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Bacteriology of Severe Periodontitis in Young Adult Humans

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A total of 78 bacteriological samples were taken from the supragingival tooth surface after superficial cleaning with toothpicks or from the periodontal sulci of 42 affected sites in 21 adolescents or young adults with severe generalized periodontitis. Of 190 bacterial species, subspecies, or serotypes detected among 2,723 isolates, 11 species exceeded 1% of the subgingival flora and were most closely associated with the diseased sulci. Eleven others were also sufficiently frequent to be suspect agents of tissue destruction. Many of these species are known pathogens of other body sites. In addition, 10 species of *Treponema* were isolated. One of these and the "large treponeme" were also more closely associated with severe periodontitis than they were with healthy sites or gingivitis. There were highly significant differences between the composition of the flora of the affected sulci and the flora of (i) the adjacent supragingival tooth surface, (ii) the gingival crevice of periodontally healthy people, and (iii) sites with a gingival index score of 0 or 2 in experimental gingivitis studies. The floras of different individuals were also significantly different. There was no statistically detectable effect of sampling per se upon the composition of the flora of subsequent samples from the same sites. The composition of the supragingival flora of the patients with severe generalized periodontitis that had serum antibody to *Actinobacillus actinomycetemcomitans* was significantly different from the supragingival flora of patients without this serum antibody. However, there was no statistically significant difference in the composition of their subgingival floras.

The bacterial species that are specific causative agents of periodontitis in humans have not been determined. In an effort to define the flora associated with active disease, we examined people under 30 years of age with severe generalized periodontitis (SP). Because these people have experienced more severe destruction than generally occurs in the population at large, and because the destruction has occurred at a young age, it is reasonable to presume that the rate of disease progression is relatively accelerated or that periods of disease exacerbation or activity occur more frequently. This more rapid progression, as compared with the usually rather slow progression of periodontitis, should relate either to the composition of the resident flora or to differences in the response of the host to the bacteria.

We know of no studies providing comprehensive descriptions of the bacterial flora in populations analogous to the SP population, although Tanner et al. (17) studied samples from two subjects described as young adults with widespread destructive lesions involving most of the dentition. They reported that the dominant organisms recovered from these subjects were *Bacteroides gingivalis* (formerly included in *Bacteroides asaccharolyticus*), *Actinobacillus actinomycetemcomitans*, and a group of unidentified anaerobic gram-negative saccharolytic rods. These results differed from results with samples from other clinical manifestations. In a study of advanced periodontal lesions in an older age group, Slots (15) found a predominance of gram-negative anaerobic rods in the flora. "*Bacteroides melaninogenicus*" and *Fusobacterium nucleatum* constituted the majority of these isolates. Neither study reported on cultivable spirochetes.

Previously reported immunological studies of the SP population have shown that this population exhibits hyperresponsiveness to stimulation of peripheral blood leukocytes by a B-cell mitogen (16) and depressed uptake of [^{3}H]thymidine in 7-day unstimulated peripheral blood leukocyte cultures as compared with a group the same age without periodontitis (18). Serum from approximately 40% of the SP population contains antibody to *Actinobacillus actinomycetemcomitans* as determined by precipitin-in-gel reactions

with sonicated cells (13). The SP population also includes some individuals with apparently depressed chemotaxis of polymorphonuclear leukocytes (11). Serum antibody to *Actinobacillus actinomycetemcomitans* and depressed chemotaxis also have been associated with localized juvenile periodontitis (JP) (2). Clinical populations similar to our SP population have been described as "generalized JP" by Genco et al. (2) and as "rapidly progressive periodontitis" by Lavine et al. (6).

The present study was designed to provide a comprehensive description of the flora associated with the SP lesions by indentification (to species or subspecies level) of all isolates that were randomly selected to represent the flora.

MATERIALS AND METHODS

Subjects. The subjects met the following criteria: (i) 30 years of age or younger (range, 12 to 30 years; mean, 24 years); (ii) at least 5 mm loss of attachment with 6 mm or more probeable depth on any surface of eight or more teeth, at least three of which were not first molars or incisors; and (iii) negative history for diabetes mellitus or any chronic or serious medical illness, current pregnancy, acute necrotizing ulcerative gingivitis, and previous periodontal therapy other than scaling.

In addition, subjects had received no antibiotics for 6 weeks before bacterial samples were taken. Clinical measurements were obtained as described previously (12).

Sample sites. Seventy-eight samples were taken from 34 affected sites in 21 subjects. Twenty-seven residual supragingival samples were taken after superficial cleaning of the gingival margin with sterile toothpicks and before the corresponding sulcus samples were taken. The residual supragingival samples were taken to provide an indicator of possible contamination of the subsequent gingival (sulcus) samples as the scalers were passed into the sulci. Repeat supra- and corresponding subgingival samples were taken from eight of the same sites in seven people after intervals of 1 to 6 months, during which time no periodontal treatment was performed and personal dental hygiene was not changed. An additional seven samples were taken from affected sulci after the supragingival area had been cleaned, but the corresponding supragingival samples were not taken. One sample was taken from an undisturbed supragingival area before superficial cleaning and resampling. For comparative purposes, subgingival samples were taken from nine sites in eight individuals who were unaffected by periodontitis and had healthy gingiva.

Sampling and isolation procedures. The residual supragingival samples and the subgingival sulcus samples were taken, as described in detail previously (10), with sterile nickel-plated Morse 00 scalers. The scaler tips were immediately transferred to oxygen-free tubes of sterile diluent. The samples were dispersed by being blended in a Vortex mixer with fine glass beads, and serial dilutions were cultured both in roll tubes of prereduced anaerobically sterilized medium and on the same medium in petri plates that had been stored in anaerobe jars, inoculated in air, and incubated in anaerobe jars. Cultures in both anaerobic tubes and jars were incubated for 5 days in an atmosphere containing 3% H_2, 10% CO_2, and 87% N_2. Separate tubes of selective medium and plates were inoculated for isolation of spirochetes and mycoplasma (10).

Media. As described previously (10), the prereduced anaerobically sterilized medium for non-spirochetes contained hemin, vitamin K_1, cysteine-hydrochloride, resazurin, fresh yeast autolysate, thiamine pyrophosphate, yeast extract, ammonium formate, brain heart infusion base (BBL Microbiology Systems), and sterile rabbit serum (for roll tubes) or rabbit blood (for plates).

The treponema isolation medium (10) contained peptone (Difco Laboratories), heart infusion broth base (BBL), yeast extract (Difco), ribose, pectin, glucose, fructose, starch, sucrose, maltose, sodium pyruvate, K_2HPO_4, NaCl, $(NH_4)_2SO_4$, cysteine-hydrochloride, hemin, vitamin K_1, resazurin, clarified rumen fluid, serum, fresh yeast autolysate, and thiamine pyrophosphate. The selective medium for spirochetes had a similar composition and also contained rifampin and polymyxin.

Colony selection. For non-spirochetes, 30 colonies were selected randomly. From most samples, 15 colonies were picked from plates and 15 from roll tubes. However, from many samples all 30 colonies were obtained from tubes, or occasionally, from plates. Approximately 60 colonies were picked from nine of the samples and 90 colonies were picked from two sulcus samples. The treponemal isolates were taken from the highest dilution cultured that produced growth after incubation for 5 to 21 days.

Identification. All isolates were characterized and identified as described previously (4) by standard morphological and cultural tests, gas chromatographic analyses of metabolic products, and electrophoretic analyses of the soluble cellular proteins without sodium dodecyl sulfate (9). Serotypes of *Actinomyces* species were determined microscopically with fluorescent antibody conjugate. Analytical results were compared with those of the type strain of described species and with the reference strain of all other species.

Statistical analyses. The coverage analysis H_9 of Good (3) was used to estimate the complexity of the floras and the adequacy of the sample sizes. It is based on the formula $1 - (n_1/N) \times 100$, where n_1 is the number of species seen once and N is the total number of isolates. This gives an estimate of the percent of the viable cells in the sample or population that are members of all of the species detected. The standard deviation is determined by $0.9\ (n_1)/N$ (3).

The minimum percent similarity analysis (S. S. Socransky, A. C. R. Tanner, and J. M. Goodson, J. Dent. Res. Spec. Issue A **60**:486, abstr. 705, 1981) was used to compare the compositions of the samples. In this test the sum of the minimum percentages (of the flora in the sample) of each species that occurred in both samples is the measure of similarity of the sample compositions (e.g., if species X is 6% of the flora of sample A and 2% of the flora in sample B, they share 2% similarity for this species, which is added to similar percentages of the other species that they share).

Statistical evaluation of the similarity values among subsets of samples (e.g., supragingival samples versus subgingival samples) was made by the L simulation analysis of Good (I. J. Good, J. Stat. Comput. Sim., in

TABLE 1. Taxa (as percent of flora) that were more numerous in the residual supragingival (RS) flora than in the subgingival (S) flora

Taxa[a]	RS	S	Taxa[a]	RS	S
Actinomyces WVa-963	1.89	0.18	*Leptotrichia buccalis*	0.26	0.18
A. israelii (−)[b]	1.12	0.53	*Leptotrichia* D-16	0.34	0.12
A. israelii I	1.54	0.82	*Leptotrichia* D-35	0.26	0.06
A. israelii II	1.20	0.47	*Propionibacterium acnes*	2.40	1.29
A. israelii X	0.17	0.06	*Peptostreptococcus anaerobius*	1.63	0.70
A. naeslundii (−)	2.49	1.40			
A. naeslundii I	3.26	0.41	*Selenomonas* D-1	0.60	0.12
A. naeslundii III	3.18	1.76	*Selenomonas* D-2	0.60	0.12
A. "naeslundii-viscosus"[c]	4.03	0.58	*Selenomonas* D-3	0.43	—
A. odontolyticus (−)	0.60	0.23	*Selenomonas* D-4	1.03	—
A. odontolyticus I	0.86	0.18	*Selenomonas* D-11	0.52	0.06
A. viscosus II	2.40	1.52	*Staphylococcus haemolyticus*	0.26	0.12
Actinomyces D-8	0.26	0.12	*S. aureus*	0.09	0.06
			S. hominis	0.09	0.06
Bacteroides disiens	0.09	0.06			
B. gingivalis	0.43	0.23	*Streptococcus constellatus*	0.77	0.64
B. gracilis	1.89	1.17	*S. intermedius* III	0.52	0.12
B. loescheii	0.43	0.29	*S. mitis*	0.86	0.53
B. melaninogenicus	0.26	0.06	*S. mutans*	0.34	0.23
B. oris	1.54	1.23	*S. sanguis* I	1.89	0.18
B. denticola	2.75	1.46	*S. sanguis* II	3.43	0.29
Bacteroides D-19	0.09	0.06	*Streptococcus* D-6	0.17	0.12
			Streptococcus D-7	0.60	0.12
Capnocytophaga ochracea	4.21	1.17	*Streptococcus* D-39	4.64	1.76
C. sputigena	0.34	0.12	*Streptococcus* SA	0.09	0.06
Coccus D-29	0.09	0.06	*Streptococcus* SM	0.60	0.12
Coccus SMI	0.43	0.18			
Eubacterium saburreum	0.69	0.06	*Veillonella atypica*	0.60	0.06
Fusobacterium naviforme	0.52	0.06	*V. dispar*	0.26	0.12
Fusobacterium D-10	0.26	0.12	*V. parvula*	5.84	1.52

[a] *Acinetobacter calcoaceticus, Actinomyces odontolyticus* (serologic cross), *Bacterionema matruchotii, Bacteroides bivius, Bacteroides buccalis, Bacteroides putredinis, Bacteroides* D-34, *Bacteroides* D-36, *Bifidobacterium* D-2, Coccus D-19, *Corynebacterium* D-2, *Eubacterium* D-21, Facultative gram-negative Coccus D-2, Facultative gram-negative Rod D-7 and D-22, *Fusobacterium* D-1, *Fusobacterium* D-3, *Fusobacterium* D-4, *Lactobacillus fermentum, Propionibacterium* D-1, *Selenomonas* D-15, *Streptococcus* D-2, *Streptococcus* D-10, and *Streptococcus* D-24 were found only in the supragingival samples, each at 0.09% of the flora.

Actinomyces WVa-963 (−), *Bacillus circulans, Eikenella corrodens,* Facultative gram-negative Rod D-14, Facultative gram-positive Coccus D-41, *Haemophilus segnis, Lactobacillus* D-9, *Streptococcus* D-11, and *Streptococcus* D-17 were found only in the supragingival samples, each at 0.17% of the flora.

Actinomyces naeslundii II, *Bacteroides* EO, Facultative gram-positive Coccus D-25, *Haemophilus paraphrophilus* were found only in the supragingival samples, each at 0.26% of the flora.

Arachnia propionica and *Streptococcus* D-16 were found only in the supragingival samples, each at 0.52% of the flora, and *Actinomyces viscosus* (−) and *Capnocytophaga gingivalis* at 0.77% of the supragingival flora.

[b] (−), no reaction with available antisera.

[c] Reacted equally with antisera for both species.

press; I. J. Good, J. Stat. Comput. Sim., in press) (10). In this test the likelihood of obtaining the observed clusters of similar samples (subsets) by chance alone is determined by obtaining the ratio (mean similarity of all between-clusters [subsets] comparisons)/(mean similarity of all within-subsets comparisons). The resulting ratio (L) is redetermined 1,000 times after redistributing the samples at random into subsets of

TABLE 2. Species that were more numerous in the subgingival (S) flora than in the residual supragingival (RS) flora

Taxa[b]	% of flora[a]		% of S samples positive	Taxa[b]	% of flora[a]		% of S samples positive
	RS	S			RS	S	
Actinomyces				*Fusobacterium*			
A. meyeri	0.26	0.53	15	*F. nucleatum*	4.38	<u>7.55</u>	85
A. meyeri (−)[c]	0.26	0.64	6	*Fusobacterium* D-2	0.34	0.76	11
Bacteroides				*Fusobacterium* D-5	−	0.29	7
B. buccae	0.09	0.64	1	*Fusobacterium* D-7	−	0.29	4
B. capillosus	0.09	0.12	13	*Fusobacterium* D-9	0.17	0.41	4
B. intermedius 4197	0.52	<u>3.92</u>[d]	35	*Fusobacterium* RD	−	0.29	9
B. intermedius 8944	0.34	0.82	20	*Lactobacillus*			
B. oralis	0.26	0.35	11	*L. catenaforme*	0.17	0.29	9
B. pneumosintes	−	0.70	13	*L. minutus*	0.94	<u>5.21</u>	48
B. zoogleoformans	−	0.41	7	*Lactobacillus* D-2	1.63	<u>1.87</u>	33
Bacteroides D1C-20	0.26	0.35	7	*Lactobacillus* D-8	0.26	0.29	4
Bacteroides D-10	0.09	0.18	7	*Lactobacillus* D-10	0.17	0.41	9
Bacteroides D-12	0.09	0.12	4	*Lactobacillus* D-12	0.09	0.35	4
Bacteroides D-22	−	0.18	7	*Peptostreptococcus*			
Bacteroides D-23	−	0.47	7	*P. micros*	2.32	<u>4.45</u>	48
Bacteroides D-25	0.17	0.29	9	*Peptostreptococcus* A2	0.26	0.35	9
Bacteroides D-28	0.26	0.88	13	*Propionibacterium*			
Bacteroides D-32	0.09	0.18	7	*P. avidum*	−	0.94	2
Bacteroides D-41	−	0.23	2	*Selenomonas*			
Bacteroides D-42	−	0.82	13	*S. sputigena*	0.60	0.82	24
Bifidobacterium				*Selenomonas* D-12	1.20	1.23	17
B. dentium	0.17	0.53	2	*Selenomonas* D-14	0.17	0.23	4
Coccus D-30	0.09	0.35	2	*Staphylococcus*			
Eubacterium				*S. epidermidis*	0.26	0.29	11
E. alactolyticum	−	0.88	26	*Streptococcus*			
E. brachy	0.17	<u>1.52</u>	24	*S. anginosus*	0.94	3.28	26
E. nodatum	0.69	<u>8.31</u>	50	*S. intermedius* IV	0.17	0.23	2
E. timidum	1.37	<u>6.21</u>	70	*S. sangius* III	0.34	0.47	4
Eubacterium D-4	0.09	0.70	9	"Vibrio" D-14	0.09	0.35	4
Eubacterium D-6	0.09	<u>1.58</u>	28	*Wolinella*			
Eubacterium D-8	0.34	<u>2.69</u>	37	*W. recta*	0.17	0.76	20
Eubacterium D-12	−	0.23	7	*Wolinella* HVS	0.34	0.58	7
Fac. G-Pos. Rod D-9	−	0.41	2	*Wolinella* X	0.34	0.58	13

[a] 1100 residual supragingival isolates and 1623 subgingival isolates.

[b] *Bacillus coagulans*, *Bacillus* D-2, *Bacteroides* D-5, *Bacteroides* D-10C, *Bacteroides* D-29, *Bacteroides* D-37, *Bacteroides* D-40, *Clostridium* D-1, *Clostridium* D-2, Coccus D-18, *Eubacterium* D-11, *Eubacterium* D-18, *Eubacterium* D-19, *Eubacterium* D-20, *Eubacterium* D-22, *Eubacterium* D-23, *Fusobacterium* D-11, *Fusobacterium* D-13, Facultative gram-negative Rod D-18, Facultative gram-positive Rod D-6, and D-8, *Haemophilus aphrophilus*, *Peptococcus* D-43, *Selenomonas* D-6, *Selenomonas* D-9, *Selenomonas* D-18, and *Streptococcus* D-44 were found only in the subgingival samples, each at 0.06% of the flora, in 1 (2%) of the samples. *Peptococcus prevotii* was found at 0.12% of the flora, in 2% of the samples, and *Lactobacillus brevis*, *Streptococcus* D-5, and *Streptococcus* D-31 at 0.18% of the flora, in 2% of the samples. *Bacteroides* D-31, *Eubacterium* D-13, *Eubacterium* D-17, *Peptococcus* D-3, and *Staphylococcus capitis* were found only in the subgingival samples, each at 0.12% of the flora, in 2 (4%) of the samples. *Campylobacter concisus* and *Eubacterium* D-15 were found at 0.18%, in 4% of the samples.

[c] (−), no reaction with available antisera.

[d] Underlined values are of species that exceed 1.0% of the subgingival flora and exceed the concentration in samples from the gingival crevice of people with healthy periodontia.

the same initial sizes each time. The observed L ratio will be low if the subgroups are quite distinct from each other (low mean between-group similarity) and if each subgroup is quite homogeneous (high within-group similarity). Therefore, the probability that the observed ratio could have occurred by chance alone from this set of samples is determined by the number + 1 of simulation L values (from random assignment of

TABLE 3. Comparative recovery of bacteria (from roll tubes or from plates in anaerobe jars) from the same sample dilutions of 44 samples from severe periodontitis

Taxa	No. of isolates from		Percent of samples positive
	tubes[a]	plates[b]	
Eubacterium nodatum	72	40	43
Lactobacillus minutus	57	34	43
Fusobacterium nucleatum	73	10	75
Eubacterium timidum	40	25	52
Bacteroides intermedius 4197	15	31	27
Capnocytophaga ochracea	31	11	45
Propionibacterium acnes	7	25	34
"Actinomyces naeslundii-viscosus"	20	10	30
Selenomonas D-12	18	8	25
Bacteroides D-42	0	10	11
Eubacterium D-4	9	0	7

[a] 108 species, 797 isolates (28 did not survive) with 95.2 ±0.70 S.D. percent coverage.

[b] 137 species, 779 isolates (37 did not survive) with 94.6 ±0.75 S.D. percent coverage.

the samples into subgroups of the same initial size) that are lower than or equal to the observed value divided by 1,000 (randomized comparisons) + 2.

RESULTS

There were 2,723 non-spirochetal isolates identified to the species or subspecies level. Of 2,730 colonies picked, 150 (5.5%) did not yield isolates that survived through identification. Colonies that yielded more than one bacterial species accounted for an additional 143 isolates.

All isolates were included in summary tabulations of the flora because they represented species associated with this disease. However, where appropriate for statistical analyses, repeat samples from the same sites were omitted so that the results would not be influenced unduly by single sites or subjects. Similar considerations were made for the single sample of undisturbed supragingival flora and for "outlier" samples associated with two sites, as described below. Among the 2,723 bacterial isolates, 190 taxa at species or subspecies level in at least 27 different genera were detected. According to the coverage analysis they accounted for 98.16 ± 0.23% (standard deviation [SD]) of the cultivable non-treponemal cells in the flora of sites affected with severe periodontitis. There were 139 kinds of bacteria among 1,100 isolates from the residual supragingival flora (coverage, 96.3 ± 0.52%) and 146 bacterial taxa among 1,623 isolates from the subgingival flora (coverage, 97.66 ± 0.34%).

Species that are equally frequent in the flora of people with healthy periodontia probably are not the agents responsible for SP, nor are those species that occur in greater numbers in the supragingival area than in the sulcus, where the destruction takes place. (Except for diseases caused by certain potent toxins, the agents responsible for tissue destruction in other body sites uniformly become predominant members of the flora.) The 94 species that were more numerous in the supra- than in the subgingival flora are listed in Table 1. The 96 species that were more numerous in the subgingival flora are listed in Table 2. Ten species (underlined) in Table 2 exceeded 1% of the subgingival flora and were not detected in greater numbers in healthy or experimental gingivitis floras. In the tables and text, species not previously described are given number or letter designations. *Actinomyces* serotypes are designated by roman numerals. *Actinomyces* species that did not fluoresce with available antibody conjugates were identified only according to their biochemical and electrophoretic characteristics and are designated with "(−)" rather than with a roman numeral.

Effect of isolation procedure. From 44 samples, an equal number of colonies was picked from plates and from roll tubes. The recovery rate of most species was similar with the two isolation methods. However, either oxidation of the plate medium during streaking or whole blood appeared to be beneficial for some bacterial species and detrimental to others. The data (Table 3) illustrate the sensitivity of the flora to relatively minor differences in isolation procedures. Except for *Bacteroides intermedius* (4197 DNA homology group; J. L. Johnson and L. V. Holdeman, Int. J. Syst. Bacteriol., in press) and *Bacteroides* D-42, which were among the pre-

dominant species, more facultative species and more species represented by only a few isolates were recovered on plates.

Sixty-one samples (29 supragingival and 32 subgingival) were cultured both in tubes and on plates. Of the 29 supragingival samples, 11 produced higher counts in tubes and 18 produced higher counts on plates. The tube counts ranged from 39 to 340% of the plate counts (mean, 94.2 ± 11% [standard error]). Of the 32 subgingival sulcus samples cultured on both tubes and plates, 17 produced higher counts in tubes, 14 on plates, and the counts were identical from one sample. The tube counts ranged from 48 to 857% of the plate counts (mean, 149.6 ± 31%).

These results suggest either that the subgingival flora usually is more anaerobic than the supragingival flora adjacent to the margin of the gums or that much of the subgingival flora is inhibited by whole blood. We used both types of cultures for most of the samples to obtain the broadest possible representation of the flora.

The composition of the medium can be expected to cause even greater differences in the relative recoveries of different bacterial species than will culture on plates or tubes. The medium used in the present study was selected on the basis of the highest total cultural count in comparisons with several other basal media and ingredient modifications. Although this medium usually produced the highest total counts, it, like all other media, does not recover every species equally well.

Sample heterogeneity. Among the 78 samples, 4 were so strikingly different from the other 74 that they were considered statistical "outliers." From one of three subgingival sites sampled in one person, 29 of 30 isolates were *Streptococcus anginosus* and one was *Actinomyces naeslundii* (−). The floras of the other two subgingival samples, like most other subgingival samples, yielded 10 and 11 species each. *Streptococcus anginosus* was isolated from the other two sites (one isolate and five isolates) and from six supragingival and nine subgingival sites in other subjects (one to seven isolates from each site).

Similarly, from an affected sulcus (tooth no. 3) of another patient, all 30 isolates (15 from a plate and 15 from a roll tube) were *Streptococcus* D-39. Twenty-six of 30 isolates from the corresponding supragingival sample and all 30 isolates from the supragingival sample of tooth no. 14 also were *Streptococcus* D-39, but *Streptococcus* D-39 was not isolated from the affected sulcus of tooth no. 14.

These results indicate an abscess-like condition in the periodontal pocket of tooth no. 3 and spreading of the organism to the supragingival area of that and other teeth. The suppuration index was 1 (pus could be expressed from the sulcus) for the site on tooth no. 3 and 0 (no pus) for the site on tooth no. 14. However, the suppuration index itself apparently is not indicative of enrichment with a single species, because this was the only outlier among eight sites with recorded suppuration index scores of 1 (or among nine with scores of 0). *Streptococcus* D-39 was not found in any other patient in this group or among approximately 8,000 periodontal isolates from several populations we have examined. It was one of many oral *Streptococcus* species we detected in periodontal floras that were unlike any type or reference strains that we examined. It fermented arabinose, cellobiose, esculin, fructose, glucose, lactose, maltose, ribose, salicin, sorbitol, starch, sucrose, trehalose, and xylose; weakly fermented inulin, mannitol, raffinose, and rhamnose; and did not ferment erythritol, glycogen, inositol, melezitose, or melibiose. It was bile-esculin positive, curded milk, grew well at 45°C and in 6.5% NaCl, and produced ammonia from peptone and from arginine. The electrophoretic pattern of soluble proteins was distinct from those of available type or reference strains of described species.

The true significance of these four unusual samples is open to conjecture. Therefore, all statistical comparisons were made both with and without these samples or repeat samples of these sites. The presence or absence of the outliers together with repeat samples had little effect upon the statistical results.

Residual supragingival versus subgingival flora. By *L* simulation analyses, the compositions of the residual supragingival and the subgingival flora of sites affected with severe periodontitis were found to be significantly different ($P = <0.001$). The observed *L* value was 11.9 SDs below the mean of 1,000 randomized similarity ratios when all samples were included and 8.1 SDs below the mean when outliers and repeat samples from the same sites were omitted. These values indicate that the probability is much less than $1/1{,}000$, but closer estimates cannot be made because the shape of the probability curve for these data at this extremity is not known. This observation suggests there was very little contamination of the subgingival samples with supragingival organisms during sampling and that the floras of those two sites are distinct.

Comparison with other populations. The composition of the subgingival flora of the sites affected with severe periodontitis also was significantly different from that of the gingival crevice of nine sites in eight persons with healthy gingiva, of 18 sites with a gingival index (GI) score of 0, and of 36 samples from sites with a GI score of 2 in an experimental gingivitis

study (10) reported earlier (P = <0.001 for all three comparisons, with or without outliers and repeat samples). These results strongly suggest a progressive change in the composition of the flora after the establishment of gingivitis.

Differences between subjects. An L similarity analysis also was made to determine whether or not the floras of sites affected with severe periodontitis were different in different subjects. In this test supra- and subgingival samples were compared within clusters representing each person. The composition of from two to nine samples in 20 persons (there was only a single subgingival sample from one person, therefore no within-person similarity value could be obtained) indicated that individuals were a significant source of variation in the experiment (P = <0.001 for all samples; P = 0.002 with two samples per person, outliers and repeat samples omitted). If there is a bacterial cause of periodontitis that is common to most patients, the statistically significant variation in the composition of the complex floras of different people will help to pinpoint the specific agents involved. On the other hand, the outlier samples may have an independent significance.

Effect of sampling. The supra- and subgingival floras of eight sites in seven people were resampled 1 to 4 months later. By L simulation analyses, there were no detectable differences between the first and second samples of the supra- and subgingival tooth flora (P = 0.88), the supragingival flora (P = 0.96), or the subgingival flora (P = 0.58). Thus there is no evidence that sampling (as opposed to thorough scaling) has any effect upon the flora that can be detected after 4 or more weeks. Considering the amount of inoculum that was left behind, it would be surprising if there were a detectable difference after a day or so unless severe trauma were induced.

Effect of amount of destruction. The flora of subgingival samples from sites with pocket depths of 5 to 7 mm was not significantly different from the flora of samples from 9- to 10-mm pockets (P = 0.64 with all samples, P = 0.42 without outliers or repeat samples). Similarly, the flora of subgingival samples from sites with 4 to 6 mm of attachment loss was not significantly different from that of sites with 8 to 10 mm of loss (P = 0.50 with all samples and 0.25 without repeat samples or outliers). Therefore, there is no evidence that a different resident flora is associated with greater amounts of destruction in this SP population.

Relationship to serological differences. Antibody to *Actinobacillus actinomycetemcomitans* Y4 was found in the sera of 5 of 14 of those subjects that were tested as part of another study (13). As nearly 80% of subjects with classical localized JP exhibit such antibody, there may be a direct relationship between the JP population and a subset of the SP population. When all bacterial samples (supra- and subgingival) from these five patients were compared by L simulation analyses with all samples from the nine known Y4 antibody-negative patients, the difference in composition of the flora was significant (P = <0.001), which indicated that there are two bacteriologically distinct groups in the SP population. The composition of the subgingival samples was not significantly different (P = 0.11 with all samples, and P = 0.32 when only one sample per person was used to remove person-difference bias due to multiple samples). Surprisingly, the composition of the flora of the residual supragingival samples was significantly different between the two groups (P = 0.007 with all samples, and P = 0.03 with one sample per person). There were supragingival samples from only three patients with antibody to Y4 and only eight patients without Y4 antibody. The difference in composition of the supragingival flora must have been great to have been detected with so few samples. This difference was confirmed by coverage analysis, which is independent of similarity simulation and is based only on the number of species seen once in each population rather than on the species they share. The coverage of all three single samples from the Y4 antibody-positive subjects was 91.67 ± 0.09% (SD) with 31 species among 96 isolates. The coverage of all eight single samples from Y4 antibody-negative subjects was 87.60 ± 0.03% (SD) with 78 species among 258 isolates. Normally, coverage is greater with more isolates. These results indicate that persons without serum antibody to Y4 had a much more complex supragingival flora than did those with serum antibody to Y4, for which fewer isolates gave greater coverage. The t test indicated that the difference was significant (t = 43 (1df), P = <0.02). Direct comparisons of the flora showed these major differences in the composition of the supragingival flora.

The two groups shared only the following 19 species in the supragingival flora at the designated minimum percentages in common: *Actinomyces israelii* (−), 1.9; *Actinomyces israelii* I, 3.1; *Actinomyces israelii* II, 0.8; *Actinomyces naeslundii* (−), 1.6; *Actinomyces naeslundii* I, 1.0; *"Actinomyces naeslundii-viscosus"* (serological cross), 2.1; *Actinomyces viscosus* (−), 1.0; *Actinomyces viscosus* II, 3.1; *Bacteroides gracilis*, 0.4; *Bacteroides intermedius* 8944, 0.4; *Capnocytophaga ochracea*, 3.1; *Eikenella corrodens*, 0.4; *Fusobacterium nucleatum*, 2.1; *Lactobacillus* D-2, 0.4; *Selenomonas sputigena*, 1.0; *Selenomonas* D-1, 1.2; *Selenomonas* D-4, 0.8; *Staphylococcus haemolyticus*, 0.4; and *Veillonella*

parvula, 3.9. There was thus 28.7% similarity based on single samples from each person.

Eleven taxa were found only in the three Y4 antibody-positive patients at the designated percentages: *Actinomyces naeslundii* II, 2.1; *Actinomyces naeslundii* III, 8.3; *Bacteroides* D-12, 1.0; *Bacteroides denticola*, 2.1; *Bacteroides* EO, 1.0; *Eubacterium* D-8, 2.1; *Eubacterium* D-21, 1.0; *Peptostreptococcus anaerobius* II, 2.1; *Selenomonas* D-11, 2.1; *Selenomonas* D-12, 2.1; *Streptococcus* D-17, 2.1; and isolates that did not survive to identification, 8.3. Thus, these species accounted for 34.4% of the antibody-positive supragingival flora.

Fifty-eight species were found only in the eight Y4 antibody-negative patients at the designated percentages: *Actinomyces* WVa-963, 0.4; *Actinomyces meyeri*, 0.8; *Actinomyces meyerii* (−), 0.8; *Actinomyces odontolyticus* (−), 0.8; *Actinomyces odontolyticus* I, 0.8; *Actinomyces odontolyticus* X, 0.4; *Actinomyces* D-8, 0.8; *Bacteroides bivius*, 0.4; *Bacteroides capillosus*, 0.4; *Bacteroides gingivalis*, 1.9; *Bacteroides loescheii*, 0.8; *Bacteroides intermedius* 4197, 0.4; *Bacteroides melaninogenicus* 2381, 0.4; *Bacteroides oris*, 2.3; *Bacteroides putredenis*, 0.4; *Bacteroides* D-10, 0.4; *Bacteroides* D-19, 0.4; *Bacteroides* D-25, 0.8; *Bacteroides* D-28, 0.8; *Bacteroides* D-36, 0.4; *Capnocytophaga gingivalis*, 1.2; *Capnocytophaga sputigena*, 0.4; Coccus SM1, 1.2; *Corynebacterium* D-2, 0.4; *Eubacterium brachy*, 0.8; *Eubacterium nodatum*, 2.7; *Eubacterium saburreum*, 0.8; *Eubacterium timidum*, 2.7; *Eubacterium* D-6, 0.4; *Fusobacterium* D-1, 0.4; *Fusobacterium* D-9, 0.8; facultative gram-negative rod D-14, 0.8; *Lactobacillus minutus*, 0.4; *Lactobacillus* D-10, 0.4; *Lactobacillus* D-12, 0.4; *Lactobacillus* D-8, 0.8; *Peptostreptococcus anaerobius*, 1.6; *Peptostreptococcus micros*, 1.6; *Propionibacterium acnes*, 3.9; *Propionibacterium* D-1, 0.4; *Selenomonas* D-2, 0.8; *Selenomonas* D-14, 0.8; *Staphylococcus epidermidis*, 0.4; *Streptococcus anginosus*, 1.9; *Streptococcus constellatus*, 1.2; *Streptococcus* D-7, 0.4; *Streptococcus* D-10, 0.4; *Streptococcus* D-24, 0.4; *Streptococcus intermedius* III, 0.4; *Streptococcus intermedius* IV, 0.4; *Streptococcus mitis*, 1.5; *Streptococcus* SA, 0.4; *Streptococcus sanguis* I, 3.9; *Streptococcus sanguis* II, 2.7; *Streptococcus* SM, 0.4; *Wolinella* HVS, 1.6; *Wolinella* X, 1.2; *"Vibrio"* D-14, 0.4; and isolates that did not survive to identification, 7.0. These species accounted for 62.0% of the antibody-negative supragingival flora.

Although predominant species of the supra- (and sub-) gingival flora of Y4 antibody-negative patients were absent from the supragingival flora of Y4 antibody-positive patients, many of these species, including predominant suspect etiological agents, were found as a high proportion of the subgingival flora of antibody-positive patients.

The data were reanalyzed, considering the antibody-positive and antibody-negative patients as different populations. As a consequence of dividing the SP population, the sample numbers became small and the statistical analyses had less power. The composition of the supragingival and subgingival floras was significantly different in the antibody-negative patients ($P = <0.001$ for all 16 samples of each, and $P = <0.004$ for 8 supra- and 8 subgingival samples, one from each person). The results were less clearcut for the antibody-positive patients, probably because there were so few samples ($P = 0.052$ with all nine supragingival and eight subgingival samples, and $P = 0.19$ with only one supra- and one subgingival sample from each of three people). The difference between individuals appeared to be greater in antibody-positive subjects ($P = <0.001$ with all samples, and $P = 0.029$ with only one pair of samples from each of four people), whereas for antibody-negative subjects, $P = 0.025$ with all 20 samples from nine people but only 0.13 (not significant) with one pair of samples from each of eight people. All of these data suggest that the primary difference in the floras of the two groups was the simplified composition of the supragingival flora of the antibody-positive subjects.

Actinobacillus actinomycetemcomitans was not detected on the non-selective medium (although it has been isolated from other populations on this medium). *Actinobacillus actinomycetemcomitans* was detected in samples from three of the four SP patients tested with the selective medium described by Mandell and Socransky (7). Representative colonies were verified as *Actinobacillus actinomycetemcomitans*. Colonies with the same morphology on the selective medium were estimated to be 0.04 to 4.0% of the counts obtained on nonselective medium in samples from two antibody-negative subjects and 15% of the subgingival flora of one patient with serum antibody to *Actinobacillus actinomycetemcomitans* strain N27. The flora of the one supragingival sample from the patient with N27 antibody was as complex as those from the antibody-negative patients. The person from whom *Actinobacillus actinomycetemcomitans* was not isolated was not tested for serum antibody.

Treponemes were isolated from 40 of the 42 subgingival SP samples examined. They were not seen microscopically in the other two samples. Because selective media were used, the concentrations of treponemes were estimated as the log value of the dilution from which they were isolated. The mean microscopic count or

TABLE 4. Frequency of treponeme isolation or detection (as percent of samples positive) from several populations[a]

Treponeme	Population (number of samples)				
	Experimental gingivitis			Severe periodontitis	
	GI=0 (18)	GI=1 (42)	GI=2 (36)	Supragingival	Subgingival
Treponeme A	16.7	21.4	33.3	17.1	54.8
Treponeme A-1	–	2.4	8.3	8.6	14.3
Treponeme D	–	–	–	5.7	14.3
T. denticola	–	–	–	8.6	21.4
Treponeme F	–	–	2.8	5.7	2.4
Treponeme G	–	–	2.8	2.9	–
Treponeme J	–	4.8	2.8	8.6	11.9
Treponeme K	5.6	–	8.3	2.9	2.4
Treponeme N	–	2.4	–	–	2.4
Treponeme S	–	–	2.8	2.9	–
"Large treponeme" (microscopic only)	–	–	–	5.7	64.3

[a] No treponemes were detected in 9 subgingival samples from 8 people with healthy gingiva.

recovery was 10% of the nonselective bacterial cultural count. From the 40 positive samples, treponemes were seen at (or isolated from) the same dilution as the total bacterial count 16 times, at one log dilution less 16 times, and at two or more log dilutions less 8 times. The treponemal cultural count equaled or exceeded the treponeme microscopic count in 14 of the 42 samples. Twelve species were detected in the SP samples (Table 4). Although several of the treponeme species exceeded 10% of the total bacterial count in one or more samples, only treponeme A, the "large treponeme," and *Treponema denticola* approached 1% of the total bacterial count of the entire population.

Treponemes were observed in, but not isolated from, the two outlier subgingival samples. There was no unusual pattern of treponemal species in the subjects that had antibody to *Actinobacillus actinomycetemcomitans*. Repetition of results was only moderately good in repeat samples from the same sites after 1 or more months. The large treponeme was seen both times in samples from four sites that were sampled twice, and once from another site that was sampled twice. Treponeme A was isolated both times in two sites, once in two other sites, and absent both times in the fifth site. Seven other species occurrences did not repeat, indicating that they were present in low numbers or that their concentration was variable within individual sites.

Mycoplasma species were isolated from 15 of 35 (43%) residual supragingival samples and 23 of 42 (55%) subgingival SP samples. Mycoplasma were never detected above 10^1 dilution, whereas the mean total bacterial cultural counts of these samples were in the range of 10^6 and 10^7 for residual supragingival and subgingival samples, respectively.

DISCUSSION

The presence of a significantly simpler supragingival flora in patients with serum antibody to *Actinobacillus actinomycetemcomitans* Y4 was surprising and suggests that these patients have a different host relationship with the oral flora. Nevertheless, there were striking similarities in the concentrations of predominant species in the subgingival floras of these patients and the antibody-negative members of the SP population. For the present, we might consider the SP population as a single group as regards agents of tissue destruction. There may be a subset related to classical localized JP, but at the SP stage of disease development those with Y4 antibody may no longer have a flora that is representative of JP. This may be clarified when we complete our analyses of a JP population currently being studied.

There is a consensus among periodontists that in affected people periodontal destruction occurs sporadically. In this respect there are interesting similarities to bacterially induced arthritis (1, 5). There are several possible relationships between the periodontal flora and episodes of tissue destruction. (i) The flora may change to induce destruction during episodes. This could

TABLE 5. Incidence (as percent of flora) of the 22 most frequent species in affected sulci of SP patients and incidence of these species in subgingival samples from persons with healthy periodontia and sites with GI scores of 0, 1, and 2 in experimental gingivitis[a]

Species	Healthy	GI=0	GI=1	GI=2	SP
Fusobacterium nucleatum	0.69	3.56	3.08	5.70	7.55
Eubacterium timidum	0.35	0.36	0.38	0.70	6.21
Eubacterium nodatum	–	–	–	0.09	8.31
Lactobacillus minutus	0.35	–	–	0.53	5.21
Peptostreptococcus micros	0.69	0.89	1.08	1.75	4.45
Bacteroides intermedius 4197	–	–	–	0.09	3.92
Eubacterium D-8	–	–	0.23	0.18	2.69
Lactobacillus D-2	0.35	0.18	1.61	3.77	1.87
Eubacterium D-6	–	–	0.08	0.44	1.58
Eubacterium brachy	–	–	–	0.09	1.52
Eubacterium alactolyticum	–	–	–	–	0.88
Selenomonas D-12	–	–	0.31	1.40	1.23
Selenomonas sputigena	–	–	0.77	0.61	0.82
Bacteroides intermedius 8944	0.35	–	0.85	0.61	0.82
Bacteroides D-28	–	–	–	–	0.88
Bacteroides D-42	–	–	–	–	0.82
Eubacterium D-11	–	–	–	–	0.82
Bacteroides pneumosintes	–	–	–	0.09	0.70
Bacteroides buccae	–	–	0.15	–	0.64
Fusobacterium D-2	–	–	–	–	0.76
Actinomyces meyeri	–	0.36	0.08	0.26	0.53
Wolinella X	–	–	0.38	0.35	0.58
Total (these species)	2.78	5.35	9.00	16.66	52.79

[a] There were 288 isolates from the healthy population, 562 from GI=0 sites, 1301 from GI=1 sites, 1141 from GI=2 sites and 1708 (including 85 that did not survive through identification) from SP sites; –, not detected.

be either an increase in the concentration of one or more agents in the flora or development of abscess-like conditions in which single species become predominant. (ii) Resistance to a relatively constant microbial pressure might change in response to other changes in systemic health. (iii) The responsible agents may be different for each person and be dependent upon chance colonization by species for which the immune response was specific in that individual.

It is evident from these possibilities that the responsible bacterial species have not been determined, but information that bears on each possibility can be obtained from inspection of the bacteriological results. Certainly, the responsible species must be present in severe periodontitis although not necessarily in all sites, because all sites may not be sites of active destruction when the samples are taken. Comparisons of the floras of sites with different degrees of existing destruction could possibly bear on this question. No differences were detected within this population.

If the etiology of severe periodontal destruction is different for each individual, then the 96 species listed in Table 2 are primary candidates. All of them were present in at least 1% of the flora of at least one affected subgingival site. Presumably, there was active destruction in at least 1 of the 50 site-sample times (or could we be that unlucky?). Ten species might be eliminated from consideration because causative agents are expected to maintain or increase their concentrations in affected sites. *Campylobacter concisus, Staphylococcus capitus, Staphylococcus epidermidis*, and *Streptococcus intermedius* IV were all found in higher concentrations in the gingival crevice of people with healthy periodontia. *Bacteroides capillosus, Bacteroides oralis, Bacteroides* D-31, *Eubacterium* D-13, *Streptococcus anginosus*, and *Wolinella recta* were found in higher concentrations in gingival crev-

ice samples from sites with a GI score of 0 in an experimental gingivitis study reported earlier (10).

If the episodes of destructive activity are (sometimes) caused by abscess-like conditions in which the flora is enriched with a single species, then *Streptococcus anginosus* and *Streptococcus* D-39 are prime candidates. *Streptococcus anginosus* is a reasonably common pathogen in deep-tissue infections of other body sites, but the incidence of *Streptococcus* D-39 is unknown.

If, however, portions of the flora increase (which can only be determined by more extensive longitudinal studies) or if, as now seems most likely, the patient response to a rather constant microbial pressure changes, then the most frequent of the species that are not found in healthy sites are the primary candidates. These species are listed in Table 5 by rank order based on their concentration and frequency in the sulcus of the 42 sites affected with severe periodontitis.

Several of the 22 most frequent species are known or suspected pathogens of other body sites (8). In our collection of clinical isolates, *Fusobacterium nucleatum* is the most common among these species from SP sulci. It is isolated from abscesses of the brain, head and neck, pleura, abdominal and pelvic regions, bite wounds, and blood (93 strains). *Bacteroides intermedius* is isolated from similar sites except brain abscesses (24 strains); *Lactobacillus minutus* (24 clinical strains), *Peptostreptococcus micros* (30 strains, 3 from brain abscesses), *Eubacterium alactolyticum* (17 clinical strains), *Selenomonas sputigena* (1 from pleural abscess), and *Bacteroides pneumosintes* (15 strains) are isolated from pleural abscesses, sinusitis, and foot and skin ulcers. "*Actinomyces meyerii*" (36 clinical strains, 12 from brain abscesses) is the oral actinomyces species most frequently associated with brain abscesses. Minimal information is available on the other frequent SP species. *Eubacterium brachy* has been isolated from one pleuropulmonary abscess with *Fusobacterium nucleatum* (14). Several periodontal species that, according to present considerations, are not primary candidates for etiology of periodontitis are frequent agents of infection at other body sites. Therefore, this criterion is not incriminating by itself. However, the established pathogenicity does indicate an ability to cause tissue destruction; in addition, it illustrates that periodontal disease sites are a significant reservoir of clinically infectious agents.

The number of gram-positive species that are included in the list may be surprising in view of many reports, based on microscopic studies, that the flora of periodontal disease sites is overwhelmingly gram negative. Only 39% of the randomly selected isolates from the sulci of SP sites were gram-negative species, as opposed to 25% of the isolates from the gingival crevice of healthy sites. However, direct microscopic studies tend to overestimate the proportions of gram-negative cells because many cells of gram-positive species stain gram negative in mature (undisturbed) culture populations. Nevertheless, the difference in the composition of the flora, including the gram-positive species, between health and severe disease is evident. The 10 most numerous gram-positive species in healthy sites were 46% of the cultivable cells but only 6% of the flora in subgingival SP sites, whereas the 10 most numerous gram-positive species in subgingival SP sites were 32% of the flora. Three of these species were absent in healthy sites, and the other seven were only 9% of the flora of healthy sites.

The mere presence of the 22 species listed in Table 5 does not indicate that they play a significant role in periodontal destruction. Several of these species may be present simply because a suitable environment exists. However, because of their predominance and the fact that several have known pathogenic potential, they appear to include the most likely etiologic agent(s) in this study. Each might contribute to pathogenesis directly or indirectly, depending on its physiology.

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