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Synthesis and Secretion of Phospholipase C by Oral Spirochetes

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Four strains of oral treponemes and *Treponema phagedenis* Reiter synthesize and secrete phospholipase C (PLC), which was detected by the hydrolysis of *p*-nitrophenylphosphorylcholine. PLC was detected in gingival crevicular fluid from diseased but not from healthy sulci. The initiation and progression of periodontal lesions may begin with the hydrolysis of membrane phospholipids by PLC.

Oral spirochetes are found in the subgingival microbiota of patients with periodontitis (1, 6, 13, 15). The spirochetes produce enzymes and toxic metabolites (14, 17–19) that may play a role in the initiation and progression of periodontitis. These bacterial products may injure host tissue directly or indirectly by activating host-mediated responses. The present investigation describes the production and secretion of phosphatidylcholine-hydrolyzing phospholipase C (PLC; phosphatidylcholine cholinephosphohydrolase [EC 3.1.4.3]) by *Treponema denticola*, *Treponema vincentii*, and *Treponema phagedenis*.

The oral spirochetes *T. denticola* ATCC 35405 and *T. denticola* ATCC 35404 were isolated and maintained in our laboratory (7, 8); *T. denticola* ATCC 33520 and *T. vincentii* ATCC 35580 were purchased from the American Type Culture Collection, Rockville, Md. *T. phagedenis* Reiter was a gift from R. George of the Center for Disease Control, Atlanta, Ga. *Clostridium perfringens* is a clinical isolate that is maintained in the Department of Microbiology and Immunology, McGill University. The spirochetes were grown as described previously (7), and *C. perfringens* and *Streptococcus mutans* ATCC 25175 were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1.8% glucose, 0.8% $NaHCO_3$, 0.3% NaCl, and 0.15% K_2HPO_4. One milliliter ($\simeq 2 \times 10^6$ bacteria per ml) was inoculated separately into 300 ml of medium. The spirochetes and *C. perfringens* were grown under anaerobic conditions (N_2-H_2-CO_2 [85:10:5]) in a glove box (Coy Laboratory Products, Ann Arbor, Mich.) at 37°C. *S. mutans* was grown aerobically at 37°C. In a preliminary investigation, the medium from exponentially growing spirochete cultures was tested for secreted PLC. PLC was present in the medium (data not shown). Consequently, the growth of the spirochetes was stopped (36 h) before the organisms entered their stationary growth phase (7), when proteases and inhibitors could accumulate in the medium. *C. perfringens* (PLC producer) and *S. mutans* (nonproducer) were grown for 24 h, and their growth media and cytosols (see below) were used to check the fidelity of the assay for PLC activity. The cultures were centrifuged at 15,000 × *g* for 30 min at 4°C, the supernatants (spent media) were collected, and the proteins were precipitated at 75% saturation with solid ammonium sulfate, dialyzed against 0.85% NaCl, and concentrated to 1/10 their original volumes. The bacteria were washed three times by centrifugation with 0.15 M Tris hydrochloride buffer, pH 7.2 (Tris buffer). The bacteria were suspended in 5 ml of Tris buffer and sonicated (at 50% of full power with 4 cycles of 30 s each and 1 min of cooling each time) in a 4°C ice bath with a sonic dismembrator (Artek, Farmingdale, N.Y.). The sonic extracts were centrifuged at 22,000 × *g* for 30 min at 4°C, and the supernatants (cytosols) were collected. The sediments were suspended in Tris buffer and centrifuged at 5,000 × *g* for 5 min, and the membrane fractions in the supernatants were sedimented and washed twice by centrifugation at 22,000 × *g* for 30 min. Zinc chloride (final concentration, 1 mM) was added to the spent media, cytosols, and membrane fractions to stabilize the enzyme (20). The spent media and cytosol fractions were filtered (1.2-μm-pore-size membrane filters; Millipore Corp., Bedford, Mass.). Phenylmethylsulfonyl fluoride (1 mM) and *N*-tosyl-L-phenylalanine chloromethyl ketone (0.5 mM) were added to all samples, which were stored at −20°C (10).

The assay for PLC activity was carried out as described by Kurioka and Matsuda (11) for PLC. The assay is based on the hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC), with the release of the chromogen, *p*-nitrophenol (NP). Spent media (150 μl), cytosol fractions (50 μl), and membrane fractions (150 μl) were added to separate wells in microdilution plates, and 100 μl of Tris buffer containing 20 mM NPPC, 1 mM Zn^{2+}, 10 mM NaF, and 45% sorbitol (NPPC-complete Tris buffer) was added to the wells. The NaF does not inhibit PLC (predetermined) but inhibits phosphatases (12). The plates were incubated at 37°C and read at 405 nm in a Multiskan Microplate Reader (Flow Laboratories, Mississauga, Ontario, Canada). Sample dilutions were selected to give an absorbance reading of $\leqq 1.6$. The instrument was blanked with NPPC-complete Tris buffer. The medium control consisted of uninoculated media in NPPC-complete Tris buffer, whereas the cytosol and membrane fraction controls were incubated in NPPC-complete Tris buffer with 2 mM disodium EDTA instead of Zn^{2+}. PLC activity is inhibited by EDTA. The concentration of NP released was determined from a standard curve with NP in a complete Tris buffer. The PLC activity of the spent media was expressed as nanomoles of NP per milliliter, since the culture media for the treponemes contained exogenous protein. The PLC activity of the cytosol fractions was expressed as nanomoles of NP per milligram of protein, which was determined by the method of Bradford (5) with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, Calif.). The cytosols and spent media of the oral spirochetes, the Reiter strain, and *C. perfringens* contained PLC activity (Table 1). The membrane fractions of the oral spirochetes, the Reiter strain, and *C. perfringens* had no PLC activity even when a macroassay (1 ml of sample and 1 ml of

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TABLE 1. Hydrolysis of NPPC by PLC from different bacterial species

Species	NP in[a]:	
	Spent media (nmol/ml per h)	Cytosol (nmol/mg per h)
T. denticola ATCC 35405	253.8 ± 6.9 (4)	1,710 ± 27 (4)
T. denticola ATCC 35404	2,091.0 ± 6.5 (4)	691 ± 28 (4)
T. denticola ATCC 33520	157.8 ± 6.5 (4)	1,709 ± 41 (4)
T. vincentii ATCC 35580	120.6 ± 7.2 (4)	742 ± 27 (4)
T. phagendenis Reiter	3,331.0 ± 47 (4)	2,936 ± 18 (4)
C. perfringens	218.1 ± 11.5 (4)	182 ± 11 (4)
S. mutans ATCC 25175	0[b] (2)	0 (2)

[a] Data represent the means ± standard deviation for the number of assays in parentheses. Duplicate cultures of each microorganism were grown, and the spent media and cytosols of each microorganism were assayed in duplicates.
[b] No enzyme detected.

NPPC-complete Tris buffer) was used. The cytosol and spent medium of *S. mutans* did not contain PLC activity. It appears that the cytosolic PLC of oral spirochetes and the Reiter strain was not membrane bound, since PLC was not detected in the membrane fractions. By contrast, PLC activity was reported in the membrane fraction but not the cytosol of *Ureaplasma urealyticum* (9). The secretion of PLC is not unique to the treponemes. Other gram-negative organisms such as *Pseudomonas aeruginosa* (4) and certain species of *Legionella* (2) also secrete PLC.

The secretion of PLC from colonies of oral spirochetes was established with *T. denticola* ATCC 35405. Tubes of new oral spirochete medium (7) containing 0.7% agar, 10 mM NPPC, and 1 mM Zn^{2+} were inoculated with a pure culture of the ATCC 35405 strain and poured into plates which were incubated anaerobically at 37°C. Agar plates with NPPC served as the control. A yellow ring (the released NP) appeared around the colonies (at ~72 h) and increased in diameter with continued incubation. Hence, oral spirochetes actively secrete PLC. A similar technique was used to identify PLC-secreting *U. urealyticum* (9). This method may be used to screen mixed bacterial populations from subgingival plaques for PLC-secreting spirochetes in the above-mentioned medium containing a selective antibiotic like rifampin (8).

A strong positive correlation has been reported between oral spirochetes and periodontal disease (1, 13). Oral spirochetes in subgingival plaques may secrete PLC into gingival crevicular fluid (GCF).

To test for PLC activity in GCF, Periopaper strips (Interstate Drug Exchange, Amityville, N.Y.) were inserted into the crevices of diseased sites (pocket depths of 7 to 10 mm) and healthy sites (pocket depths of 2 to 3 mm) and left in place for 2 min. The strips were removed and incubated at 37°C with 20 μl of 10 mM NPPC in complete Tris buffer. The strips with absorbed GCF which contained PLC hydrolyzed NPPC, and the released NP colored the strip yellow. The GCF obtained from diseased sites of 14 patients with periodontitis contained PLC, but the GCF from 3 individuals without periodontal disease and from healthy sites of 7 patients with periodontitis did not contain PLC. Saliva was collected from adults with healthy periodontal tissue and quantified for PLC activity. No PLC activity was detected in the saliva samples. The assay is qualitative but serves as a rapid screening test for PLC in GCF. The PLC in GCF could have originated from spirochetes or other bacterial species in diseased periodontal pockets as well as from lysed leukocytes that migrated into diseased pockets. The presence of PLC in diseased pockets might have contributed to the progression of periodontal lesions. It is known that PLC can hydrolyze membrane phospholipids, and this can lead to the destruction of crevicular epithelial cells or the release of arachidonic acid that is utilized in the production of prostaglandins and leukotrienes (3). The eicosanoids may be responsible for inflammation, loss of tooth attachment, and bone loss (16, 21, 22). Our finding implicates oral spirochetes as important agents of periodontal diseases. An investigation is in progress to determine by means of specific antibodies the amount of spirochete PLC in diseased pockets.

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