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Antagonistic Effect of Oral Bacteria towards *Treponema denticola*

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This study was designed to isolate oral bacteria exhibiting antagonism towards *Treponema denticola* and to characterize the inhibitory activity. Eleven bacterial isolates obtained from subgingival sites and identified as either *Staphylococcus aureus* or *Streptococcus mutans* were found to inhibit the growth of *T. denticola*. When the activity spectra of these isolates were analyzed, two additional periodontopathogens (*Porphyromonas gingivalis* and *Prevotella intermedia*) were found to be affected, whereas most gram-positive bacteria were not. Strains of *S. aureus* produce a bacteriocin-like inhibitory substance (heat stable and protease sensitive), whereas the inhibitory effect of *S. mutans* appears to be related to the production of lactic acid. The negative interactions reported in this paper may govern population shifts observed in subgingival sites.

Bacterial interactions, including synergism, commensalism, and antagonism, are likely to play an important role in the ecology of the microflora found in subgingival areas (11, 12, 15, 16). These phenomena may govern the population shifts observed during the development of periodontal disease (1, 17, 18, 26). Of particular interest is the fact that antagonistic mechanisms may prevent overgrowth of specific periodontopathogens and be involved in the maintenance of periodontal health. This hypothesis has been discussed by Hillman et al. (7, 8) in regard to *Actinobacillus actinomycetemcomitans* and *Streptococcus sanguis*. They suggested that the strong negative association between these two bacterial species that has been found in microbiological studies of subgingival plaque samples from healthy and affected sites may be related to antagonistic relationships. Indeed, they reported an in vitro suppression of growth of *A. actinomycetemcomitans* by *S. sanguis*. The inhibition appears to be the result of hydrogen peroxide production by *S. sanguis*. On the other hand, it has also been shown that *A. actinomycetemcomitans* can produce a bacteriocin able to inhibit the growth of *S. sanguis* (5).

Negative interactions among oral bacteria may be related to specific molecules known as bacteriocins (5, 19, 21, 22) or to metabolic by-products, including hydrogen peroxide (28) and acids (3, 21). The aim of this study was to isolate oral bacteria exhibiting antagonism towards the periodontopathic bacterium *Treponema denticola* and to characterize the inhibitory compounds.

MATERIALS AND METHODS

Sampling and cultural conditions. Subgingival plaque samples were collected by insertion of sterile absorbent paper points (Johnson and Johnson, East Windsor, N.J.) in the sulci of five individuals having healthy periodontia showing no clinical signs of inflammation. The sites of collection were randomly selected for each subject. When necessary, the supragingival plaque was removed with a sterile cotton swab prior to sampling. Each sample was placed in a prereduced one-fourth-strength Ringer's solution, dispersed with a Vortex mixer for 2 min, and subjected to 10-fold serial dilutions. The dilutions were then plated on new oral spirochete (NOS) medium (13) which had been spread with *T. denticola* ATCC 35405 (approximately 5.0×10^8 cells, as determined with a Petroff-Hausser counting chamber) immediately beforehand. The plates were incubated anaerobically (N_2-H_2-CO_2, 80:10:10) for 7 days at 37°C. Distinct colonies (in each sample) which formed an evident inhibitory zone (no growth) of *T. denticola* were picked and subcultured on NOS agar plates until pure cultures were obtained.

Bacterial identification. Bacterial isolates inhibiting the growth of *T. denticola* were Gram stained and examined by phase-contrast microscopy. Bacterial identification was conducted with the API 20S system (API Laboratory Products, Ltd., St.-Laurent, Quebec, Canada) and with standard procedures (fermentation tests, catalase and oxidase activities, and growth in 15% NaCl) as described by MacFaddin (14).

Determination of inhibitory spectrum. The inhibitory spectra of the antagonistic clinical isolates were determined by measuring inhibition of growth of additional oral bacterial species. Indicator bacteria to be tested were plated (approximately 5.0×10^8 cells, as determined with a Petroff-Hausser counting chamber) on brain heart infusion medium (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 2.5% human blood, hemin (10 μg/ml), and vitamin K (1 μg/ml). Inhibitory strains were immediately spotted onto the surface of the plates. After 4 days of incubation at 37°C in the anaerobic chamber, the zones of inhibition were measured from the edge of the growth of inhibitory strains to the margin of the inhibitory zone. The inhibition scores were as follows: +, radius of ≥1 mm; ±, radius of <1 mm; and −, no inhibition.

Characterization of the inhibitory substances. The inhibition of *T. denticola* by clinical isolates was tested by incorporating 0.03% catalase, 20 μM $FeCl_3$, 1% $CaCO_3$, or 0.75% arginine in the NOS agar plates. In order to further characterize the inhibitory compounds, an attempt was made to extract soluble active inhibitors from lawn cultures (on NOS plates) of inhibitory strains. After cultivation for 3 days, the bacterial growth was removed and the plates were frozen (−80°C for 4 h). After thawing, the liquid phase was harvested, subjected to centrifugation ($8,000 \times g$ for 15 min), concentrated 20 times by lyophilization, and finally passed through a filter with a membrane pore size of 0.45 μm. The preparations were then assayed for growth inhibition of *T. denticola* as follows. Paper discs (7-mm diameter, grade 3MM; Whatman, Inc., Clifton, N.J.) were moistened with 50-μl samples of the fractions and allowed to dry at 37°C. NOS agar plates were spread with *T. denticola*, and the paper discs were then applied. After incubation for 7 days under anaerobiosis, inhibition of *T. denticola* was recorded. Samples were also treated with heat (100°C for 10 min) as well as proteolytic enzymes (trypsin or pronase at a final concentration of 500 μg/ml; 16 h at 37°C) prior to being tested for growth-inhibitory effects on *T. denticola*. The relative sizes of the inhibitors were estimated by ultrafiltration through membranes with molecular weight cutoffs of 10,000, 50,000, 100,000, and 300,000. Finally, solutions of lactic acid, ranging from 0.25 to 5% (wt/vol), were prepared with the pH adjusted to either 4.0 or 6.0. The solutions (50 μl) were applied to paper discs, and their ability to inhibit the growth of *T. denticola* was determined as described above.

RESULTS AND DISCUSSION

Bacterial colonies surrounded by a clear halo indicating inhibition of growth of *T. denticola* are shown in Fig. 1 (sample from subject 3). Analysis of the five subgingival plaque samples (from five individuals) allowed the recovery of a total of 11

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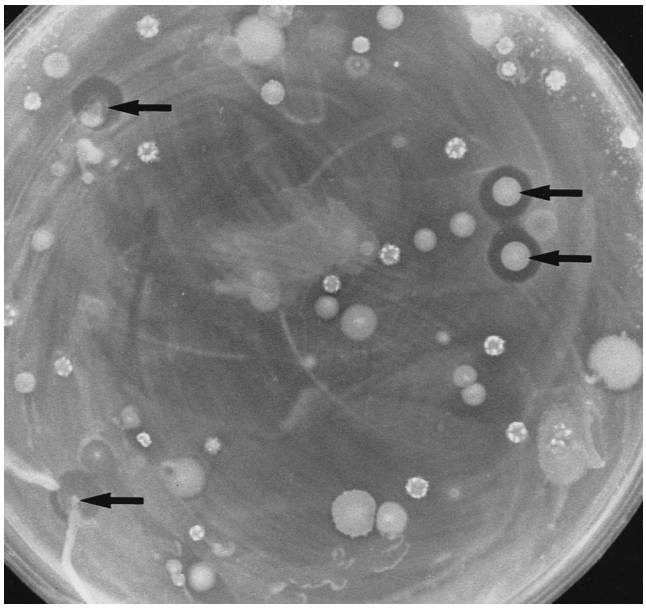

FIG. 1. Demonstration of subgingival bacterial isolates exhibiting inhibition of growth of *T. denticola* (arrows).

clinical isolates (called IN_1 to IN_{11}) having the ability to inhibit growth of *T. denticola*. The radius of the inhibition zones ranged from 2.5 to 4 mm. All bacteria were found to be gram-positive cocci, and eight of these strains possessed catalase activity. The catalase-positive isolates were all identified as *Staphylococcus aureus*, on the basis of fermentative metabolism, growth in 15% NaCl, and mannitol fermentation. With the API 20S system, the catalase-negative, gram-positive cocci (three strains) were found to belong to the species *Streptococcus mutans*. This identification was confirmed on the basis of the criteria of Coykendall (2) and the fact that mannitol, sorbitol, raffinose, and melibiose were fermented. All sampled subjects harbored at least one isolate of *S. aureus* (three subjects had two colonial morphotypes), whereas *S. mutans* isolates were recovered from subgingival plaque samples from only three of the five individuals.

The results for suppression of growth of additional oral bacterial species are presented in Table 1. In addition to *T. denticola* ATCC 35405 and D11, the cell growth of *Porphyromonas gingivalis* ATCC 33277 and 23A4 was affected by all inhibitory bacterial isolates. To a lesser extent, some inhibition was also noted for *Prevotella intermedia*, *Prevotella loescheii*, *Capnocytophaga ochracea*, *Eubacterium saburreum*, and *Actinomyces viscosus*. The *S. mutans* strains showed a larger spectrum of activity than the *S. aureus* strains. The strains within the *S. aureus* group or the *S. mutans* group were not affected by each other.

The inhibition of growth of *T. denticola* did not result from the production of hydrogen peroxide or siderophores by *S. aureus* and *S. mutans*, since similar zones of inhibition were obtained when the NOS agar plates contained catalase or $FeCl_3$, respectively. Preparations of *S. aureus* (IN_1 and IN_{10}) gave an inhibition zone of 3 mm, whereas no inhibition was obtained with preparations of *S. mutans* (IN_2 and IN_5). Boiling the *S. aureus* preparations did not affect their ability to suppress growth of *T. denticola*. However, inhibition of *T. denticola* growth was completely prevented by the proteolytic treatments, suggesting a proteinaceous moiety of the inhibitory compound produced by *S. aureus*. The results of ultrafiltration indicated a high molecular weight for the compound, since the inhibitory component passes through only the membrane with the 300,000-molecular-weight cutoff. These results suggest that the inhibitory compound produced by the *S. aureus* strains is a true bacteriocin-like inhibitory substance that is a heat-stable molecule with a protein moiety. The production of a heat-stable and protease-sensitive bacteriocin, active against gram-positive bacteria, by an oral isolate of *S. aureus* has been previously reported (19). Since the molecular mass of that particular bacteriocin was 5 kDa, it is likely to be different from the one demonstrated in the present study. However, it is

TABLE 1. Inhibition of growth of selected oral bacteria by clinical isolates of *S. aureus* and *S. mutans*

Target bacterium	Inhibition zone produced by[a]:										
	S. aureus								*S. mutans*		
	IN_1	IN_3	IN_4	IN_6	IN_7	IN_8	IN_{10}	IN_{11}	IN_2	IN_5	IN_9
Treponema denticola											
ATCC 35405	+	+	+	+	+	+	+	+	+	+	+
D11	+	+	+	+	+	+	+	+	+	+	+
Porphyromonas gingivalis											
ATCC 33277	+	+	+	+	+	+	+	+	+	+	+
23A4	+	+	+	+	+	+	+	+	+	+	+
Prevotella intermedia BMH	±	+	+	+	±	+	±	±	−	+	+
Prevotella loescheii ATCC 15930	−	−	−	−	−	−	−	−	±	±	−
Capnocytophaga ochracea 1956c	±	−	+	+	±	±	−	−	+	+	+
Eubacterium saburreum 162.4	−	−	−	−	−	−	−	−	+	+	+
Fusobacterium nucleatum XV.156	−	−	−	−	−	−	−	−	−	−	−
Wolinella recta ATCC 33238	−	−	−	−	−	−	−	−	−	−	−
Actinomyces viscosus 54.2	−	−	−	−	−	−	−	−	+	+	+
Actinomyces naeslundii 85.1	−	−	−	−	−	−	−	−	−	−	−
Staphylococcus aureus											
IN_1	−	−	−	−	−	−	−	−	+	+	+
IN_{10}	−	−	−	−	−	−	−	−	+	+	+
Streptococcus mutans											
IN_2	−	−	−	−	−	−	−	−	−	−	−
IN_5	−	−	−	−	−	−	−	−	−	−	−

[a] +, radius ≥ 1 mm; ±, radius < 1 mm; −, no inhibition.

TABLE 2. Effects of lactic acid and pH on growth of *T. denticola*

% Lactic acid (wt/vol)	pH	Inhibition zone[a]
5	6	−
0	4	−
0.25	4	−
0.5	4	±
1	4	+
2	4	+
5	4	+

[a] +, radius ≥ 1 mm; ±, radius < 1 mm; −, no inhibition.

possible that the bacteriocin-like inhibitory substance present in the *S. aureus* preparation may be complexed with other bacterial components.

The fact that no soluble inhibitory activity was recovered from *S. mutans* suggests that the inhibition of growth of *T. denticola* could be related to the ability of *S. mutans* to produce large amounts of acids and to consequently decrease the pH (20). Since some bacterial species, including *S. mutans* (3, 27), have been previously shown to produce lactic acid, which interferes with bacterial growth, the effect of lactic acid on *T. denticola* was investigated. Data presented in Table 2 indicate that inhibition by lactic acid was possible only at a low pH. It is likely that at a low pH the lactic acid, which is in its nonionized form, is better able to penetrate the cell envelope of *T. denticola* and affect the bacteria. The minimal concentration of lactic acid required to produce significant growth inhibition was approximately 1% (wt/vol). In the absence of lactic acid, the low pH did not interfere with growth of *T. denticola*. Despite the fact that it is difficult to accurately determine the local concentration of lactic acid around bacterial colonies, a concentration of 0.65% (wt/vol) has been previously reported (3). The above results indirectly suggest that the high level of production of lactic acid by *S. mutans* may contribute to the inhibition of growth of *T. denticola*. This is reinforced by the fact that incorporation in NOS agar plates of $CaCO_3$ or arginine, two compounds that can prevent a pH decrease (21), significantly reduced the inhibition zone produced by *S. mutans* strains (IN_2 and IN_5). However, the possibility that another mechanism of inhibition, such as the production of mutacin-like substances, is involved in the inhibition of *T. denticola* should not be excluded. Indeed, because of the high degree of instability of some mutacins produced by *S. mutans* (21), it may be rather difficult to prepare an active fraction.

This study reports for the first time that bacteria found in subgingival areas may interfere with the growth of *T. denticola*. In particular, it was found that *S. aureus* could produce a bacteriocin-like inhibitory substance active against this periodontopathogen. Although *S. aureus* does not represent a predominant bacterial species in the oral cavity, it has been often isolated from dental plaque and saliva (19, 23). Few studies have found bacteriocins which are produced by gram-positive bacteria and active against gram-negative organisms (4, 9, 10). Fujimura and Nakamura (4) isolated a bacteriocin (molecular weight, 280,000) from *S. sanguis* which was active against gram-negative bacteria, including *Prevotella melaninogenica*, formerly *Bacteroides melaninogenicus*. An additional bacteriocin (molecular weight, 4,800) active against *P. melaninogenica* and produced by *S. mutans* has also been reported (9).

In view of the great diversity of bacterial species found in subgingival sites (17, 18), it is not surprising that such bacterial antagonism exists. The inhibition of growth of suspected periodontal pathogens such as *T. denticola* and *P. gingivalis* may be one of the factors involved in the maintenance of periodontal health. As *T. denticola* has been associated with periodontal disease (24, 25), studies of the incidence of strains inhibitory for this bacterium in diseased and healthy sites deserve consideration. Interestingly, Hillman and Socransky (6) showed that organisms which are inhibitory to the periodontopathic bacterium *A. actinomycetemcomitans* were found in high numbers in subgingival plaque from healthy individuals, whereas affected sites of juvenile periodontitis subjects did not usually harbor these inhibitory bacteria.

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REFERENCES

1. **Armitage, G. C., W. R. Dickinson, R. S. Jendersek, 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. **Coykendall, A. L.** 1989. Classification and identification of the viridans streptococci. Clin. Microbiol. Rev. **2:**315–328.
3. **Donoghue, H. D., and J. E. Tyler.** 1975. Antagonisms amongst streptococci isolated from the human oral cavity. Arch. Oral Biol. **20:**381–387.
4. **Fujimura, S., and T. Nakamura.** 1979. Sanguicin, a bacteriocin of oral *Streptococcus sanguis*. Antimicrob. Agents Chemother. **16:**262–265.
5. **Hammond, B. F., S. E. Lillard, and R. H. Stevens.** 1987. A bacteriocin of *Actinobacillus actinomycetemcomitans*. Infect. Immun. **55:**686–691.
6. **Hillman, J. D., and S. S. Socransky.** 1982. Bacterial interference in the oral ecology of *Actinobacillus actinomycetemcomitans* and its relationship to human periodontosis. Arch. Oral Biol. **27:**75–77.
7. **Hillman, J. D., and S. S. Socransky.** 1989. The theory and application of bacterial interference to oral diseases, p. 1–17. *In* H. M. Myers (ed.), New biotechnology in oral research. S. Karger, Basel.
8. **Hillman, J. D., S. S. Socransky, and M. Shivers.** 1985. The relationships between streptococcal species and periodontopathic bacteria in human dental plaque. Arch. Oral Biol. **30:**791–795.
9. **Ikeda, T., T. Iwanami, M. Hirasawa, C. Watanabe, J. M. McGhee, and T. Shiota.** 1982. Purification and certain properties of a bacteriocin from *Streptococcus mutans*. Infect. Immun. **35:**861–868.
10. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
11. **Johansson, A., A. Bergenholtz, and S. E. Holm.** 1994. Bacterial interference *in vitro*: comparison between a quantitative kinetic and a cocultivation blood agar test method. APMIS **102:**810–816.
12. **Kornman, K. S.** 1982. Age, supragingival plaque, and steroid hormones as ecological determinants of the subgingival flora, p. 132–138. *In* R. J. Genco and S. E. Mergenhagen (ed.), Host-parasite interactions in periodontal diseases. American Society for Microbiology, Washington, D.C.
13. **Leschine, S. B., and E. Canale-Parola.** 1980. Rifampin as a selective agent for isolation of oral spirochetes. J. Clin. Microbiol. **12:**792–795.
14. **MacFaddin, J. F.** 1980. Gram-positive bacteria, p. 345–370. *In* Biochemical tests for identification of medical bacteria, 2nd ed. Williams and Wilkins Co., Baltimore.
15. **Marsh, P. D.** 1989. Host defenses and microbial homeostasis: role of microbial interactions. J. Dent. Res. **68:**1567–1575.
16. **Mayrand, D.** 1985. Virulence promotion by mixed bacterial infections, p. 281–291. *In* Bayer-Symposium VIII. The pathogenesis of bacterial infections. Springer-Verlag, Berlin.
17. **Moore, W. E. C.** 1987. Microbiology of periodontal disease. J. Periodontal Res. **22:**335–341.
18. **Moore, W. E. C., L. V. Holdeman, R. M. Smibert, D. E. Hash, J. A. Burmeister, and R. R. Ranney.** 1982. Bacteriology of severe periodontitis in young adult humans. Infect. Immun. **38:**1137–1148.
19. **Nakamura, T., N. Yamazaki, H. Taniguchi, and S. Fujimura.** 1983. Production, purification, and properties of a bacteriocin from *Staphylococcus aureus* isolated from saliva. Infect. Immun. **39:**609–614.
20. **Onose, H., and H. J. Sandham.** 1976. pH changes during culture of human dental plaque streptococci on mitis-salivarius agar. Arch. Oral Biol. **21:**291–296.
21. **Parrot, M., M. Charest, and M. C. Lavoie.** 1989. Production of mutacin-like substances by *Streptococcus mutans*. Can. J. Microbiol. **35:**366–372.
22. **Parrot, M., M.-F. Dréan, L. Trahan, and M. C. Lavoie.** 1990. Incidence of bacteriocinogeny among fresh isolates of *Streptococcus mutans*. Can. J. Microbiol. **36:**507–509.
23. **Rams, T. E., D. Feik, and J. Slots.** 1990. Staphylococci in human periodontal diseases. Oral Microbiol. Immunol. **5:**29–32.

24. **Simonson, L. G., C. H. Goodman, J. J. Bial, and H. E. Morton.** 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56:**726–728.
25. **Simonson, L. G., C. H. Goodman, and H. E. Morton.** 1990. Quantitative immunoassay of *Treponema denticola* serovar c in adult periodontitis. J. Clin. Microbiol. **28:**1493–1496.
26. **Socransky, S. S., A. D. Haffajee, J. L. Dzink, and J. D. Hillman.** 1988. Associations between microbial species in subgingival plaque samples. Oral Microbiol. Immunol. **3:**1–7.
27. **Tramer, J.** 1966. Inhibitory effect of *Lactobacillus acidophilus*. Nature (London) **211:**204–205.
28. **Willcox, M. D. P., and D. B. Drucker.** 1988. Partial characterisation of the inhibitory substances produced by *Streptococcus oralis* and related species. Microbios **55:**135–145.