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Evidence for the Absence of Hyaluronidase Activity in *Porphyromonas gingivalis*

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The aim of the present study was to evaluate the ability of *Porphyromonas gingivalis* to degrade hyaluronic acid. No hyaluronidase activity was detected using a turbidimetric method, whereas a standard plate assay showed a positive reaction for *P. gingivalis*. We postulated that the high proteolytic activity of *P. gingivalis* may account for this observation. A modified plate assay was designed to avoid false-positive reactions caused by proteolytic bacteria. The new assay, based on the formation f a water-insoluble salt between hyaluronic acid and the polyanion cetylpyridinium chloride, indicated that *P. gingivalis* does not have hyaluronidase activity. By this modified plate method, it was found that among 24 different oral bacterial species tested, *Propionibacterium acnes* and *Prevotella oris* were the only species that possess hyaluronidase activity.

Hyaluronidases, or more generally mucopolysaccharidases, catalyze the breakdown of glycosaminoglycans, which are major structural components of connective tissue and are essential for maintaining the integrity of the extracellular matrix. Microbial hyaluronidases should be considered an important virulence factor in disease, as they may act as a spreading factor and thus enhance the dissemination of bacteria and toxins within tissues. Numerous pathogenic bacteria, including *Propionibacterium acnes* (6, 14), *Clostridium perfringens* (14), and *Treponema pallidum* (1, 2), have been shown to produce hyaluronidases, either extracellular or cell bound.

Porphyromonas gingivalis is considered the primary etiologic agent of chronic adult periodontitis (8, 10, 13). This bacterial species is known to have a large array of putative virulence factors, including hydrolytic enzyme production (4, 8, 9). By using a standard plate assay based on the precipitation of hyaluronic acid and bovine serum albumin under acidic conditions (14), a number of researchers have suggested that *P. gingivalis* produces a hyaluronidase (12, 15, 18). However, nothing is known about the nature and the characteristics of the enzyme. Several attempts by our laboratory to purify the hyaluronidase from *P. gingivalis* were unsuccessful. The present report provides evidence suggesting that *P. gingivalis* in fact does not produce a hyaluronidase and proposes a modified plate assay for the detection of hyaluronidase activity.

P. gingivalis ATCC 33277 and W50, *Porphyromonas asaccharolytica* BM4, and *P. acnes* UD were used in most experiments. Additional oral bacterial species, representing type strains and clinical isolates, were also tested for hyaluronidase activity and are listed in Table 1. Bacteria were cultured in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with hemin (10 μg/ml) and vitamin K (1 μg/ml). Cultures were incubated in an anaerobic chamber (N_2-H_2-CO_2, 80:10:10) at 37°C. Unless indicated otherwise, bacteria were grown for 2 days.

Hyaluronidase activity was first measured by a turbidimetric assay according to Mathews (7). Hyaluronic acid (from human umbilical cord; Sigma Chemical Company, St. Louis, Mo.) was dissolved in 0.3 M phosphate buffer (pH 6.0) at a concentration of 0.2 mg/ml. The assay mixture consisted of 100 μl of the hyaluronic acid solution, 40 μl of 20 mM phosphate buffer (pH 6.0) containing 0.45% NaCl and 0.01% bovine serum albumin (BSA) fraction V, and 60 μl of the bacterial fractions. The fractions tested were the culture supernatant, the bacterial cells (A_{660} = 2.0 in distilled water), and a sonic extract of the cells (5×, 30 s each time, 50% duty cycle; Sonic dismembrator model 150; Artek Systems Corporation, Farmingdale, N.Y.). After an incubation period of 1 h at 37°C, 1 ml of an acid albumin solution (1% BSA–0.3 M sodium acetate buffer, pH 3.75) was added, which was followed by vigorous mixing of the mixture. The tubes were incubated at room temperature for 10 min before measuring the A_{600}.

The sensitivity of the turbidimetric method was determined with a commercial source of bovine testis hyaluronidase (Sigma). By this assay, a final concentration of 7.5 national formulary units per ml allowed complete degradation of the hyaluronic acid (20 μg) within a 1-h incubation period at 37°C, whereas 50% degradation was obtained with 2.5 national formulary units per ml. Culture supernatant from *P. acnes* UD, a strain isolated from a diseased periodontal site, completely digested the hyaluronic acid. A hyaluronidase produced by strains of *P. acnes* has been previously purified and characterized (6). On the other hand, culture supernatants and whole cells as well as sonic extracts from *P. gingivalis* ATCC 33277 and W50 and *P. asaccharolytica* BM4 did not degrade hyaluronic acid. Negative results were also obtained when (i) mid-log-phase and 5-day-old cultures were used, (ii) the assay mixtures were incubated at 37°C for 16 h, and (iii) the assays were carried out in a pH range of 6.0 to 9.0. To ensure that the hyaluronidase activity was not due to an inducible enzyme, *P. gingivalis* was grown in the presence of hyaluronic acid (1 mg/ml). Residual hyaluronic acid in the culture supernatant was measured using the turbidimetric assay. The hyaluronic acid was not degraded by either strain of *P. gingivalis*.

A plate assay was then performed, as hyaluronidase production by *P. gingivalis* has previously been reported by this method (12, 15, 18). Plates were prepared by the method of Smith and Willett (14). Briefly, brain heart infusion agar plates were supplemented with hemin (1 μg/ml), vitamin K (1

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TABLE 1. Hyaluronidase activities of selected oral bacterial species determined by the modified plate assay

Organism	Hyaluronidase activity[a]
Porphyromonas gingivalis ATCC 33277, ATCC 49417, W50, BH18/10, CR2A	–
Porphyromonas endodontalis ATCC 35406	–
Porphyromonas asaccharolytica ATCC 27067, BM4	–
Prevotella denticola ATCC 33185	–
Prevotella intermedia ATCC 25611, NCTC 9336	–
Prevotella loescheii ATCC 15930	–
Prevotella melaninogenica ATCC 25845	–
Prevotella buccae ATCC 33574	–
Prevotella oralis ATCC 33269	–
Prevotella oris ATCC 33573	+
Fusobacterium nucleatum CM33MB-5	–
Capnocytophaga ochracea 1956c	–
Campylobacter rectus ATCC 33238	–
Actinobacillus actinomycetemcomitans ATCC 29522, Y4	–
Veillonella parvula VeA2	–
Eubacterium saburreum 162.4	–
Actinomyces viscosus RA	–
Actinomyces naeslundii SA	–
Actinomyces odontolyticus XD	–
Propionibacterium acnes UD, T1, NA	+
Staphylococcus aureus Tpro2	–
Peptostreptococcus micros 89A	–
Streptococcus mutans ATCC 10449	–
Streptococcus sanguis StB4	–

[a] Duplicate assays were performed.

μg/ml), hyaluronic acid (0.4 mg/ml), and BSA (10 mg/ml). Plates were spot inoculated with bacteria from a 2-day broth culture. After incubation under anaerobic conditions for 7 days, the plates were flooded with 2 N acetic acid for 10 min. A clear zone surrounding bacterial growth was indicative of a hyaluronidase-producing bacterial isolate. Figure 1A shows the presence of a clear halo surrounding *P. acnes* and *P. gingivalis*, whereas no zone was produced by *P. asaccharolytica*. This method relies on the formation of a water-insoluble complex, under acidic conditions, between the hyaluronic acid and BSA incorporated into the culture medium. As a result of the negative result obtained by using the turbidimetric assay, we postulated that the strong proteolytic activity of *P. gingivalis* may account for the false-positive reaction obtained with the plate assay. *P. gingivalis* can indeed degrade the BSA (4) and thus is able to prevent the formation of the hyaluronic acid-BSA complex.

A modified plate assay was designed to avoid the false-positive reactions that may be caused by proteolytic bacteria such as *P. gingivalis*. Plates were prepared as described above, except that the BSA was omitted. Following growth, hyaluronidase-producing bacterial isolates were revealed by flooding the plate with 10% cetylpyridinium chloride. This cationic agent has been previously shown to form a water-insoluble salt with polysaccharides (11). Hyaluronidase-producing bacteria (clear zone) became apparent after 10 min. By using this modified assay, *P. acnes* was found to be positive, while *P. gingivalis* and *P. asaccharolytica* were negative (Fig. 1B). This observation clearly suggests that the proteolytic activity of *P. gingivalis* was likely responsible for the clear zone in the plate method of Smith and Willett (14). By using the modified plate assay, a variety of oral bacteria were assayed for the production of hyaluronidase. Table 1 indicates that among the bacterial species tested, *P. acnes* (UD, T1, and NA) and *Prevotella oris* (ATCC 33573) were the only species that possess true hyaluronidase activity. Taniguchi et al. (16) had previously characterized and purified a hyaluronidase from an oral strain of a *Bacteroides* sp. isolated from the exudate around experimentally introduced crevice materials in guinea pigs. On the basis of a comparison of the biochemical characteristics of their strain and those of *P. oris* (5), it is likely that the isolate used by Taniguchi et al. (16) was in fact *P. oris*.

The present report provides evidence clearly suggesting that *P. gingivalis* in fact does not produce a hyaluronidase. The ability of *P. gingivalis* and several other oral bacterial species to degrade hyaluronic acid has been previously suggested on the basis of results from spectrophotometric and plate assays (12, 15, 18). The positive reactions obtained by the spectrophotometric method (12, 18) may have been caused by bacterium-derived substances, such as nucleotides, which absorb at 230 nm and strongly interfere with the determination of hyaluronidase activity (3). On the other hand, the strong proteolytic activity of *P. gingivalis* may have been responsible for the positive reactions previously reported with the standard plate method (12, 15, 18). A modified plate assay which avoids the false-positive reac-

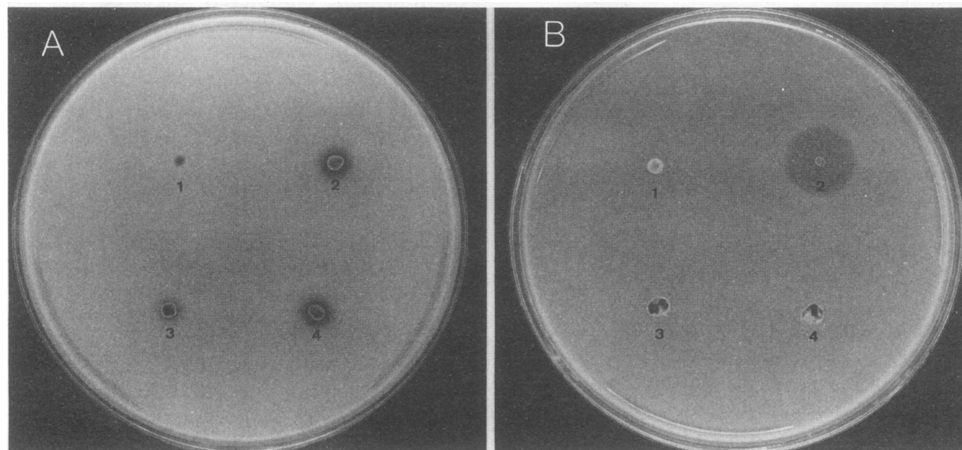

FIG. 1. Detection of hyaluronidase activity by conventional (A) and modified (B) plate methods. Bacteria tested were *P. asaccharolytica* BM4 (1), *P. acnes* UD (2), *P. gingivalis* ATCC 33277 (3), and *P. gingivalis* W50 (4).

tions caused by proteolytic activity is proposed. By this method, it was demonstrated that among 24 different oral bacterial species tested, only *P. acnes* and *P. oris* produce a hyaluronidase. These two bacterial species, which have been associated with active periodontal pockets (17), may thus contribute to periodontal disease by degrading structural components of connective tissues.

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