Biochemical properties of *Streptococcus sobrinus* reisolates from the gastrointestinal tract of a gnotobiotic rat

M. D. P. WILLCOX,¹* R. J. FITZGERALD,² B. O. ADAMS,² M. PATRIKAKIS¹ and K. W. KNOX¹

¹Institute of Dental Research, 2 Chalmers Street, Surry Hills, NSW 2010, Australia

²Veterans Affairs Medical Center, 1201 Northwest 16th Street, Miami, Florida 33125, USA

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Streptococcus sobrinus strain 6715-13-201 was inoculated into the oral cavity of a gnotobiotic rat and then reisolated from different portions of the gastrointestinal tract. Fourteen isolates, selected on the basis of their colonial morphology, were then screened for their ability to adhere to saliva-coated hydroxyapatite (SHA) *in vitro*, and their ability to produce extracellular polysaccharide from sucrose, and low pH in glucose broth. Certain isolates were also tested for their cariogenic potential as monoinfectants in gnotobiotic rats. All isolates differed in their abilities to adhere to SHA, with most showing an increased level of adhesion in the presence of sucrose, but this did not correlate with their ability to be aggregated by dextran. Most isolates were capable of producing glucosyltransferases (with only one exception) and dextranases (also one exception). There was more variability in the production of dextranase inhibitor. No isolate was capable of producing dextranase inhibitor in the absence of dextranase production. There were no correlations between the ability of isolates to adhere *in vitro* or produce/utilize polysaccharides and their ability to produce caries *in vivo*. Due to the differences between strains in their abilities to adhere, produce polysaccharides, utilize polysaccharides or produce a low pH and the lack of correlation between any of these parameters and cariogenicity, the results suggest that the ability of strains to colonize and produce caries depends on a number of different characteristics, no one of which is essential.

Introduction

Dental caries occurs when tooth enamel is dissolved by acids, particularly lactic acid produced by the fermentation of carbohydrates by plaque bacteria. The mutans streptococci have been associated with the development and progression of dental caries in humans (Shklair *et al.*, 1974; Huis in't Veld *et al.*, 1979), with the species *S. mutans* and *S. sobrinus* being most frequently isolated from human dental plaque. Strains of mutans streptococci have been examined for their ability to produce caries in gnotobiotic animals (Willcox *et al.*, 1990; Fitzgerald *et al.*, 1960; Drucker & Green, 1981) and certain strains can produce caries on all surfaces of the teeth.

Dental plaque formation, and ultimately the production of carious lesions on the teeth, requires that bacteria adhere to the tooth surface or accumulate by adhering to other (adherent) bacteria, or to bacterial or

host products. For mutans streptococci, adhesion is mediated by bacterial surface components, and considerable emphasis has been given to the protein variously called P1, I/II, IF and B (Forester *et al.*, 1983) which has been implicated in the adhesion of mutans streptococci to the tooth surface (Lee *et al.*, 1989; Takahashi *et al.*, 1989). A cross-reacting antigen in *S. sobrinus* has been named SpaA (Goldschmidt & Curtiss, 1990). Attention has also been directed towards the extracellular polysaccharides that streptococci can produce from sucrose using either the glucose moiety to produce glucan or the fructose moiety to produce fructan (Birkhed *et al.*, 1975; Trautner *et al.*, 1981). Early work with *S. sobrinus* strains demonstrated that they required glucan synthesis for adhesion. The fructans or glucans may also act as carbohydrate reserves, and some members of the *S. mutans* group produce fructanase and dextranase (Jacques *et al.*, 1985; Walker *et al.*, 1981) which degrade the polysaccharides. Studies using mutants of mutans streptococci have shown that adhesion, production of extracellular polysaccharides and degradative enzymes may be important cariogenic traits (reviewed by Tanzer *et al.*, 1985).

S. sobrinus strain 6715-13-201 has been used

* Author for correspondence. Tel. 61 2 211 3091; fax 61 2 282 0368.

Abbreviations: EPS, extracellular polysaccharide; SHA, saliva-coated hydroxyapatite; BHI, brain heart infusion.

extensively in animal caries experiments (Tanzer *et al.*, 1985) and accordingly was chosen for this study where reisolates from the gastrointestinal tract of a rat were compared for their cariogenicity, *in vitro* adhesion and polysaccharide production. The hypothesis to be tested was that adhesion and polysaccharide production are key elements in caries production by *S. sobrinus*.

Methods

Bacterial strains. *Streptococcus sobrinus* strain 6715 was derived from passage through hamsters of strain K1-R, a streptomycin-resistant mutant of the original human isolate K1. Strain 6715-13WT was isolated after passages of strain 6715 through rats and hamsters (Freedman & Tanzer, 1974; personal communication, J. M. Tanzer). Strain 6715-13-201 is a mutant of 6715-13WT, selected because it lacks dextranase activity, and was constructed by Freedman & Tanzer (1982) using chemical mutagenesis.

Selection of isolates from the gastrointestinal tract of a gnotobiotic rat monoinfected with *S. sobrinus* 6715-13-201. An outbred germ-free Sprague-Dawley rat was maintained in a sterile isolator (Fitzgerald *et al.*, 1989) and fed radiation-sterilized diet 2000 (Keyes & Jordan, 1964). Strain 6715-13-201 was inoculated (0.2 ml) into the mouth of the rat and allowed to establish for 60 d. The animal was killed humanely after 60 d. Sections of the gastrointestinal tract were then removed aseptically, and the contents were removed and weighed. The tissue was then washed in sterile saline, bottled and weighed. The contents and tissue were separately homogenized and serial dilutions were plated on Mitis-Salivarius agar (MS; Difco) containing 200 µg streptomycin ml⁻¹. After 48 h incubation, the morphology (rough or smooth) of colonies was recorded and isolates were selected for further examination (Table 1).

Growth and biochemical characterization. Strains were grown in brain-heart infusion (BHI; Oxoid) supplemented with 0.3% yeast extract (Difco), and 0.074 MBq ml⁻¹ ³H-labelled thymidine for adhesion experiments, at 37 °C for 24 h. Cells were then washed three times in phosphate-buffered saline (PBS (g l⁻¹): NaCl, 8; KCl, 0.2; NaH₂PO₄, 0.2; KH₂PO₄, 2.9) and finally resuspended at OD₆₆₀ = 1.0 (1 × 10⁹ c.f.u. ml⁻¹) unless otherwise stated. The final pH produced in glucose broth was recorded after growth for 18 h at 37 °C. To determine the ability of strains to produce intracellular, iodophilic polysaccharides, they were grown in 0.5% glucose broth for 18 h at 37 °C. Cells were removed by centrifugation (10000 g, 10 min) and the cell pellet was then stained with Lugol's iodine. If cells have produced intracellular polysaccharide, they stain blue-black or dark brown (a negative result is indicated by no change from the iodine colouration). The possession of serotype polysaccharides was measured by incubating washed cells with fluorescein-labelled rabbit IgG specific for serotype polysaccharides *a*, *b*, *c*, *d*, *e*, *f* and *g*, and then examining them by fluorescence microscopy.

Adhesion to saliva-coated hydroxyapatite (SHA). This method has been published previously (Willcox & Knox, 1990, 1991). Briefly, whole unstimulated saliva was collected from one of us (M. D. P. W.), clarified by centrifugation (5000 g, 15 min) and allowed to coat 20 mg hydroxyapatite at ambient temperature for 1 h. Unadsorbed salivary material was removed by washing three times in PBS. Radiolabelled bacteria were allowed to interact with SHA at 37 °C for 2 h. After 2 h, non-absorbed bacteria were removed by washing three times in PBS and the amount of radioactivity associated with the SHA was measured. Each strain was tested on two separate occasions, each of three assays. To assess the effect of sucrose on adhesion, bacteria were resuspended in half the volume of buffer that gave OD₆₆₀ = 1.0; an

equal volume of 10% sucrose was then added and the assays were repeated.

Aggregation of cells by dextran and sucrose. Cells were washed three times in buffer and then resuspended to OD₆₆₀ = 1.0 in the assay buffer (100 mM-sodium phosphate, pH 6.5, 40 mM-sucrose, 10 mM-sodium fluoride, 0.1 mM-histidine and 0.04% dextran T10). The optical density was compared to that of control cells resuspended in buffer lacking dextran and sucrose at time zero and after 2 h incubation at ambient temperature. Experiments were repeated three times.

Possession of cell surface protein P1. The method of Wyatt *et al.* (1988), boiling in SDS, was used to extract cell-surface P1. The extract was then subjected to SDS-PAGE on 4–15% (w/v) gradient gels, blotted onto nitrocellulose and probed with antisera to P1 (Forester *et al.*, 1983). Reactive bands were seen by secondary probing with goat alkaline-phosphatase-labelled anti-rabbit IgG and developing with fast blue BB salt (Sigma). The experiments were repeated on two separate occasions.

Glucosyltransferase, dextranase and dextranase inhibitor production. Cultures were grown in BHI for 18 h and centrifuged, and the supernatant was concentrated six-fold by placing in dialysis tubing and then immersing in polyethylene glycol flakes. Cells were washed once in 0.85% NaCl and then incubated in a 1% (w/v) solution of sodium lauroylsarcosinate (SLS; Jenkinson, 1986) for 60 min and then removed by centrifugation. The cell extract and concentrated culture fluid were heated at 100 °C for 5 min and subjected to SDS-PAGE in a 7.5% (w/v) gel at constant 120 V. All experiments were repeated.

Production of cell-bound and extracellular glucosyltransferase (GTF). Following electrophoresis of proteins from either the supernatants or cell-surface, the gels were shaken for 90 min in 50 mM-Tris/HCl, pH 7.5, containing 1 mM-CaCl₂, transferred to a sucrose solution (50 mM-NaPO₄, pH 6.5, 1% (w/v) sucrose, 1% (v/v) Triton X100, 1 mM-CaCl₂, 0.02% dextran T10) and incubated for 24 h at 37 °C with shaking. The gel was then fixed in 75% (v/v) ethanol for 30 min and stained with periodic acid/Schiff's reagent to detect GTFs synthesizing both insoluble and soluble polysaccharides.

Production of dextranase and dextranase inhibitor. The proteins were separated on 7.5% (w/v) SDS-PAGE gels containing 5 mg blue dextran 2000 ml⁻¹ (Pharmacia). Gels were incubated for 18 h at 37 °C with shaking, in 0.2 M-citrate buffer, pH 6.0, containing 1% (v/v) Triton X100. At the end of the incubation, dextranase bands were seen as transparent bands in a blue gel. In order to detect dextranase inhibitor bands, the gels were incubated for 18 h at 37 °C in 0.2 M-citrate buffer, pH 6.0, containing 1% (v/v) Triton X100 and 500 mU dextranase. (Dextranase was obtained by ammonium sulphate precipitation of the culture supernatant of *S. sobrinus* K1-R, a known dextranase producer (Walker *et al.*, 1981), and kindly supplied by Dr G. J. Walker, Institute of Dental Research). After incubation, the inhibitor bands were seen as blue bands in a clear gel.

The levels of GTF, dextranase and dextranase inhibitor production were assessed independently by three people and expressed as a range from +++ (high levels) to 0 (no detectable level).

Caries assay of reisolates. The cariogenic potential of strains was assessed in germ-free Sprague-Dawley rats using an abbreviated caries test procedure (Fitzgerald *et al.*, 1981). Briefly, weanling germ-free animals were infected orally by swabs of the respective strains and maintained on irradiated diet 2000 (Keyes & Jordan, 1964) for 30 d. The animals were killed humanely by chloroform inhalation and the jaws were defleshed. The severity of caries was assessed in the first two mandibular molars using scores from 0 to 4 to cover the range from no caries to complete loss of the tooth.

Statistical analysis of data. For experiments designed to measure the effect of a particular treatment on a property of all strains, Wilcoxon's

Table 1. Site of isolation of *S. sobrinus* strains, colonial morphology, adhesion to hydroxyapatite and aggregation of strains in the presence of dextran

Strain	Site of isolation	Colonial morphology*	10 ⁻⁵ × Adhesion to SHA (cells mg ⁻¹)		Aggregation by dextran plus sucrose‡
			Buffer†	Sucrose†	
6715	—	—	2.3 ± 0.8	8.3 ± 6.0	94
6715-13WT	—	—	4.3 ± 1.8	7.3 ± 1.5	0
6715-13-201	Parent strain	Large smooth	3.3 ± 1.5	3.8 ± 0.3	47
A-1a	Oral cavity	Large smooth	1.8 ± 0.5	2.8 ± 1.3	29
A-2b	Oral cavity	Large rough	5.5 ± 1.3	13.0 ± 5.3	0
SC-1b	Stomach contents	Small rough	1.8 ± 0.8	5.8 ± 2.3	4
SC-3§	Stomach contents	Large mucoid	1.0 ± 0.3	7.8 ± 2.3	85
ST-1	Stomach tissue	Large grainy	4.3 ± 1.5	6.0 ± 3.5	70
ST-2b	Stomach tissue	Small watery	1.8 ± 0.5	3.0 ± 0.8	0
DC-1a§	Duodenal contents	Small rough	4.5 ± 1.3	5.0 ± 1.5	0
IC-1	Ileum contents	Small rough	4.5 ± 1.3	3.0 ± 0.8	64
IT-1a	Ileum tissue	Small rough	0.8 ± 0.3	12.8 ± 4.3	0
IT-2	Ileum tissue	Large rough flat	1.5 ± 0.8	1.8 ± 1.0	0
CC-1	Caecum contents	Small rough	1.3 ± 0.8	2.5 ± 1.3	0
CC-3	Caecum contents	Small rough	6.5 ± 2.0	14.3 ± 8.0	0
CT-3	Caecum tissue	Large watery	2.0 ± 0.8	2.5 ± 1.0	0

* Examined after growth on Mitis-Salivarius agar.

† Cells incubated in the absence (buffer) or presence (sucrose) of sucrose. Figures are the means of two cultures and three replicates per culture ± SD.

‡ 100% aggregation was indicated by an OD₆₆₀ after settling of aggregates, equal to that of sterile buffer. Results are means of three separate experiments. Standard deviation was always less than 10% of the mean value.

§ Strains which autoaggregate.

sum rank test was employed. For correlations between different tests, Pearson's correlation coefficient was calculated. Newman-Keuls multiple range test ANOVA was used to test for differences between caries values.

Results

Isolation of strains and preliminary characterization

Reisolated strains of *S. sobrinus* 6715-13-201 were obtained from MS plates inoculated with the gastrointestinal tissues or luminal contents of a gnotobiotic rat monoinfected with the micro-organism for 60 d. The total concentration in terms of c.f.u. per gram of tissue (T) or contents (C) of the stomach (S), duodenum (D), ileum (I) or caecum (C) were: ST, 3.5×10^3 ; SC, 2.0×10^5 ; DT, 1.0×10^3 ; DC, 2.9×10^5 ; IT, 8.9×10^4 ; IC, 4.0×10^8 ; CT, 1.4×10^7 ; CC, 2.2×10^9 . An oral swab (A) contained 3.0×10^3 c.f.u. Subcultures made from these plates on the basis of predominant colony morphology are listed in Table 1. Approximately equal numbers of different colonial variants were seen, regardless of site of isolation, and in separate experiments similar findings were noted with other rats. The colonial morphologies were stable upon passage of strains *in vitro*.

Variation in the rate of fermentation of mannitol and sorbitol was noted for isolates (unpublished). All strains produced a final pH of 4.1 after growth in glucose broths

and no strain was capable of producing iodophilic-intracellular polysaccharides. All strains reacted with fluorescein-labelled rabbit antiglobulin to *S. sobrinus* B13 (serotype g), through we have since shown that the amount of the serotype g polysaccharide may differ (data not shown). No strains cross-reacted with antisera to serotypes a, b, c, d, e or f.

Adhesive characteristics

Adhesion to SHA. The relative abilities of strains to adhere to SHA are shown in Table 1. All strains were fairly poor adherers to SHA, with strain CC-3 giving the highest adhesion of 6.5×10^5 cells (mg SHA⁻¹), and strain IT-1a giving the lowest adhesion of 8.0×10^4 cells (mg SHA⁻¹). The site of isolation of the strains did not correlate with adhesion to SHA, with strains isolated from the oral cavity of rats showing a range of adhesive values similar to that for strains isolated elsewhere. Sucrose in the buffer increased the adhesion of most strains ($P < 0.025$) and in no case did sucrose significantly decrease adhesion.

Aggregation of cells by dextran and sucrose. Table 1 also shows that both stock strains and reisolates displayed differing aggregating abilities. For the reisolates, only 4 out of 13 gave > 10% aggregation, with strains SC-3, ST-1 and IC-1 giving values greater than their parent strain (6715-13-201).

Fig. 1. Production of extracellular GTF, dextranase and dextranase inhibitor. GTF activity (band approx. 170 kDa) was measured by incubating SDS-polyacrylamide gels first in sucrose buffer and then in Schiff's reagent. Dextranase activity (approx. 170 kDa) and its inhibitor (approx. 50 kDa) were measured by incubating gels in either buffer, or buffer containing cell-free dextranase, respectively. See Methods for details.

Table 2. Production of GTF, dextran and dextranase inhibitor

Strain	Production of:		
	GTF*	Dextranase†	Dextranase inhibitor‡
6715	+	+++	+/-
6715-13WT	+++	+++	++
6715-13-201	+++	+++	+++
A-1a	++	+++	+/-
A-2b	++	+++§	+§
SC-1b	+++	++	0
SC-3	+++	+++	++
ST-1	++	+++	+
ST-2b	+++	+++	++
DC-1a	0	0	0
IC-1	+++	+++	+
IT-1a	+++§	0	0
IT-2	++	+++§	+§
CC-1	+++	++	+++
CC-3	+++	+++	+/-
CT-3	++	+++	+

* GTF production was estimated from the degree of Schiff's staining in Fig. 1 by three independent observers.

† Dextranase production was estimated from the degree of destaining of blue dextran in Fig. 1 by three independent observers.

‡ Dextranase inhibitor production was assessed as the amount of blue dextran remaining (Fig. 1) after incubation of SDS-polyacrylamide gels with dextranase by three independent observers.

§ Activity or inhibitor observed in SLS extracts of cells.

Table 3. Cariogenicity of strains in monoinfected germ-free rats

Germ-free rats were infected orally with an overnight broth culture of the appropriate strain and maintained on a high sucrose diet for 30 d. Data are means of 5 experiments $\pm$ SD.

Strain	Caries Score
6715-13-201	1.28 $\pm$ 0.31
SC-3	0.68 $\pm$ 0.28
ST-2b	0.43 $\pm$ 0.12
DC-1a	0.54 $\pm$ 0.11
CC-1	0.68 $\pm$ 0.14
CT-3	0.53 $\pm$ 0.17

Detection of cross-reacting P1. The Western blot for cell-surface SpaA protein demonstrated that all strains, with the exception of SC-3 and ST-1, carried the protein. The reactive band was approximately 200 kDa in molecular mass, and this corresponded to a positive control of purified P1 from *S. mutans* Ingbritt (Forester *et al.*, 1983).

Production of glucosyltransferase, dextranase and dextranase inhibitor

Glucosyltransferase production. Fig. 1 demonstrates that all strains, with the exception of strain DC-1a, released glucosyltransferase into the culture medium.

The level of activity of both the stock strains and reisolates varied, with strain 6715 producing a low activity, strain 6715-13WT producing high activity, and strain CC-1 producing the highest activity for the reisolates. Only strain IT-1a showed activity in gels after SLS extraction and staining with Schiff's reagent, indicating the production of cell-bound GTF activity producing soluble dextran (Table 2).

Production of dextranase and dextranase inhibitor.

Most strains produced extracellular dextranase activity associated with a band of approximately 170 kDa (Fig. 1; Table 2), although five of the reisolates had a reduced activity compared with the parent strain. After SLS extraction of surface proteins, only strains A-2b and IT-2 showed dextranase activity (Table 2), and the dextranase of strain A-2b was approximately 97 kDa.

The ability of strains to produce extracellular dextranase inhibitor varied widely between the stock strains and the reisolates (Fig. 1; Table 2). After SLS extraction of proteins, only strains A-2b and IT-2 showed inhibitor bands (Table 2), each with an estimated molecular mass of approximately 50 kDa.

Cariogenicity of selected strains in gnotobiotic rats

Table 3 shows the relative cariogenicity of six strains selected on the basis of differences in the above *in vitro* tests. The highest activity score was given by strain 6715-13-201 and the lowest score by strain ST-2b. Strain 6715-13-201 gave a statistically ($P < 0.05$) greater caries score than the reisolates which were statistically similar to each other.

Statistical correlations between data sets

The following pairs were tested for correlations: aggregation by dextran and increase in adhesion in the presence of sucrose (i.e. [adhesion to SHA plus sucrose] — [adhesion to SHA]); possession of P1-CRA and adhesion to SHA; possession of P1-CRA and increase in adhesion to SHA in the presence of sucrose; and (in specific instances) cariogenicity and adhesion to SHA or production/utilization of polysaccharides. There were no correlations between any of the data sets.

Discussion

This study clearly demonstrates that passage through and colonization of the gastrointestinal tract of a gnotobiotic rat by *S. sobrinus* 6715-13-201 profoundly affects phenotypic traits and that these traits are revealed after a relatively short colonization time (60 d). The strains used in this study were all derivatives of *S. sobrinus* strain K1, an isolate from the human oral cavity

that has been subjected to animal passage on numerous occasions (Freedman & Tanzer, 1974). Whilst there is no information in the literature on the occurrence of variants after animal passage of strain K1, alterations could have occurred but not been noted. Strain 6715-13-201 does not show variants upon subculture *in vitro* (data not shown) and therefore it can be concluded that the variants shown in this study must be the direct result of *in vivo* passage. Although the isolates were selected from the gastrointestinal tract of one rat, other experiments have shown that strain 6715-13-201 shows similar results in other rats.

The isolation of strains from specific sites in the gastrointestinal tract was not reflected in their ability to adhere to surfaces *in vitro*. The ability to adhere directly to the tooth surface may not be essential for strains to become part of the oral flora. Previous studies have demonstrated that *S. sobrinus* strains require sucrose for attachment *in vitro* to SHA (Koga & Inoue, 1978) and the present investigation demonstrates that sucrose increases adhesion of the *S. sobrinus* strains. However, the strains were able to adhere in the absence of sucrose, indicating that they must possess adhesins capable of interacting with molecules in the pellicle other than GTF or dextran. Mechanisms of specific adhesion have focused on protein P1 that is found on the surface of *S. mutans* strains (Wyatt *et al.*, 1988). Although protein P1 has been shown to mediate adhesion to SHA for *S. mutans* strains, there was no correlation between possession of P1-CRA (SpaA) by *S. sobrinus* and adhesion or site of isolation. There is information suggesting that *S. sobrinus* strains attach to several different salivary pellicle molecules (Gibbons *et al.*, 1986) and this may be mediated by molecules other than SpaA. SpaA of *S. sobrinus* strains may be involved in the sucrose-induced aggregation of this species (Goldschmidt & Curtiss, 1990; Takahashi *et al.*, 1989), although in the present investigation we found no correlation between the possession of P1-CRA and increase in adhesion in the presence of sucrose.

Adhesion to surfaces is important in initial colonization, whereas subsequent accumulation may depend on other factors and lead to the successful incorporation of non-adherent strains or species in an ecosystem. Bacteria may accumulate by synthesizing extracellular molecules and subsequently binding to these. With one exception, all strains were capable of synthesizing dextrans from sucrose, whereas only 47% of strains were aggregated by dextran, suggesting that the binding of preformed dextran is not mediated by glucosyltransferase, indeed *S. sobrinus* strain 6715 has been shown to produce a glucan-binding protein (Lu Lu *et al.*, 1992).

The production of dextrans and their subsequent utilization as energy reserves has been postulated to be

important in maintaining a low pH and thus in demineralizing enamel. Therefore, the ability of strains to produce dextranase and their ability to regulate the production of dextranase may be important traits in caries production. Most strains produced dextranase, and even strain 6715-13-201, that had been selected on the basis of its inability to produce dextranase, was shown to have dextranase activity on SDS-PAGE. However, strain 6715-13-201 produced large amounts of dextranase inhibitor, which may explain why no dextranase activity was seen on blue dextran agar plates. There was a wide variation in the ability of strains to produce dextranase inhibitors but no strain was shown to be able to produce a dextranase inhibitor without the production of dextranase.

Comparisons were made between selected strains for their ability to produce caries as monoinfectants in gnotobiotic rats. The caries experiments were conducted for 30 d, as this was a minimal time for the detection of fissure caries lesions, and it was hoped that variations in the bacteria would be minimal, indeed oral swabs of rats after 30 d showed no rough/smooth colonial variants. Although there were variations in the ability of strains to cause caries, there was no clear relation between the ability to produce the disease and any *in vitro* characteristics. Therefore, *S. sobrinus* strains may be able to inhabit dental plaque and produce dental caries using a variety of different strategies.

In conclusion, this series of experiments has demonstrated that changes in a wide range of phenotypic traits may occur in *S. sobrinus* on passage through the gastrointestinal tract of germ-free rats. Whilst only a single strain has been studied in detail, it has been noted (data not presented) that other strains of *S. sobrinus* also demonstrate colonial variation after passage in a gnotobiotic rat. The study also draws attention to the different mechanisms *mutans* streptococci strains employ to become part of the normal flora, where loss of a single characteristic may not preclude successful colonization. The results indicate that no single trait, of those studied, is dominant in the ability of the strains to produce caries; a number of factors acting in concert may be essential and the nature of these factors may differ between strains.

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