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Nutritional Interactions between Two Suspected Periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*

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A mutual symbiotic enhancement of growth of *Porphyromonas gingivalis* and *Treponema denticola* is described in this report. Brain heart infusion broth supplemented with vitamin K did not support the individual growth of *P. gingivalis* or *T. denticola*. However, when inoculated as a mixture, both bacterial species did grow significantly. The growth-stimulating factors produced by *P. gingivalis* and *T. denticola* were dialyzable and heat stable and were further identified as isobutyric acid and succinic acid, respectively. Since some forms of periodontal disease are associated with the presence, in affected sites, of high numbers of *P. gingivalis* and spirochetes, it is suggested that the bacterial interaction described in this report might be of utmost importance in the initiation and progression of the disease.

Periodontal diseases affecting the tissues surrounding and supporting the teeth are considered to be the result of mixed anaerobic infections. Microbial analyses of affected periodontal sites indicate the presence of a complex microflora mainly consisting of spirochetes and gram-negative anaerobic rods (10, 14, 15, 19, 20). Since most of these bacterial species are only weakly virulent on an individual basis, it has been suggested that interbacterial cooperation might promote the infectious process of periodontal disease (17). Several types of bacterial interactions are probably important for the establishment of a potentially pathogenic subgingival plaque. One major mechanism of interaction involves the production of growth-stimulating factors. Previous studies have found nutritional relationships between *Porphyromonas gingivalis* and some oral bacterial species (7, 8, 16, 18). Other nutritional interactions between oral bacteria involve the enhancement of growth of *Bacteroides forsythus* by *Fusobacterium nucleatum* (5) and of *Treponema denticola* by a bacterial mixture containing a diphtheroid-shaped bacterium and a fusiform-shaped bacterium (22) as well as by *Bacteroides intermedius* or *Eubacterium nodatum* (23). In a survey for nutritional interactions among oral bacteria, a consortium composed of *P. gingivalis* and *T. denticola* displayed a mutualistic relationship. The aim of the present study was to characterize the nutritional interaction between these two suspected periodontopathogens.

The type strains *P. gingivalis* ATCC 33277 and *T. denticola* ATCC 35405 were obtained from the American Type Culture Collection (Rockville, Md.). Growth of *P. gingivalis* was carried out in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) containing hemin (10 μg/ml) and vitamin K (1 μg/ml) (BHI-HK). In some cases, hemin was omitted (BHI-K) in order to obtain hemin-depleted *P. gingivalis* cells. *T. denticola* was grown in fluid NOS medium as previously described (12). Cultures were incubated in an anaerobic chamber (N_2-H_2-CO_2, 80:10:10) at 37°C. The mixed culture of *P. gingivalis* and *T. denticola* was grown in BHI-K, which does not contain all necessary nutritional factors for the bacteria. This medium was inoculated with a 2.5% volume of both an overnight hemin-depleted *P. gingivalis* culture and a 3-day *T. denticola* culture. The cell growth was monitored by recording the A_{660} and by direct cell counts using a Petroff-Hausser counting chamber according to the manufacturers' instructions.

As shown in Table 1 and Fig. 1, the BHI-K medium did not support the individual growth of *P. gingivalis* or *T. denticola*. However, when inoculated as a mixture, both bacterial species did grow significantly in this medium. Total cell counts, determined with a Petroff-Hausser counting chamber, indicate a mutual symbiotic enhancement of growth for *P. gingivalis* and *T. denticola* (Table 1). The growth of *T. denticola* was enhanced from the time of inoculation in the mixed culture (Fig. 1). On the other hand, the development of *P. gingivalis* appeared to be stimulated once the number of *T. denticola* cells had reached a certain level (about 2×10^8 bacteria per ml). Observation of the mixed culture by phase-contrast microscopy showed the presence of *P. gingivalis* cells attached to the spirochetes (Fig. 2). Very few *P. gingivalis* cells were found to be free in the culture medium. Small- and intermediate-size aggregates were still observed after 30 s of vigorous vortex agitation of the mixed culture, which indicates the relative strength of attachment of *P. gingivalis* to *T. denticola*. The stability of the mixed culture was evaluated by serial transfers, using an inoculum of 10% (vol/vol), in BHI-K (Table 1). The number of *T. denticola* cells was constant, at about 1.0×10^9 to 1.2×10^9 bacteria per ml, throughout all mixed cultures. However, the number of *P. gingivalis* cells tended to decrease following the initial transfer. A stable concentration of 4.5×10^8 to 4.8×10^8 bacteria per ml appeared to be reached after the third transfer.

Bacterial culture filtrates were then assayed for stimulatory effect. *P. gingivalis* and *T. denticola* were grown in BHI-HK and NOS, respectively. The cultures were incubated for 5 days and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant fluid was removed, adjusted to pH 7, and sterilized by filtration through a 0.22-μm-pore-size membrane filter. The filtrate was then mixed with an equal volume of fresh BHI-K and inoculated with either *T. denticola* (for culture filtrate from *P. gingivalis*) or hemin-depleted *P. gingivalis* (for culture filtrate from *T. denticola*).

TABLE 1. Cell counts during mixed and individual cultures of *P. gingivalis* and *T. denticola*

Culture[a]	A_{660}	Total cell count[b] (10^8 cells/ml)	
		P. gingivalis	*T. denticola*
P. gingivalis	0.023	0.7	
T. denticola	0.032		0.8
P. gingivalis + *T. denticola* (first growth)	0.628	15	10
P. gingivalis + *T. denticola* (second growth)	0.465	6.0	12
P. gingivalis + *T. denticola* (third growth)	0.415	4.5	11
P. gingivalis + *T. denticola* (fifth growth)	0.424	4.8	11

[a] Three days in BHI-K medium.
[b] Determined with a Petroff-Hausser counting chamber.

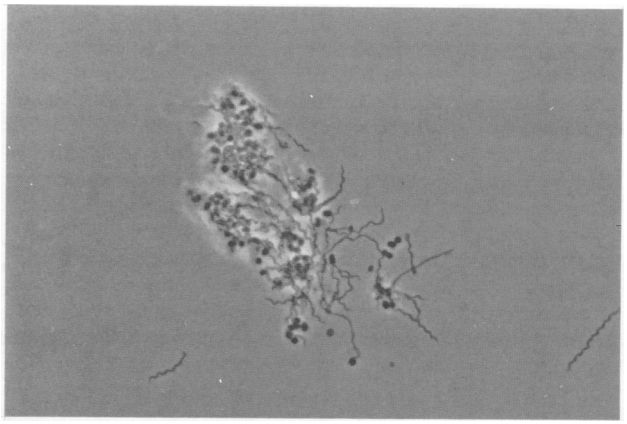

FIG. 2. Phase-contrast micrograph of mixed culture of *P. gingivalis* and *T. denticola*.

The stimulatory effect was determined by reading the A_{660} after 4 days of incubation. Bacterial culture filtrates of *P. gingivalis* and *T. denticola* were found to contain growth-stimulating activities for *T. denticola* and *P. gingivalis*, respectively (Table 2). No enhancement of growth was observed when the BHI-K medium was supplemented with heat-killed, washed whole bacterial cells. Some characteristics of the growth-stimulating factors found in the culture filtrates of *P. gingivalis* and *T. denticola* are presented in Table 2. The nutritional effect of both culture filtrates was unchanged following heating at 70°C for 30 min or boiling for 5 min. Growth-stimulating activities were removed following dialysis (molecular weight cutoff [MWCO] = 6,000 to 8,000) or ultrafiltration (MWCO = 1,000), indicating a low molecular weight for both factors.

Fatty acids present in the culture filtrates from *P. gingivalis* and *T. denticola* were analyzed by gas-liquid chromatography, using a Hewlett-Packard 5830A chromatograph equipped with a hydrogen flame ionization detector. Extraction and methylation of volatile and nonvolatile fatty acids were done according to the methods outlined in the *Anaerobe Laboratory Manual* (11). The metabolic by-products found in the culture supernatant of *P. gingivalis* grown in the complete medium (BHI-HK) were acetic acid (7.1 mM), butyric acid (13.2 mM), isobutyric acid (1.8 mM), isovaleric acid (4.1 mM), phenylacetic acid (1.6 mM), and propionic acid (2.3 mM). Growth of *T. denticola* in this bacterial culture filtrate did not significantly reduce the amount of any of the fatty acids. The final bacterial concentration of *T. denticola* obtained during this culture (4 days) was 8×10^8 bacteria per ml, as compared with 0.8×10^8 bacteria per ml at zero time (Table 2). On the other hand, the culture supernatant of *T. denticola* grown in NOS broth medium contained acetic acid (40 mM) and succinic acid (1.9 mM). When *P. gingivalis* was cultivated (4 days) in this bacterial culture filtrate, the succinic acid totally disappeared. The number of *P. gingivalis* cells was evaluated to be 4×10^8/ml (0.9×10^8 bacteria per ml at zero time) (Table 2), which is lower than the concentration obtained during the mixed culture with *T. denticola* (15×10^8/ml).

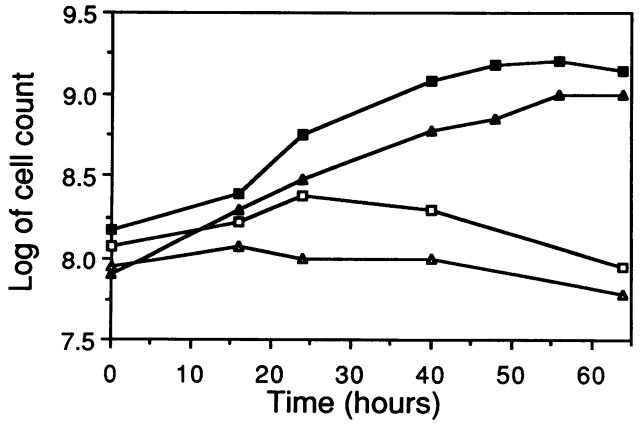

FIG. 1. Cell counts of *P. gingivalis* and *T. denticola* during mixed (■ and ▲, respectively) and individual (□ and △, respectively) cultures in BHI-K broth medium.

The various fatty acids detected in both culture filtrates were individually assayed for their ability to stimulate the growth of *P. gingivalis* and *T. denticola*. BHI-K containing fatty acids at concentrations ranging from 0.01 to 5 mM was inoculated with either *T. denticola* or hemin-depleted *P. gingivalis* cells. The enhancement of growth was determined by reading the A_{660} after 4 days of incubation. The succinic acid and the isobutyric acid were found to promote the growth of *P. gingivalis* and *T. denticola*, respectively (Tables 2 and 3). The minimal optimum concentration of isobutyric acid required to stimulate the growth of *T. denticola* was found to be about 0.2 mM, which is much less than the amount found in the culture filtrate of *P. gingivalis* grown in BHI-HK medium. Succinic acid at 5 mM was efficient in promoting the growth of *P. gingivalis*, whereas a concentration of less than 1 mM did not significantly enhance growth. No additive effect of growth stimulation of *P. gingivalis* or *T. denticola* was observed when isobutyric acid or succinic acid was used in combination with the other fatty acids produced by the bacteria.

In the present study, a mutual symbiotic enhancement of growth of *P. gingivalis* ATCC 33277 and *T. denticola* ATCC 35405 was noted. This phenomenon was found to be reproducible (triplicate experiments) and was also observed for *P. gingivalis* W83 and *T. denticola* D11 (data not shown). The growth-stimulating factors produced by *P. gingivalis* and *T. denticola* were identified to be isobutyric acid and succinic acid, respectively. The stimulatory effect of isobutyric acid on the growth of *T. denticola* has been previously reported

TABLE 2. Growth of *P. gingivalis* and *T. denticola* in bacterial culture filtrates

Bacteria[a]	Culture filtrate	Treatment	A_{660}	Total cell count[b] (10^8 cells/ml)	
				P. gingivalis	*T. denticola*
P. gingivalis	NOS (control)	None	0.045	0.8	
	NOS + 1.9 mM succinic acid	None	0.178	5.5	
	T. denticola	None	0.142	4	
		70°C for 30 min	0.110	3.5	
		100°C for 5 min	0.123	3.5	
		Dialysis (MWCO = 6,000–8,000)	0.020	0.7	
		Ultrafiltration (MWCO = 1,000)	0.010	0.6	
T. denticola	BHI-HK (control)	None	0.067		1.0
	BHI-HK + 1.8 mM isobutyric acid	None	0.32		8.0
	P. gingivalis	None	0.315		8.0
		70°C for 30 min	0.294		8.5
		100°C for 5 min	0.305		8.2
		Dialysis (MWCO = 6,000–8,000)	0.052		0.9
		Ultrafiltration (MWCO = 1,000)	0.048		0.9

[a] Four-day culture.
[b] Determined with a Petroff-Hausser counting chamber.

by Socransky et al. (22). In their study, a maximal stimulation was obtained in the presence of sodium isobutyrate at 2 μg/ml (0.023 mM). The exact role of isobutyric acid in the metabolism of *T. denticola* is not known. However, in the case of rumen bacteria, fatty acids have been demonstrated to provide a carbon skeleton for the biosynthesis of branched-chain amino acids and lipids (1, 2). Thiamine PP_i, which is produced by several bacterial species found in subgingival sites, has also been reported to stimulate the growth of oral spirochetes (4). However, incorporation of thiamine PP_i (5 μg/ml) into BHI-K medium did not stimulate the growth of *T. denticola* (data not shown). The growth factor produced by *T. denticola* is thought to be succinic acid. Hespell and Canale-Parola (9) already demonstrated the ability of *T. denticola* to produce succinic acid by fermentation of amino acids. The growth stimulation of *P. gingivalis* by succinic acid has been previously observed (7, 13, 18). This compound appears to be incorporated into the lipids and phospholipids of the *P. gingivalis* cell envelope (13). In the present study, the amount of succinic acid produced by *T. denticola* was found to be low. For this reason, it is suggested that the observed interbacterial coaggregation between *P. gingivalis* and *T. denticola* might favor growth enhancement of the microbial consortium. By this way, the succinic acid produced by *T. denticola* could be more easily accessible to *P. gingivalis* cells, instead of being diluted in the culture medium. A similar concept has been suggested for the coaggregation of *Bacterionema matruchotii* and *Streptococcus sanguis*, in which *B. matruchotii* could derive energy by metabolizing lactate produced by the streptococci (24). Studies in our laboratory have demonstrated and characterized a bimodal bacterial coaggregation reaction between *P. gingivalis* and *T. denticola* (6). Experiments devoted to the demonstration of the importance of bacterial coaggregation in the observed synergy are presently being carried out in our laboratory.

Various studies have shown that the development of some forms of periodontal disease is associated with significantly increased numbers of *P. gingivalis* and spirochetes (3, 14, 15). Loesche et al. (15) and Wolff et al. (25) have suggested that a succession *P. gingivalis* and the spirochetes may exist in subgingival plaque. More recently, Simonson et al. (21) presented data indicating that the occurrence of *T. denticola* in affected periodontal sites requires the presence of a detectable level of *P. gingivalis*. The present investigation yielded data that support and reinforce the above studies (15, 21, 25). The bacterial interaction between *P. gingivalis* and *T. denticola* may thus be of utmost importance in the initiation and progression of disease.

TABLE 3. Growth of *P. gingivalis* and *T. denticola* in fatty acid-containing BHI-K medium

Bacteria[a]	Fatty acid (final concentration [mM])	A_{660}	Total cell count[b] (10^8 cells/ml)	
			P. gingivalis	*T. denticola*
P. gingivalis	Succinic acid (5)	0.408	25	
	Succinic acid (2)	0.184	6.5	
	Succinic acid (1)	0.086	2.5	
	Succinic acid (0.5)	0.055	0.9	
	None	0.040	0.7	
T. denticola	Isobutyric acid (0.5)	0.307		12
	Isobutyric acid (0.2)	0.312		12
	Isobutyric acid (0.1)	0.243		9.4
	Isobutyric acid (0.01)	0.104		4.2
	None	0.086		0.7

[a] Four-day culture.
[b] Determined with a Petroff-Hausser counting chamber.

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