INFECTION AND IMMUNITY, Dec. 1995, p. 4584–4588 Vol. 63, No. 12
0019-9567/95/$04.00+0

Intergeneric Coaggregation of Oral *Treponema* spp. with *Fusobacterium* spp. and Intrageneric Coaggregation among *Fusobacterium* spp.

PAUL E. KOLENBRANDER,[1]* KENNETH D. PARRISH,[2] ROXANNA N. ANDERSEN,[1] AND E. PETER GREENBERG[2]

Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, Maryland,[1] *and Department of Microbiology, University of Iowa, Iowa City, Iowa*[2]

Received 20 June 1995/Returned for modification 10 August 1995/Accepted 12 September 1995

A total of 22 strains of *Treponema* spp. including members of all four named human oral species were tested for coaggregation with 7 strains of oral fusobacteria, 2 strains of nonoral fusobacteria, and 45 strains of other oral bacteria, which included actinobacilli, actinomyces, capnocytophagae, eubacteria, porphyromonads, prevotellae, selenomonads, streptococci, and veillonellae. None of the treponemes coaggregated with any of the latter 45 oral strains or with the two nonoral fusobacteria. All treponemes, eight *Treponema denticola* strains, eight *T. socranskii* strains, four oral pectinolytic treponemes, one *T. pectinovorum* strain, and one *T. vincentii* strain coaggregated with at least one strain of the fusobacteria tested as partners. The partners consisted of one strain of *Fusobacterium periodonticum*, five *F. nucleatum* strains including all four subspecies of *F. nucleatum*, and a strain of *F. simiae* obtained from the dental plaque of a monkey. In the more than 100 coaggregations observed, the fusobacterial partner was heat inactivated (85°C for 30 min), while the treponemes were unaffected by the heat treatment. Furthermore, the fusobacteria were usually inactivated by proteinase K treatment, and the treponemes were not affected. Only the *T. denticola* coaggregations were inhibited by lactose and D-galactosamine. None were inhibited by any of 23 other different sugars or L-arginine. Intrageneric coaggregations were seen among the subspecies of *F. nucleatum* and with *F. periodonticum*, and none were inhibited by any of the sugars tested or by L-arginine. No intrageneric coaggregations were observed among the treponemes. These data indicate that the human oral treponemes show a specificity for oral fusobacteria as coaggregation partners. Such cell-to-cell contact may facilitate efficient metabolic communication and enhance the proliferation of each cell in the progressively more severe stages of periodontal disease.

Fusobacteria are found in significant numbers in dental plaque from healthy sites and in increased numbers in samples from periodontally diseased sites (20). Treponemes become a significant proportion of the population only in periodontally affected sites (11). This later appearance of the treponemes may be due to a requirement for growth stimulation by metabolic and enzymatic activities of other periodontal microorganisms (30). For example, *Treponema denticola* Ny375 is unable to grow in human serum, but in the presence of *Fusobacterium nucleatum* Ny373 and three other species of oral bacteria, it shows vigorous growth to a density of 3×10^9 cells per ml (30). An oligopeptidase unable to hydrolyze complete proteins has been found in *T. denticola* ATCC 35405 (19), and peptidases active on 19 tested short (3 to 6 amino acids) peptides (27) as well as transport of at least one dipeptide, L-cysteinylglycine, have been found in *F. nucleatum* (2). Other oral bacteria like porphyromonads (18) may provide the proteases that degrade serum proteins and immunoglobulins to peptides for the fusobacteria and treponemes (12). By such metabolic interactions, it is presumed that the members of microbial consortia communicate.

In the oral cavity, it is not sufficient to communicate metabolically. Because they are in a flowing environment, the nonmotile oral bacteria must adhere to an available surface (17). Collectively, various strains of *F. nucleatum* coaggregate with all species of oral bacteria so far tested (15). Only cell-to-cell interactions between the motile treponemes and *Porphyromonas gingivalis* (10), *Bacteroides forsythus*, or *Streptococcus crista* (34) and between treponemes and host cells have been reported (7, 24). Coaggregations between the fusobacteria and treponemes have not been reported; this study was designed to examine the possible coaggregations between these periodontally important bacteria. Direct cell-to-cell interaction is presumed to enhance potential communication between cells and may be especially effective in mediating metabolic communication.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used were of human oral origin except where noted and were grown as described previously (15). Selenomonads were grown in complex broth medium consisting of brain heart infusion broth supplemented with yeast extract, vitamin K_1, cysteine, and hemin. Porphyromonads were grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.). All other nontreponeme strains were grown in modified Schaedler medium with glucose or lactate as the source of energy. Cells were grown at 37°C under anaerobic conditions with a GasPak system (BBL Microbiology Systems), harvested in the late exponential or early stationary phase of growth, washed three times, and suspended in coaggregation buffer (5).

The treponemes were grown in GM-1 Pectin medium (33) in a GasPak Plus system (BBL Microbiology Systems) at 37°C to late exponential phase and then harvested, washed, and suspended in coaggregation buffer as described above. The 22 treponemes tested were *T. denticola* TD-4 (ATCC 35404), GM-1, N16B1, N17A1, ATCC 33520, ATCC 33521, ATCC 35405, and MS25, *Treponema pectinovorum* ATCC 33768, pectinolytic oral treponemes P2, P3, P5, and P8, *Treponema socranskii* subsp. *buccale* ATCC 35534, N5B, and N3A2A, *T. socranskii* subsp. *paredis* ATCC 35535, *T. socranskii* subsp. *socranskii* ATCC 35536 and N3B1A, *T. socranskii* subsp. SSD N18B and N7A, and *Treponema vincentii* ATCC 35580. The pectinolytic oral treponemes were from E. Canale-Parola; strains TD-4, GM-1, and MS25 were from S. C. Holt; the strains with ATCC

* Corresponding author. Mailing address: National Institutes of Health, Building 30, Room 310, 30 Convent Dr. MSC 4350, Bethesda, MD 20892-4350. Phone: (301) 496-1497. Fax: (301) 402-0396. Electronic mail address: kolenbrander@yoda.nidr.nih.gov.

TABLE 1. Coaggregation of *Fusobacterium* spp. with *Treponema* spp.

Treponema spp. strain	Coaggregation score with *Fusobacterium* spp. strain[a]						
	PK1594	PK1909	ATCC 10953	ATCC 33693	ATCC 49256	NCTC 11326	ATCC 33568
T. denticola GM-1	**3**	0	1	2	2	3	ND
T. denticola N16B1	**2**	0	0	0	0	0	ND
T. denticola N17A1	**2**	0	0	0	0	0	ND
T. denticola ATCC 35404 (TD-4)	**2**	0	0	0	3	3	ND
T. denticola ATCC 33520	4	0	3	**2**	3	3	ND
T. denticola ATCC 33521	**2**	0	4	0	2	3	ND
T. denticola MS25	**3**	3	2	3	3	4	ND
T. denticola ATCC 35405	**2**	0	0	0	1	3	ND
T. pectinovorum ATCC 33768	4	4	4	3	4	4	4
Pectinolytic oral treponeme P2	4	3	4	3	3	4	4
Pectinolytic oral treponeme P3	4	4	4	3	4	4	ND
Pectinolytic oral treponeme P5	3	3	4	3	4	4	ND
Pectinolytic oral treponeme P8	4	4	4	4	3	3	4
T. socranskii subsp. *buccale* ATCC 35534	4	1	4	2	4	4	3
T. socranskii subsp. *buccale* N5B	4	2	3	3	3	4	4
T. socranskii subsp. *buccale* N3A2A	4	0	4	1	3	4	3
T. socranskii subsp. *paredis* ATCC 35535	4	3	4	2	3	4	4
T. socranskii subsp. *socranskii* ATCC 35536	1	0	ND	ND	ND	ND	ND
T. socranskii subsp. *socranskii* N3B1A	4	2	4	3	4	4	4
T. socranskii subsp. SSD N18B	4	3	4	3	3	4	4
T. socranskii subsp. SSD N7A	4	1	4	2	3	4	3
T. vincentii ATCC 35580	3	1	0	0	1	2	ND

[a] PK1594 = *F. nucleatum* subsp. *nucleatum* PK1594; PK1909 = *F. nucleatum* subsp. *polymorphum* PK1909; ATCC 10953 = *F. nucleatum* subsp. *polymorphum* ATCC 10953; ATCC 33693 = *F. periodonticum* ATCC 33693; ATCC 49256 = *F. nucleatum* subsp. *vincentii* ATCC 49256; NCTC 11326 = *F. nucleatum* subsp. *fusiforme* NCTC 11326; ATCC 33568 = *F. simiae* ATCC 33568. Scores in boldface indicate reversibility by addition of 60 mM galactosamine or 200 mM lactose to a coaggregating pair. ND, not done.

numbers were from the American Type Culture Collection, Rockville, Md.; all other treponemes were from L. V. H. Moore.

The 54 strains tested for potential partners of the treponemes included 9 fusobacteria and 45 other strains representing 11 genera. They were *Actinobacillus actinomycetemcomitans* Y4 and N27, *Actinomyces israelii* PK14, *Actinomyces naeslundii* PK2, T14V, ATCC 12104, PK25, PK29, PK947, PK606, and PK984, *Actinomyces* serovar WVA963 strain PK1259, *Actinomyces odontolyticus* PK48, *Capnocytophaga gingivalis* ATCC 33624, *Capnocytophaga ochracea* ATCC 33596, *Capnocytophaga sputigena* ATCC 33612, *Enterococcus faecalis* EBH-1, *Eubacterium nodatum* PK1934 and PK1937, *Fusobacterium mortiferum* ATCC 25557 (from human maxillary abscess), *F. nucleatum* subsp. *fusiforme* NCTC 11326, *F. nucleatum* subsp. *nucleatum* PK1594, *F. nucleatum* subsp. *polymorphum* PK1909 and ATCC 10953, *F. nucleatum* subsp. *vincentii* ATCC 49256, *Fusobacterium periodonticum* ATCC 33693, *Fusobacterium simiae* ATCC 33568 (from *Macaca arctoides* periodontium), *Fusobacterium ulcerans* ATCC 49185 (from human skin ulcer), *P. gingivalis* PK1924 and W50, *Prevotella denticola* PK1277, *Prevotella intermedia* PK1511, *Prevotella loescheii* PK1295, *Rothia dentocariosa* PK44, *Selenomonas flueggei* PK1958, *Selenomonas infelix* PK1956, *Streptococcus constellatus* PK2819, *Streptococcus cricetus* AHT, *Streptococcus gordonii* DL1, PK488, and Blackburn, *Streptococcus intermedius* PK2821, *Streptococcus mutans* ATCC 33534 and NCTC 10449, *Streptococcus oralis* ATCC 55229, 34, and J22, *Streptococcus parasanguis* FW213, *Streptococcus rattus* BHT, *Streptococcus sanguis* 12, *Streptococcus sobrinus* ATCC 27351, *Streptococcus* SM PK509, *Veillonella atypica* PK1910, and *Veillonella dispar* PK1950.

Coaggregation assay. The visual assay has been described in detail previously (14, 16). Briefly, suspensions with a final cell density of about 10^9 cells per ml (Klett unit value of 260 with a 660-nm [red] filter in a Klett-Summerson colorimeter; Klett Manufacturing Co., Inc., New York, N.Y.) were prepared for each cell type. Equal volumes (0.1 ml) of each cell type were added to a glass test tube (10 by 75 mm) and vortex mixed for 5 s. A coaggregation score ranging from 0 (no change in turbidity and no visible coaggregates) to +4 (maximum coaggregation; i.e., large coaggregates settled immediately, leaving a water-clear supernatant) was given for each pair. A score of +3 indicated the formation of large settling coaggregates but a slightly turbid supernatant; a +2 score was given when definite coaggregates were visible but did not settle immediately. The weakest score (+1) indicated finely dispersed coaggregates in a turbid background.

Reversal of coaggregation was determined by the addition of lactose to a final concentration of 200 mM, all other sugars to a final concentration of 60 mM, or L-arginine at 200 mM and rescoring the coaggregating pair. The sugars tested were lactose, L-mannose, D-mannose, L-rhamnose, sucrose, D-fructose, D-glucose, 2-deoxy-D-glucose, L-fucose, D-fucose, D-galacturonic acid, stachyose, D-xylose, methyl-α-D-galactoside, methyl-β-D-galactoside, methyl β-D-mannoside, 6-*O*-methyl-D-galactose, D-galactosamine, D-galactose, D-glucose-6-phosphate, D-mannoheptulose, D-melezitose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine. The effect of temperature was determined by heating a cell suspension at 85°C for 30 min before mixing it with heated or unheated cells of the partner. Protease treatment of cells was done by incubation of cell suspensions (10^9 cells per ml) in coaggregation buffer with 0.45 mg of protease K (catalog no. P-0390; Sigma Chemical Co., St. Louis, Mo.) per ml, 0.45 mg of trypsin (catalog no. T-8253; Sigma) per ml, or 0.5 mg of pronase (Calbiochem, San Diego, Calif.) per ml at 50°C for 60 min. The cells were washed three times with coaggregation buffer and suspended to the original volume with coaggregation buffer.

RESULTS

Survey of coaggregation partners of treponemes. The 22 strains of treponemes were mixed pairwise with 52 strains of oral bacteria to test their potential coaggregation partnership. Two nonoral fusobacteria, *F. mortiferum* ATCC 25557 and *F. ulcerans* ATCC 49185, were also tested. Only the seven oral fusobacteria were partners of the treponemes, and all treponemes exhibited coaggregation (Table 1). Each of the coaggregating pairs was tested for sensitivity to addition of various sugars and L-arginine, which have been reported to inhibit coaggregations between fusobacteria and other oral bacteria (10, 13, 16, 29). None of the interactions was reversed by addition of L-arginine, and only the coaggregations with strains of *T. denticola* were completely reversed by addition of 60 mM galactosamine or 200 mM lactose (Table 1). The coaggregations between the strains of *T. denticola* and *F. nucleatum* subsp. *nucleatum* PK1594 exhibited all but one of the observed lactose-reversible coaggregations (Table 1). The fusobacterial isolate from monkey plaque also coaggregated with several of the treponemes (Table 1).

Effect of heat treatment on coaggregation. Each of 10 treponemes, *T. pectinovorum* ATCC 33768, pectinolytic oral treponemes P2 and P8, *T. socranskii* subsp. *buccale* ATCC 35534, N5B, and N3A2A, *T. socranskii* subsp. *paredis* ATCC 35535, *T. socranskii* subsp. *socranskii* N3B1A, and *T. socranskii* subsp.

TABLE 2. Effect of protease treatment on coaggregation of *Fusobacterium* spp. with *Treponema* spp.

Treponema spp. strain	Coaggregation score with *Fusobacterium* spp. strains[a]												
	Untreated							Proteinase K treated					
	PK1594	PK1909	ATCC 10953	ATCC 33693	ATCC 49256	NCTC 11326	ATCC 33568	PK1594	PK1909	ATCC 10953	ATCC 33693	ATCC 49256	NCTC 11326
Proteinase K treated													
T. pectinovorum ATCC 33768	4/3	4/3	4/3	3/3	4/3	4/4	4/3						
Pectinolytic oral treponeme P2	4/4	3/3	4/4	3/3	3/3	4/4	4/4						
Pectinolytic oral treponeme P8	4/3	4/0	4/0	4/3	3/0	ND	4/0						
Untreated													
T. socranskii subsp. *buccale* N3A2A								4/0	0	4/3	1/0	3/3	4/0
Pectinolytic oral treponeme P2								4/0	3/0	4/2	3/0	3/3	4/0
T. pectinovorum ATCC 33768								4/0	4/0	4/3	3/0	4/3	4/0
T. socranskii subsp. *buccale* N5B								4/0	2/0	3/2	3/0	3/2	4/0
T. socranskii subsp. SSD N18B								4/0	3/0	4/2	3/0	3/3	4/0
T. socranskii subsp. *paredis* ATCC 35535								4/0	3/0	4/2	2/0	3/3	4/0
T. socranskii subsp. *socranskii* N3B1A								4/0	2/0	4/2	3/0	4/3	4/0
T. socranskii subsp. *buccale* ATCC 35534								4/0	1/0	4/2	2/0	4/3	4/0

[a] PK1594 = *F. nucleatum* subsp. *nucleatum* PK1594; PK1909 = *F. nucleatum* subsp. *polymorphum* PK1909; ATCC 10953 = *F. nucleatum* subsp. *polymorphum* ATCC 10953; ATCC 33693 = *F. periodonticum* ATCC 33693; ATCC 49256 = *F. nucleatum* subsp. *vincentii* ATCC 49256; NCTC 11326 = *F. nucleatum* subsp. *fusiforme* NCTC 11326; ATCC 33568 = *F. simiae* ATCC 33568. The score on the left side of the slash mark is the control value obtained when untreated cells were tested against untreated cells; the score on the right of the slash mark is obtained when treated cells were tested against untreated cells. ND, not done.

SSD N18B and N7A, was tested with each of six fusobacteria, *F. nucleatum* subsp. *nucleatum* PK1594, *F. nucleatum* subsp. *polymorphum* PK1909 and ATCC 10953, *F. nucleatum* subsp. *vincentii* ATCC 49256, *F. periodonticum* ATCC 33693, and *F. simiae* ATCC 33568 for the effect of prior treatment at 85°C for 30 min on the ability to coaggregate. When treponemes were heated, there was no change in the coaggregation, but heating the fusobacteria abolished coaggregation in every partnership (data not shown).

Effect of protease treatments on coaggregation. Three treponemes were treated with proteinase K and tested for the ability to coaggregate with each of seven fusobacteria (Table 2). Only pectinolytic strain P8 was inactivated and unable to coaggregate with four of the fusobacteria. Identical results were obtained when pectinolytic strain P8 was treated with pronase (data not shown).

Proteinase K treatment of fusobacteria usually abolished their ability to coaggregate with the treponemes (Table 2). *F. nucleatum* subsp. *vincentii* ATCC 49256 was resistant to proteinase K, and *F. nucleatum* subsp. *polymorphum* ATCC 10953 was partially resistant. The other four fusobacteria tested were completely inactivated by proteinase K treatment. Both pronase and trypsin treatments were done with *F. nucleatum* subsp. *nucleatum* PK1594; pronase partially inhibited and trypsin had no effect on the fusobacterium's ability to coaggregate with the treponemes (data not shown). Pronase treatment of two other fusobacteria, strains PK1909 and ATCC 33693, gave results identical to those for proteinase K treatment (data not shown).

Intrageneric coaggregations. All 22 treponemes were tested pairwise with each other, and none coaggregated with any other treponemes (data not shown). However, when the type strains for each of the four subspecies of *Fusobacterium* as well as *F. nucleatum* subsp. *nucleatum* PK1594 and *F. periodonticum* ATCC 33693 were paired with each other, several intrageneric coaggregations were observed (Table 3). None was reversed by addition of lactose, galactosamine, or L-arginine. All four coaggregations involving *F. nucleatum* subsp. *nucleatum* PK1594 (Table 3) are unimodal coaggregations, with strain PK1594 as the heat-sensitive partner. The two coaggregations between *F. nucleatum* subsp. *polymorphum* ATCC 10953 and either *F. periodonticum* ATCC 33693 or *F. nucleatum* subsp. *vincentii* ATCC 49256 are bimodal in that coaggregation is prevented only if both partners are heated. None of the coaggregations involving heated cells was reversed by the addition of 200 mM lactose.

DISCUSSION

Extensive coaggregations between oral treponemes and fusobacteria but not with more than 40 other oral bacterial strains indicates the high specificity of this unusual coaggregation involving a motile bacterium. The fact that motile treponemes adhere to nonmotile bacteria suggests that cell-to-cell

TABLE 3. Intrageneric coaggregation among species and subspecies of the genus *Fusobacterium*[a]

	PK1594	PK1909	ATCC 10953	ATCC 33693	ATCC 49256	NCTC 11326
PK1594		0	3	3	3	3
	PK1909		0	1	0	0
		ATCC 10953		4	3	0
			ATCC 33693		0	0
				ATCC 49256		0
					NCTC 11326	

[a] PK1594 = *F. nucleatum* subsp. *nucleatum* PK1594; PK1909 = *F. nucleatum* subsp. *polymorphum* PK1909; ATCC 10953 = *F. nucleatum* subsp. *polymorphum* ATCC 10953; ATCC 33693 = *F. periodonticum* ATCC 33693; ATCC 49256 = *F. nucleatum* subsp. *vincentii* ATCC 49256; NCTC 11326 = *F. nucleatum* subsp. *fusiforme* NCTC 11326.

interactions within the oral community may be important for additional reasons than attachment.

Metabolic communication among the population is likely to be advantageous to the residents of the community and may be most effective when cells are directly attached to one another. The periodontal region in the oral cavity, where the treponemes and fusobacteria reside, is bathed in crevicular fluid, which is of serous origin. When tested for growth in human serum, *T. denticola* could not grow unless other bacteria like *F. nucleatum* were added (30). Other oral bacteria express proteolytic enzymes (3, 4). The cell surface location of an oligopeptidase of *T. denticola* (19) may be a reason for the cell to attach to a ready source of peptides produced by other members of the oral microbial community. In this way, motility may position the treponeme in the microbial consortium so that adherence to an active source of nutrient can occur.

The idea that a motile bacterium can use direct cell-to-cell interaction with partner cells to its advantage encompasses two factors: coordinate presence of treponemes and fusobacteria, and ability to coaggregate. Both increase in numbers concomitant with increased severity of stages of periodontal disease (11, 20). Late-colonizing bacteria coaggregate primarily with fusobacteria and very infrequently with any of the early colonizers such as streptococci and actinomyces (17). As an extension of our earlier proposal that the fusobacteria as a group coaggregate with all varieties of oral bacteria (17), we can add the oral treponemes to the list of fusobacterial partners. However, the two nonoral fusobacteria failed to coaggregate with any of the treponemes, indicating additional specificity by the treponemes for oral fusobacteria as coaggregation partners. It should be noted that these two nonoral fusobacteria are the first fusobacteria we have examined that have failed to coaggregate (15).

T. denticola has been reported to coaggregate with *Porphyromonas gingivalis* (10), another late-colonizing bacterium, although we did not detect such a coaggregation in our survey of potential partners. Also, L-arginine had been noted as an inhibitor of the treponeme-porphyromonad coaggregation (10) and of several coaggregations between a fusobacterium and oral streptococci (29). We did not observe any effect of this amino acid on any of the treponeme-fusobacterium coaggregations. We did observe inhibition by lactose, which supports the observation that fusobacteria express a galactose-binding lectin (21) and are blocked from attachment to various host cells by galactose (8, 28, 31). We previously reported a galactose-sensitive lectin activity on *F. nucleatum* PK1594 that mediates coaggregation with many oral gram-negative bacteria (13, 15). Accordingly, in the present study, it appears that the heat- and protease-inactivated potential lectin is expressed by the fusobacterium and that its complementary receptor is on the treponeme.

The intrageneric coaggregations among the subspecies of *F. nucleatum* were unexpected, since we had tested 28 strains of *F. nucleatum* in an earlier survey and found that none coaggregated with the other (15). At the time of the earlier survey, all of the fusobacteria were classified as *F. nucleatum* without a subspecies epithet. Most may have been from the same subspecies, which may be less likely to coaggregate. Only two strains, PK1594 and PK1909, from the earlier study were included in the current study, and these strains do not coaggregate (Table 3). The strains of *F. nucleatum* are a heterogeneous group (22), and they have been reclassified as several subspecies (6). Clearly, from the results presented here, coaggregations are prevalent among the various subspecies, including *F. periodonticum* (Table 3). Only oral streptococci and a few oral actinomyces have been observed previously to exhibit intrageneric coaggregation (15).

The coaggregations of treponemes and fusobacteria add to the growing list of human oral bacteria that interact by cell-to-cell contact. Additional reports of coaggregations involving uropathogenic bacteria (23), bacteria from the chicken gastrointestinal tract (32), and morphological observations of cell-to-cell associations in the oral cavities of cats and dogs (25, 26), termite gut (1), as well as alpine streams (9) indicate that coaggregations occur in a variety of econiches. Every genus of human oral bacteria that has been examined for the ability to coaggregate has members that coaggregate with strains from other genera. Microscopic observation of dental plaque readily demonstrates cell-to-cell interactions, but in vivo experiments to test the potential role of coaggregation in colonization must still be done. On the basis of the fact that fusobacteria are coaggregation partners of all oral bacteria, it is hypothesized that fusobacteria play a key role in the establishment of oral microbial communities and subsequent developmental stages of severity of periodontal disease.

ACKNOWLEDGMENTS

We thank E. Canale-Parola, S. C. Holt, and L. V. H. Moore for supplying many of the human oral treponemes, and we thank A. Tanner for the type strains of *F. nucleatum* and *F. periodonticum*. We thank J. London and S. Robrish for advice on writing the manuscript.

This work was supported by grant R01-DE10730 from the National Institutes of Health. K.D.P. was a dental scientist trainee supported by USPHS training grant DE00175 from the National Institute for Dental Research.

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Editor: J. R. McGhee