene. The *T. denticola* neutral phosphatase gene was isolated from a *Sau*3AI library with plasmid vector pMCL19. Three blue transformants were obtained among 2,000 colonies on XP plates containing isopropyl-β-D-thiogalactopyranoside (IPTG). Both restriction analysis and Southern blot analysis indicated that the three positive clones harbored plasmids with the identical 6-kb *Sau*3AI fragment (data not shown). One of these plasmids was designated pTph14. This chimeric plasmid proved to be unstable in *E. coli* when cultured at 37°C and was therefore routinely grown at 30°C. A restriction map of the treponemal DNA in pTph14 revealed that the insert contained two *Pst*I sites (Fig. 1), as well as single *Sac*I, *Sph*I, and *Kpn*I sites and three *Hin*dIII sites. Southern blot analysis (data not shown) indicated that positive bands of the same size were detected from strain ATCC 35405 DNA and pTph14 following cleavage with *Hin*cII and *Pst*I or with *Sau*3AI with the *Hin*cII-*Hin*dIII fragment as a probe. To localize the phosphatase gene, DNA fragments from pTph14 were subcloned into the medium-copy-number plasmid pMCL19 and the low-copy-number plasmid pGD103 and analyzed for expression of phosphatase activity (Fig. 2). The phosphatase activities were expressed from both plasmids pTph14 and pZU. The lower activities expressed from plasmid pZU reflected the low copy number of the plasmid. With pZU, but not pTph14, phosphatase activity was enhanced following IPTG induction. This result and the instability of the latter clone suggested that high-level expression of phosphatase activity from pTph14 is toxic for growth of *E. coli*. In the subclones other than pTph14 and pZU, no phosphatase activity was expressed even in the presence of IPTG. These results suggested that the neutral phosphatase activity was produced from the 1.9-kb *Sau*3AI-*Pst*I fragment and that the gene is larger than the 600-bp *Sau*3AI-*Hin*dIII fragment.

Sequence analysis of the *phoN* gene. From the resultant DNA sequence (Fig. 3), one open reading frame capable of coding for neutral phosphatase activity with a putative initiating codon near the *Sau*3AI site was identified on the 1.9-kb *Sau*3AI-*Pst*I fragment. The phosphatase gene was designated as *phoN* (neutral phosphatase). The open reading frame begins with an ATG codon (position 1) and terminates with a TAA



Clone	BCIP hydrolysis activity		p-NPP hydrolysis activity	
	without IPTG	with IPTG	without IPTG	with IPTG
			(10-3 U/mg)	
Tph14	++	++	62.4 ± 2.81	48.1 ± 1.31
CHI	-	-	9.0 ± 0.81	7.0 ± 0.24
ZU	+	++	21.6 ± 0.65	31.4 ± 0.58
O1	-	-	18.6 ± 0.38	10.6 ± 0.29
MCL19	-	-	14.4 ± 1.50	12.0 ± 0.17

FIG. 2. Phosphatase activities of pTph14 subclones. Each sample was assayed by the standard assay system. Symbols: ++, clone expressed strong blue color on Luria-Bertani (LB) plates containing 200 μg of potassium phosphate per ml; +, clone expressed weak blue color on LB plates containing 200 μg of potassium phosphate per ml; −, clone expressed no blue color on LB plates containing 200 μg of potassium phosphate per ml.

codon (position 1028). An *E. coli* Shine-Dalgarno sequence (34) was identified 8 to 12 bp upstream from the beginning ATG codon. The deduced protein contains 343 amino acids and has a calculated M_r of 37,950. The G+C content of the open reading frame is 38.7%, which agrees well with the data for the chromosomal DNA of several *T. denticola* strains (37). The codons AGA for Arg and CCC for Pro are not used frequently in *E. coli* (23) but are common in *phoN* (data not shown). Furthermore, TTT and TTC coding for Phe are used at the same frequency in *E. coli*, but in *phoN* codon bias for TTT is observed. In *phoN*, codons TTT for Phe, CTT for Leu, and GAA for Glu are frequently used; they are also common in the *atc* (15) and *tdpA* (26) genes of *T. denticola*. In *phoN*, codons AAA for Lys, TTT for Phe, and TAT for Tyr are frequently used, but in the *flaB2* gene of *T. pallidum* (30), AAG for Lys, TTC for Phe, and TAC for Tyr are most frequent. The estimated pI of the phosphatase, 10.10, indicates that it is a basic protein. The hydrophobicity plot by the method of Kyte and Doolittle (17) suggested that the recombinant phosphatase is a somewhat hydrophobic protein (data not shown).

An examination of the NBRF protein database revealed no other protein with extensive homology with the *phoN* phosphatase.

Transcription of the *phoN* gene. To determine whether the *phoN* gene was transcribed independently or as part of polycistronic mRNA, Northern blot analysis was carried out. The results clearly indicated that a single mRNA band of approximately 1.4 kb was detected (Fig. 4). This is large enough to contain the entire *phoN* gene and suggests that the gene is transcribed as a monocistronic mRNA species. Additional sequencing both upstream and downstream of the *phoN* gene will be required to confirm this suggestion.

Purification and characterization of the neutral phosphatase. Since the results from both the hydrophobicity analysis of the phosphatase and the distribution of the activity in the crude extracts of the clone suggested that the enzyme may be membrane associated, various detergents were used in an attempt to solubilized the activity. Triton X-100, octylglucoside, and 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) did not solubilize the neutral phosphase activity. However, treatment of the crude extracts with lysophosphatidylcholine did result in solubilization of the enzyme. Following treatment with this phospholipid, the enzyme was partially purified by ion-exchange chromatography. The activity could be purified approximately threefold by anion exchange chromatography on MonoQ columns (Table 1). The active fractions were then pooled and chromatographed on a MonoS cation-exchange cartridge. The phosphatase activity was eluted from the columns with a NaCl gradient at approximately 0.4 M NaCl. The resultant enzyme was purified 388-fold over the crude extract. SDS-PAGE analysis revealed the presence of several major protein bands in addition to that of 38 kDa corresponding to the phosphatase. This band was not detected in the fraction which was fractionated from extracts of subclone O1 by the same method (data not shown). Further purification of the enzyme by a variety of approaches was not successful in enhancing the purity of the active phosphatase.

The pH profiles of the partially purified enzyme (Fig. 5) revealed a pH optimum of 6.4. Negligible activity was detected at acidic pHs below pH 5.0 and clearly distinguished this enzyme from the acidic phosphatase of *T. denticola* identified recently (28). Neither the activity isolated from the *E. coli* clone nor that in crude extracts of *T. denticola* was stimulated by reducing agents such as dithiothreitol (data not shown). In addition, no stimulation by cations such as Na^+, K^+, Mg^{2+}, Ca^{2+}, or Mn^{2+} could be detected, but Zn^{2+} inhibited phosphatase activity strongly.

Since the neutral phosphatase was detected with the synthetic substrate *p*-NPP, it was of interest to attempt to identify the natural substrate for this activity. An examination of the hydrolysis of a number of phosphate-containing compounds suggested that the enzyme was most active with nucleoside di- or triphosphates (Table 2). In addition, the enzyme displayed strong pyrophosphatase activity. Interestingly, nucleoside monophosphates were relatively poor substrates for the enzyme.

DISCUSSION

Phosphatase activity has been observed in several periodontopathic bacteria, including *T. denticola* (19, 36, 39). However, only one of these phosphatases has been extensively purified until now (44). In the present investigation, we detected a major neutral phosphatase activity in sonicates of *T. denticola*. Previously, Norton-Hughes and Yotis (28) characterized an acid phosphatase activity from *T. denticola*. In the present study, both acidic and neutral phosphatase (optimal pH 6.2) activities were detected in crude preparations from *T. denticola*. The present investigation yielded an optimal pH of 6.4 for the neutral phosphatase, which was slightly lower than the phosphatase activity of crude extracts reported previously (28). In a previous study, Tris-HCl and acetate buffers were used to determine the optimal pH. These buffers apparently do not have strong buffering capacity in the pH range around 6.0 to

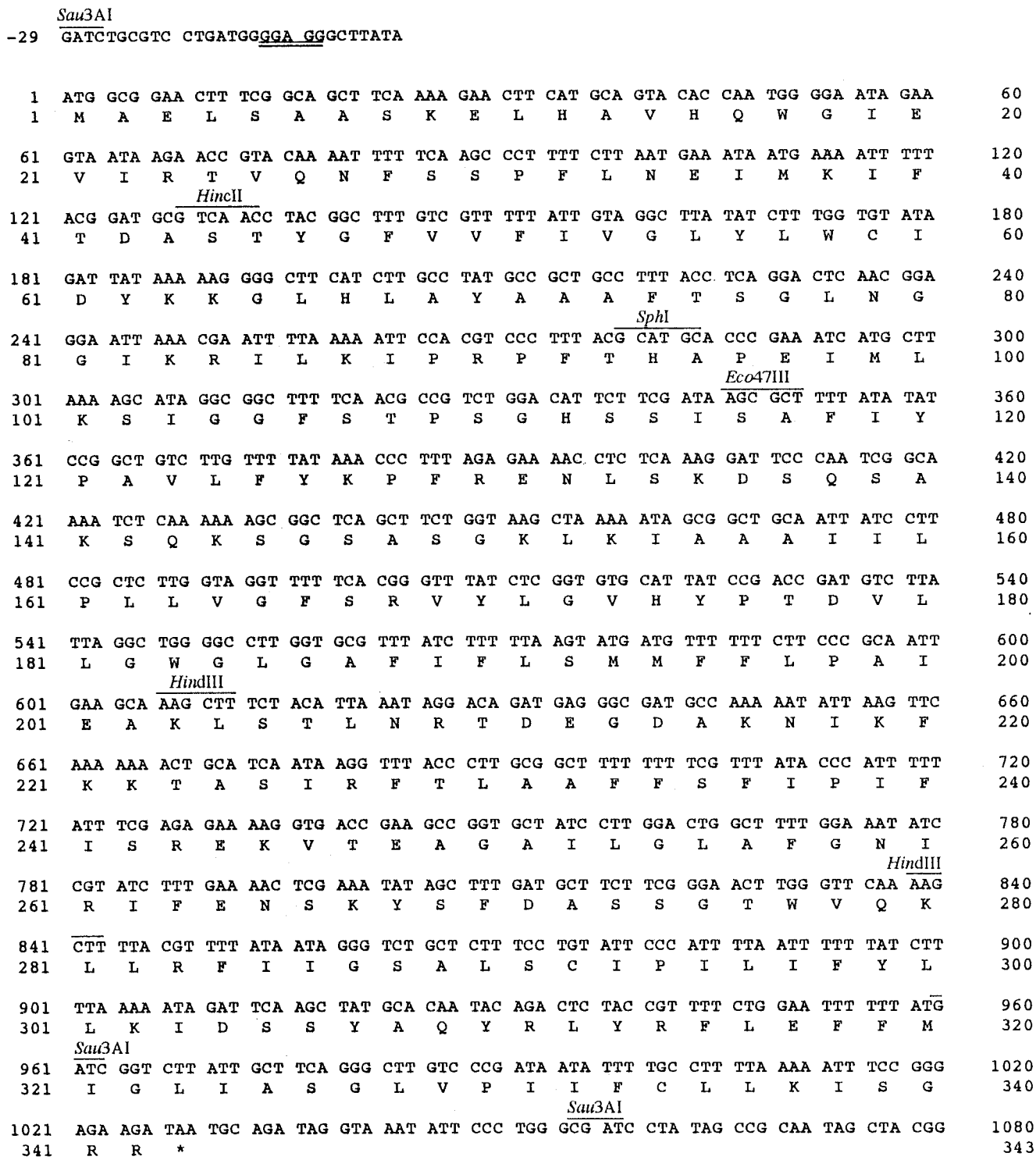


FIG. 3. DNA sequence and deduced amino acid sequence of the *phoN* gene. A potential Shine-Dalgarno sequence is underlined, and restriction sites are overlined.

7.0. Becker and Hurwitz (2) indicated that 3′-nucleotidase activity exhibited slightly different pH optima depending on the buffers used. This could explain the slightly different pH optimum previously reported for the neutral phosphatase compared with the present results. Therefore, it is likely that the *phoN* gene isolated in the present investigation codes for the phosphatase which was identified previously (28). Confirmation of this relationship will require further purification of the phosphatase and comparison of the partial amino acid sequence of the protein with the deduced sequence of the *phoN* gene.

The analysis of deduced amino acid sequence indicated that

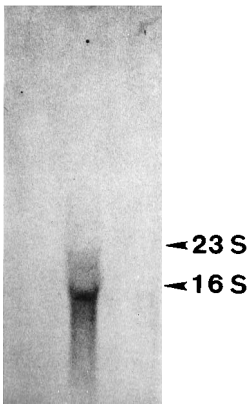


FIG. 4. Northern blot analysis. The 23S on the right indicates the 23S rRNA (2.9 kb), and the 16S indicates the 16S rRNA (1.5 kb). RNA was extracted and analyzed with the 447-bp *Hin*cII-*Hin*dIII fragment as a probe, as described in Materials and Methods.

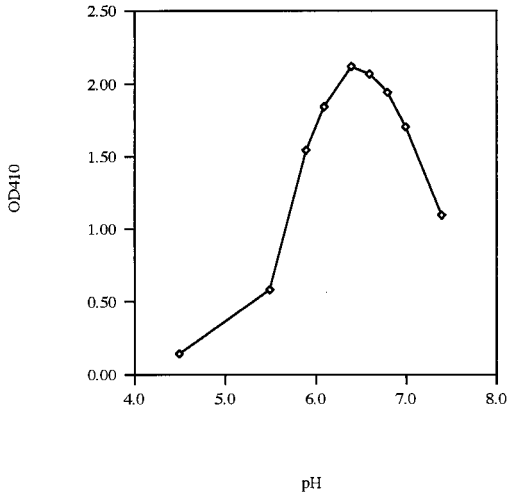


FIG. 5. pH optimum of the cloned neutral phosphatase. The activity of the partially purified enzyme was measured at the indicated pHs in cacodylate buffer. OD410, optical density at 410 nm.

the neutral phosphatase is hydrophobic and basic. These results suggested that the neutral phosphatase is possibly a membrane-associated protein. In addition, the results from the purification of the recombinant protein indicated that the phosphatase is associated with the *E. coli* membrane fraction. Furthermore, the neutral phosphatase activity from *T. denticola* is found exclusively in the membrane fraction (Table 1). Taken together, these results strongly suggest that the *phoN* gene encodes the membrane-associated neutral phosphatase. Previous reports have also characterized bacterial membrane-associated phosphatase activity (3, 13, 14, 42). The neutral phosphatase from *T. denticola* hydrolyzed nucleoside di- and triphosphates. A membrane-bound alkaline phosphatase from *Bacillus licheniformis* hydrolyzes AMP but only weakly cleaves ATP and glycerol 2-phosphate. However, the *phoN* neutral phosphatase does not hydrolyze glycerol phosphate and hydrolyzes ATP strongly. An outer membrane-associated protein from *Lysobacter enzymogenes* also hydrolyzes glucose phosphate and glycerol phosphate (43). Other membrane-associated phosphatases apparently are concerned with the metabolism of membrane phospholipids. The membrane-associated phosphatase of *E. coli* catalyzes cleavage of phosphatidylglycerophosphate, phosphatidic acid, and lysophosphatidic acid. These phosphatases hydrolyze monophosphate bonds, whereas the *T. denticola* neutral phosphatase hydrolyzes PP_i bonds. These differences in substrate specificity emphasize the uniqueness of the *phoN* phosphatase of the present investigation relative to other membrane-associated phosphatases. A 3′-deoxynucleoside phosphatase from *E. coli* showed a similar optimal pH compared with the cloned neutral phosphatase, but the 3′ deoxynucleotidase does not hydrolyze *p*-NPP whereas the neutral phosphatase does not hydrolyze nucleoside monophosphates. In addition, several other phosphatases from other oral microorganisms have been described (16, 44). The phosphatase from *Streptococcus mutans* hydrolyzes only *p*-NPP. The phosphatase from *Porphyromonas gingivalis* (44) hydrolyzes β-glycerophosphate and *o*-phosphoserine but not PP_i. However, the effects of cations, molecular weights, and optimal pHs of these enzymes distinguish them from the *T. denticola* neutral phosphatase. These differences suggest that the neutral phosphatase belongs to a unique category of phosphatases compared with these other enzymes.

The *phoN* phosphatase hydrolyzes nucleoside di- and triphosphates and also displays pyrophosphatase activity. However, it does not strongly cleave glucose-6-phosphate or glycerol phosphate. The low glucose-6-phosphatase activity of the enzyme is reasonable since *T. denticola* displays negligible glucose-metabolizing activity (37). The association of the neutral phosphatase with the membranes of *T. denticola* suggests that the enzyme could play a role in the transport of these nucleoside phosphates into the cells. However, no information relating to the purine or pyrimidine requirements of these cells is

TABLE 1. Purification of the neutral phosphatase from *E. coli* pTph14[a]

Fraction	Total amt of protein (mg)	Total units of activity	% Recovery	Sp act (U/mg)	Fold purification
Sonicate	144.1	12.67	100	0.088	1
MonoQ	29.7	8.10	64	0.273	3
MonoS	0.030	1.02	8	34.23	388

[a] Phosphatase activity was measured in the standard assay system.

TABLE 2. Substrate specificity of the neutral phosphatase from pTph14[a]

Substrate	Activity (U/mg)
ATP	27.1
CTP	40.5
GTP	30.95
Deoxy-UTP	30.95
ADP	40.5
GDP	55.36
AMP	7.9
GMP	5.4
Cyclic AMP	4.45
Glucose 6-phosphate	0.39
PP_i	14.58
Glycerophosphate	0.39
Casein	0
Tyrosine-*o*-phosphate	0
p-NPP	34.2

[a] Activity of the partially purified enzyme was measured in the standard assay system.

currently available. Further investigation is required to define the role of this enzyme in the physiology of the oral spirochete.

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REFERENCES

1. **Armitage, G. C., W. R. Dickenson, R. J. Jenderseck, S. M. Levine, and D. W. Chambers.** 1982. Relationship between the percentage of subgingival spirochetes and the severity of periodontal disease. J. Periodontol. **53:**550–556.
2. **Becker, A., and J. Hurwitz.** 1966. The enzymatic cleavage of phosphate termini from polynucleotides. J. Biol. Chem. **242:**936–950.
3. **Bell, R. M., R. D. Mavis, M. J. Osborn, and P. R. Vagelos.** 1971. Enzymes of phospholipid metabolism: localization on the cytoplasm and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*. Biochim. Biophys. Acta **249:**628–635.
4. **Binder, T. A., J. M. Goodson, and S. S. Socransky.** 1987. Gingival fluid level of acid and alkaline phosphatase. J. Periodontal Res. **22:**14–19.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Cheng, S. I., R. Siboo, T. Chin Quee, J. L. Johnson, W. R. Mayberry, and E. C. S. Chan.** 1985. Comparative study of six random oral spirochete isolates. Serological heterogeneity of *Treponema denticola*. J. Periodontal Res. **20:**602–612.
8. **Dawson, J. R., and R. P. Ellen.** 1990. Tip-oriented adherence of *Treponema denticola* to fibronectin. Infect. Immun. **58:**3924–3928.
9. **Fleisch, H., and S. Bisaz.** 1962. Mechanism of calcification: inhibitory role of pyrophosphate. Nature (London) **195:**911.
10. **Frank, R. M., and J. C. Voegel.** 1978. Bacterial bone resorption in advanced cases of human periodontitis. J. Periodontal Res. **13:**251–261.
11. **Gopalsami, C., W. Yotis, K. Corrigan, S. Schade, J. Keene, and L. Simonson.** 1993. Effect of outer membrane of *Treponema denticola* on bone resorption. Oral Microbiol. Immunol. **8:**121–124.
12. **Green, B. A.** Personal communication.
13. **Hulett-Cowling, F. M., and L. L. Campbell.** 1971. Purification and properties of an alkaline phosphatase of *Bacillus licheniformis*. Biochemistry **10:**1364–1371.
14. **Icho, T., and R. H. Raetz.** 1983. Multiple genes for membrane-bound phosphatases in *Escherichia coli* and their action on phospholipid precursors. J. Bacteriol. **153:**772–730.
15. **Ishihara, K., M. Ishihara, I. Takazoe, and K. Okuda.** 1992. Cloning and expression of the aspartate carbamoyltransferase gene from *Treponema denticola*. Appl. Environ. Microbiol. **58:**3399–3403.
16. **Knuuttila, M. L. E., and K. K. Makinen.** 1972. Purification and characterization of a phosphatase specifically hydrolyzing *p*-nitrophenyl phosphate from an oral strain of *Streptococcus mutans*. Arch. Biochem. Biophys. **152:**685–701.
17. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **Laughon, B. E., S. A. Syed, and W. J. Loesche.** 1982. API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp., and spirochetes of oral origin. J. Clin. Microbiol. **15:**97–102.
20. **Listgarten, M. A.** 1965. Electron microscopic observations on the bacterial flora of acute necrotizing ulcerative gingivitis. J. Periodontol. **36:**328–339.
21. **Loesche, W. J., S. A. Syed, E. Schmidt, and E. C. Morrison.** 1985. Bacterial profiles of subgingival plaques in periodontitis. J. Periodontol. **56:**447–456.
22. **Maeshima, M., and S. Yoshida.** 1989. Purification and properties of vacuolar membrane proton translocating inorganic pyrophosphatase from mung bean. J. Biol. Chem. **264:**20068–20073.
23. **Maruyama, T., S. Gojobori, S. Aota, and T. Ikemura.** 1986. Codon usage tabulated from the gene bank genetic sequence data. Nucleic Acids Res. **14**(Suppl.)**:**r151–r197.
24. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miyamoto, M., S. Noji, S. Kokeguchi, K. Kato, H. Kurihaha, Y. Murayama, and S. Taniguchi.** 1991. Molecular cloning and sequence analysis of antigen gene *tdpA* of *Treponema denticola*. Infect. Immun. **59:**1941–1947.
27. **Nakano, Y. J., and H. K. Kuramitsu.** 1992. Mechanism of *Streptococcus mutans* glucosyltransferases: hybrid-enzyme analysis. J. Bacteriol. **174:**5639–5646.
28. **Norton-Hughes, C. A., and W. W. Yotis.** 1990. A study of the acid phosphatase of *Treponema denticola*. Int. J. Med. Microbiol. **274:**195–202.
29. **Ohta, K., K. K. Makinen, and W. J. Loesche.** 1986. Purification and characterization of an enzyme from *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. **53:**213–220.
30. **Pallesen, L., and P. Hindersson.** 1989. Cloning and sequencing of a *Treponema pallidum* gene encoding a 31.3-kilodalton endoflagellar subunit (FlaB2). Infect. Immun. **57:**2166–2172.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Shenker, B. J., M. A. Listgarten, and N. S. Taichman.** 1984. Suppression of human lymphocyte response by oral spirochetes: a monocyte-dependent phenomenon. J. Immunol. **132:**2039–2045.
34. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
35. **Shiroza, T., and H. K. Kuramitsu.** 1993. Construction of a model secretion system for oral streptococci. Infect. Immun. **61:**3745–3755.
36. **Slots, J.** 1981. Enzymatic characterization of some oral and nonoral gram-negative bacteria with the API ZYM system. J. Clin. Microbiol. **14:**288–294.
37. **Smibert, R. M.** 1984. Genus III. *Treponema* Schaudinn 1905, 1728^{AL}, p. 49–57. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
38. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
39. **Syed, S. A., S. L. Salvador, and W. J. Loesche.** 1988. Enzyme profiles of oral spirochetes in RapID-ANA system. J. Clin. Microbiol. **26:**2226–2228.
40. **Uitto, V. J., D. Grenier, E. C. Chan, and B. C. McBride.** 1988. Isolation of a chymotrypsinlike enzyme from *Treponema denticola*. Infect. Immun. **56:**2717–2722.
41. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
42. **von Tigerstrom, R. G., and S. Stelmaschuk.** 1985. Localization of the cell-associated phosphatase in *Lysobacter enzymogenes*. J. Gen. Microbiol. **131:**1611–1618.
43. **von Tigerstrom, R. G., and S. Stelmaschuk.** 1986. Purification and characterization of the outer membrane associated alkaline phosphatase of *Lysobacter enzymogenes*. J. Gen. Microbiol. **132:**1379–1387.
44. **Yamashita, Y., K. Toyoshima, M. Yamazaki, N. Hanada, and T. Takehara.** 1990. Purification and characterization of alkaline phosphatase of *Bacteroides gingivalis* 381. Infect. Immun. **58:**2882–2887.