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Studies on strains of *Streptococcus mutans* isolated from caries-active and caries-free individuals in Iceland

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Background: Dental caries has been strongly associated with mutans streptococci, particularly *Streptococcus mutans* and *S. sobrinus*. Many studies have linked these organisms to the carious process and counts of mutans streptococci have been used to monitor caries risk. The high levels of caries generally found in Iceland have enabled several studies to be performed on the variation within strains of *S. mutans*.
Methods: This paper reports some studies showing phenotypic differences between strains of *S. mutans* that were related to whether the strain was isolated from an individual with active caries or from a caries-free subject.
Results: Strains from individuals with active caries generally adhered better to apatite, were more vigorous in decalcifying apatite and had bacteriocin-like activity that was likely to help the strain compete successfully with other strains, for example in the dental plaque biofilm.
Conclusions: Phenotypic differences exist between strains of *S. mutans* depending on the caries activity of the individual from whom the strain was isolated.

Keywords: *Streptococcus mutans*; *dental caries*; *bacteriocin*; *decalcification*; *hydroxyapatite*

*S*treptococcus mutans has been recognised for many years as the predominant microorganism in the aetiology of dental caries. It is the key cariogenic organism according to the 'specific plaque hypothesis' (1) and a considerable amount of research has been carried out on the role of this organism in causing caries and in predicting a higher caries risk (2, 3). Counts of mutans streptococci from clinical samples, usually salivary samples, have been extensively used to predict and monitor caries risk (4–6) Furthermore *S. mutans* has been shown to be transmitted from mother to child, leading to the potential early onset of caries (7, 8); to colonise the mouths of children during 'windows of infectivity' that also open up the possibility of caries (9). Early colonisation of the mouth with mutans streptococci results in more caries later in life (3) and high counts of mutans streptococci in samples of dental plaque or saliva have been shown to predict a higher incidence of caries (4, 5). The considerable amount of clinical data linking mutans streptococci to dental caries is further backed up by a vast amount of laboratory and animal studies, not least the production of a vaccine (10) that has been shown to protect against caries induced in

experimental animals, including monkeys, by *S. mutans* and a highly cariogenic diet.

Dental caries has been a major problem in Iceland for several decades. The prevalence of caries was higher than in other Nordic countries (11) and the decline in caries recorded in N. America, Europe and elsewhere in the latter part of the 20th century did not occur in Iceland until later and, according to the latest surveys, caries prevalence seems to have risen again (12). A number of studies of mutans streptococci have been carried out in Iceland that have served to confirm a number of conclusions that fit with the specific plaque hypothesis (13–16). Interestingly, one longitudinal study of the development of approximal caries on originally healthy approximal tooth surfaces showed that *S. mutans* sometimes only appeared on the particular approximal tooth surface after the development of caries as detected radiographically. In all cases the count of *S. mutans* increased following the onset of caries (16).

Streptococcus sobrinus belongs to the group termed mutans streptococci and was originally a serotype (d, g, h) of *S. mutans*, later being reclassified as a species and one that is known to be particularly cariogenic due to

its enhanced production of acid and tolerance of low pH. *S. sobrinus* is, consequently, often associated with particularly severe caries both in studies of communities (17) and individuals (18, 19) and its frequency in the oral flora was found to be high in Iceland and associated with more observed caries (18–20).

Streptococcus mutans has been isolated from individuals in Iceland over at least three decades and a number of microbiological factors have been investigated. It has been of particular interest that *S. mutans* strains were sometimes isolated from individuals that had no caries although high counts of mutans streptococci in saliva samples were usually isolated from individuals with much caries. The salivary count of *S. mutans* was a significant component in a caries activity test developed from the analysis of salivary, microbiological and dietary data collected in a longitudinal study of Icelandic children aged 4–6 years (5, 15) However, the mutans count alone was not sufficient to give a reliable indication of caries risk for any particular individual. Köhler and Krasse (21) demonstrated in a hamster model, greatly increased carious destruction of teeth when the experimental animal was fed a cariogenic diet including a strain of *S. mutans* from an Icelandic patient compared with an experimental animal receiving the same diet but including the *S. mutans* strain Ingbritt, that has been widely used in microbiological studies of caries. This finding (21) prompted further studies of the role of mutans streptococci in caries that have subsequently been carried out in the research laboratory of the Faculty of Odontology, University of Iceland. In particular one aim was to determine if any differences were present between those strains of *S. mutans* that were isolated from individuals with no caries compared with those strains isolated from individuals with much caries. These investigations were used to determine phenotypic differences between strains in these two categories. Studies on this collection of strains over a number of years have included investigation of adherence of the strains to hydroxyapatite and decalcification of hydroxyapatite. Both these properties can be envisaged to play a role in the caries process and represent areas where strain differences may be significant with respect to pathogenicity. Later, bacteriocin-like activity was investigated as such bacterial interaction might play a role in determining which bacteria can become established in the dental plaque biofilm (22, 23).

Materials and methods

Strains of *S. mutans* were collected regularly from subjects of different ages from 4 years up to adulthood as part of one of a number of research projects aimed at assessing caries risk (15, 16, 18, 20). Ethical permission for each study was obtained from the relevant authority in Iceland as appropriate. Following the completion of these individual studies a collection of 38 strains of *S. mutans* was established (24 from subjects with active caries and 14 from individuals that were caries-free according to the examination criteria used in each particular study). All strains were stored frozen at − 80°C in a preservative medium (Nunc cryotubes, Fisher Scientific Loughborough, UK) All personal identifiers were removed and the strain collection was merely grouped into the identification of the species as *S. mutans* or *S. sobrinus* and the disease category as either caries-free or considerable active caries above average for the age group.

Adherence to hydroxyapatite

Test strains were removed from the freezer, cultured for 48 h in Todd-Hewitt broth (Difco) and subsequently incubated overnight in Todd-Hewitt broth containing tritiated thymidine and hydroxyapatite powder (Sigma-Aldrich) (24). The cultures were shaken gently on a Gyro rocker (Stuart Scientific, Keison International Ltd, UK). Following this treatment, the cultures were centrifuged and washed to remove non-adherent bacteria. The apatite powder was dissolved in 1N HCl and 0.25 mL aliquots transferred to a scintillation tube to which were added 1 mL Hionic fluor scintillation fluid and the radioactive count recorded in a scintillation counter (Packard instruments, Rockville, Md, USA).

Decalcification of apatite

The ability of strains of different *S. mutans* to decalcify hydroxyapatite was assessed using the method described by Chestnutt et al. (25). Test strains from caries-active individuals (24 strains) and caries-free individuals (14 strains) aged 7–20 years were obtained from the strain collection and cultured for 24 h in Todd-Hewitt broth (Difco). Cultures were centrifuged and 40 mg of bacterial deposit was resuspended in 400 μL glucose to which 40 mg hydroxyapatite slurry (Sigma) was added. This culture was incubated for 5 h at 37°C on a Gyro rocker (Stuart Scientific, Keison International Ltd, UK). Cultures were then centrifuged for 20 min at 15,000 rpm (Microspin 12S, Sorvall Instruments, UK). Then the pH of the supernatant fluid was determined and the calcium concentration of the supernatant was determined using 10 μL aliquots tested in a clinical chemistry autoanalyser (Kodak, Rochester, USA) at 680 nm.

Bacteriocin-like activity

The bacteriocin-like inhibitory effect was tested, using the methods of Rogers (22) and Parrot et al. (23). All eight test strains of *S. mutans* from caries-active individuals with open caries and the eight test strains from caries-free individuals were investigated against each other. Thus each strain was tested for bacteriocin-like activity against 15 strains of *S. mutans*. In addition, 14 *S. mutans* strains (seven from caries active and seven from caries-free

individuals) were tested using the same methodology against a laboratory collection of 25 oral microorganisms as listed in Table 3. All test strains were grown overnight in Todd-Hewitt broth, yielding approximately 10^5/cfu/mL, and 1 mL of each culture was used to inoculate pour-plates of semi-solid tryptic-soy-yeast-extract agar. In this role the strains were indicator strains of bacteriocin-like production. After the pour plates had set all of the test strains were stab-inoculated into the agar surface of each pour plate. These pour plates were then incubated at 37°C in a candle jar for 48 h and zones of inhibition of the indicator strain around the stab inoculations were viewed in a bifocal microscope and the diameter of the zone of inhibition measured. Zones of inhibition of the indicator strains around the stab inoculated test strain ranged from 7 to 12 mm and all zones of diameter 7 mm or greater were recorded as positive for bacteriocin activity.

Results

Adherence of S. mutans strains to hydroxyapatite

Strains of *S. mutans* isolated from caries-active individuals were found to adhere significantly better to apatite than did those strains isolated from caries-free subjects (Student′s *t*-test; $p < 0.05$), see Table 1.

Decalcification of hydroxyapatite by strains of S. mutans

Strains of *S. mutans* from individuals with active caries were found to release significantly more calcium from hydroxyapatite than strains isolated from caries-free individuals (Student′s *t*-test; $p < 0.02$), see Table 2.

The pH of the individual cultures was measured at the end of the incubation period. Considerable strain variation in the final pH was observed with pH values from caries-active individuals ranging from 4.1 to 4.9 (mean 4.4; SD 0.23) and from caries-free individuals 4.2–5.1 (mean 4.6; SD 0.19) but the difference in final pH between strains was significantly lower in the cultures containing strains from caries-active individuals (Student′s *t*-test; $p < 0.01$).

Bacteriocin-like activity

Strains isolated from caries-active subjects showed greater bacteriocin-like activity against other isolates of

Table 1. Scintillation counts for strains of *S. mutans* from caries-active and caries free subjects and adhering to hydroxyapatite

Strains from individuals	Mean scintillation count (cpm)	SD
Caries active	1639	961
Caries free	603	103

Table 2. Calcium released from hydroxyapatite by strains of *S. mutans* from caries-active and caries free subjects

Strains isolated from individuals	Mean calcium release (mmol/L)	SD
Caries free	10.5	4.1
Caries active	15.7	6.6

*S. mutans (*average 3.4 strains*)* than did strains isolated from subjects that were caries free (average 1.0 strains; $p < 0.01$(chi^2); see Fig. 1). Furthermore 7/8 strains from caries active individuals inhibited ≥ 2 other strains of *S. mutans* whereas only 1/8 strains from a caries-free individual inhibited ≥ 2 other strains of *S. mutans.*

When bacteriocin-like inhibition of 25 oral commensal organisms by *S. mutans* producer strains was tested (seven strains from caries active individuals and seven strains from caries-free individuals) the strains of *S. mutans* from caries-free individuals were found to be significantly more inhibitory to other oral commensals than the strains from caries active individuals ($p < 0.001$; see Table 3).

Thus the seven *S. mutans* strains from caries-free individuals inhibited other oral commensals in a total of 88/126 tests whereas the seven *S. mutans* strains inhibited from caries-active individuals inhibited oral commensals in only 58/126 tests (*t*-test; $p < 0.05$)

Discussion

Intitially studies of the role of *S. mutans* in caries in Iceland was expected to confirm the pathogenic role of
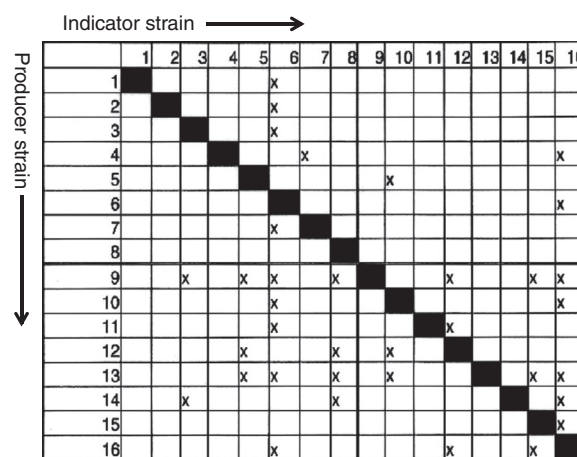

Fig. 1. Pattern of bacteriocin-like activity within strains of *S. mutans* (strains 1–8 isolated from caries-free subjects and strains 9–16 from caries-active subjects). Note that the strains from caries-active subjects produce bacteriocins that inhibit growth of more indicator strains than do strains isolated from caries-free subjects.

Table 3. Bacteriocin-like activity against oral commensals by 14 strains of S. mutans, seven from caries-active subjects and seven from caries-free subjects

Indicator organism	No. of strains tested	Total number of indicator oral bacteria inhibited by the producer strains for each test group of *S. mutans* (number inhibited/ number of tests)	
		Caries free ($N = 7$)	Caries active ($N = 7$)
S. salivarius	3	15/21	5/21
S. bovis	1	4/7	2/7
S. sanguinis	1	5/7	7/7
S. mitis	4	16/28	10/28
S. sobrinus	2	7/14	4/14
'Viridans streptococci'	3	16/21	13/21
S. pneumoniae	4	25/28	18/28
Staphylococci coag.+	3	11/21	5/21
Staphylococci coag.−	2	8/14	4/14
Lactobacillus sp.	2	8/14	9/14

this organism and indeed it was hoped to add to our knowledge of the pathogenic capacity of this bacterium in the light of the high levels of caries that were being investigated. The findings of Köhler and Krasse (21) that an Icelandic strain of *S. mutans* was particularly cariogenic in an animal model reinforced this line of thought. Laboratory studies then demonstrated that Icelandic strains of *S. mutans* from subjects with active caries indeed adhered significantly better to hydroxyapatite and decalcified hydroxyapatite significantly more than strains isolated from subjects that were caries free. Furthermore the prevalence of *S. sobrinus* had been found in several studies (18–20) to be relatively high in the Icelandic population, reinforcing the view that the teeth of Icelanders were in a rather more cariogenic environment than was found in many other studies being carried out at this time.

With the development of the ecological plaque hypothesis (27, 28) the emphasis on the differing cariogenic potentials of strains of mutans streptococci began to wane. Studies of bacteriocin-like activity among the Icelandic test strains suggested indeed that the strains of *S. mutans* from caries active subjects were more aggressive at inhibiting other strains of *S. mutans* than those strains isolated from caries-free individuals. Thus *S. mutans* from caries-active individuals may dominate the less cariogenic strains of the same species and succeed in finding space to adhere and multiply within the cariogenic dental plaque biofilm. In caries-free individuals, however, the apparently less cariogenic strains of *S. mutans* appear to compete more successfully against other oral commensals than do the more cariogenic strains of *S. mutans*. These less cariogenic strains then occupy the niche for *S. mutans*-like organism within the dental plaque biofilm and thus help maintain its low cariogenicity. Several studies of bacteriocin-like proper-

ties have suggested a significant role for this activity in the ecology of dental plaque (7, 29–31). It is quite possible that the differing degrees of cariogenicity seen in *S. mutans* strains from caries-active and caries-free subjects could be a reflection of the oral/ dental environment from which the organisms were originally isolated rather than a genetically determined pathogenic potential. Thus *S. mutans* bacteria in a dental biofilm that has ready access to sucrose could have several metabolic pathways working that contribute to the cariogenicity of the organism whereas *S. mutans* strains from a biofilm that is less exposed to sucrose, for example, may have to compete with other organisms to retain a place in the biofilm but this possibly results in less expression of the cariogenic potential of the *S. mutans* strain.

In developing this hypothesis further the authors were fortunate in being asked to submit strains of *S. mutans* from this collection to an international study of possible genetic differences between strains isolated from caries-active and caries-free individuals. This investigation was carried out by recently by Do et al. (32) and showed no genetic differences between strains and thus reinforces somewhat the ecological plaque hypothesis and the idea that the increased cariogenic properties found in strains of *S. mutans* from caries-active subjects are manifestations of phenotypic characteristics related to the environment from which the strains were sampled. It may also serve to illustrate the considerable potential for adaptability of strains of *S. mutans* in the oral cavity.

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