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Impaired Colonization of Gnotobiotic and Conventional Rats by Streptomycin-Resistant Strains of *Streptococcus mutans*

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Colonization of streptomycin-resistant mutants derived from *Streptococcus mutans* strain LB1, a human isolate, and strain FA-1, a rodent isolate, was studied in gnotobiotic and conventional rats. Mutants resistant to 2.0 mg of streptomycin per ml were isolated by using both stepwise (suffix "R"M) and one-step (suffix "R"1) selections. Rats were infected with mixtures of parental and streptomycin-resistant strains, and the proportions of each strain present in samples from the intestinal canal, tongue dorsum, teeth, and fissure plaque were determined. Combinations of strains investigated were LB1 and FA-1"R"M; FA-1 and LB1"R"M; LB1 and LB1"R"1; FA-1 and FA-1"R"1. In gnotobiotic rats, nonresistant strains predominated in every oral sample studied at 7 and 21 days after infection. Similarly, when conventional exgermfree rats were infected with FA-1 and FA-1"R"1, FA-1 dominated in all samples. Streptomycin-sensitive revertants were not detected in rats monoinfected with strains LB1"R"1 and FA-1"R"1 for 21 days. No antagonistic interactions were observed between the strains in in vitro experiments. Streptomycin-resistant mutants attached to hydroxyapatite treated with rat or human saliva in equal or higher numbers than did parental strains. However, parental strains appeared to grow faster in Trypticase soy broth than streptomycin-resistant mutants. These observations indicate that induction of streptomycin resistance frequently impairs the colonization properties of *S. mutans* strains, possibly by altering their rate of growth.

Organisms of the *Streptococcus mutans* group have been studied extensively because they are associated with the etiology of dental caries (14). Streptomycin-resistant (SR) mutants of *S. mutans* have been used in many investigations because they can be easily and sensitively quantitated in samples of mixed bacterial populations by use of media containing streptomycin (10, 11, 19, 30, 33). Studies which have relied upon use of SR mutants of *S. mutans* include those investigating: (i) implantation of the organism in the mouths of humans, primates, and rodents (6, 8, 11, 19, 21, 24, 36); (ii) its transmission between individuals (17); (iii) its interdental transmission (7); (iv) its ability to attach to teeth and other oral surfaces (6, 31, 33, 35); (v) its cariogenic potential in animal models (3, 10, 11, 19, 24, 29); and (vi) the effects of antibodies on its colonization and virulence (8, 22, 24, 25, 29). During the course of studying the colonization of gnotobiotic rats by *S. mutans* strains isolated from humans and rodents, we observed that SR mutants colonized less effectively than wild-type strains; the present report describes these studies.

MATERIALS AND METHODS

Cultures and cultural conditions. *S. mutans* strain LB1 (2), a human isolate, and strain FA-1 (9), a rat isolate, were studied; both belong to Bratthall serogroup b. The organisms were propagated in Trypticase soy (TS) broth (Baltimore Biological Laboratory) and in fluid thioglycolate medium (Baltimore Biological Laboratory). Cultures were incubated in Brewer jars containing an atmosphere of 80% N_2–10% H_2–10% CO_2 for 24 to 48 h at 35°C.

SR mutants were isolated by two procedures. The first was a stepwise selection, previously used by several investigators (10, 11, 13, 19, 33), which consisted of sequential transfer in TS broth containing 2 to 2,000 μg of streptomycin per ml. Mutants obtained upon initial inoculation were not resistant to concentrations of streptomycin greater than 50 μg/ml, and several transfers were generally required to obtain mutants resistant to 2 mg/ml. Such multiple-step mutants were designated LB1"R"M and FA-1"R"M. The second procedure was a one-step selection in which approximately 10^9 cells were spread on the surface of TS agar plates containing 2 mg of streptomycin per ml; mutants isolated in this manner were designated LB1"R"1 and FA-1"R"1. When cultivated on TS agar, colonies of strains LB1, LB1"R"M, and LB1"R"1 were smooth. Colonies of wild-type strain FA-1 and mutant

FA-1"R"1 were rough, whereas those of mutant FA-1"R"M were smooth.

For experimental purposes, the streptococci were grown in a chemically defined medium as previously described (12). Twice-washed streptococcal suspensions in 0.01 M phosphate-buffered saline (pH 7.2) were prepared from 18-h cultures; these were standardized to contain approximately 10^8 organisms per ml. Mixtures of SR and streptomycin-sensitive strains were prepared, and the number of colony-forming units of each strain present was determined by plating appropriate dilutions in modified Ringer solution (32) on TS agar plates. After 48 h of incubation, all colonies present on an entire plate, or on a marked segment, were picked and spotted on mitis salivarius agar and on mitis salivarius agar containing 200 μg of streptomycin per ml; between 50 and 100 colonies were tested from each sample. Two colonies of each parental and mutant strain were also spotted on the media to serve as controls. Colonies which failed to grow on streptomycin-containing mitis salivarius agar were considered parental types; colonies which grew on both media were considered SR mutants. This procedure was more laborious than performing differential counts on the two media; however, it provided a more reproducible estimation of the proportions of each strain in experimental and natural mixtures.

Colonization of parental and SR strains of *S. mutans* in the intestinal canal and oral cavity of germfree rats. Germfree Sprague-Dawley rats were maintained in flexible plastic isolators and fed diet 2000 (20). Samples (0.1 ml) of the streptococcal mixtures were placed in the mouths of each of six 21-day-old rats comprising a group. Fecal samples and swab samples from the tongue dorsum were obtained from each of three rats 7 and 21 days later. Three upper molar teeth on one side of the mouth were carefully extracted, and, in addition, samples of bacterial deposits present in the occlusal fissures of the three upper molar teeth present on the opposite side of the mouth were taken with a sterile explorer. The fecal samples were suspended in Ringer solution and ground in a tissue grinder; the tongue swabs were mixed in Ringer solution for 1 min with a Vortex mixer; the extracted molar teeth were ground in a tissue grinder with Ringer solution, and the samples of fissure plaque were suspended in Ringer solution by blending in a Vortex mixer in the presence of small glass beads for 2 to 3 min. The proportions of parental and SR mutant strains present in the suspensions were determined as described.

Groups of three animals were also monoinfected with strains LB1"R"1 and FA-1"R"1. Samples of feces and upper molar teeth were obtained 21 days later; these were analyzed for streptomycin-sensitive revertants as described.

Colonization of *S. mutans* strains FA-1 and FA-1"R"1 in conventional rats. Twenty-one-day-old rats derived from conventional exgermfree parents were caged separately and fed diet 2000. Fecal and oral samples collected from each animal over a 2-week period were cultured on mitis salivarius agar and on mitis salivarius bacitracin medium (15) to confirm that the animals were not infected with *S. mutans*. Six rats free of detectable levels of this organism were then orally inoculated (0.1 ml) with approximately 10^8 cells each of strains FA-1 and FA-1"R"1. Three animals were sacrificed 13 and 21 days after infection, and the proportions of the parent and mutant strain present in fecal, tongue, tooth, and fissure plaque samples were determined as described. Non-inoculated animals were used as controls; these remained free of detectable levels of *S. mutans*.

Adsorption of parental and SR strains of *S. mutans* to saliva-treated HA. [^{3}H]thymidine-labeled *S. mutans* cells were prepared as previously described (4, 6). Whole rat saliva was obtained from 20 exgermfree rats (21 days old) injected intraperitoneally with 33 to 66 mg of Ketamine hydrochloride (Bristol Laboratories) per kg of body weight and 10 mg of pilocarpine nitrate (ICN Pharmaceuticals, Inc.) per kg of body weight. The saliva was pooled, heated at 60°C for 30 min, and clarified by centrifugation. Samples of whole clarified human saliva were prepared as described previously (4, 6). The adsorption of ^{3}H-labeled streptococci to spheroidal hydroxapatite (HA) beads pretreated with either rat or human saliva was determined as described previously (6).

In vitro growth experiments. The growth rates of pure cultures of parental and mutant strains were compared in tubes of TS broth given a 10% (vol/vol) inoculum. The tubes were flushed with 80% N_2–10% H_2–10% CO_2, sealed with rubber stoppers, and incubated in a water bath at 37°C. Growth was monitored turbidimetrically at 550 nm.

Growth of the strains as mixtures was also compared. Tubes of TS broth were inoculated with 5×10^6 cells of the parent and mutant strains. Samples taken periodically were cultured to determine the ratio of each strain present. The proportions of each strain recovered at a given sampling period were multiplied by the reciprocal of their ratio present in the mixture used as inoculum to reflect equal opportunity for growth; these ratios were then converted into percentages. The pH of each sample was also determined.

RESULTS

Colonization of parental and SR mutants of *S. mutans* in germfree rats. In initial experiments designed to compare the ability of human and rodent isolates of *S. mutans* to colonize gnotobiotic rats, animals were inoculated with a mixture of strains LB1 and FA-1"R"M. Analyses of fecal and oral samples taken 7 and 21 days after infection indicated that strain LB1 comprised more than 95% of the streptococcal populations in the rats, even though LB1 was a human isolate and FA-1 was derived from rats (Table 1). Similar results were obtained with rats fed diet L-356, which does not contain high levels of sucrose (data not shown). However, when animals were inoculated with strain FA-1 and LB1"R"M, the results were quite different. Nonresistant rodent strain FA-1 predominated in tongue, tooth, and fissure samples, whereas strain LB1"R"M only predominated in fecal samples (Table 1).

These observations suggested that streptomycin resistance impaired the colonization properties of these *S. mutans* strains. To investigate this possibility further, the ability of SR mutants obtained by one-step selection to compete with their respective parental strains was studied. In animals inoculated with strains LB1 and LB1"R"1, streptomycin-sensitive isolates, presumably derived from parent strain LB1, were recovered in somewhat higher proportions than SR mutants in all samples 7 days after infection, and the differences between strains became greater 21 days after infection (Table 2). Even larger differences were noted with parental and SR strains of FA-1 (Table 2); parental type organisms accounted for almost 100% of the bacterial populations in every sample.

The predominance of nonresistant organisms did not appear to be due to the emergence of revertants because analyses of 199 isolates of strain LB1"R"1 and 245 isolates of strain FA-1"R"1 obtained from rats monoinfected for 21 days did not detect any streptomycin-sensitive revertants.

Colonization of FA-1 and FA-1"R"1 in conventional rats. The ability of strains FA-1 and FA-1"R"1 to colonize conventional rats harboring a potentially competing indigenous flora was also studied. As observed in gnotobiotic rats, parental strain FA-1 dominated over its SR mutant in all samples studied (Table 3).

Adsorption to saliva-treated HA and in vitro growth of parental and SR strains of *S. mutans*. SR mutants of strain LB1 adsorbed to HA pretreated with either rat or human saliva comparably to their parent strain; mutants of strain FA-1 adsorbed in somewhat higher numbers than their parent strain (Table 4). Higher numbers of all strains adsorbed to HA pretreated with human saliva than with rat saliva, irrespective of whether the organisms were derived from humans or from rats.

The approximate doubling times of TS broth

TABLE 3. *Colonization of conventional rats by S. mutans strain FA-1 and SR strain FA-1"R"1*

Sample	% FA-1 recovered	
	13 days	21 days
Feces	87.0[a]	97.8 ± 0.3
Tongue	94.3 ± 1.2[b]	83.0 ± 14.8
Ground teeth	96.2 ± 1.1	84.2 ± 7.7
Fissure plaque	89.2 ± 3.9	92.8 ± 1.1

[a] Only one animal sampled.
[b] Standard error of mean.

TABLE 4. *Adsorption of S. mutans strains to saliva-treated HA*

Strain	No. of streptococci adsorbed ($\times 10^{-4}$) per mg of HA treated with rat saliva[a]	No. of streptococci adsorbed ($\times 10^{-4}$) per mg of HA treated with human saliva[a]
LB1	1.5 ± 0.4[b]	8.0 ± 0.5
LB1"R"M	ND[c]	10.6 ± 1.1
LB1"R"1	1.5 ± 0.3	8.6 ± 0.8
FA-1	0.9 ± 0.1	3.5 ± 0.2
FA-1"R"M	ND	9.3 ± 0.2
FA-1"R"1	6.1 ± 0.8	14.6 ± 0.4

[a] Reaction mixtures containing 7.3×10^5 *S. mutans* cells per mg of HA.
[b] Mean and standard error of mean.
[c] ND, Not determined.

TABLE 1. *Colonization of germfree rats by parental and SR mutants of S. mutans LB1 and FA-1*

Sample	% LB1 recovered in animals inoculated with LB1 and FA-1"R"M		% FA-1 recovered in animals inoculated with FA-1 and LB1"R"M	
	7 days	21 days	7 days	21 days
Feces	96.6 ± 3.3[a]	100.0	10.7 ± 4.1	0.0
Tongue	99.3 ± 0.7	95.1 ± 1.8	64.0 ± 9.6	54.7 ± 3.1
Ground teeth	99.3 ± 0.6	79.2 ± 2.8	77.3 ± 4.7	70.7 ± 13.4
Fissure plaque	100.0	82.6 ± 2.6	79.5 ± 5.8	71.1 ± 9.2

[a] Standard error of mean.

TABLE 2. *Colonization of germfree rats by parental and SR mutants of S. mutans strains LB1 and FA-1*

Sample	% LB1 recovered in animals inoculated with LB1 and LB1"R"1		% FA-1 recovered in animals inoculated with FA-1 and FA-1"R"1	
	7 days	21 days	7 days	21 days
Feces	55.8 ± 3.7[a]	80.8 ± 4.1	99.4 ± 0.5	100.0
Tongue	66.0 ± 3.3	81.5 ± 4.0	100.0	100.0
Ground teeth	69.5 ± 4.9	71.6 ± 6.7	100.0	95.7 ± 4.3
Fissure plaque	77.1 ± 6.6	69.6 ± 3.6	99.7 ± 0.3	97.0 ± 3.0

[a] Standard error of mean.

cultures in early log phase were 60 and 66 min for strains LB1 and LB1"R"M, respectively, and 70 min for each of strains FA-1 and FA-1"R"M, under the static conditions used. However, the doubling times of all strains increased in mid- and late log phase, presumably due to the developing acidity; this made it difficult to definitively detect differences between strains. Consequently, the growth of combinations of strains tested in experimental animals was compared in mixed culture in TS broth. In these experiments, the proportions of parental strains increased over SR mutants in all cases studied (Table 5). Because domination tended to begin before the pH of the medium fell markedly (Table 5), it would appear that parental strains grew at a somewhat higher rate than the mutants and were not necessarily more tolerant to the acidic conditions which progressively developed. Parental and mutant strains were examined for the production of bacteriocins and other growth-inhibiting substances by procedures previously described (1, 18), but no evidence of antagonistic interactions was obtained.

DISCUSSION

Investigations of the acquisition and prevention of *S. mutans* infections have frequently used SR mutants. We have observed that SR mutants derived by either single-step or multiple-step selections colonized gnotobiotic and conventional rats less effectively than parental strains. Although the magnitude of impaired colonization varied among strains, all of the four SR mutants studied colonized the tongue dorsum and teeth less effectively than parental strains.

Hints of impaired colonization of antibiotic-resistant streptococci have previously appeared in the literature (11, 19). Keyes and Fitzgerald (11, 19) observed that SR or erythromycin-resistant strains of *S. mutans* could not be regularly isolated from albino hamsters inoculated with mixtures of resistant and nonresistant strains. Similarly, SR strains of *S. faecalis* implanted in germfree rats were quickly replaced by nonresistant enterococci when the animals were conventionalized (16). We have also observed that the minimum dose required for implantation of *S. mutans* LM-7, a streptomycin-sensitive strain, was approximately 10^4 colony-forming units for conventional rats (5), whereas the minimum dose for an SR mutant derived from strain LM-7 has been reported to be in excess of 2×10^7 colony-forming units (31).

The predominance of nonresistant streptococci observed in animals in the present study does not appear to be due to loss of antibiotic resistance because no revertants could be detected among isolates derived from the feces and teeth of rats monoinfected with strains LB1"R"1 and FA-1"R"1. Others have also noted that *S. mutans* strains retain streptomycin resistance for prolonged periods while colonizing rodents and humans (10, 28a), and Fitzgerald and Keyes (10) showed that strains retained resistance when cultured in the absence of this drug for over 125 transfers.

Mutants resistant to, or dependent upon, streptomycin possess genetically altered ribosomal subunits (23). Thus, it may be postulated that protein synthesis is in some way altered in resistant mutants, which results in cells with altered permeabilities, different rates of metabolism and growth, and different surface properties. SR mutants were found to adsorb to saliva-treated HA surfaces thought to mimic the teeth in numbers comparable to or higher than parental strains; thus, their adsorptive behavior does not explain their impaired colonization. However, resistant mutants appeared to grow more slowly than parental strains in mixed static cultures.

The increased adherence of some mutants suggests that they formed increased quantities

TABLE 5. *Growth of mixtures of parental and SR strains of S. mutans in TS broth*

Mixture	Strain	Relative % of each strain in mixed culture at:					pH of mixed culture at:			
		0 h	7 h	13 h	20 h	27 h	7 h	13 h	20 h	27 h
1	LB1	50.0	82.1	92.0	90.0	86.0	6.6	5.6	5.55	5.55
	FA-1"R"M	50.0	17.9	8.0	10.0	14.0				
2	LB1	50.0	53.9	59.1	56.5	62.8	6.5	5.5	5.35	5.35
	LB1"R"1	50.0	46.0	40.9	43.4	37.2				
3	FA-1	50.0	92.0	84.0	90.3	89.1	6.65	5.6	5.6	5.6
	LB1"R"M	50.0	8.0	16.0	9.7	10.9				
4	FA-1	50.0	58.9	64.7	68.1	66.6	6.95	6.4	5.7	5.7
	FA-1-"R"1	50.0	41.0	35.3	31.9	33.4				

of surface components capable of interacting with the salivary film on HA. This could be a manifestation of a slower growth rate because the development of thick cell walls and altered synthesis of surface components has been associated with slow growth occurring during nutrient limitation (26-28, 34).

Natural colonization of a host tissue requires that bacteria first attach to the tissue and then proliferate. Based upon the in vitro observations made, it seems that the most likely reasons for impaired colonization of SR mutants of *S. mutans* relates to parameters affecting their growth. However, although the basis of impaired colonization has not been firmly established, it seems clear that the physiological, ecological, and possibly virulence properties of SR mutants of *S. mutans* may not be identical to parental strains.

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