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Stability of the Resident Microflora and the Bacteriocinogeny of *Streptococcus mutans* as Factors Affecting its Establishment in Specific Pathogen-Free Rats

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The outcome of the experimental implantation of *Streptococcus mutans* strains in humans and animals is unpredictable, and neither success nor failure can be explained. It seems logical to assume that, apart from dietary and host factors, the characteristics of the *S. mutans* strain involved and those of the resident plaque microflora are important in colonization. For example, previous work in this laboratory suggested that bacteriocin production accounts at least in part for the establishment of an invading bacterium in a microbial ecosystem. In the present study, a complex specific pathogen-free Ny plaque ecosystem was obtained by the inoculation of specific pathogen-free rats with *Actinomyces viscosus* Ny1 and *S. sanguis* Ny101, and the establishment of *S. mutans* in such rats was then examined. It was found that bacteriocinogenic (*bac*$^+$) strains generally colonized in much higher proportions than non-bacteriocinogenic (*bac*$^-$) strains. Moreover, the longer the delay in introducing *S. mutans*, the poorer was its establishment. Shortly after inoculation of strains Ny1 and Ny101, there is probably a transient state in which microbial equilibrium has not been reached, but later the specific pathogen-free Ny system attains a stable climax community which more strongly resists invaders. The ability of a number of *S. mutans* strains to establish in such a climax community was then examined, and it was found that *bac*$^+$ strains generally established at a higher level than did *bac*$^-$ strains. In summary, it was concluded that, although the *bac*$^+$ state is an important property in the successful invasion of a plaque by *S. mutans*, the stability of the resident microflora is also an important factor.

In recent years, various aspects of the establishment of *Streptococcus mutans* in the mouth have been investigated. For instance, the presence of hard surfaces as a necessary requirement for the establishment of *S. mutans* has been demonstrated (3). Molecular mechanisms of the process by which of *S. mutans* adheres to hard tissues have been studied in detail but not yet completely clarified (17). The role of dietary carbohydrates such as sucrose and glucose on the establishment of *S. mutans* has also been investigated (21-23). Despite this knowledge, the outcome of experimental implantation of *S. mutans* in humans or animals is unpredictable (4, 6, 8, 19). Success or failure of establishment cannot be explained, but it is clear that the initial adherence of *S. mutans* (at a site) does not imply its final success in becoming established (21). Very little is known of those factors that influence the proliferation of *S. mutans* in the ecosystem of dental plaque, but they include host factors as well as the characteristics of the strain and of the resident microflora. One possibility is that the production of bacteriocin accounts for the successful establishment of a bacterium (1). It is well known that a high proportion of the *S. mutans* strains isolated from human dental plaque exhibit bacteriocin activity (5, 14). Like the bacteriocins of other gram-positive species, the *S. mutans* bacteriocins are also active against unrelated gram-positive bacteria (14), the oral actinomycetes being particularly sensitive (J. S. van der Hoeven, unpublished data).

Our previous studies have strongly suggested that *S. mutans* bacteriocins are produced in vivo and are active against sensitive species in the plaque of gnotobiotic rats (16). Other studies in this laboratory have involved the use of specific pathogen-free (SPF) rats inoculated with *Actinomyces viscosus* Ny1 and *S. sanguis* Ny101, giving the so-called "SPF-Ny" microflora—a complex plaque ecosystem more closely approaching that of the natural situation. It was

found, for example, that the non-bacteriocinogenic *S. mutans* strain OMZ 176 could not colonize such a system, although it was readily established in normal SPF rats (9). Studies of this type further indicated the possibility that bacteriocinogeny plays an ecological role in dental plaque. The main aim of the present study was, therefore, to test *S. mutans* strains differing in bacteriocin activity for their ability to establish in the SPF Ny plaque ecosystem. Before this, however, the kinetics of the establishment of parent bacteriocinogenic (bac^+) and non-bacteriocinogenic (bac^-) mutants were examined, and so was the implantation of selected *S. mutans* strains in the developing SPF-Ny system.

MATERIALS AND METHODS

Microflora of the experimental animals. Young (25 to 30 days old) SPF Osborne Mendel rats from different litters were divided among the inoculation groups, each consisting of 12 animals. Occasionally, more animals were involved. On two successive days, the animals were individually inoculated with 0.1 ml of overnight cultures of *A. viscosus* Ny1 and *S. sanguis* Ny101 in *Actinomyces* broth (Baltimore Biological Laboratory [BBL]), the inocula being applied with a tuberculin syringe. From the day of the first inoculation, the experimental diet 516S, containing 16% sucrose (10), was administered to the rats. The microflora harbored by these rats were referred to as the SPF-Ny microflora.

***S. mutans* strains.** Strains IB, Fa-1, OMZ 175, B2, B13, OMZ 65, and K1R were kindly provided by D. Brathall, strains T2, T8 and SM6 were human isolates (13), and strains O1H1, C 67-1, OMZ 176, E49, LM7, and SW31 (24), were kept in our stock culture collection. Strains T2 bac^- and SW31 bac^-, mutagen-induced non-bacteriocogenic mutants of T2 and SW31, respectively, were isolated in our laboratory (16). All strains were made streptomycin resistant by exposure to increasing concentrations of streptomycin in brain heart infusion broth (Difco Laboratories). *S. sanguis* Ny101, also used as an indicator for bacteriocin activity, was made resistant to erythromycin and designated Ny101 E.

The inocula of *S. mutans* consisted of overnight cultures in brain heart infusion broth (Difco). Both the absorbance at 550 nm and the number of colony-forming units on blood agar of these cultures were found to be very similar, giving viable counts of 2×10^9 to 4×10^9 CFU/ml. The animals were inoculated on two successive days with 0.1 ml of broth culture of the appropriate strain, each animal thus receiving 2×10^8 to 4×10^8 CFU. *S. mutans* inoculations were begun 1, 3, 5, or 6 days after the second inoculation of *A. viscosus* Ny1 and *S. sanguis* Ny101, as indicated below.

Bacteriological sampling. After the inoculation of *S. mutans*, plaque samples were taken from four animals per group at various times, as indicated below. Plaque was sampled from the first molar of the lower jaw, being taken from the mesial surface and the central fissure. In experiment I, the samples were treated separately, whereas in experiments II and III they were pooled (see below). They were then suspended in 100 μl of saline solution (0.85%) and ultrasonically dispersed (Kontes K 881440, 30 s at maximum output). A 10-μl amount of the dispersed plaque was plated on Trypticase soy-streptomycin (TSS) agar, which consisted of the following (per liter): Trypticase soy broth (BBL), 30 g; yeast extract (Difco), 10 g; streptomycin, 100 mg; and agar (Difco), 15 g for the recovery of the streptomycin-labeled *S. mutans* strains. Tests had shown that the recovery of *S. mutans* on TSS agar was similar to that on blood agar. Tenfold dilutions of the dispersed plaque were also plated on blood agar and TSS agar, and the plates were incubated for 48 h at 37°C in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2.

On blood agar, *A. viscosus* Ny1, *S. sanguis* Ny101, and the inoculated *S. mutans* strains could be easily distinguished from other components of the SPF-Ny flora. The counts of each of the species mentioned above were expressed as a proportion of total counts on blood agar. The other components of the SPF-Ny microflora were not counted separately. Representative colonies from this flora were isolated and designated as SPF biotypes 1, 2, and 3; they occurred with decreasing proportions in that order. Biotype 1 was a *Streptococcus* species, as yet unidentified; biotype 2 was a gram-negative coccus, probably *Veillonella*, and biotype 3 was an unidentified gram-negative rod.

Test for bacteriocin activity in vitro. The bacteriocin activity of *S. mutans* against the main components of the SPF-Ny microflora was tested as follows. The test strain was grown for 24 h on TSS agar to give separate colonies. The indicator strains were grown in TS broth or *Actinomyces* broth (BBL) in the case of *A. viscosus* Ny1. An overlay of the indicator strain was prepared by mixing 0.2 ml of an overnight culture of the indicator strain with 5 ml of Trypticase soy broth plus 0.75% agar, kept at 50°C just before pouring the mixture over the surface of the *S. mutans* plate. Inhibition zones were read after 18 h of incubation at 37°C under an atmosphere of N_2, H_2, and CO_2.

Demonstration of bacteriocin activity in plaque. From a number of SPF-Ny rats previously inoculated with strain T2 or T2bac^- on day 1 or day 6 after infection with strain Ny1 and Ny101 (experiment I), 28-day-old pooled plaque was collected. One portion was used for the determination of lactic and acetic acids present (20), whereas the remainder was used to test for the presence of inhibitory activity as follows. Samples were transferred directly onto the surface of TS agar plates containing 200 μg of erythromycin per ml. The effectiveness of erythromycin in preventing growth of plaque organisms was demonstrated by streaking a small portion over the surface of Trypticase soy-erythromycin agar. Strain Ny101 E was used as the indicator, and the overlay also contained 200 μg of erythromycin per ml. After overnight aerobic incubation at 37°C, plates were examined for zones of inhibition. In addition, direct measurements of the pH in areas adjacent to the plaque samples were made to determine any differences between those showing zones of inhibition and those producing no such inhibition.

Experiment I. Kinetics of the establishment of

S. *mutans* parent (*bac*$^+$) and *bac*$^-$ mutants in SPF-Ny rats. The following *S. mutans* strains were tested in experiment I: T2, T2*bac*$^-$, SW31, and SW31*bac*$^-$. They were inoculated 1 day after the second of two inoculations with Ny1 and Ny101. Plaque was sampled 3, 6, 13, and 28 days after inoculation with *S. mutans*.

The percent contribution of *S. mutans* to the total SPF-Ny flora in both fissure and smooth-surface plaques was determined as described above.

Experiment II. Implantation of *S. mutans* in the developing SPF-Ny microflora. The following strains were used in experiment II: T2, SW31, and OMZ 176. Rats were inoculated either 1, 3, 5, or 6 days after the introduction of Ny1 and Ny101. Plaque was sampled and pooled from each animal 28 days after they had been infected with *S. mutans*, and the proportion of this organism was determined as described above.

Experiment II. Ability of various *S. mutans* strains to establish in SPF-Ny rats. The *S. mutans* strains used in experiment III are listed in Table 2. Animals were infected 5 days after the introduction of Ny1 and Ny101, and plaque was sampled and pooled from each rat 28 days after they had been infected with *S. mutans*. The proportion of *S. mutans* was determined as described above.

RESULTS

As has been pointed out previously (16), there is little point in tabulating the absolute number of *S. mutans* and other organisms in the plaque samples, since they are meaningless unless expressed in terms of a standard amount of plaque or per animal (21). Results have therefore been expressed in terms of the median percent contribution of *S. mutans* to the total plaque flora.

Kinetics of the establishment of *S. mutans* in SPF-Ny rats (experiment I). The results of experiment I are shown graphically in Fig. 1 and 2. Strain T2 rose to a constant level in about 13 days, being more dominant in fissures as compared with smooth-surface plaque (Fig. 1). In contrast, although the T2*bac*$^-$ mutant was consistently recovered from both sites, its proportion remained 1%. The results for strain SW31 were essentially the same as those for T2, but the SW31*bac*$^-$ mutant, although its establishment was far less pronounced than that of its parent, reached proportions of some 20% of the total plaque flora (Fig. 2).

Demonstration of bacteriocin activity in plaque. As can be seen (Table 1), antibacterial activity could not be demonstrated at all in plaque containing strain T2*bac*$^-$ or a low level of strain T2 but was detected in plaque harboring much higher levels of the latter strain. The similarity in the pH measurements adjacent to the plaque samples and within the inhibition zones was not a pH effect, and this is supported by the fact that the acid end products in plaque

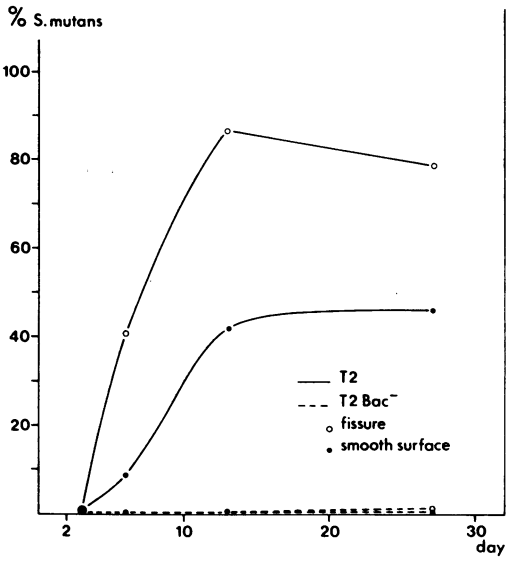

FIG. 1. *Kinetics of the establishment of S. mutans T2 and its non-bacteriocinogenic mutant T2bac⁻ in smooth-surface and fissure plaque of SPF-Ny rats. The S. mutans strains were inoculated 1 day after A. viscosus Ny1 and S. sanguis Ny101. Plaque was sampled 3, 6, 13 and 28 days after S. mutans inoculation. Proportion of S. mutans was expressed as a median percentage of the total cultivable plaque flora.*

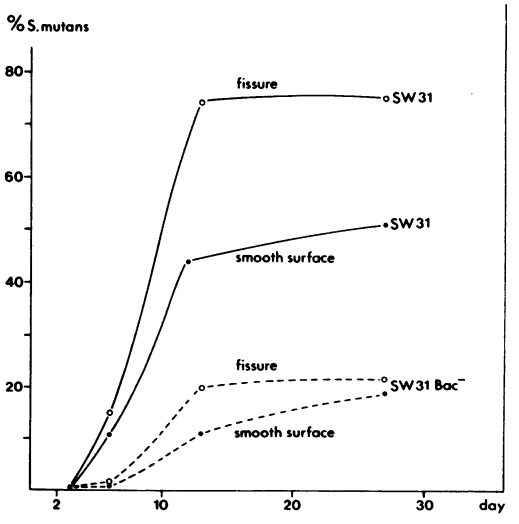

FIG. 2. *Kinetics of the establishment of S. mutans SW31 and its non-bacteriocinogenic mutant SW31bac⁻ in smooth-surface and fissure plaque of SPF-Ny rats. The S. mutans strains were inoculated 1 day after A. viscosus Ny1 and S. sanguis Ny101. Plaque was sampled 3, 6, 13, and 28 days after S. mutans inoculation. S. mutans was expressed as a median percentage of the total cultivable plaque flora.*

TABLE 1. *Bacteriocin activity in plaque harboring S. mutans T2 and T2 bac⁻*

S. *mutans* strain	Inoculation[a]	Contribution[b]	Bacteriocin activity in plaque sample	pH	Fermentation end products (nmol of DNA per mg)[c]	
					Lactic	Acetic
T2	1	62	+	6.4	110	150
	6	1	−	6.4	ND	ND
T2bac⁻	1	0.8	−	6.3	100	190
	6	6	−	6.4	ND	ND

[a] Each value indicates day(s) after Ny1 and Ny101.
[b] Each value indicates median percent contribution of *S. mutans* to total plaque flora.
[c] ND, Not determined.

samples were not significantly different, regardless of whether the *S. mutans* harbored was *bac*⁺ or *bac*⁻.

Implantation of *S. mutans* in the developing SPF-Ny microflora (experiment II). Figure 3 shows the establishment of *S. mutans* strains T2, SW31, and OMZ 176 in relation to the time interval between inoculation of Ny1 and Ny101 and the subsequent introduction of *S. mutans*. The bacteriocinogenic strains T2 and SW31 again reached quite high levels in plaque when they were inoculated only 1 day after Ny1 and Ny101 but, the longer this introduction was delayed, the lower their eventual proportions in the plaque flora. On the other hand, the non-bacteriocinogenic strain OMZ 176 established poorly even when introduced only 1 day after Ny1 and Ny101.

Ability of various *S. mutans* strains to establish in SPF-Ny rats. In Table 2 are listed a number of *S. mutans* strains together with the ability of each to establish in the plaque of SPF-Ny rats and the in vitro bacteriocin activity of each against the components of the SPF-Ny microflora. Parenthetically, it might be noted that, in a 5-day-old SPF-Ny fissure plaque, the relative proportions in this flora were as follows: Ny, 1 to 20%; Ny101, 5%; and the sum of the SPF biotypes, 75% (J. S. van der Hoeven, unpublished data). In addition, although the proportions of *S. mutans* are listed only for 28 days, samples were in fact also taken at 3 and 13 days after infection with *S. mutans*, and these showed that the kinetics of establishment were similar to those found for strain T2 (Fig. 1). In general, bacteriocinogenic strains, irrespective of serotype, established at a much higher level than those without detectable levels of activity.

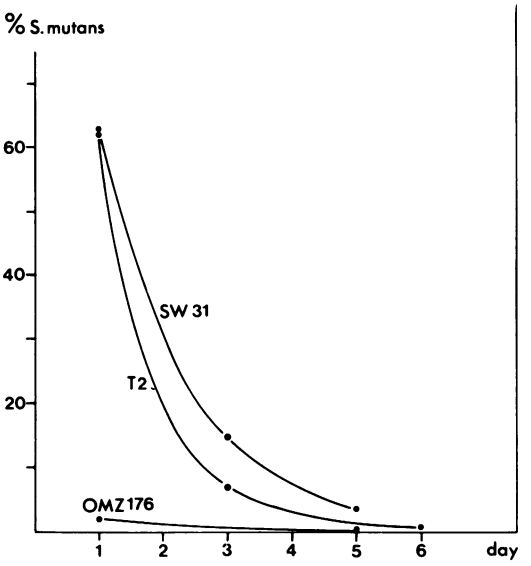

FIG. 3. *Implantation of S. mutans in the developing SPF-Ny ecosystem. Rats were inoculated either 1, 3, 5, or 6 days after the introduction of A. viscosus Ny1 and S. sanguis Ny101. Plaque was sampled 28 days after S. mutans inoculation. Proportion of S. mutans was expressed as a median percentage of the total cultivable plaque flora.*

DISCUSSION

Previous studies (16) using simple ecosystems in germ-free rats have shown that *bac*⁺ *S. mutans* strains inhibit sensitive species (including homologous *bac*⁻ mutants). Furthermore, plaque harboring the parent *bac*⁺ *S. mutans* strain T2 but not the corresponding T2*bac*⁻ mutant contained inhibitory component(s) against sensitive indicator strains. Although these results indicated that bacteriocins are produced and are active in dental plaque in vivo, their overall ecological role remains obscure. For instance, it is well known that the presence of *bac*⁺ *S. mutans* strains in human dental plaque does not lead to the elimination of sensitive species (7, 12, 24), and this suggests that bacteriocins, if produced at all under such conditions, function on a microecological scale and would thus affect only the sensitive organisms in the immediate vicinity of the producing cell.

Nevertheless, the present study, using rats harboring the more complex SPF-Ny microflora, again indicates that bacteriocin production by *S.*

TABLE 2. *Ability of S. mutans strains with various bacteriocin activities to establish in SPF-Ny rats*

S. mutans strain[a]	Serotype	Bacteriocin activity *in vitro* against:					Establishment of *S. mutans*[b]
		A. viscosus Ny1	*S. sanguis* Ny101	SPF biotype			
				1	2	3	
T8	*c*	+	+	+	+	+	15
T2	*c*	+	+	+	+	+	1
SW31	*c*	+	+	+	+	+	4
C 67-1	*c*	+	+	+	+	+	0.1
SMG	*c*	+	+	+	−	+	5
O1H1	*d*	+	+	+	−	+	7
IB	*c*	±	+	+	+	+	18
FA-1	*b*	−	+	+	−	−	<0.01
OMZ 175	*f*	−	−	+	−	−	<0.01
LM7	*e*	−	−	+	+	+	3
B2	*e*	−	−	+	−	+	0.01–0.1
B13	*d*	−	−	−	−	−	<0.01
OMZ 65	*d/g*	−	−	−	−	−	3
K1R	*d/g*	−	−	−	−	−	<0.01
OMZ 176	*d*	−	−	±	−	−	<0.01
E49	*a*	−	−	−	−	−	<0.01
T2bac$^-$	*c*	−	−	−	−	−	<0.01
SW31bac$^-$	*c*	−	+	+	−	−	0.01–0.1

[a] Strains were inoculated 5 days after strain Ny1 and Ny101, except that T2 was inoculated 6 days after Ny1 and Ny101.

[b] Each value indicates percentage of total flora, as determined from pooled (fissure and smooth-surface) plaque sampled 28 days after inoculation of *S. mutans*.

mutans occurs in vivo and that this property may be ecologically advantageous to the invading strain (experiment I). The *bac*$^+$ parent strains reached stable and high proportions in about 14 days, whereas the corresponding *bac*$^-$ mutants remained at fairly constant but low levels. That such an effect was not so pronounced in the case of SW31 may well be due to the fact that the SW31*bac*$^-$ mutant, while having no in vitro activity against *A. viscosus* Ny1, did in fact retain such activity against *S. sanguis* Ny101 and SPF biotype 1 (Table 2). The T2*bac*$^-$ mutant has no in vitro activity against either strain.

The state of the resident microflora can also have an effect on the establishment of invading bacteriocinogenic *S. mutans* strains, since the longer their introduction was delayed, the lower their eventual proportions in the plaque flora (experiment II). It seems logical that shortly after the inoculation of Ny1 and Ny101 there is a transient state in the SPF-Ny system because microbial equilibrium has not yet been reached and even *bac*$^-$ strains can establish. However, with the passage of time, a climax community (1) is attained. An invading organism, in this case *S. mutans*, would find possible niches occupied by the well-integrated members of such a community, and even *bac*$^+$ strains would establish poorly, if at all. This is of interest in relation to a study of the colonization of *S. mutans* in artificial tooth fissures in humans (19). Here, too, it was found that the establishment of invading *S. mutans* organisms was rare once the fissures had been occupied, within 1 day, by a large number of indigenous oral bacteria. In another recent human study (18), it was also found that, although *S. mutans* established quite well on cleansed smooth tooth surfaces, its implantation was greatly impaired by the presence of 2-day-old plaque.

In experiment III, the ability of a series of *S. mutans* strains to establish in 5-day-old SPF-Ny plaque, a stable climax community, was assessed. Previous work in this laboratory (J. S. van der Hoeven and F. H. M. Mikx, unpublished data) has shown that all tested strains of *S. mutans*, regardless of bacteriocinogenic state, colonize the SPF system. However, as already mentioned, the phenotypically *bac*$^-$ strain OMZ 176 failed to colonize the whole complex SPF-Ny system (9). The present study confirms the latter finding and extends it to include a number of other *S. mutans* strains. In general, *bac*$^+$ strains established at a higher level than did those showing no detectable in vitro bacteriocin activity. This would support the thesis that the *bac*$^+$ state may enable an invading *S. mutans* strain to disturb the equilibrium in microenvironments in the climax community and thereby become established.

Nevertheless, factors other than bacteriocin-

ogeny may be involved in successful colonization, since OMZ 65, a strain with poor or no detectable bacteriocin activity, established quite well. Bacteriocin production in vitro is known to be influenced by the composition of the medium (11), and indeed this strain has been shown to be bacteriocinogenic, albeit weakly, on a different medium (15). Furthermore, the present results (Table 2) do not indicate any relationship between successful establishment and bacteriocin activity against any single component of the SPF-Ny flora. The work of van Houte (21), in which he showed that *S. mutans* strains varied considerably in the ease with which they could be established in conventional rats, is also relevant. For example, he found that strains BHT and LM 7 implanted poorly despite the fact that they are both bacteriocinogenic (15), whereas E-49 implanted quite readily although it appears to be non-bacteriocinogenic (15). The failure of strain C 67-1 to establish (Table 2) is more difficult to explain, since it has been shown to produce bacteriocin(s) in vivo using the gnotobiotic system (16). This would indeed indicate that factor(s) other than bacteriocinogeny may be involved in the successful colonization of an invading organism. Although additional data are needed, there appears to be no correlation between serotype and the ability of *S. mutans* to colonize the SPF-Ny ecosystem. From the results of another study (21) it would, however, appear that serotype *c* strains established consistently better than those belonging to other groups. This is of interest, since serotype *c* is by far the most prevalent in most human populations (2). In summary, then, the current investigation indicates that bacteriocinogeny is a factor in the successful colonization of *S. mutans* strains in the SPF-Ny microflora.

It appears that the stability of such a plaque ecosystem, in addition to its qualitative microbial composition, is also an important factor. Attempts to prevent dental caries by manipulation of the existing plaque microflora should take account of findings such as these.

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