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Mutacin production in *Streptococcus mutans* genotypes isolated from caries-affected and caries-free individuals

R. U. Kamiya, M. H. Napimoga, R. T. Rosa, J. F. Höfling, R. B. Gonçalves
Microbiology and Immunology Laboratory, Dentistry School of Piracicaba, State University of Campinas, Brazil

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Relationships between genetic diversity and mutacin production in *Streptococcus mutans* were evaluated in 319 clinical isolates from eight caries-affected and eight caries-free individuals. The isolates were submitted to mutacin typing and AP-PCR (arbitrarily primed polymerase chain reaction) assay. The mutacin production was detected for 12 *Streptococcus* sp. indicator strains. Results showed significant variations in the mutacin production profiles and the inhibitory spectra of both groups. A possible association was seen between mutacin activity and the distinct patterns of *Streptococcus* sp. colonization in the two groups. Genotyping by AP-PCR using the primers OPA-02 and OPA-13 revealed 101 distinct genotypes against 48 phenotypes identified by mutacin typing. No correlation was observed between the inhibitory spectra of mutacin and genotypic similarities based on AP-PCR analyses. According to our results, strains of the same *S. mutans* genotype showed different mutacin profiles, suggesting a high degree of interstrain diversity. In conclusion, mutacin production seems to be of clinical importance in the colonization of *S. mutans* and is highly diversified in the *S. mutans* species.

Key words: AP-PCR; dental caries; genotyping; mutacin; *Streptococcus mutans*

Reginaldo Bruno Gonçalves, Microbiology and Immunology Laboratory, Dentistry School of Piracicaba, State University of Campinas, Brazil
E-mail: reginald@fop.unicamp.br
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Streptococcus mutans is believed to be the principal etiologic agent of dental caries (3, 15). Most strains of *S. mutans* produce mutacins (bacteriocins), which are antimicrobial substances that inhibit the growth of closely related species (9, 26) as well as other gram-positive bacteria (26). The role of mutacins *in vivo* is unknown; however, the antimicrobial activity of these substances may confer an ecologic advantage for the producing strain in bacterial communities such as the dental biofilm (3). Mutacins may also be important for the establishment of *S. mutans in vivo* (3, 10).

Several studies suggested that the mutacin activity of *S. mutans* can be related to the prevalence of this species in the bacterial biofilm, saliva and dental caries (4, 12, 23), increasing the risk of caries (9).

Fabio et al. (7) demonstrated that mutacin production can increase the proportion of *S. mutans* in oral streptococci. However, Alaluusua et al. (2) and Longo et al. (16) did not find any positive correlation between mutacin production and caries activity.

The possible association between the biodiversity of *S. mutans* and caries activity deserves further study because the scarce literature has reported contradictory data about the impact of the genetic/phenotypic diversity of cariogenic species in the development and progress of dental caries (1, 13). Studies of virulence factors of *S. mutans*, as well as the mutacin production and its correlation with species biodiversity, are

fundamental to the understanding of the role played by different genotypes colonizing the same individual, and the expression of characteristics that may or may not influence their virulence capacity and survival ability under different environmental pressures.

This study determined the production of mutacin of *S. mutans* genotypes isolated from caries-affected and caries-free individuals.

Materials and methods

Subjects

The study population consisted of 16 individuals aged 18–29 years. Eight subjects were assigned to each group, one caries-free and the other caries-affected (dmft = 12.0 ± 3.07). Ethical approval for this study was granted by the Ethics Committee for Human Subjects at Piracicaba School of Dentistry.

Bacterial strains

None of the subjects had used antibiotics for the past 6 months. A total of 319 *S. mutans* strains had previously been isolated from the saliva, dental biofilm and tongue dorsum of these 16 individuals (141 and 178 isolates, respectively, in the caries-affected and caries-free groups) (19).

To test mutacin activity, 12 *Streptococcus* sp. stock cultures were used as indicator strains: *S. mutans* CCT 3440, *S. mutans* 32 K, *Streptococcus sobrinus* ATCC 27607, *S. sobrinus* 6715, *Streptococcus mitis* A, *S. mitis* ATCC 903, *Streptococcus salivarius* ATCC 25975, *S. salivarius* 66.4, *Streptococcus sanguis* CR 311, *S. sanguis* M 5, *S. sanguis* ATCC 10556 and *Streptococcus oralis* PB 182.

Microbiological processing

For the detection of mutans streptococci, samples were previously diluted and cultured on Mitis Salivarius agar plates (MSA; Difco Laboratories, Sparks, MD) with 0.2 units·ml^{-1} of bacitracin. The plates were incubated at 37°C for 48 h in a 10% CO_2 atmosphere (Walter-Jacked CO_2 Incubators/Cole Parmer Instruments, Vernon Hills, IL, USA). When samples exhibited growth on the MSB plates, 10–15 isolates, if available, representing all morphologic types per sample. were taken and inoculated in brain heart infusion broth (BHI; Difco Laboratories).

All isolates of *S. mutans* were identified by biochemistry tests (fermentation of mannitol, sorbitol, raffinose and melibiose, production of hydrogen peroxide, arginine hydrolysis and resistance to bacitracin) (11, 28), and had their molecular identification confirmed by polymerase chain reaction (PCR) with specific primers of GTFB-F (5′-ACTACACTTTCGGGTGG CTTGG-3′) and GTFB-R (5′-CAGTATAA GCGCCAGTTTCATC-3′) (21).

Mutacin typing

Mutacin production by clinical isolates was tested by modification of the deferred-antagonism method, the stab culture method (10). The frozen *S. mutans* cultures were reactivated in 5 ml BHI and incubated at 37°C in 10% CO_2 for 24 h. The strain cultures (10^8 colony forming units (CFU)/ml through McFarland Scale) were inoculated in Trypticase Soy agar 1.5% (w/v) (TSA; Difco) with a 0.6-mm-thick needle. After a 48-h incubation at 37°C and 10% CO_2, the plates were overlaid with 4.5 ml of soft TSA 0.8% (w/v) containing 0.5 ml (10^8 CFU/ml) of an overnight Trypticase Soy broth (TSB; Difco) culture of the indicator strain. After overnight incubation at 37°C, the diameter of the inhibition zone was measured. The isolates were recorded as mutacin active against the indicator strain if the diameter was 4 mm or greater. The breakpoint of 4 mm was based on earlier studies (2, 9, 10). The isolates were tested in duplicate for mutacin activity. The mean size of the inhibition zone was used in the fingerprinting of *S. mutans*.

Mutacin activity profiles

Twelve indicator strains were used. For every three indicator strains, one score between 0 and 7 was given to the isolates depending on which of the isolates were sensitive to the mutacin produced (Table 1). In this way a 4-figure profile characterized every isolate. These profiles were regarded as distinct when one or more of the figures were different (27).

Table 1. System used to transform bacteriocin production against three indicator strains into a unique numerical classification (27)

	Indicator strains		
Score	1	2	3
0	–	–	–
1	+	–	–
2	–	+	–
3	+	+	–
4	–	–	+
5	+	–	+
6	–	+	+
7	+	+	+

– = no bacteriocin production against indicator strain.
+ = bacteriocin production against indicator strain.

S. mutans AP-PCR typing

The strains identified as *S. mutans* by biochemical tests and the PCR method were submitted to AP-PCR typing. PCR amplification was performed with single-stranded 10-mer oligonucleotide primers OPA-02 (5′-TGCCGAGCTG-3′) (14) and OPA-13 (5′-CAGCACCCAC-3′) (25). The method used for amplification of *S. mutans* was performed as described by Saarela et al. (25) with some modifications. The DNA used as template was obtained by lyses (10 min/100°C) of bacterial cell with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Briefly, each PCR mixture contained 350 μg of the DNA template, 5.0 μl of 10× PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 0.2 mM of dNTP, 0.4 μM *primer* OPA-02 or OPA-13, 3.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Gibco, Grand Island, NY, USA) together with sterile distilled water to make up a final volume of 50 μl.

The temperature profile in a thermocycler (Gene Amp PCR System 2400, Perkin Elmer Inc. Shelton, CT) for 35 cycles was carried out as follows: an initial denaturation at 94°C for 5 min, followed by denaturation at 95°C for 1 min, annealing (36°C for 2 min), extension (72°C for 2 min) and the final extension (72°C for 5 min) (16). Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gel in Tris-borate-EDTA running buffer (TBE), stained in 0.5 μg/ml ethidium bromide and visualized with ultraviolet light. The images of gels from either AP-PCR pattern were photographed and scanned by a high-resolution imaging system (Image Master-LISCAP, VDS; Pharmacia Biotech, Piscataway, NJ). A 100 bp DNA ladder (Gibco) was run as a molecular-size marker in the gel.

The molecular weights for each band or amplicon were computed and analyzed by the SIGMA GEL software program. In order to assess the clonal diversity of the *S. mutans* isolates, a matrix of similarity using Dice's similarity coefficient was built based on data obtained with the two *primers* in the AP-PCR. The dendograms were constructed using the UPGMA method across NTSYS (numerical taxonomic and multivariate analysis system) program (Exeter Soft Ware, Setauket, NY). This was done for all DNA patterns

produced by the AP-PCR method. To assure reproducibility, all PCR reactions were carried out with *S. mutans* CCT3440 chromosomal DNA. The genotypes were considered identical when there was 100% similarity.

Statistics

The results of the deferred antagonism method were statistically analyzed using the Chi-squared test to compare the mutacin production against different indicator strains. The Pearson correlation was used to analyze the relationships between number of genotypes/phenotypes with the number of strains used and the number of genotypes/phenotypes with caries activity (dmft). Simpson's index of diversity was used to test the discriminatory index of each technique (8).

Results

A total of 48 different phenotypes and 101 genotypes were detected among the 319 of *S. mutans* isolates. Based on mutacin typing, 75.23% (240/319) of the isolates produced mutacins against one or more of the indicator strains. Of the *S. mutans* strains isolated in the caries-affected and caries-free groups, respectively, 26.24% (37/141) and 23.59% (42/178) did not produce mutacin against any of the 12 indicator strains used. The inhibition zone sizes for producer strains varied from 4 to 24 mm in diameter. On average, the isolates were able to inhibit 33% of the indicator strains used. Nine isolates, representing five *S. mutans* genotypes, were active against all the indicator strains. Strains with higher mutacin activity (profile [7777]) were infrequent among the isolates in both caries-free and caries-affected groups. In addition, two caries-affected and two caries-free individuals had a high incidence of no producer strains (biotype [0000]).

The activity and profile of mutacin production between the groups analyzed showed opposing results (Fig. 1). The *S. mutans* strains of caries-affected individuals had a higher mutacin production and broad inhibitory spectrum against the different indicator strains (Fig. 1). Mutacin production profiles between groups were distinct. The isolates of caries-free individuals had statistically higher inhibitory activity against initial colonizers, represented by *S. sanguis*, *S. oralis* and *S. mitis* and low antagonist activity against the cariogenic species *S. mutans* and *S. sobrinus* (Fig. 1).

The *S. mutans* strains with similar profiles were classified and grouped according to their intraindividual phenotypic and genotypic heterogeneity, using mutacin typing and AP-PCR, respectively. Table 2 shows the number of phenotypic and genotypic profiles found in both groups.

There were no associations between the number of isolates tested and the number of genotypes (Pearson correlation test, $P = 0.67$; $r = -0.11$) or phenotypes ($P = 0.10$; $r = 0.68$) in either group.

The Pearson correlation test showed a strong association between dmft and genotypic diversity ($r = 0.88$, $P = 0.003$); however, no correlation between dmft and phenotypic diversity ($r = 0.47$, $P = 0.23$) was found.

The AP-PCR typing of *S. mutans* performed with the random primers OPA-02 and OPA-13 showed a greater discriminatory ability (0,975) than mutacin typing (0,905). Epidemiologically unrelated individuals did not share any AP-PCR identical types. Mutacin typing with 12 indicator strains revealed 48 different mutacin types among these isolates and identical phenotypes were shared by epidemiologically unrelated individuals.

Table 2. Number of phenotypes and genotypes of *S. mutans* isolated from caries-free and caries-affected individuals

Individuals	Isolates (*n*)	Mutacin typing phenotype	AP-PCR genotype	dmft
Caries-affected				
A	27	5	4	8
B	19	6	13	16
C	19	6	9	14
D	16	4	8	12
E	11	5	5	8
F	16	5	10	13
G	16	6	4	10
H	17	9	8	15
Total	141	46	61	–
Caries-free				
I	29	6	7	–
J	11	5	5	–
K	27	6	4	–
L	22	4	5	–
M	21	8	6	–
N	27	4	4	–
O	25	7	7	–
P	16	3	2	–
Total	178	43	40	–

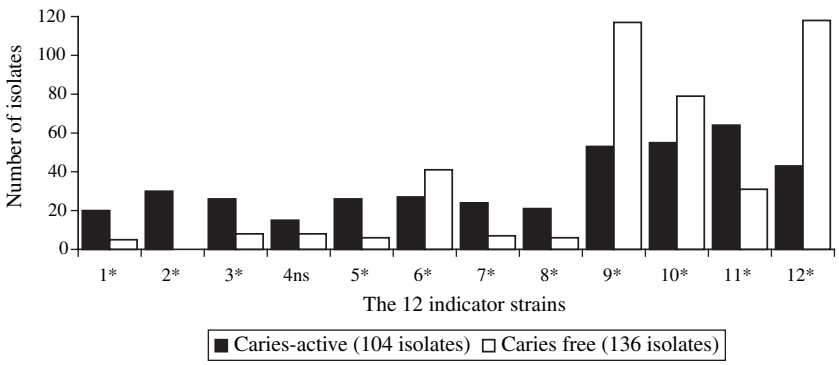

Fig. 1. Number of mutacin-producing isolates from caries-affected and caries-free individuals. Axis: 1 – *S. mutans* CCT 3440; 2 – *S. mutans* 2K; 3 – *S. sobrinus* ATCC 27607; 4 – *S. sobrinus* 6715; 5 – *S. mitis* A; 6 – *S. mitis* ATCC 903; 7 – *S. salivarius* ATCC 25975; 8 – *S. salivarius* 66.4; 9 – *S. sanguis* CR 311; 10 – *S. sanguis* M5; 11 – *S. sanguis* ATCC 10556; 12 – *S. oralis* PB182. * Differed among themselves by Chi-squared test ($p < 0.05$). ns, did not differ among themselves by Chi-squared test ($p > 005$).

Discussion

Inside the oral ecosystem, the development of the bacterial community generally involves a succession of populations and competition for receivers of adhesion, foods, and the production of inhibitory substances such as the bacteriocins. These factors are among the mechanisms involved in the modulation of the colonization and the microbial growth (17). The present study evaluated the mutacin production in different genotypes of *S. mutans* isolated from caries-free and caries-affected individuals.

The frequency of mutacin production in *S. mutans* may vary from 70 to 100% (4, 5, 9, 10, 22). In the present study, 79.62% (254/319) of *S. mutans* strains analyzed showed mutacin activity against one or more indicator strains. These differences in the frequency of production described in the literature are probably caused by different conditions in the tests and the use of distinct indicator strains (24). The existence of producer or nonproducer

strains in the same niche of the oral cavity may suggest a possible synergistic activity of mutacins, favoring the stability *in situ* of nonproducer bacteria of mutacin.

According to Grönroos et al. (9), the bacteriocin activity of *S. mutans* may increase the ratio of this species in the dental biofilm, contributing to the increased risk of caries. However, there is conflicting and controversial information regarding mutacin production and the risk of caries. Alaluusua et al. (2) did not find a positive correlation between mutacin activity and the number of cariogenic streptococci in dental biofilm (risk of caries), whereas Fabio et al. (7) showed a positive association between the proportion of *S. mutans*/ total of oral streptococci and mutacin potential. Longo et al. (16) reported no association between mutacin inhibitory spectrum and infecting levels of mutans streptococci or caries incidence, suggesting that the mutacin production may not be relevant for *S. mutans* to be able to colonize the host and to induce disease.

In the present study, we show distinct mutacin production profiles between *S. mutans* isolated from caries-affected and caries-free individuals, which can be related to the different colonization profiles described in these individuals. According to the findings of Nyvad & Kilian (20), caries-affected individuals are more easily infected and colonized by cariogenic streptococci, whereas caries-free individuals harbor a higher prevalence of *S. sanguis* and other streptococci of the mitis group, which may explain the more intense mutacin activity against the predominant colonizers in the respective studied populations.

Besides ecology studies, bacteriocin typing can be used in the taxonomic classification of bacteria. To get more precise results in the fingerprinting of *S. mutans,* the isolates were submitted to the AP-PCR technique. In none of 16 individuals analyzed was the phenotypic grouping identical to the genotypic grouping. Genotypes that were identical did not present the same mutacin production profile as traced by mutacin typing, as well as distinct genotypes grouped in the same phenotypic profile, showing that the patterns of inhibitory spectra produced by distinct *S. mutans* genotypes are independent of the degree of genetic similarity of the strains tested.

Various factors can influence these different grouping results. The use of cell culture supernatant instead of previously purified bacteriocins can result in a limited bacteriocin production profile; a strain can therefore synthesize one or more bacteriocin types (6). In view of the wide diversity of mutacins synthesized by *S. mutans,* the arbitrary primers OPA-02 and OPA-13 were not capable of detecting the polymorphism of the gene locus of mutacin production, on the basis of identical genotypes with distinct phenotypic profiles. In addition, slight changes in amino acid composition can account for differences in the spectra of inhibitory activity (18).

According to Balakrishnan et al. (3), phylogenic analysis of the mutacin producer *S. mutans* through genotypic characteristics accessed by multilocus enzyme electrophoresis revealed that the mutacin groups are associated with distinct evolutionary lineages of species. The existence of strains with the same phenotypic profile that were not genetically related supports the hypothesis of horizontal transmission of bacteriocin production genes. In the present study, identical mutacin production profiles were detected in strains that were genetically not related, as judged by AP-PCR results. In addition, some mutacin producer strains were grouped in the same nonproducer strain phylogenic group (profile [0000]) (data not shown), suggesting that genetic mutations not detected through AP-PCR technique may have led to loss of inhibitory activity.

AP-PCR showed a greater discriminatory ability than the mutacin typing, and different genotypes were not distinguished using the phenotypic technique, in agreement with Grönroos et al. (9), who showed a lower discrimination of mutacin typing in relation to ribotyping of *S. mutans*.

There are controversial studies of *S. mutans* clonal diversity relationship with caries activity. While Alalusuua et al. (1) detected a greater genotypic diversity of cariogenic streptococci in nursing-bottle caries children, using ribotyping, Kreulen et al. (13) did not show the same results using AP-PCR technique in *S. mutans* isolates, suggesting that children with nursing-bottle caries experience clonal selection of potentially cariogenic strains and, consequently, less genetic diversity.

The phenotypic diversity of *S. mutans* detected through mutacin typing was not correlated with previous caries experience; however, the genotypic diversity was positively correlated with the dmft index of caries-affected individuals. It is difficult to compare these data with previous results from previous studies because the parameters, such as type of method used, genetic marker, number of isolates, sites analyzed and age of samples, are different.

In conclusion, mutacins could play an important biological role in the regulation and composition of dental biofilm due to their synergistic or antagonist activity. The patterns of mutacin production are independent of the AP-PCR genotypic similarity, which suggests high genetic polymorphism in the gene *locus* of mutacin biosynthesis. Further studies are needed to identify specific mutacins that might be associated with different production phenotypes identified among the *S. mutans* isolated in the present study.

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