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Proportional Distribution and Relative Adherence of *Streptococcus miteor* (*mitis*) on Various Surfaces in the Human Oral Cavity

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A group of streptococci possessing the characteristics of *Streptococcus miteor* (*S. mitis*) was found to predominate on nonkeratinized human oral mucosa. These organisms averaged from 76 to 89% of the total flora cultivable on anaerobically incubated blood agar plates from cheek, lip, and ventral tongue surfaces. They averaged 34, 40, and 18% of the streptococci in dental plaque, in saliva, and on the tongue dorsum, respectively. Their ability to adhere to oral surfaces was studied by introducing mixtures of streptomycin-resistant strains of *S. miteor*, *S. salivarius*, and *S. mutans* into the mouths of volunteers. Samples of oral surfaces taken 1 hr later indicated *S. miteor* adhered far better than the other streptococci to buccal mucosa and to teeth, but *S. salivarius* showed a higher affinity to the tongue dorsum. Glucose-grown cells of *S. mutans* adhered feebly to all oral surfaces studied and were rapidly cleared from the mouth. Cells of *S. miteor* and *S. salivarius* present naturally in saliva adhered to cleaned teeth comparable to in vitro cultivated strains. Electron microscopy of cells of *S. miteor* attached to buccal epithelial cells obtained from germfree rats indicated that the organisms possessed a fibrillar "fuzzy" coat which appeared to mediate their attachment to the epithelial cell membrane. This "fuzzy" coat was removed by treatment with trypsin, and it appears to be similar to that previously observed on cells of *S. pyogenes* and *S. salivarius*.

Different bacterial species are known to preferentially colonize various sites within the human oral cavity (2, 4, 13, 16, 21). The oral streptococci, *Streptococcus sanguis* and, when present, *S. mutans*, are found in highest proportions on the tooth surface, whereas *S. salivarius* favors the dorsal surface of the tongue (2, 4, 23, 24). Collectively these three species comprise approximately one-half of the streptococci cultivable from the tooth surface and tongue, but only 15% of the streptococci recovered from the vestibular mucosa (2, 4, 13, 23, 24). The present investigation describes a group of streptococci, possessing the characteristics of *S. miteor* (*S. mitis*), which predominate on human vestibular mucosa. The proportional distribution of these organisms within the human oral cavity was determined, and their ability to adhere to surfaces in the mouth as related to their ecological localization was studied.

MATERIALS AND METHODS

Cultures. Swabbings of human vestibular mucosa obtained from five individuals were cultured on blood agar plates which were incubated anaerobically. Twenty-one strains of streptococci possessing comparable colonial morphology were isolated, cloned to assure purity, and characterized by standard biochemical procedures. Rantz and Randall (18) antigen extracts were prepared from each strain and tested for reactivity against Lancefield streptococcal grouping sera (Difco) for groups A through T and Mg. All positive reactions were confirmed by agar double-diffusion analyses.

S. mutans strains 6715 and BHT, *S. salivarius* strains 9GS2 and Su, and *S. miteor* strains 26 and B1 were obtained from the culture collection of the Forsyth Dental Center. Streptomycin-resistant mutants were prepared by repeated transfer of the strain in Trypticase soy broth (BBL) containing increasing amounts (up to 2,000 μg/ml) of streptomycin as previously described (24, 16). No apparent colonial or biochemical differences were observed between the parent strains and streptomycin-resistant mutants. All strains were transferred weekly on mitis-salivarius agar (Difco) plates and in tubes of Trypticase soy broth. Cultures of streptomycin-resistant strains were examined periodically for the appearance of streptomycin-sensitive cells by comparing viable counts on

mitis-salivarius agar with and without 200 μg of streptomycin per ml.

Subjects and sampling procedures. Fifteen males and five females 20 to 39 years of age participated in the study. No restrictions were placed upon dietary or other habits, unless noted otherwise. All oral surfaces were rinsed with saline prior to sampling. Epithelial surfaces and precleaned teeth were sampled by vigorous swabbing with Calgiswabs. Samples of dental plaque were collected from surfaces of anterior and posterior teeth by using sterile curettes. Care was taken to avoid touching the gingiva throughout the sampling procedures. Saliva was collected in sterile tubes.

Cultural methods. Calgiswab samples from teeth and epithelial surfaces, as well as dental-plaque samples, were dispersed in modified Ringer solution as previously described (23). Appropriate dilutions were spread with bent glass rods on duplicate plates of mitis-salivarius agar for the enumeration of *Streptococcus* species and on brain heart infusion agar (Difco) supplemented with 5% horse blood for determining the total cultivable count. All plates were incubated anaerobically for 2 to 3 days at 35 C. Plates for the enumeration of *S. mutans* were left aerobically at room temperature for 1 day prior to enumeration. Colonies of *S. miteor* were recognized by their distinctive morphology on mitis-salivarius agar as described in Results. Colonies of *S. sanguis*, *S. salivarius*, and *S. mutans* were identified by the morphological criteria suggested by Carlsson (2), Krasse (13), and Jordan, Englander and Lim (11).

Adherence of streptomycin-resistant streptococci to oral surfaces. Streptomycin-resistant strains of *S. miteor*, *S. mutans*, and *S. salivarius* were cultivated for 24 hr anaerobically in Trypticase soy broth containing 2.5% glucose. The organisms were collected by centrifugation, washed twice, and suspended and standardized in sterile Ringer solution as previously described (16). The streptococcal suspensions were exposed to 15 sec of sonic oscillation to reduce chain length and increase colony-forming units. Mixtures containing approximately 5×10^8 colony-forming units of each species were prepared, and 1-ml samples were introduced into the mouths of six volunteers. The subjects were instructed to distribute the mixture throughout their oral cavity for 10 min. All oral fluid was then expectorated. Samples of dental plaque and swabbings of the tongue and vestibular mucosa were collected 60 min later. The proportion of each streptomycin-resistant species in the samples was determined by culturing on plates of mitis-salivarius agar containing 200 μg of streptomycin per ml, as described (16, 24).

Adherence naturally occurring by S. miteor to cleaned tooth surfaces. The facial surfaces of upper incisors and molars of five subjects were cleaned to reduce their bacterial populations to negligible levels as previously described (16, 23). The subjects were asked to refrain from eating or drinking. After 15 min, the same tooth surfaces were rinsed with sterile saline and sampled for the presence of cells of *S. miteor*, *S. salivarius*, and *S. sanguis* which had adsorbed to the surface from saliva.

Adherence of S. miteor to epithelial cells in vitro. The adherence of *S. miteor* to cheek cells obtained from germfree rats was determined as previously described (7, 8). Trypsin treatment was performed by adding 1% trypsin (×3 salt-free crystalline, Nutritional Biochemicals Corp.) to exponential-phase cultures growing in Trypticase soy broth (8). Untreated cultures served as controls.

Electron microscopy. Bacteria and epithelial-cell bacterial mixtures were prefixed in a modified Palades buffer containing 3.3% gluteraldehyde (*p*H 7.3) for 18 hr. Postfixation was accomplished in Kellenberger fixative (12) for 4 hr. The cells were dehydrated and embedded in Epon 812 by a modification of the procedure of Luft (17). Thin sections were prepared on an LKB Ultratome, mounted on unsupported grids, and stained with a saturated solution of uranyl acetate followed by Reynold's lead citrate (19). All electron micrographs were taken with an RCA-3G electron microscope.

RESULTS

Characteristics of oral isolates of S. miteor. During study of the distribution of various bacteria in different sites in the oral cavity, we observed that samples obtained from human vestibular mucosa which had been cultured on anaerobically incubated blood agar plates contained a predominating colonial type. In contrast, samples from the tongue dorsum or tooth surface contained complex mixtures of bacterial colonies. Twenty-one strains possessing the same characteristic alpha hemolytic colonial morphology were isolated from the vestibular mucosa of five individuals. All isolates consisted of gram-positive, catalase-negative cocci. They formed unusually long chains, which frequently contained over 200 cells when cultured in Trypticase soy broth. The isolates proved relatively homogeneous in terms of their biochemical characteristics. All fermented glucose, fructose, sucrose, maltose, and lactose, and all formed iodine-staining intracellular glycogen. None fermented arabinose, melibiose, inulin, cellobiose, mannitol, sorbitol, or trehalose. Raffinose was fermented by two strains. None reduced nitrate or grew in broth at *p*H 9.6 or in the presence of 4 or 6.5% NaCl. No strain produced ammonia from arginine, and H_2S was not detected. Alcohol-precipitable polysaccharides were not detected after growth in sucrose broth. All strains aggregated with whole, clarified human saliva when tested as previously described (6). The isolates proved serologically heterogeneous, and three strains reacted with Lancefield group H serum, three with group O serum and one with group M serum. These characteristics fit the description of *S. miteor* (*mitis*) based on the criteria of *Bergey's Manual*, of Carlsson (2), and of Guggenheim (9).

Although the strains of *S. miteor* stud-

ied formed colonies of homogeneous morphology on anaerobically incubated blood agar plates, they produced three distinctive colonial types when cultured on mitis-salivarius agar. On this medium the colonies were flat with regular margins and were either light blue or brown. Strains forming light-blue colonies had a tendency to develop a "warty" appearance after 3 days of incubation, whereas this transformation was infrequent in strains forming brown colony types. No correlation was observed between the blue, brown, or warty colonies and other characteristics studied. The colonies of *S. miteor* on mitis-salivarius agar could be readily distinguished from those of *S. salivarius*, *S. mutans*, and *S. sanguis*.

Distribution of S. miteor in various sites in the human oral cavity. Data concerning the proportional distribution of *S. miteor* in various sites in the mouths of 10 subjects are listed in Table 1. *S. miteor* was found to predominate on the nonkeratinized epithelial surfaces of the mouth, including the lip, cheek, and ventral surface of the tongue. On these surfaces, it averaged approximately 60% of the total anaerobically cultivable flora and from 76 to 89% of the streptococci. *S. miteor* comprised a much smaller percentage of the total cultivable bacteria on the dorsal surface of the tongue, and it represented about one-third of the streptococcal populations colonizing teeth.

Relative adherence of streptomycin-resistant strains of S. miteor to oral surfaces. The adherence of streptomycin-resistant strains of *S. miteor* artificially introduced into the mouth was studied. For comparative purposes, streptomycin-resistant strains of *S. salivarius* and *S. mutans* were included in the bacterial mixtures. It was found that strains of *S. miteor* possessed a high affinity for the vestibular mucosa in comparison to *S. salivarius* and *S. mutans*, because the proportions of *S. miteor* recovered from cheek surfaces were higher than those present in the mixture introduced into the subjects' mouth in all cases (Table 2). Comparable data were obtained with samples of dental plaque scraped from tooth surfaces. Because *S. miteor* was found to comprise approxi-

TABLE 1. *Proportional distribution of Streptococcus miteor (mitis) in the human oral cavity*

	Total cultivable flora (%)[a]		Streptococci (%)[a]	
	Mean	Range	Mean	Range
Lip	68	(30–98)	89	(60–96)
Cheek	65	(37–82)	76	(37–97)
Tongue ventral	59	(20–86)	79	(41–97)
Tongue dorsum	9	(1–28)	17	(6–38)
Plaque	20[b]	(1–45)	34	(1–83)
Saliva	22	(8–39)	40	(17–74)

[a] Mean percentages from 10 subjects are expressed as percentage of bacteria on anaerobically incubated plates.

[b] Mean percentages from 20 subjects.

TABLE 2. *Streptomycin-resistant Streptococcus miteor (mitis), S. salivarius, and S. mutans adhering to dental plaque of unknown age, tongue dorsum, and vestibular mucosa*

Subject	Strain	Mixture (%)	Vestibular mucosa (%)	Tongue dorsum (%)	Plaque samples (%)[a]			
					1	2	3	4
J.S.	*S. miteor* B1-R	35	77	38	85	72	86	84
	S. salivarius Su-R	28	18	51	11	19	11	8
	S. mutans BHTR	37	5	11	4	9	3	8
D.K.	*S. miteor* 26R	19	92	22	67	92	83	27
	S. salivarius Su-R	33	7	65	3	8	10	62
	S. mutans BHTR	48	1	13	30	1	7	11
L.O.	*S. miteor* 26R	26	60	4	97	96	99	41
	S. salivarius Su-R	68	38	87	3	3	1	46
	S. mutans 6715	6	2	9	1	1	1	13
A.F.	*S. miteor* 26R	14	91	8	83	72	59	70
	S. salivarius Su-R	68	1	89	11	21	26	1
	S. mutans BHTR	18	6	3	6	7	15	29
W.L.	*S. miteor* 26R	41	85	44	82	71	75	95
	S. salivarius Su-R	51	10	48	1	8	1	1
	S. mutans 6715	8	5	8	17	21	24	4
C.S.	*S. miteor* B1-R	18	57	7	90	50	73	72
	S. salivarius 9GS2	7	30	86	9	28	18	13
	S. mutans 6715	75	13	7	1	22	9	15

[a] Each sample was obtained from a different site.

mately three-fourths of the streptococci present indigenously on the vestibular mucosa and one-third of the streptococci present in dental plaque (Table 1), and because *S. salivarius* and *S. mutans* have been previously shown to average less than 2% of the streptococci present in these sites (4, 23, 24), it is evident that the relative adherence of *S. miteor* to vestibular mucosa and dental plaque correlates with the proportions of the organism found indigenously.

In striking contrast to these observations, swabbings from the dorsum of the tongue contained high proportions of labeled *S. salivarius* relative to its proportions in the original mixture and to the proportions of *S. miteor* and *S. mutans*. *S. salivarius* has been previously found to comprise approximately one-half of the streptococci indigenous on the tongue dorsum (4, 13), whereas *S. miteor* (Table 1) and *S. mutans* (2, 7, 23) are present in smaller proportions.

Adherence of naturally occurring Streptococcus species to clean tooth surfaces. It is possible to reduce the bacterial populations on teeth to negligible levels by thorough cleaning and swabbing (16, 23). Because *S. miteor* and *S. salivarius* comprise 30 to 40% of the streptococci in saliva, whereas *S. sanguis* generally averages 10 to 20% (Table 1) (4, 13, 23, 24), it was possible to determine their affinity for the tooth surface by comparing their proportions which adsorbed to clean teeth with those in saliva. It was found that naturally occurring cells of *S. miteor* adhered to cleaned teeth comparably to those of *S. sanguis*, whereas cells of *S. salivarius* adhered poorly (Table 3). Thus, the relative adherence of these *Streptococcus* species, as they exist naturally in saliva, agrees well with the adherence of artificially introduced streptomycin-resistant strains to teeth.

TABLE 3. *Distribution of Streptococcus miteor, S. sanguis, and S. salivarius in the total streptococci adherent to the teeth 15 min after cleaning*

Subject	Tooth	Percentage of total streptococci		
		S. sanguis	*S. miteor*	*S. salivarius*
A	Molar, incisor	35	56	1
		26	55	2
B	Molar, incisor	60	20	<1[a]
		79	8	<1
C	Bicuspid, incisor	16	79	<1
		12	34	<1
D	Molar, incisor	54	23	8
		33	50	2
E	Bicuspid, incisor	17	27	1
		18	14	2

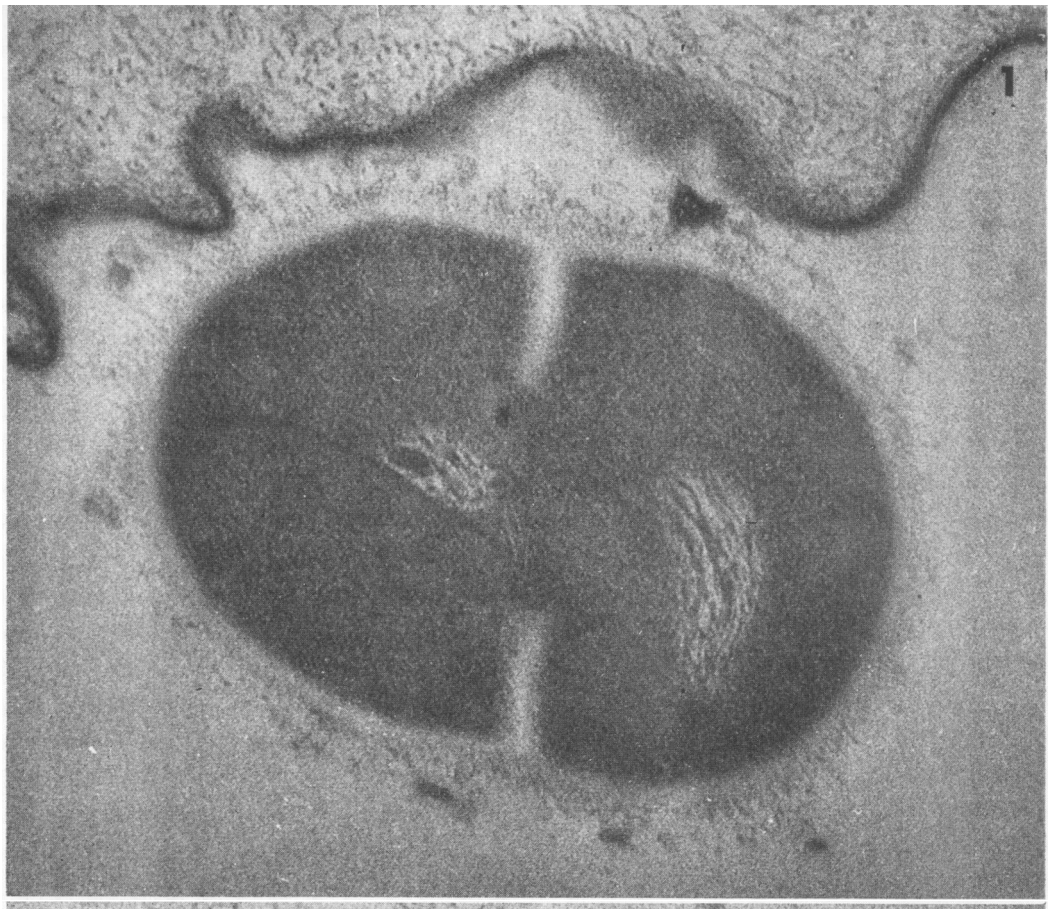
[a] <, No *S. salivarius* on the plates.

In vitro studies of the adherence of S. miteor to epithelial cells. The ultrastructural morphology of cells of *S. miteor* attached to cheek epithelial cells obtained from germfree rats is shown in Fig. 1 and 2. The streptococci were found to be surrounded by a dense, fibrillar material which gave the appearance of a "fuzzy" surface coating. This fibrillar material appeared to bind the streptococci directly to the epithelial cell membrane. Cells of *S. miteor* treated with trypsin were found to lose much of their fuzzy surface coat, as shown in Fig. 3 and 4. This enzyme did not otherwise detectably affect the integrity of the cells and, in fact, *S. miteor* grew well in broth supplemented with trypsin.

DISCUSSION

Previous studies of the distribution of *Streptococcus* species in various sites in the mouth have focused upon *S. salivarius*, *S. sanguis*, and *S. mutans*. These species differentially synthesize extracellular glucans and fructans from sucrose and, consequently, they form characteristic colonies on mitis-salivarius agar, which facilitates their identification. Collectively, these species have been found to account for 50% of the streptococci cultivable on mitis-salivarius agar from the surfaces of teeth and from the dorsum of the tongue. However, they comprise only 15% of the streptococci cultivable from vestibular mucosa (2, 4, 13, 23, 24). The present investigation has shown that a large percentage of bacteria referred to as "other streptococci" in these studies are strains of *S. miteor*. This species averaged 76% of the streptococci recoverable from vestibular mucosa and 34% of the streptococci recoverable from dental plaque. *S. miteor* was found to be the predominant bacterial species colonizing human vestibular mucosa and other nonkeratinized oral surfaces, and it also comprises a significant percentage of the bacterial populations present on other oral surfaces. The 21 isolates of *S. miteor* studied were not observed to synthesize extracellular polysaccharides from sucrose, and consequently their colonies could be readily distinguished from *S. salivarius*, *S. sanguis*, and *S. mutans* on mitis-salivarius agar. The isolates proved biochemically homogeneous, but differed serologically. They formed three distinctive colony types on mitis-salivarius agar. It is not known if additional colony types of *S. miteor* exist. If other types do occur, they were considered in the present investigation.

Mixtures of in vitro-cultivated streptomycin-resistant cells of *S. miteor*, *S. salivarius*, and *S. mutans* introduced into the mouths of volunteers indicated that these organisms possess widely different abilities to adhere to oral surfaces. *S.*



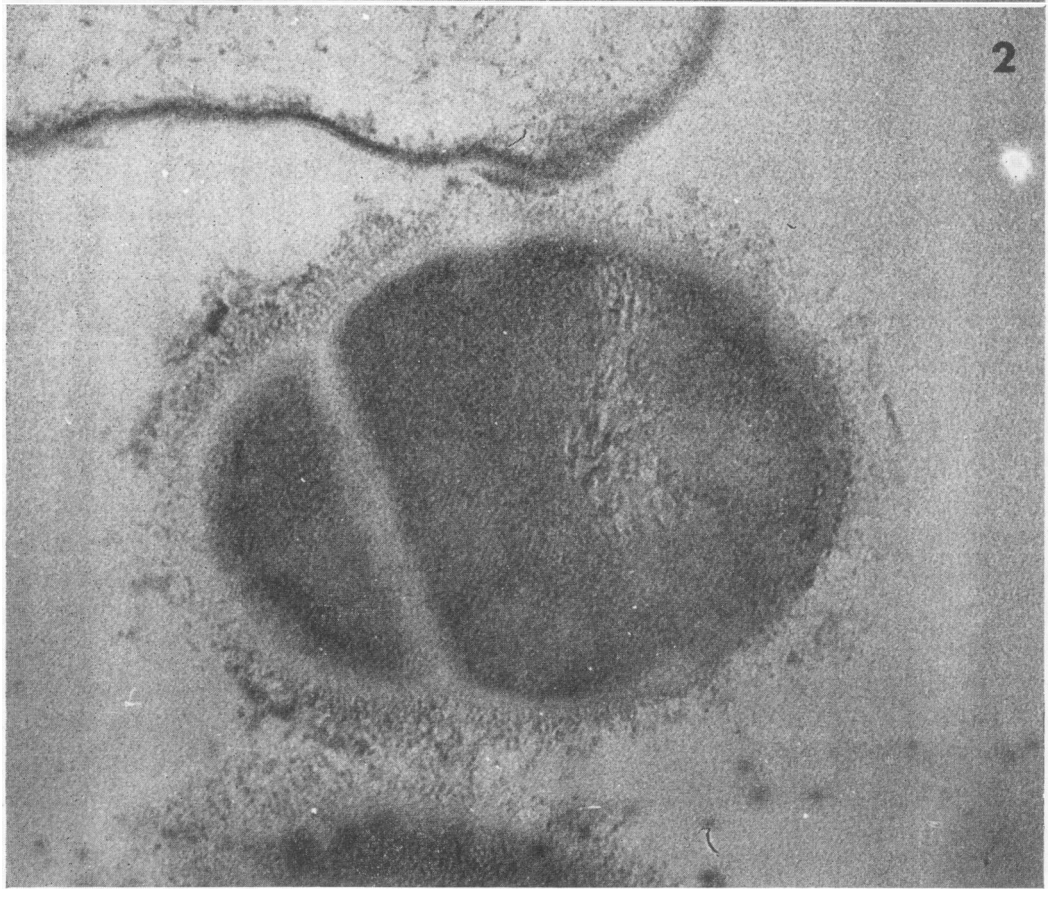
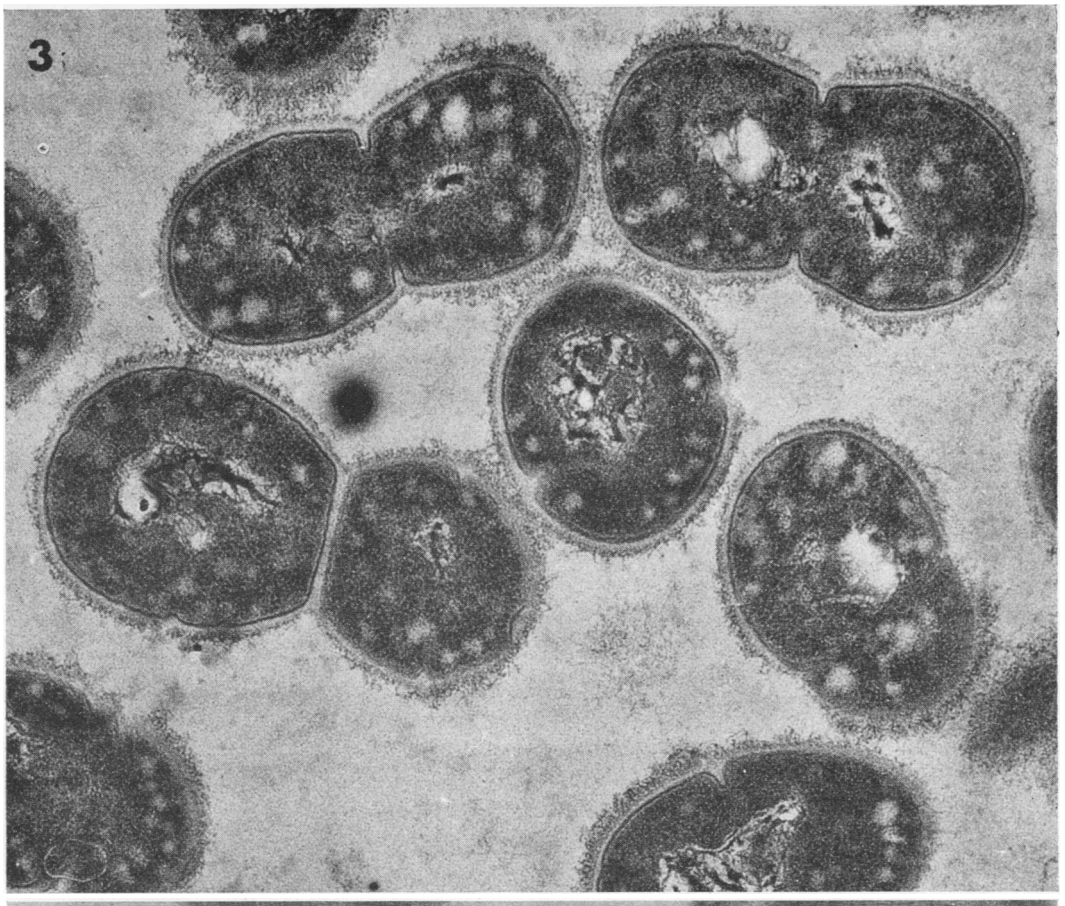


FIG. 1–2. *Streptoccus miteor 26 cells attached to germfree rat cheek cells show that the fibrillar "fuzzy" coat of the bacteria mediates the adherence to the epithelial cell membrane.* ×78,000.



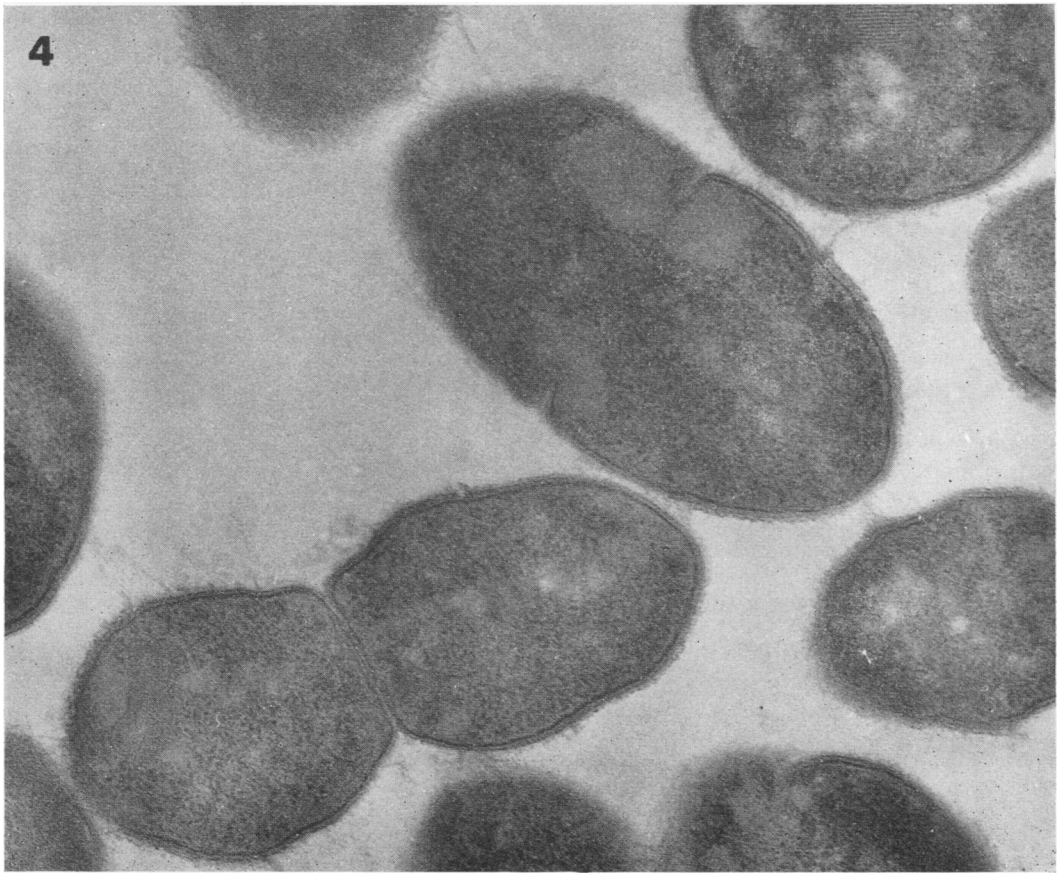


FIG. 3. *Untreated control cells of Streptococcus miteor show the surface "fuzzy" coat. ×41,000.*
FIG. 4. *Streptococcus miteor after trypsin treatment shows the loss of its "fuzzy" coat. ×46,000.*

miteor adhered far better than *S. salivarius* to cheek surfaces, where *S. salivarius* exhibited a high afflnity for the dorsum of the tongue. The adherence of streptococci, naturally present in saliva, to cleaned tooth surfaces was found to be comparable to that of the in vitro-cultivated streptomycin-resistant strains, suggesting that profound differences do not exist in the adherence of the organisms as they grow naturally in the mouth. As has been shown for other bacteria studied (6, 16, 23, 24), the proportions of *S. miteor* found naturally in sites within the mouth seem to be directly related to the organisms' ability to attach to the respective surface. Bacteria which do not attach to a surface are simply washed away by bathing fluids and swallowed. The striking differences in adherence observed between species indicates that marked differences must exist on the surfaces of cheek and tongue epithelial cells. This type of specificity has been observed previously (1, 7, 16, 20, 24), and the data imply that there are epithelial cell "receptor sites" for bacteria which are quite analogous to those involved in virus attachment.

The adherence of *S. mutans* to teeth in vivo has not been previously studied. This organism possesses a high cariogenic potential and is known to colonize teeth selectively (2, 15). *S. mutans* does not comprise a significant proportion of the streptococci on oral epithelial surfaces (2, 24), and in the absence of dietary sucrose, it is generally unable to effectively colonize teeth (14, 15). Sucrose is required for synthesis of glucans and fructans which are involved in the adherence of *S. mutans* to hard surfaces (5, 7). The feeble adherence of glucose-grown cells of *S. mutans* to teeth and oral surfaces (7, 24) suggests that the organism would be rapidly cleared from the mouth. This appears to explain why this *Streptococcus* does not readily colonize the oral cavity of man and animals in the absence of sucrose.

It has been suggested that the adherence of certain bacterial species to tooth surfaces is influenced by high-molecular-weight salivary glycoproteins which absorb to hydroxyapatite and enamel powder (6, 10). All strains of *S. miteor* studied were found to aggregate with saliva, and they exhibited a high relative adherence to tooth surfaces. These data support the hypothesis that salivary glycoproteins, which adsorb both to teeth and to bacteria, may influence dental plaque formation.

Cells of *S. miteor* attached to cheek cells obtained from germfee rats indicated that the organisms possessed a trypsin-sensitive, fibrillar surface coating which appeared to bind them directly to the epithelial cell membrane. This surface component is similar to that previously observed on cells of *S. pyogenes*, which represents M protein (3, 22), and on *S. salivarius* (8). The fuzzy coating of these organisms is also trypsin sensitive and has been shown to be involved in their adherence to epithelial surfaces (3, 8). Virtually all of the gram-positive bacteria indigenously present on human cheek cells possess a similar fuzzy coat which mediates their attachment (7). Thus, this surface component appears to serve a vital ecological function in a wide variety of bacteria.

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