Predominant Cultivable Microflora of Human Dental Fissure Plaque

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Plaque developed in 10 occlusal fissures from unerupted third molars during implantation for 200 to 270 days in lower molars of dental students was studied. To characterize the predominant cultivable flora, 592 isolates (51 to 67 from each fissure) were subcultured from anaerobic roll tubes. Twenty-eight of the isolates were lost. Streptococci constituted 8 to 86% (median, 45%) of the isolates, *Streptococcus mutans* constituted 0 to 86% (median, 25%) and *S. sanguis* constituted 0 to 15% (median, 1%). A few isolates of "*S. mitior*" and "*S. milleri*" were found, but no *S. salivarius*. Staphylococci made up 0 to 23% (median, 9%). Gram-positive rods constituted 6 to 59% (median, 35%). Of these, 0 to 46% (median, 18%) were *Actinomyces naeslundii* and *A. viscosus*, but no anaerobic actinomyces were isolated. Arachnia and propionibacteria made up small proportions, lactobacilli were isolated from two fissures, constituting 10 and 29%, and eubacteria were isolated from one fissure (27%). Gram-negative cocci made up 0 to 46% (median, 4%). Only two isolates of gram-negative rods were found, both facultative anaerobes. Although 8 of the 10 fissures had large proportions of *S. mutans*, lactobacilli, or both, no caries was found even with microradiography. The large individual variation probably reflects differences in initial colonization from saliva and in growth conditions in each fissure.

Only little information is available on the microflora of plaque in occlusal fissures of human teeth, most likely because the narrow fissure presents a sampling problem. The development of model systems involving implantation of natural or artificial fissures into teeth in humans allows the sampling of plaque from the entire depth of the fissure (7, 14). Earlier microbiological studies using such model systems demonstrated the presence of various organisms such as *Streptococcus mutans*, *S. sanguis*, *Lactobacillus*, and *Veillonella* in young fissure plaque developing in artificial or natural fissures implanted in the mouth for up to 21 days (18, 21, 22, 25). We previously published preliminary data on old fissure plaque formed in occlusal fissures from unerupted third molars implanted into occlusal fillings in dental students for 200 to 270 days (20). No caries was found in any of these fissures by either clinical or microradiographic methods. Gram-positive cocci and rods were the predominant organisms seen by microscopy of suspensions of the fissure plaque samples. From colony counts on a nonselective medium and various selective media, it appeared that lactobacilli, haemophili, and veillonellae constituted only minor and variable proportions of the flora. On the other hand, streptococcal counts on mitis salivarius agar corresponded to 6 to 94% of total viable counts, and 23 to 100% of the streptococcal colonies appeared to be *S. mutans*. This study presents a bacteriological characterization of the predominant cultivable flora of the same fissure plaque samples.

MATERIALS AND METHODS

Fissures. Ten blocks (2 by 2 by 3 mm) comprising occlusal fissures were prepared from unerupted, surgically removed human third molars and implanted in occlusal surfaces of preexisting amalgam fillings in lower molars in 10 dental students as previously described (7). The subjects continued their usual diet and tooth brushing with a fluoride containing dentifrice. A drop of 20% sucrose solution was placed at the entrance of the implanted fissures twice daily during the experimental period of 200 to 270 days in order to favor the development of a cariogenic flora. Before removal of the fissures, the teeth containing them were rinsed with a water spray and isolated from saliva, using cotton rolls and suction.

Microbiological methods. After removal, the fissure blocks were divided along the fissure, and the two fragments were immediately dropped into 5 ml of reduced transport fluid (19). The fissure content was suspended by treatment with ultrasound for 10 s in a crushed-ice bath, using a PG 100 MSE 150-W ultrasonic disintegrator with Titan microprobe at an amplitude of 6 μm. Appropriate 10-fold serial dilutions were prepared in reduced transport fluid, and anaerobic viable counts were performed in triplicate by two

TABLE 1. Characterization of streptococci[a]

Strain	Fermentation of:			H₂O₂ production	Acetoin (Voges-Proskauer)	Hydrolysis of:		Dextran from sucrose
	Sorbitol	Mannitol	Salicin			Arginine	Esculin	
S. sanguis	−	− −	+ +	+ +	− −	+ +	+	+ +
"*S. mitior*"								
Dextran positive	− −	− −	−	+ +	−	− −	− −	+ +
Dextran negative	− −		−	+ +	−	− −	− −	− −
"*S. milleri*"	− −	− −	+ +	− −	+	+ +	+	− −
S. salivarius	− −	− −	+ +	− −	−	− −	+	−
S. mutans	+	+ +	+ +	−	+ +	−	+	+ +

[a] + +, All strains positive; − −, all strains negative; +, most strains positive, but some negative; −, most strains negative, but some positive.

methods. In the first, 1 ml of the diluted suspensions was added to melted agar in anaerobic roll tubes, and the agar was then solidified as a layer on the inner surface by spinning the tube. The medium was a tryptone-yeast extract agar containing glucose, starch, cysteine, menadione, and hemolyzed blood (13). Reduced transport fluid and roll tubes were prereduced by gassing with an oxygen-free mixture of 85% N₂, 10% H₂, and 5% CO₂ and anaerobically autoclaved in stoppered tubes in a press. The tubes were gassed again when opened. Roll tube viable counts were calculated from colony counts after 10 days of incubation at 37°C. In the second method, 0.1 ml of the dilutions was spread in air on agar plates of the same medium. The plates had been prereduced by storage for 24 h in Baird & Tatlock jars in 85% N₂, 10% H₂, and 5% CO₂ before inoculation. Anaerobic plate viable counts were carried out after 3 days of incubation in the same gas mixture at 37°C.

From each of the 10 samples, 50 colonies were subcultured from a roll tube, and 50 colonies were subcultured from a plate, each having 50 to 500 colonies. Subcultures from roll tubes were carried out in gassed tubes, and those from plates were transferred in air and incubated in anaerobic jars. Some colonies yielded two to three pure cultures. From the roll tubes, 564 bacterial isolates (34 to 67 from each fissure) were purified and characterized, and 28 were

lost. From the plates, 514 isolates (43 to 65 from each fissure) were characterized, and 19 were lost. Each isolate was initially studied for cell morphology, Gram-staining reaction, catalase, aerobic growth, and growth on mitis salivarius agar (Difco Laboratories).

Streptococci were classified according to a simple scheme (Table 1) based on information from Carlsson (3), Colman and Williams (5), Mejare and Edwardsson (16), and Hardie and Bowden (10). Fermentation tests were performed in phenol red broth (Difco) with 1% Tween 80. Production of H₂O₂ was tested according to Whittenbury (26). Acetoin production was demonstrated with α-naphthol and KOH (6). Arginine hydrolysis was tested by the method of Cowan (6). The esculin broth of Carlsson (2) was used for esculin hydrolysis, and dextran production from sucrose was tested by precipitation with 1 part of ethanol on the supernatant of 7-day cultures in the Trypticase medium of Gibbons and Banghart (9).

Other gram-positive, facultative cocci were classified after tests for catalase, oxidase, coagulase, acetoin production, and fermentation or oxidation. Gram-negative, facultative cocci were tested for catalase, oxidase, acid production from glucose, and a fermentative or oxidative carbohydrate metabolism. Gram-negative, anaerobic cocci were tested for catalase and for the production of acid in glucose broth. Tests and classification were performed according to Cowan (6).

TABLE 2. Characterization of gram-positive rods (other than lactobacilli)

Strain	Growth		Filamentous microcolonies	Branching	Catalase	Nitrate reduction	Indole	Acetoin (Voges-Proskauer)
	Aerobic	Anaerobic						
Actinomyces viscosus	+	+	+	+	+	+	−	−
A. naeslundii	+	+	+	+	−	+	−	−
A. odontolyticus	±	+	−	+	−	+	−	−
A. israelii	(+̄)	+	+	+	−	+	−	−
Propionibacterium acnes	−	+	−	±	+	+	+	−
Arachnia propionica	(−̟)	+	+	+	−	+	−	−
Rothia dentocariosa	+	±	+	+	+	+	−	+
Bacterionema matruchotii	+	+	+	+	+	+	−	+
Bifidobacterium species	−	+	−	+	−	−	−	−
Eubacterium species	−	+	−	−	−	−	±	−

Gram-positive rods were examined for branching and filamentous forms by microscopy of microcolonies on brain heart infusion agar (Difco). They were tested for growth on Rogosa selective lactobacillus agar and also for nitrate reduction (6). Acids produced by fermentation in a prereduced, anaerobically sterilized peptone-yeast extract-glucose broth (11) were identified by the gas chromatographic procedure of Carlsson (4).

Lactobacilli were classified according to Cowan (6) after further tests in MRS lactobacillus broth (6) for production of gas from glucose and gluconate, ammonia from arginine, esculin hydrolysis, and acid from arabinose, galactose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, and trehalose.

For other gram-positive rods, tests for fermentation of arabinose, cellobiose, lactose, mannitol, mannose, raffinose, salicin, sorbitol, and xylose were performed in thioglycolate medium (Difco) as recommended for *Actinomyces* by Slack and Gerencser (17). Tests for production of acetoin and hydrolysis of esculin were done as for streptococci. Indole production was tested with Kovács reagent. SSR urea medium was used in tests for urea hydrolysis (6). The strains were then classified according to Table 2.

RESULTS

Viable counts in the anaerobic roll tubes ranged from 0.4×10^6 to 9.7×10^6 colony-forming units per fissure sample, with a median value of 3.7×10^6. Viable counts on the anaerobic plates were lower for all 10 samples, constituting 19 to 81% of the roll tube counts (median, 38%).

Table 3 shows classification of the 564 bacterial isolates representing the predominant cultivable bacteria from anaerobic roll tubes. Several species were present, the majority being gram positive. The most predominant species were *S. mutans* (30% of the isolates) and *Actinomyces naeslundii* (15%). The isolates listed as *Veillon-*

ella parvula (12%) were gram negative, anaerobic, and catalase positive, reduced nitrate, and did not produce acid from glucose. Staphylococci constituted 9%. Nearly all of these were similar to *S. epidermidis*, as they were catalase positive, oxidase negative, fermentative, and coagulase negative and produced acetoin. In one sample, *S. aureus* (coagulase positive) was found.

Great individual variation was seen in the percentage distribution of predominant bacteria isolated from the 10 samples (Table 3, Fig. 1). *S. mutans* constituted 0 to 100% (median, 66%) of the streptococcal isolates from the 10 samples. It was isolated from only seven samples, but in all of these it made up more than 50% of the streptococci and 25 to 86% of the total cultivable flora. *S. sanguis* made up 86 and 91% of the streptococci in two of the samples which had no *S. mutans*. On the other hand, *S. sanguis* was absent from the predominant flora of five samples and made up 0 to 91% (median, 3%) of the streptococcal isolates from each sample.

A. naeslundii was found in seven samples, and in four of these it constituted more than 50% of the gram-positive rods. *A. viscosus* was cultured from eight samples, and in two it constituted more than 50% of the gram-positive rods. *Arachnia propionica* was found in six samples, and in three of these it made up more than 25% of the gram-positive rods. In six samples, a small proportion of the isolates were identified as *Propionibacterium* species.

The predominant cultivable flora isolated from conventional anaerobic plates (data not shown) was largely similar to that isolated from the more strictly anaerobic roll tubes, although the latter gave higher viable counts. Thus, the proportional distributions were similar for the two methods for total streptococci, for individ-

TABLE 2—*Continued*

Hydrolysis of:		Fermentation of:									Acid end products[a]
Urea	Esculin	Arabinose	Cellobiose	Lactose	Mannitol	Mannose	Raffinose	Salicin	Sorbitol	Xylose	
(+̄)	+	(+̄)	(+̄)	+	−	±	+	±	−	±	ALS
(+̄)	+	−	(+̄)	+	−	±	+	±	−	±	ALS
−	±	(+̄)	−	±	−	−	−	±	−	±	ALS
−	+	(+̄)	±	+	±	(+̄)	+	(+̄)	−	+	ALS
−	−	−	−	±	±	+	−	−	(+̄)	−	AP
−	−	(+̄)	−	(+̄)	+	+	±	−	±	±	AP
−	+	−	−	(+̄)	−	±	−	±	−	−	AL
+	+	−	−	−	−	+	±	+	−	−	APL
−	+	±	+	+	+	+	+	+	−	+	AL
	±	±	±	±	±	±	±	−	±	−	AB

[a] A, Acetic acid; L, lactic acid; S, succinic acid; P, propionic acid; B, butyric acid.

TABLE 3. Predominant cultivable flora of 10 samples of 200- to 270-day-old fissure plaque (isolates from anaerobic roll tubes)

| Isolate	No. of isolates in each sample										Range		Median		Total isolates	
	1	2	3	4	5	6	7	8	9	10	No.	%	No.	%	No.	%
Streptococci	5	37	44	25	7	11	17	35	29	23	5–44	7.9–86.3	24	44.9	233	39.4
S. mutans		33	44	14			16	26	29	13	0–44	0–86.3	15	24.7	175	29.6
S. sanguis		4				10	1	4			0–10	0–14.9	0.5	0.8	25	4.2
"S. mitior" (dextran positive)	3			1	6						0–3	0–4.7	0	0	5	0.9
"S. mitior" (dextran negative)				7	1						0–7	0–13.0	0	0	7	1.2
"S. milleri"	2										0–2	0–3.2	0	0	2	0.3
Not classified				3		1		5		10	0–10	0–18.9	0	0	19	3.2
Staphylococci	3	1		6	14	5	12	7		5	0–14	0–23.0	5	8.5	53	9.0
S. epidermidis	3	1		6	14	5	12	4		5	0–14	0–23.0	5	7.1	50	8.5
S. aureus								3			0–3	0–5.0	0	0	3	0.5
Gemella					1						0–1	0–1.6	0	0	1	0.2
Actinomyces	7			3	28	26	23	10	12	11	0–28	0–45.9	11	18.2	120	20.2
A. naeslundii	2				27	25	22	2	9	2	0–27	0–44.3	2	3.3	89	15.0
A. viscosus	5			3	1	1	1	8	3	9	0–9	0–17.0	2	3.3	31	5.2
Arachnia propionica					5	1	1	4	6	11	0–11	0–20.8	1	1.6	28	4.7
Propionibacteria					3	5	2	1		2	0–5	0–7.5	0.5	0.9	13	2.2
Eubacteria		16									0–16	0–27.1	0	0	16	2.7
Lactobacilli	18		5								0–18	0–28.6	0	0	23	3.9
L. plantarum	18										0–18	0–28.6	0	0	18	3.0
L. casei			5								0–5	0–9.8	0	0	5	0.9
Veillonella parvula	28				1	18	8	3	14		0–28	0–44.4	2	3.3	72	12.2
Unidentified gram-negative																
Anaerobic cocci	1	5			1						0–1	0–1.6	0	0	2	0.3
Facultative cocci						1					0–1	0–1.5	0	0	1	0.2
Facultative rods					1					1	0–1	0–1.9	0	0	2	0.3
Isolates lost	1	2	2	20							0–20	0–37.0	0	0	28	4.7
Total isolates	63	59	51	54	61	67	63	60	61	53	51–67		61		592	100
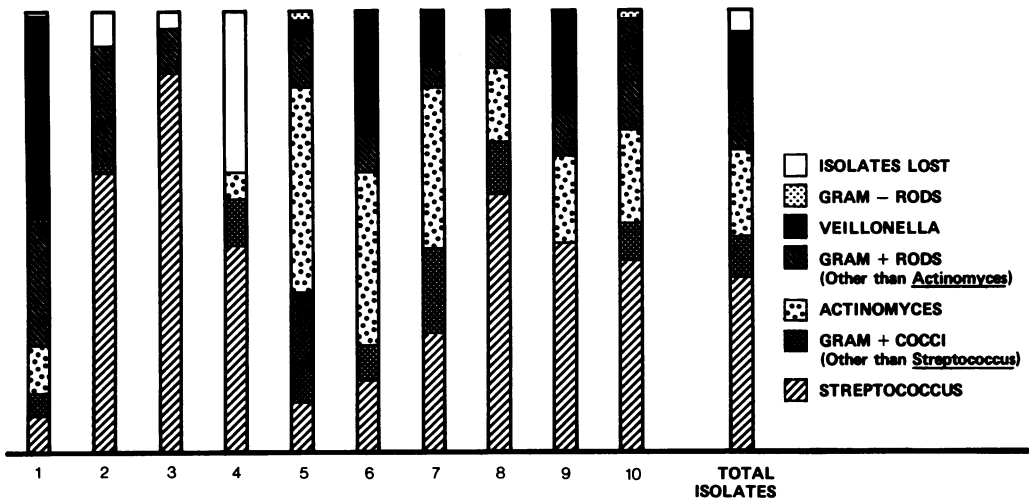
FIG. 1. Predominant cultivable flora of 10 fissures implanted in 10 dental students. Percentage distribution of 51 to 67 isolates from each sample and of total isolates.

ual species of streptococci, for staphylococci, for *Actinomyces naeslundii*, for *Arachnia propionica*, for gram-negative, facultative rods, and for isolates lost before identification. The plates yielded slightly higher proportions than the roll tubes (Table 3) of *A. viscosus* (14% of the total isolates versus 5%), *Gemella* species (3% versus 0.2%), and gram-negative, facultative cocci (1% versus 0.2%). On the other hand, the plates gave slightly lower proportions than the roll tubes of veillonellae (2% of total isolates versus 12%), lactobacillus (2% versus 4%), propionibacteria (0.6% versus 2%), and eubacteria (0.4% versus 3%).

DISCUSSION

Although the technique of studying only the predominant cultivable flora eliminated bacteria constituting small proportions of the flora, most samples yielded several species (2 to 11 species; median, 8; Table 3). The species present together as well as their proportions varied greatly from one sample to another. Although the roll tube counts were consistently higher than the plate counts, no fastidious anaerobes were isolated. All roll tube isolates subsequently grew on anaerobic plates, and most were facultative. Most commonly found were *A. viscosus*, *S. epidermidis*, *S. mutans*, *A. naeslundii*, *V. parvula*, *A. propionica*, *Propionibacterium*, and *S. sanguis*, which were isolated from at least half of the samples. The distribution of species correlates well with the microscopic (20) and ultrastructural (8, 23, 24) observations reported earlier. These indicate, however, that the numerous actinomyces isolates probably occur as short rods or coccoid forms in the depth of the fissure and as long rods or filaments in the orifice.

S. mutans as identified according to Table 1 constituted 0 to 86% (median, 25%) of the predominant cultivable flora of each sample. In a previous study of the same samples (20), colony counts of *S. mutans* on mitis salivarius agar constituted 0 to 75% (median, 11%) of the viable counts on a nonselective medium, whereas *S. mutans* counts on MSB agar (mitis salivarius agar with bacitracin and extra sucrose) constituted only 0 to 20% (median, 4%). These differences may be due to a suppression of *S. mutans* on the selective media or a failure to recognize atypical colonies on mitis salivarius agar. It should, however, also be remembered that the method of isolating the predominant cultivable flora eliminates all species present in small proportions. The species isolated will therefore constitute a larger proportion of the predominant cultivable flora than of the total flora.

Eight of our ten fissures showed a predominance of species that have been associated with fissure caries (12, 15). Six had high proportions of *S. mutans*, one had high proportions of *S. mutans*, and *Lactobacillus casei*, and one had high numbers of *L. plantarum*. Still, all fissures were clinically and microradiographically caries-free. There are several possible explanations for this: (i) the period of implantation may have been too short, although fissure caries often develops within the 1st year after eruption (1); (ii) the subjects were dental students, who were probably relatively caries inactive; and (iii) the subjects probably did not consume sugar very frequently. The two daily applications of sucrose solution to the fissure may have been insufficient to change this pattern. Our finding of cariogenic bacteria in intact fissures is also in agreement with the results of longitudinal stud-

ies in children (12, 15), where some fissures had high *S. mutans* levels throughout observation periods of up to 4 years without developing caries.

On the basis of the data available from ultra-structural and cultural studies (18, 20–25), it may be suggested that occlusal fissures after implantation or eruption in the mouth are rapidly colonized with the bacteria which predominate in the saliva of that particular mouth. In a few days the bacteria proliferate to occupy the whole fissure space, replacing the food debris initially seen. After 7 days, areas with degenerating bacteria as well as foci of mineralization are found. Viable counts, however, are similar after 7 days and after 200 to 270 days (20, 21). It must be difficult for new bacteria to enter the confined space once it is colonized, and the flora remains largely gram positive and facultatively anaerobic. Certain changes in microbial proportions with time have been noticed, such as an increase in *S. mutans* (18, 20), decrease in *S. sanguis* and *S. salivarius* (20, 21), increase in gram-positive rods, and decrease in acid-forming bacteria (25). Such changes may be due to proliferation at different rates and to varying tendencies to degeneration and mineralization of the different species present. The ecology of each fissure is most likely dependent on the nutrients available from saliva and food and on microbial synergistic and antagonistic phenomena.

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