Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

UNICAMP

Regianne Umeko Kamiya

Cirurgiã Dentista

"ANÁLISE DA FREQÜÊNCIA E DA EXPRESSÃO DE GENES DE BIOSSÍNTESE DE MUTACINAS EM ISOLADOS DE *Streptococcus mutans*"

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para a obtenção do título de Doutor em Biologia Buco Dental, Área de Microbiologia e Imunologia.

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Orientador: Prof. Dr. Reginaldo Bruno Goncalves

Banca examinadora:
Prof. Dr. Antônio Olavo Cardoso Jorge
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- Confúcio

SUMÁRIO

RESUMO

Esta tese, apresentada na forma de 3 artigos, teve por objetivos: (1) analisar a freqüência dos genes de produção de mutacinas I, II, III e IV, em genótipos de *S. mutans* isolados de indivíduos cárie-ativos e livres de cárie, (2) analisar a freqüência dos genes de produção das mutacinas I, II, III, IV, N, B-Ny 266, 1140 e genes homólogos às bacteriocinas, identificadas em outras espécies bacterianas, em cepas de *S. mutans* isolados de crianças, bem como detectar a expressão diferencial dos genes identificados, em células de *S. mutans* crescidas na condição planctônica e séssil, (3) analisar a expressão dos genes de produção das mutacinas I, II e proteínas kinases CiaH, Dgk e ComD, em diferentes fases do crescimento planctônico e séssil.

O rastreamento e a freqüência dos genes estruturais de diferentes mutacinas em isolados de *S. mutans,* foram realizados pela técnica de PCR e a análise da expressão gênica, pela técnica de RT-PCR semi-quantitativa.

Os estudos, apresentados nesta tese, demonstraram o papel das mutacinas como um fator de virulência, altamente diversificado entre a espécie *S. mutans*, e relacionado com o risco de cárie. Este fator de virulência, pode ser regulado por mecanismos *quorum-sensing*, sendo assim, dependente da condição de crescimento planctônica ou séssil. A regulação da produção de mutacinas, por mecanismos *quorum-sensing*, pode representar uma vantagem seletiva à espécie produtora, principalmente em ambiente complexo, como o biofilme dental e lesões de cárie.

Futuramente, mais estudos serão necessários para identificar novos determinantes genéticos, necessários para a síntese de substâncias semelhantes às mutacinas, bem como, identificar os mecanismos e componentes, que modulam a expressão deste importante fator de virulência em *S. mutans*.

ABSTRACT

This thesis, comprised of 3 manuscripts was designed (1) to analyse the frequency of biosynthesis genes of the mutacins types I, II, III and IV, in *S. mutans* isolated from caries-affected and caries-free individuals, (2) to analyse the frequency of biosynthesis genes of the mutacins types I, II, III, IV, N, B-Ny 266, 1140 and genes homologues to bacteriocins identified in other bacterial species, in *S. mutans* isolated from children, in addition, to detect the differential expression of these genes, in *S. mutans* cells grown in planktonic and sessil conditions, (3) and to analyse the expression of the mutacins I and II production genes and kinase proteins genes (*cia*H, *dgk* e *com*D), in different phases of the planktonic and sessile growth.

The *screening* and frequency of the mutacins structural genes in *S. mutans* isolates were realized by PCR technique and the analysis of genetic expression, by RT-PCR semi-quantitative method.

The studies, presented in this thesis, demonstrate the role of mutacins as a virulence factor, highly diverse among *S. mutans*, and related to risk of dental caries. The mutacins production may be regulated by *quorum-sensing* mechanisms and is dependent on planktonic and sessile conditions. The modulation by *quorum-sensing* mechanisms may represent a selective advantage for producer *S. mutans* strain, mainly in complex environments as the dental biofilm and caries.

Hereafter, more studies will be necessary to identify new genetic determinants for synthesis of mutacin-like substances and elucidate the mechanisms and components that modulate the genetic expression of this important virulence factor in *S. mutans*.

I - INTRODUÇÃO GERAL

Os estreptococos grupo mutans, em destaque os *Streptococcus mutans* são considerados os principais agentes etiológicos da cárie dental [Whiley & Beighton, 1998]. Vários fatores, predisponentes da doença cárie, estão relacionados com características de virulências de *S. mutans*. A produção de glucanos insolúveis em água e de proteínas ligantes de glucanos (Gbps), em presença de sacarose, aumenta a aderência de *S. mutans* na superfície dental promovendo a formação do biofilme cariogênico [Smith *et al.*, 1994; Sato *et al.*, 1997].

Neste biofilme, o acúmulo de metabólitos ácidos produzidos por *S. mutans*, provoca a desmineralização da superfície do esmalte e a formação de cavitações, tornando o meio seletivo para espécies mais acidúricas [Li *et al.*, 2002]. A resistência ao pH ácido e a síntese de bacteriocinas, denominadas mutacinas, podem representar vantagens ecológicas à espécie de *S. mutans*, através do controle e diminuição da população de microrganismos competidores na cavidade oral [Kitamura *et al.*, 1989; Gronröos *et al.*, 1998; Li *et al.*, 2001b; Napimoga *et al.*, 2005].

Uma característica marcante de *S. mutans* é que a maioria dos isolados clínicos produz substâncias semelhantes às mutacinas capazes de inibir o crescimento de bactérias genética e ecologicamente relacionadas [Jacob *et al.*, 1953; Tagg *et al.*, 1976; Grönroos *et al.*, 1998; Kamiya *et al.*, 2005a – trabalho realizado no mestrado].

1.1. Atividade biológica das mutacinas e a cárie dental

Existem evidências de que as mutacinas podem exercer um importante papel na proteção e colonização de *S. mutans* na cavidade oral, principalmente em ambientes complexos, como o biofilme dental e lesões de cárie [Balakrishnan et al., 2002; Kamiya *et al.*, 2005a]. A produção destas substâncias *in vivo* sugere vantagens ecológicas à cepa produtora através da substituição da microbiota residente ou prevenção da invasão de microrganismos exógenos [Ikeda *et al.*, 1982; Hillman *et al.*, 1984].

O papel ecológico das mutacinas *in vivo* foi demonstrado após a implantação e estabilidade de cepas de *S. mutans* produtoras de mutacinas de amplo espectro, na cavidade oral de animais e de humanos [van der Hoeven & Rogers, 1979; Kitamura *et al.*, 1989; Hillman *et al.*, 1987, 2000].

Baseando-se nas propriedades seletivas e ecológicas das mutacinas *in vivo*, foi desenvolvida a terapia de substituição bacteriana para prevenção do desenvolvimento da cárie dental [Hillman *et al.*, 2000]. Através da construção de uma cepa mutante *S. mutans* BCS3-L1, produtora de bacteriocina de amplo espectro *in vitro* (mutacina 1140) e defectiva na síntese de ácido láctico, foi possível efetivar a colonização da cepa produtora de baixo potencial cariogênico, na cavidade oral de ratos. A atividade biológica da mutacina produzida *in vivo* reduziu o índice de cárie possivelmente devido à inibição de *S. mutans* competidores mais cariogênicos.

Embora existam evidências da atividade biológica e seletiva das mutacinas *in vivo,* estudos não relataram uma correlação positiva entre o espectro inibitório das mutacinas e os níveis de infecção por *S. mutans* ou incidência de cárie [Longo *et al.*, 2003].

Entretanto, substâncias semelhantes às mutacinas de amplo espectro, foram mais freqüentes em genótipos de *S. mutans,* isolados de indivíduos cárie-ativos em relação aos genótipos isolados de voluntários livres de cárie [Kamiya *et al.*, 2005a]. O espectro de atividade das mutacinas, identificado neste estudo, foi direcionado contra colonizadores predominantes nas respectivas populações estudadas, sugerindo uma possível correlação entre o perfil de produção de mutacinas (espectro inibitório) e o padrão de colonização microbiana observado em indivíduos cárie-ativos e livres de cárie, descrito previamente por Nyvad & Kilian (1990).

O rastreamento e a identificação dos determinantes genéticos, relacionados com os diferentes fenótipos produtores de mutacinas, identificados entre os isolados do grupo cárie-ativo e livre de cárie, foi o principal objetivo do primeiro trabalho [Kamiya *et al.*, 2005b - capítulo 1].

1.2. Classificação das Mutacinas Caracterizadas

As bacteriocinas sintetizadas pelas diversas espécies de bactérias apresentam atividades e estruturas muito distintas, porém, preservam entre si propriedades em comum. Estas proteínas consistem em 20 a 60 resíduos de aminoácidos, com cargas elétricas positivas, podendo ser hidrofóbicas e/ou anfifílicas, atuando na formação de poros na membrana celular ou reduzindo o metabolismo de bactérias sensíveis [Nes & Holo, 2000].

Baseando-se na modificação pós-traducional, as bacteriocinas de bactérias Gram-positivas foram classificadas em 2 grupos: classe I, de bacteriocinas modificadas após a tradução ou lantibióticos, e classe II, de bacteriocinas não modificadas ou não lantibióticos.

Os lantibióticos são peptídeos que contém lantionina, β-metil-lantionina e resíduos dehidratados, são sintetizados por ribossomos e sofrem modificações após a tradução (SAHL *et al.*, 1995). Dentre as mutacinas bem caracterizadas, as mutacinas I, II, III, 1140, B-Ny 266, SmbA e SmbB são classificadas como lantibióticos [Mota-meira *et al.*, 1997; Hillman *et al.*, 1998; Qi *et al.*, 1999b, 2000; Yonezawa & Kuramitsu, 2005]. Já as mutacinas tipo IV (composta por dois peptídeos de atividades sinérgicas, NlmA e NlmB) e tipo N foram classificadas como não lantibióticos [Qi *et al.*, 2001; Balakrishnan *et al.*, 2000].

Os locus gênicos de biossíntese das mutacinas lantibióticos I, II e III foram completamente seqüenciados [Qi *et al.*, 1999a, 1999b e 2000], e a análise seqüencial envolve vários genes, incluindo aqueles envolvidos na produção (*mut*A), regulação (*mut*R), modificação (*mut*M), clivagem *(mutBC*), transporte (*mut*T) e auto-imunidade (*mut*FEG) (figura 1). Atualmente, foram seqüenciados fragmentos abertos de leitura (ORFs) que flanqueiam os genes estruturais das mutacinas IV, N, SmbA e SmbB e que possivelmente estão relacionados com a regulação da expressão e produção das respectivas substâncias [Qi *et al.*, 2001; Hale *et al.*, 2004, 2005; Yonezawa & Kuramitsu, 2005] (figura 1). A proximidade dos genes estruturais da mutacina (*mut*N) e do peptídeo sinal de competência (*com*C) sugere correlação entre a produção de mutacina N e o desenvolvimento da competência [Hale *et al.*, 2004].

Estudos prévios [Longo *et al.*, 2003; Kamiya *et al.*, 2005b; Li *et al.*, 2005] têm demonstrado a baixa freqüência dos genes estruturais das mutacinas caracterizadas, em diferentes isolados clínicos de *S. mutans*, fenotipicamente identificados como produtores de substâncias semelhantes às mutacinas. Estes dados sugerem a existência de grande diversidade de mutacinas produzidas pela espécie, das quais muitas ainda não foram identificadas.

A baixa freqüência dos genes de produção de mutacinas, ratreados em nossos estudos [Kamiya *et al.*, 2005b], impossibilitou a identificação dos determinantes genéticos

relacionados aos diferentes fenótipos de produção das mutacinas, identificados entre os isolados do grupo cárie-ativo e livre de cárie.

A hipótese da existência de alta diversidade nos determinantes genéticos necessários para a síntese das mutacinas, foi reavaliada em estudos posteriores utilizando genótipos de *S. mutans* isolados de crianças [Kamiya *et al.* – submetido - capítulo 2]. O estudo, descrito no capítulo 2, realizou o rastreamento adicional dos genes estruturais das mutacinas tipos 1140, BNy-266 e N, além dos genes homólogos às bacteriocinas identificadas em diferentes espécies bacterianas.

Em adição, neste estudo, foi verificada a expressão dos genes estruturais rastreados, em isolados portadores dos mesmos, na condição séssil e planctônica, através da técnica de RT-PCR semi-quantitativa. Os resultados obtidos com a análise transcripcional do segundo estudo, juntamente com as recentes pesquisas sobre os mecanismos regulatórios envolvidos com a síntese de mutacinas, nos conduziram para o desenvolvimento do terceiro trabalho, descrito no capítulo 3.

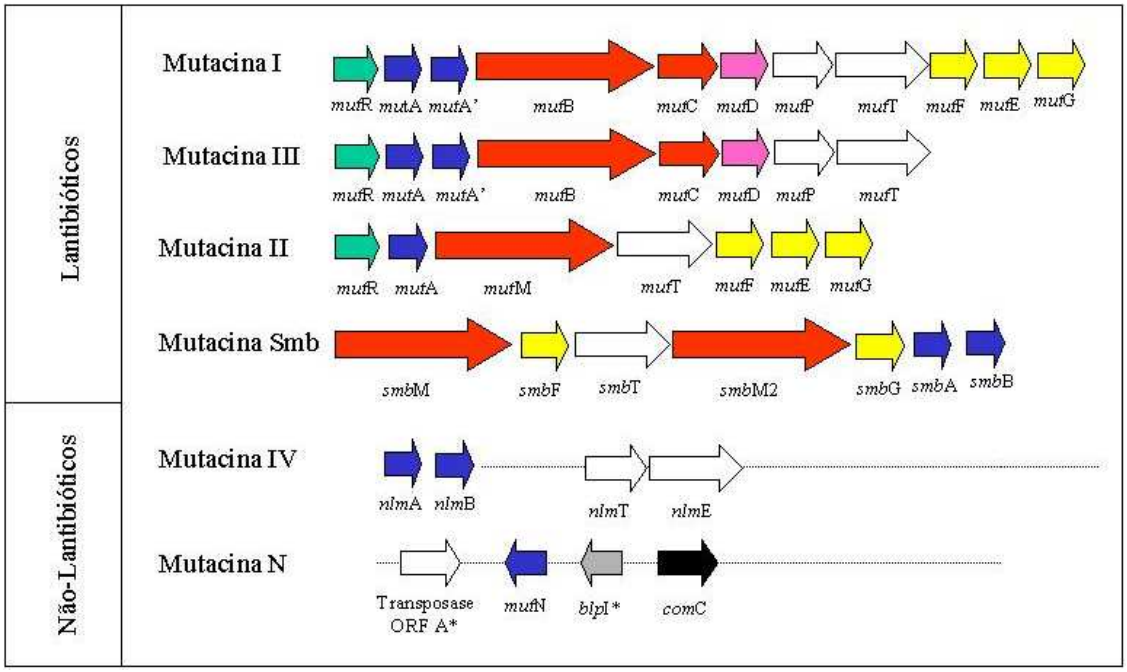

Figura 1 - Organização dos clusters de genes de biossíntese das mutacinas caracterizadas e isoladas de *S. mutans*. Genes com funções similares apresentam as mesmas cores (verde de regulação, azul de produção de peptídeo precursor, vermelho de modificação, branco de transporte/processamento, amarelo de imunidade, e rosa representa um gene adicional - *mut*D).
* seqüenciamento do cluster de biossíntese da mutacina N, envolve uma ORF com homologia à transposase e o gene *blp*I homólogo ao gene estrutural da bacteriocina de *Streptococcus pneumoniae*.

1.3. Análise da expressão dos determinantes genéticos de produção das mutacinas

Estudos *in vitro*, que analisam a expressão e os mecanismos regulatórios da produção de mutacinas, têm elucidado o papel destes antimicrobianos em saliva e/ou biofilme *in vivo.* Com este propósito foram realizados os estudos, descritos posteriormente com maiores detalhes, nos capítulos 2 e 3 da tese.

A maior atividade inibitória das mutacinas detectada em meios sólidos (condição reminiscente de biofilme) bem como a significante expressão gênica em células sésseis versus planctônicas, sugere maior atividade biológica destas substâncias em biofilme dental em relação à saliva [Kreth *et al.*, 2004].

Em trabalhos prévios [Kamiya *et al.* – submetido – capítulo 2] foram detectadas a expressão positiva dos genes de produção das mutacinas I e II, em células planctônicas, e a atividade antibacteriana em meio líquido, sugerindo o importante papel destas mutacinas na inibição de microrganismos competidores na saliva, possibilitando a subseqüente colonização de *S. mutans* produtor, no biofilme dental.

Neste mesmo trabalho, foi observada que a expressão do gene estrutural da mutacina tipo I (*mut*AI) foi maior em células aderidas ou sésseis em relação às células planctônicas, *in vitro,* entretanto os mesmos resultados não foram observados para o gene estrutural da mutacina II (*mut*AII) [Kamiya *et al.* – submetido – capítulo 2]. Segundo alguns estudos, os perfis de expressão gênica e conseqüentemente fenótipos desenvolvidos no estado planctônico e de biofilme são diferenciados, devido às diferenças ambientais as quais as células bacterianas foram expostas [Costerton *et al.*, 1995; Burne at al., 1997; O´Toole et al, 2000].

Estudos recentes têm relacionado a produção de peptídeos antimicrobianos com o sistema *quorum-sensing* – TCSTS (Sistema de Transdução de Sinal via Dois Componentes), demonstrando o aumento da expressão de genes estruturais em condições de maior densidade celular ou controle da expressão gênica através de mecanismos regulatórios pertencentes à este sistema [Qi *et al.*, 2004; Hale *et al.*, 2004; van der Ploeg, 2005; Yonezawa & Kuramitsu, 2005].

Condições no biofilme, como alta densidade celular e aumento do estresse ambiental, entre outros fatores ativam o sistema *quorum-sensing*, através do aumento da concentração de peptídeos ferormônios ou sinais que coordenam funções protetoras e de sobrevivência

para a célula, que incluem desenvolvimento da competência celular, resistência ao pH ácido e produção de mutacinas [Appleby *et al.*, 1996; Li *et al.*, 2001a, b; van der Ploeg, 2005].

O Sistema TCSTS é realizado através de dois componentes regulatórios consistindo de uma proteína histidina kinase, associada à membrana citoplasmática, e uma segunda proteína regulatória, situada no citoplasma. A proteína histidina kinase detecta sos peptídeos sinais do ambiente e transmite este sinal através da fosforilação da proteína regulatória, ao qual ativa a transcrição de genes alvos envolvidos na adaptação, sobrevivência e mecanismos de virulência [Appleby *et al.*, 1996; Fabret & Hoch, 1999; van der Ploeg, 2005]. Este sistema funciona como modulador da expressão de diferentes genes em resposta às alterações ambientais.

Dentre as mutacinas bem caracterizadas, as mutacinas I, II, IV e Smb têm a expressão gênica controlada indiretamente pelo aumento da densidade celular ou envolvem mecanismos de regulação *quorum-sensing* (estudos *in vitro*) [Chen *et al.*, 1998; Qi *et al.*, 2004; van der Ploeg, 2005; Yonezawa & Kuramitsu, 2005]. Foi demonstrado que as proteínas kinases CiaH, Dgk and ComD são importantes componentes regulatórios que coordenam a expressão dos genes de produção das mutacinas I, II e IV, respectivamente (Chen *et al.*, 1998; Qi *et al.*, 2004; van der Ploeg, 2005). Estas proteínas também foram correlacionadas com a maior adaptação ao estresse ácido, resistência à bacitracina e desenvolvimento da competência celular. Estes mecanismos regulatórios coadjuvantes podem representar um importante meio de sinalização da expressão de fatores de virulência, relacionados com a sobrevivência das cepas produtoras, principalmente em ambientes complexos e competitivos como o biofilme dental e lesões de cárie.

Para elucidar o papel das proteínas kinases CiaH, Dgk e ComD com a síntese de mutacinas tipos I e II, foi desenvolvido o trabalho, descrito no capítulo 3. Neste estudo, foi realizada a análise da expressão dos genes estruturais das mutacinas I e II (*mut*AI e *mut*AII) e das proteínas kinases (*cia*H, *dgk* e *com*D) durante diferentes períodos do crescimento planctônico de uma cepa produtora, visando relacionar a expressão gênica com o aumento gradual da densidade celular, em culturas estáticas. Em adição, realizou-se a mesma análise durante o crescimento séssil (formação de biofilme dependente de sacarose).

Através da análise da transcrição dos genes, concluiu-se que: a síntese das mutacinas I e II foi dependente da condição de crescimento planctônica ou séssil e

apresentou um perfil de expressão similar aos genes de proteínas kinases, sugerindo regulação por mecanismos dependentes da densidade celular ou do sistema *quorum-sensing*. Entretanto, mais estudos serão necessários para identificar os mecanismos (diferentes dos estudados), que podem modular a síntese destas substâncias antimicrobianas em estado planctônico ou de biofilme, uma vez que o aumento da expressão dos genes regulatórios estudados não induziu o aumento da expressão dos genes estruturais das mutacinas I e II, necessariamente.

Em adição, a manutenção da produção das mutacinas I e II, durante o crescimento celular séssil, pode representar um importante fator de virulência de *S. mutans,* relacionados com a sobrevivência e competição da espécie, em ambientes complexo como o biofilme dental e lesões de cárie.

II – PROPOSIÇÃO

Os objetivos desse estudo foram:

1. Detectar a freqüência dos genes de produção (estruturais) de mutacinas caracterizadas (tipos I a IV) em diferentes genótipos de *Streptococcus mutans* isolados de indivíduos cárie-ativos e livres de cárie.

2. Detectar a freqüência e expressão dos genes de produção de mutacinas caracterizadas (tipos I a IV, N, BNy-266 e 1140) bem como genes homólogos às bacteriocinas identificadas em outras espécies bacterianas, em diferentes genótipos de *Streptococcus mutans* isolados de crianças. Além de quantificar a expressão dos genes estruturais rastreados nas condições de crescimento planctônico e séssil.

3. Analisar a expressão dos genes estruturais das mutacinas I e II e genes de proteínas histidina kinase em diferentes períodos do crescimento planctônico e séssil, visando relacionar a produção das mutacinas I e II com o mecanismo *quorum-sensing* e determinar o papel destes genes na formação de biofilme mono-espécie, em culturas estáticas.

III – CAPÍTULOS

Esta tese foi aprovada pelo Comitê de Ética em Pesquisa da FOP – UNICAMP parecer n° 125/2003 (Anexo 1). Adicionalmente, ela está baseada na Informação CCPG/001/98/Unicamp que regulamenta o formato alternativo para teses de Mestrado e Doutorado e permite a inserção de artigos científicos de autoria do candidato Assim sendo, esta tese é composta de três capítulos contendo um artigo publicado e dois em fase de submissão em revistas científicas, conforme descrito abaixo:

✓ Capítulo 1

"Frequency of four different mutacin genes in *Streptococcus mutans* genotypes isolated from caries-free and caries-active individuals". Kamiya, R.U.; Napimoga, M.H.; Höfling, J.F.; Gonçalves, R.B. Esse artigo foi publicado no periódico **Journal of Medical Microbiology** 54: 599-604, 2005.

✓ Capítulo 2

"Frequency and expression of mutacins biosynthesis genes in planktonic and sessile cells of *Streptococcus mutans* isolates". Kamiya, R.U.; Höfling, J.F.; Gonçalves, R.B. Esse artigo será submetido à publicação no periódico **Journal of Medical Microbiology.**

✓ Capítulo 3

"Expression analysis of mutacins biosynthesis and kinase proteins genes in *Streptococcus mutans*". Kamiya, R.U.; Höfling, J.F.; Gonçalves, R.B. Esse artigo será submetido à publicação no periódico **Microbiology**.

CAPÍTULO 1

FREQUENCY OF FOUR DIFFERENT MUTACIN BIOSYNTHESIS GENES IN *Streptococcus mutans* GENOTYPES ISOLATED FROM CARIES-FREE AND CARIES-ACTIVE INDIVIDUALS

Artigo publicado no **Journal of Medical Microbiology** 54: 599-604, 2005.

(Disponível no *MedLine*)

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Running title: Mutacin genes in *S. mutans* isolates from caries-free and caries-active individuals

Key words: *Streptococcus mutans*, mutacin, oral biofilm

ABSTRACT

The ability of *S. mutans* to produce mutacins, combined with other virulence factors as lactic acid production, may contribute to the pathogenesis of this bacterium. In the present study, the detection of genes coding mutacins type I/III, II and IV was performed by PCR with specific primers to each type of biosynthesis genes in a total of 63 genotypes of *S. mutans* isolated from caries-active and caries-free individuals. In the caries-free group, PCR screening for mutacin IV revealed 31.81% positive genotypes to this mutacin. PCR for other three mutacins (I/III and II) tested did not yield amplicons in any *S. mutans* genotypes in this group. The PCR with primers of mutacin IV showed 68.29% positive genotypes, in the caries-active group. The Chi-square test showed significant differences in the number of positive strains to mutacin IV when compared to caries-free and caries-active genotypes of *S. mutans* ($p = 0.01$). On the other hand, the amplicons of mutacins I/III revealed 41.46% positive strains that carried these genes in the caries-active group. Statistical analyses revealed significant differences between the positive genotypes in the caries-active group that carried mutacins IV and mutacins I/III ($p = 0.02$). All tested *S. mutans* genotypes were negative by PCR for mutacin II. The low frequencies of detection of some mutacins genes suggest the existence of high diversity and polymorphism in production genetic determinants of the mutacins-like substances. In addition, the mutacins production of wide spectrum can play an important biological role in colonization of S. *mutans* strains, mainly in niche of high-complexity microbial communities.

INTRODUCTION

Mutans streptococci are generally accepted as one of the principal etiological agents of dental caries [Loesche, 1986; Becker *et al.*, 2002]. The dental biofilm consists of a complex bacterial community and the ability of specific strains of *Streptococcus mutans* to compete with other strains may be essential for colonization. Alaluusua *et al.* [1996] suggested that some strains of *S. mutans* might be able to colonize the host and induce dental caries better than other strains. Alternatively, dietary patterns of the host may be an important factor, since a high salivary mutans streptococci count does not necessarily exert a cariogenic challenge [van Palenstein Helderman *et al.*, 1996].

Numerous factors affect the equilibrium among oral populations of microorganisms, and several inhibitory substances have been identified, including mutacins [Delisle, 1976; Caufield *et al.*, 1985; Fukushima *et al.*, 1985]. Mutacins are peptide or protein antibiotics that are mainly bactericidal for other bacteria of the same or closely related species, as well as for other Gram-positive microrganisms, and are likely to confer ecological advantage in diverse bacterial communities such as dental biofilm [Parrot *et al.*, 1990; Balakrishnan *et al.*, 2002]. Some studies demonstrated that the mutacin activity of *S. mutans* could be related to the prevalence of this species in the dental biofilm, saliva and dental caries [Berkovitz and Jordan, 1975; Hillman *et al.*, 1987].

Classification of mutacin-producer strains based on their bactericidal activity divides mutacins into four types, I, II, III and IV. The antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci, while that of mutacin I, II and III is broader [Qi *et al.*, 1999a, b; Qi *et al.*, 2001].

The relationship between caries activity and the higher synthesis of some virulence factors by different genotypes of *S. mutans* has been demonstrated in the literature [Mattos-Graner *et al.*, 2000]. In a previous study, we observed a statistically significant positive association between the level of synthesis of water-insoluble glucan by *S. mutans* clinical genotypes and adherent cells in the presence of sucrose in caries-active subjects, but not in caries-free subjects [Napimoga *et al.*, 2004]. In addition, the genotypes of caries-active individuals produced mutacins of wide spectrum in comparison with genotypes of caries-free individuals [Kamiya *et al.*, 2005 – anexo 3], suggesting that isolates from subjects with

high caries activity were better at colonizing and accumulating on teeth, and consequently, inducing caries.

In the present study, we analyze the relationship between the frequency of detection of four different mutacins (mutacin I, II, III and IV) from *S. mutans* genotypes, isolated from caries-free and caries-active individuals.

MATERIALS AND METHODS

Bacterial Strains

A total of 63 genotypes and 3 reference strains, as positive control, of *S. mutans* were used. The clinical *S. mutans* were previously isolated from individuals aged 18 to 29 years (meand ± SD, 23.5 ± 3.9). Clinical strains were identified by PCR and genotyped by arbitrarily primed PCR [Napimoga *et al.*, 2004] and included 41 genotypes from eight caries-active individuals and 22 strains from eight caries-free individuals.

The isolates were previously evaluated for the production of mutacin-like substances [Kamiya *et al.*, 2005] by a modification of the deferred antagonism method [Hamada and Ooshima, 1975]. For mutacin activity testing, the following 12 Streptococcus sp were used as indicator strains: *Streptococcus mutans* CCT3440, *Streptococcus mutans* 32K, *Streptococcus sobrinus* ATCC27607, *Streptococcus sobrinus* 6715, *Streptococcus mitis* A, *Streptococcus mitis* ATCC903, *Streptococcus salivarius* ATCC25975, *Streptococcus salivarius* 66.4, *Streptococcus sanguinis* CR311, *Streptococcus sanguinis* M5, *Streptococcus sanguinis* ATCC10556 and *Streptococcus oralis* PB182. These strains were acquired from the respective collections of bacterial strains.

Extraction of chromosomal DNA

The strains were grown planktonically in brain heart infusion broth (Difco). DNA from all strains was extracted using a simpler DNA preparation in which the cells from an overnight culture in Brain Heart broth (BHI, Difco Laboratories, Detroit, Mich, USA) were washed and boiled for 10 minutes with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) modified from Welsh and McClelland [1990] and Saarela *et al.*, [1996], the debris pelleted and the supernatant used for detection of mutacin genes I/III, II and IV by PCR.

PCR screening of mutacin genes

The detection of biosynthesis genes coding mutacins type I, II, III and IV [Qi *et al.*, 1999a,b, 2001] was performed by PCR using specific primers to each type of gene. Only a

pair of primers was used to detect genes coding mutacins types I and III, due to high homology between them [Qi *et al.*, 1999b].

Primers to the genes coding mutacin types II and IV were designed based on the sequences obtained from the GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). The homologous genes to the mutacins types I and III were detected by a pair of primers based in conserved aminoacid sequence of mutacin 1140, mutacin NY266, epidermin and gallidermin [Qi *et al.*, 1999b].

Amplification by PCR was performed in the GeneAmp® PCR System 2400 (Perkin Elmer). The 50 μL PCR reactions consisted of 1 X PCR buffer containing 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.3μM of each oligonucleotide primer, 1.25U of Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil) and 50 ng of template DNA. Besides the strains tested, positive and negative controls were used in each PCR reaction: purified genomic DNA from *S. mutans* UA159 was used as positive control to mutacin genes type IV and two genotypes previously isolated from a mother/child pair [Klein *et al.*, 2004] were used as positive control to mutacins genes types I/III and II, respectively (unpublished results). Distilled water was used as negative control.

The PCR conditions were optimized for control strains. The PCR conditions included 35 cycles of initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 45 sec, annealing at 52ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 7 min. The PCR products were analyzed by electrophoresis in 1.0% agarose gel using Tris-borate-EDTA buffer (pH 8.0). A 250 bp DNA ladder was included in each gel. The DNA was stained with 0,5 μg mL^{-1} ethidium bromide and visualized under UV illumination.

Statistical analysis

The Chi-square test was applied to detect differences in the frequency of mutacins genes.

RESULTS

In the caries-free group, among the 22 *S. mutans* genotypes analyzed, 4 (18.2 %) showed inhibitory activity against mutans streptococci, 20 (90.9 %) showed inhibitory activity against mitis streptococci and 5 (22.7 %) against *S. salivarius* (Table 2). In the caries-active group, among 41 genotypes, 13 (31.7 %) showed inhibitory activity against mutans streptococci, 33 (80.5 %) against mitis streptococci and 7 (17.1 %) showed

inhibitory activity against *S. salivarius* (Table 3). Two *S. mutans* genotypes from the caries-free and four from the caries-active group, showed no inhibitory activity against any of the 12 indicator-strains.

In the caries-free group, PCR screening with primers of mutacin IV revealed 7/22 (31.8 %) genotypes positive to this mutacin. PCR with other three mutacins (I/III and II) tested did not yield amplicons in any *S. mutans* genotypes in this group (Table 2).

The PCR with primers of mutacin IV showed 28 out of 41 (68.3 %) strains were positive in the caries-active group; on the other hand, the amplicons of the mutacin I/III genes revealed that 17 out 41 (41.5 %) strains carried these genes (Fig. 1). Significant differences were found in the number of positive strains that carried the mutacin IV gene when comparing the caries-free and caries-active genotypes of *S. mutans* ($p = 0.01$).

In both groups, there were some strains that produced mutacin and showed inhibitory activity agains one indicator strain but did not yield amplicons to mutacin genes. On the other hand, in the caries-active group, some genotypes that showed amplicon to mutacin IV and to mutacin I/III did not reveal inhibitory activity against any of the indicator strains tested (Table 3).

DISCUSSION

Mutacins have been implicated as virulence factors in dental caries [Novák *et al.*, 1994]. In this study, we investigated the detection frequency of mutacins genes in *S. mutans* strains isolated from caries-free and caries-active individuals. We found that *S. mutans* strains recovered from caries-active individuals showed higher frequency of detection to mutacin IV compared to *S. mutans* genotypes recovered from caries-free individuals. In addition, only *S. mutans* in the caries-active group showed amplicons corresponding to mutacins I/III genes.

The oral biofilm is subjected to variable environmental stress, including the availability of nutrients, acidic pH [Carlsson, 1989] and mutacins activity [Qi *et al.*, 2001]. Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental biofilm: the mutacin-producing strains might colonize more easily and suppress nonproducing or sensible strains [Hillman *et al.*, 1987].

Longo *et al.* [2003] analyzed 19 strains isolated from children and found only one genotype that showed an amplicon homologous to gene for mutacin II. Qi *et al.* [2001], searched clinical isolates of *S. mutans* for the presence of mutacin IV genes by PCR, found > 50% positive results. According to Qi *et al.* [2001], mutacin IV is produced by planktonic cells while mutacin I is produced by biofilm-like cells. Different mutacins may serve different purposes during the process of colonization by *S. mutans*. For instance, production of mutacin IV by planktonic cells in saliva may help *S. mutans* kill the primary colonizers on the tooth surface to make room for its own population. Supporting this hypothesis, the antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci [Qi *et al.*, 2001]. Nevertheless, our study suggests that the increasing of the oral microbiota complexity, as found in caries-active individuals, and the mutacin-producing *S. mutans* of wide spectra as mutacins I, II and III, could became prevalent in most oral sites.

Longo *et al.* [2003] related no association between mutacins inhibitory spectrum and infecting levels of mutans streptococci or caries incidence in the host, suggesting that the mutacin production may not be relevant in the ability of the strain to colonize the host and induce disease. In a previous study we showed distinct mutacin production profiles between *S. mutans* isolated from caries-active and caries-free individuals, which can be related to the different colonization profiles described in these individuals [Kamiya *et al.*, 2005].

In the caries-active individuals the sites from which *S. mutans* were recovered were more diverse, probably because production of organic acids and mutacins within the bioiflm resulted in a more complex community compared to caries-free individuals [Paddick *et al.*, 2003]. Probably, due to this complexity. *S. mutans* genotypes recovered from caries-active individuals presented higher frequencies of mutacin IV and a wide spectrum of mutacins, such as I/III, and presented greater mutacin activity *in vitro* compared to *S. mutans* recovered from caries-free individuals [Kamiya *et al.*, 2005].

We also found some mutacin-producing strains in both groups that showed *in vitro* inhibitory activity against at least one indicator strain but did not yield amplicons to mutacin genes. These data suggest a high genetic diversity at the mutacin locus or absence of the structural genes encoding these substances. One possible explanation is that the

polymorphism at the mutacins locus may have compromised primer annealing in the PCR, which suggestes that there are different mutacin-coding genes that have a similar phenotype.

On the other hand, in the caries-active group, some genotypes showed amplicons to mutacin IV and to mutacin I/III, but did not reveal inhibitory activity against none of the indicator strains. One possible explanation is that the modification of only one aminoacid has already been shown to alter or to lose the activity of certain bacteriocins [Mulders *et al.*, 1991; Rollema *et al.*, 1995; Chan *et al.*, 1996]. The inhibition assay previously performed [Kamiya *et al.*, 2005] could also result from the production of more than one inhibitory substance, showing that the understanding of the genetic determinants of several mutacins still needs to be improved.

This study evaluated the frequency of mutacins I, II, III and IV in *S. mutans* strains recovered from caries-active and caries-free individuals, and compared them with phenotypic profiles of these substances *in vitro*. Our results suggest the high diversity and polymorphism in the production of genetic determinants of the mutacins-like substances. In addition, the production of a wide spectrum of mutacins can play an important biological role in colonization of *S. mutans* strains, mainly in the niche of high-complexity microbial communities.

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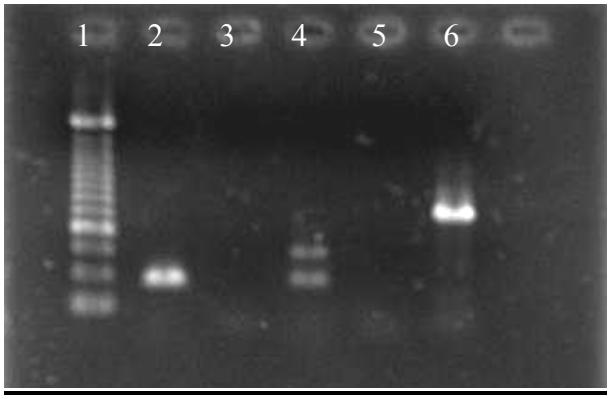

Figure 1: PCR screening of four different mutacin biosynthesis genes in *Streptococcus mutans*. Lane 1: Molecular Weight (250 bp); Lanes 3 and 5: Negative control (distilled water); Lane 2: Mutacin II (444 bp); Lane 4: Mutacin I/III (750/450 bp) and Lane 6: Mutacin IV (1344 bp).

Table 1: PCR primers of four mutacins biosynthesis genes.

Primer Sequence	Primers	GenBank	Reference
5′- AGTTTCAATAGTTACTGTTGC - 3′	*mut* I/III forward	AF 267498 and	Qi *et al.*, 1999b
5′- GCCAAACGGAGTTGATCTCGT – 3′	*mut* I/III reverse	AF 154675	
5′- AACGCAGTAGTTTCTTTGAA – 3′	*mut* II forward	U40620	Novák *et al.*, 1994
5′- TTCCGGTAAGTACATAGTGC – 3′	*mut* II reverse		
5′- ATGGGATATTTAAAGGGAAA – 3′	*mut* IV forward	NC 004350	Qi *et al.*, 2001
5′- TCAGAGCAGCTACAAAAACT – 3′	*mut* IV reverse		

Table 2: Mutacin inhibition number against 12 indicator strains, and detection of mutacin IV, mutacins I/III and mutacin II genes by PCR in 22 *S. mutans* genotypes from caries-free group.

Genotype (*S. mutans*)	Mutans group (n=4)	Mitis group (n=6)	*S. salivarius* (n=2)	PCR screening		
				Mutacin IV	Mutacin I/III	Mutacin II
AD13	2	6	2	-	-	-
AD8	0	6	2	-	-	-
AS4	0	6	2	-	-	-
AS38	0	5	1	-	-	-
CD35	0	0	0	-	-	-
CS2	1	5	1	-	-	-
CD34	0	3	0	-	-	-
DD31	0	1	0	-	-	-
DS37	0	3	0	-	-	-
DP38	0	3	0	-	-	-
ES31	0	1	0	-	-	-
ED33	0	2	0	+	-	-
GD2	0	2	0	+	-	-
GS33	0	0	0	-	-	-
GS43	0	3	0	-	-	-
IP35	0	3	0	+	-	-
OS31	0	3	0	+	-	-
OS40	0	2	0	+	-	-
OP35	0	1	0	+	-	-
OD32	0	4	0	+	-	-
RP2	1	4	0	-	-	-
RS7	2	4	0	-	-	-
Total : 22	4	20	5	7	0	0

Table 3: Mutacin inhibition number against 12 indicator strains, and detection of mutacin IV, mutacins I/III and mutacin II genes by PCR in 41 *S. mutans* genotypes from caries-active group.

Genotype (*S. mutans*)	Mutans group (n=4)	Mitis group (n=6)	*S. salivarius* (n=2)	PCR screening		
				Mutacin IV	Mutacin I/III	Mutacin II
C1S2	0	3	0	+	+	-
C1D3	0	3	0	+	+	-
C2D2	0	4	0	+	-	-
C2D12	0	4	0	+	-	-
C2S13	0	0	0	+	-	-
C2S4	4	6	2	-	+	-
C2D8	0	0	0	+	-	-
C2D6	0	0	0	+	-	-
C2D13	0	3	0	+	-	-
C2D7	0	4	0	-	-	-
C3D15	1	1	0	+	-	-
C3D14	1	1	0	+	-	-
C3D9	2	0	0	+	-	-
C3P13	1	1	0	+	-	-
C3S13	1	0	0	+	-	-
C4P4	0	1	0	+	-	-
C4P5	0	1	0	+	-	-
C4D3	0	0	0	-	-	-
C4S3	0	1	0	-	-	-
C4D6	1	0	0	-	-	-
C5P5	0	2	0	+	-	-
C5D5	0	2	0	+	-	-
C5D15	0	2	0	+	-	-
C5S2	4	6	2	+	+	-
C7D10	0	1	0	-	+	-
C7D1	0	2	0	-	+	-
C7P3	0	3	0	+	-	-
C7D11	0	1	0	+	-	-
C7D9	0	1	0	+	-	-
C7S4	0	1	0	-	+	-
C7S5	0	3	0	+	+	-
C8D7	4	6	2	-	+	-
C8P12	2	2	2	-	+	-
C8S4	0	0	0	+	-	-
C8D10	4	6	2	-	+	-
C9D3	4	6	1	-	+	-
C9S14	4	6	1	-	+	-
C9S15	0	1	0	+	+	-
C9S10	0	3	0	+	+	-
C9S2	0	3	0	+	+	-
C9S9	0	3	0	+	+	-
Total : 41	13	33	7	28	17	0

CAPÍTULO 2

FREQUENCY AND EXPRESSION OF MUTACINS BIOSYNTHESIS GENES IN PLANKTONIC AND SESSILE CELLS OF *Streptococcus mutans* ISOLATES

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Key words: *Streptococcus mutans*, mutacin, bacteriocin, planktonic and sessile cells

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ABSTRACT

The aims of this study were to detect the frequency and expression of biosynthesis genes of mutacins I, II, III, IV, N and homologues to B-Ny 266, 1140 and non-lantibiotics (subclass IIa and IIb) in 23 mutacin-like substances producing *S. mutans* isolates (Sm 1-to-23), using PCR and semi-quantitative RT-PCR methods. In addition, this study detected the inhibitory activity of mutacins I and II in liquid preparation and analyzed the expression of respective structural genes in planktonic and sessile cells, *in vitro*.

Among 23 mutacin producing strains, only 5 were biosynthesis genes carrier of characterized mutacins (I, II and IV). One strain carried *mut*A coding both mutacins I and II. The low frequency of structural genes of mutacins I, II and IV and the absence of isolates carrying genes coding non-lantibiotics and mutacins B-Ny266, 1140 and III suggests high diversity of genetic determinants of mutacin-like substances in *S. mutans.*

The analysis of expression by semi-quantitative RT-PCR showed than only 2 *S. mutans* strains (Sm 7 and Sm 17) expressed *mut*A coding mutacins I (*mut*AI) and II (*mut*AII), and one of these genotypes (Sm 7) produced both mutacins, in planktonic and sessile cells. The partially purified mutacins (crude extract) obtained from cells cultures in THB sucrose (0,5%) showed inhibitory activity in liquid media against *S. sanguinis* and presented higher concentrations of inhibitory substances (about 8-fold increase) in comparison to supernatant of cultures in THB without sucrose, on the respective final log phase.

There was significant difference between the transcription levels of *mut*AI in planktonic and sessile cells grown in THB sucrose (test t, $p = 0,02$). This result was not observed for *mut*AII gene.

In conclusion, there is a high diversity in the genetic determinants needed for the synthesis of mutacins in *S. mutans* isolates. Mutacins I and II could be produced in planktonic and sessile cells and showed inhibitory activity in liquid cultures. In addition, the mutacin I production was dependent on growth in sessile conditions and may be part of the stress response that *S. mutans* displays under adverse conditions, as the dental biofilm.

INTRODUCTION

S. mutans is considered a major etiological agent of human dental caries due to its capacity of adherence, acid resistance, acidogenicity, resistance to other stress conditions and the mutacin production [Bowden & Hamilton, 1998; Napimoga *et al.*, 2005]. Mutacins are peptide or protein antibiotics that are mainly bactericidal against other bacteria of closely related species as well as a surprisingly wide of other gram-positive bacteria [Tagg *et al.*, 1976; Qi *et al.*, 1999b]. The mutacins production may likely confer ecological advantage to *S. mutans* in diverse bacterial communities such as saliva and dental biofilm [Parrot *et al.*, 1990; Balakrishnan *et al.*, 2002].

In the previous survey, we showed that the majority of the *S. mutans* strains produced mutacin-like substances [Kamiya *et al.*, 2005a], out of 319 *S. mutans* isolated from caries-active and caries-free individuals, more than 70% were able to produce one or more bacteriocin-like substances *in vitro*. However, the low frequency of biosynthesis genes of mutacins previously characterized in these isolates suggested high diversity of the bacteriocins genetic determinants in *S. mutans* [Kamiya *et al.*, 2005b]. Besides this great diversity, little has been revelead about the biochemical structures of mutacins. In addition, many mutacins have not been identified yet.

Most bacteriocins from *S. mutans* characterized up to now belong to the class I lantibiotics (mutacin I, II, III, 1140, BNy-266 and SmbA and SmbB) [Novák *et al.*, 1994; Hillman *et al.*, 1998; Qi *et al.*, 1999a; Qi *et al.*, 1999b; Qi *et al.*, 2001; Yonezawa & Kuramitsu, 2005]. These lantibiotics present ample inhibitory spectrum when tested in solid media, which may suggest high antimicrobial activity in dental biofilm [Qi *et al.*, 2004].

The bacteriocins of Class I include two subgroups according to their primary peptide sequences [de Vos *et al.*, 1995; Sahl & Bierbaum, 1998]. Subgroup AI contains the nisin-like lantibiotics, as well as subtilin, epidermin, pep5, mutacins I and III as the most thoroughly characterized members. Subgroup AII consists of lantibiotics as lacticin 481, SA-FF22, salivaricin, variacin and mutacin II [de Vos *et al.*, 1995; Sahl & Bierbaum, 1998; Qi *et al.*, 2000].

The bacteriocins class II include non-lantibiotic, divided in Subclass IIa that contain the pediocin-like substances, and Subclass IIb is composed of synergic two-peptides [Nes & Holo, 2000]. A nonlantibiotic class IIb bacteriocin, mutacin IV, was purified from *S.*

mutans UA140, which is a producer of the lantibiotic mutacin I [Qi *et al.*, 2001]. Mutacin IV is composed of two synergic peptides encoded by *nlm*A and *nlm*B genes, which are probably organized in the same operon [Qi *et al.*, 2001], however no pediocin-like mutacin has been identified up to now. In this study, we tried to identify by PCR possible mutacins homologues to non-lantibiotic subclass IIa and IIb among the isolates *S. mutans*, using degenerate primers based on consensus of different bacteriocins already characterized in the other species.

In the present study, we evaluated the titre of mutacin-like substances and the expression of biosynthesis genes of some well characterized mutacins in planktonic cells of *S. mutans*. In addition, we detected the mutacin inhibitory activity in liquid media.

During the biofilm formation, the bacteria express phenotypes that distinguish these sessile cells from planktonic cells in response to environmental signals [Costerton *et al.*, 1995; O´Toole *et al.*, 2000]. In this study we analyzed and compared the gene expression profiles of characterized mutacins in planktonic and sessile cells by sqRT-PCR technique.

MATERIALS AND METHODS

1. *S. mutans* isolates and Antagonism Method: We selected for this study 23 different genotypes of *S. mutans* (Sm 1 to Sm 23) from caries-free children, isolated in the previous studies [Flório *et al.*, 2004; Klein *et al.*, 2004]. These strains were characterized as producer of mutacin-like substances by Antagonism Method as described previously by our group [Kamiya *et al.*, 2005a].

For mutacin activity testing, the following 30 *Streptococcus* sp were used as indicator strains: *S. mutans* UA159, *S. mutans* UA130, *S. mutans* CCT3440, *S. mutans* 32K, *S. sobrinus* ATCC27607, *S. sobrinus* 6715, *S. mitis* A, *S. mitis* ATCC903, *S. salivarius* ATCC25975, *S. salivarius* 66.4, *S. sanguinis* CR311, *S. sanguinis* M5, *S. sanguinis* ATCC10556, *S. oralis* PB182 and more 15 *S. mutans* isolates.

2. Extraction and purification of DNA:

Total DNA was extracted using the Master Pure^{TM} DNA Purification kit, EPICENTRE, according to the manufacturer's instructions. The mutacin producing strains were or grown in 3 ml of Brain Heart Infusion broth (BHI, OXOID Ltd, Hampshire, England) for 18h, 37^{o}C and CO_2 (10%). Aliquots of 1,5 ml of culture were submitted to

centrifugation (13.000g, at 4°C, for 5min) and the pellet submitted to DNA extraction. The DNA purity was obtained spectrophotometrically by proportion A_{260} / A_{280} nm.

3. *PCR screening of mutacin genes*:

The detection of biosynthesis genes coding mutacins type I, II, III and IV [Qi *et al.*, 1999a; Qi *et al.*, 1999b; Qi *et al.*, 2001] was performed by PCR using specific primers to each type of genes. Primers to the genes coding mutacins were designed based on the sequences obtained from the GenBank (http://www.ncbi.nlm.nih.gov) (table 1) using Primer 3 Program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For detection of the mutacins homologues to 1140 and B-Ny 266, one pair of primers designed by QI *et al.* [1999b] was used. The forward primer for the structural gene LanA (5' – agtttccaatagttactgttgc – 3') was based on conserved amino acid sequence, S-F-N-S-Y-C-C of the mutacin 1140, mutacin Ny-266, epidermin and gallidermin and the codon preference in *S. mutans.* The reverse primer (5' – gccaaacggagttgatctcgt –3') was based on the conserved amino acid sequence T-R-S-T-P-F-G of LanB from mutacin 1140, subtilin, nisin and epidermin. PCR amplification was performed with chromosomal DNA of one control strain detected in previous studies and the PCR conditions were optimized using this control strain [Kamiya *et al.*, 2005b].

The biosynthesis genes homologue to mutacin N was identified using forward primers (5' – ccgtcaagctgctgaatacattcc – 3') and reverse primers (5' – cgatatttaaacctgcaccgactacg –3') and PCR conditions developed by Hale *et al.* [2004].

For detection of mutacins homologues to bacteriocins non-lantibiotics (subclass IIa and IIb), the degenerate primers were designed based on consensus of bacteriocins produced by different bacterial species (table 2). The alignments of the peptides were developed by Clustal W Program (http://www.mbio.ncsu.edu/BioEdit) (figure 1).

Amplification by PCR was performed in the GeneAmp® PCR System 2400 (Perkin Elmer). The 50 μL PCR reactions consisted of 1 X PCR buffer containing 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.3 μM of each oligonucleotide primer, 1.25 U of Taq DNA polymerase (Life Technologies - Gibco, Grand Island, NY) and 50 ng of template DNA. Besides the strains tested, purified genomic DNA from *S. mutans* UA159 was used as positive control to mutacin genes type IV and distilled water was used as negative control in each PCR reaction.

The PCR conditions were optimized using control strain *S. mutans* UA159 and the parameters were used to amplify structural genes of mutacins I, II and III. The PCR conditions included 35 cycles of initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 45 sec, annealing at 50ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 7 min. For degenerate primers, the annealing temperature varies between 45 and 47^{o}C. The PCR products were analyzed by electrophoresis in 1.0% agarose gel stained with ethidium bromide. A 100 bp DNA ladder was included in each gel.

5. RNA extraction:

Mutacin producing strains, which presented screened structural genes by PCR were submitted to the RNA extraction and to the semi-quantitative RT-PCR technique, to verify the possible expression of these genes in planktonic and sessile cells.

The strains were grown in 40 mL of Todd-Hewitt Broth (THB, DIFCO Laboratories, Sparks, MD) supplemented with sucrose (0,5%) at 37^{o}C, 10% CO_2 up to final log phase, 17-18 h. This media was selected previously because it promoted a higher number of adhered cells to polyethylene tube used (Greiner Labortechnik Gmbh) (data not showed).

The planktonic cells were obtained by centrifugation and the sessile cells were removed mechanically after the tube had been dried three times with saline. The planktonic and sessile cell pellets were submitted to RNA extraction by phenol-chloroform method [Qi *et al.*, 1999a].

6. Synthesis of cDNA and semi-quantitative RT-PCR

The residual DNA of extracted RNA was purified with Deoxyribonuclease I (Gibco), according to the manufacturer's instructions. The cDNA synthesis was realized with 24 ng of total RNA using random primers Mix (20 μM) Ea1, Ea7, Es1, Es3 and Es8 [Chia *et al.*, 2001] and the enzyme Super Transcrispt RT III (Gibco), following the manufacturer's protocol, with some modifications.

In the first phase, to the total RNA was added random primers Mix (20 μM), dNTPs Mix (10 mM) and DEPC to 10 μL. The solution was heated at 65^{o}C for 5 min and cooled at 4^{o}C for 1 min. Subsequently, it was added to the reaction mixture: 10x RT buffer, $MgCl_2$ (25 mM), DTT (0,1 M), RNase OUT (40 U) and Super Transcript RT III (200 U). The total solution containing cDNA (20 μL) was submitted to the following thermal cycle:

25°C/10min; 50°C/50min; 85°C/5min e 4°C/1min. For each RNA sample, the cDNA synthesis reaction was also carried out without reverse transcriptase in order to identify contamination by residual genomic DNA. For the maximum efficiency, RT-PCR primers were designed to generate amplicons ranging from 150 to 200 bp in size (table 1).

A 1 μL volume of cDNA was used as template in the PCR (25 μL) with 50 mM of $MgCl_2$, 0.3 mM of each specific primer (table 1), 200 μM of dNTPs, 1.25 U of Taq polymerase (Gibco) and 10x PCR buffer. All samples of cDNA were tested with primers to the house-keeping gene 16S, a constitutively transcribed control gene whose expression was invariant under the experimental conditions used. The primer set *16S*/forward (5'-cggcaagctaatctctgaaa-3') and *16S*/reverse (5'-gcccctaaaaggttacctca-3'), designed based on *S. mutans* UA159 genome (NC004350), was used to normalize the expression mutacin production genes (*mut*A). Controls for RT-PCR included reaction mixtures without template cDNA, to effectively rule out the presence of contaminating DNA and/or the formation of primers dimmers.

The cDNA was used to generate critical threshold (Ct) values for specific primer sets. The Ct values were determined as the cycle at which fluorescence was intermediary between the first cycle that gave rise to a detectable PCR product above the background and the first cycle in which there is saturation of PCR reaction.

The numbers of the cycles included 25 cycles for 16S gene and 37 for other genes. The thermal cycles of PCR for cDNA were identical to cycles used on *screening* PCR with specific primers and DNA samples (previously described). The amplicons were visualized in agarose gel (2%) with ethidium bromide (0,3 mg/mL), and the densitometry was realized by KODAK Program (Gel logic 100 Imaging System, Eastman Kodak Co., USA).

7. Isolation of mutacins and detection of inhibitory activity in liquid media

Simultaneously to RNA extraction, the mutacins were partially isolated (crude extract) from respective culture supernatants according to previous studies [Novák *et al.*, 1994; Hillman *et al.*, 1998; Qi *et al.*, 1999a; Qi *et al.*, 1999b] with some modifications. Briefly, the cultures of identified mutacins producing strains were centrifuged for 10 min at 10.000 g and 4°C. The supernatant was filtrated in milli-pore membrane (0,45 μm pore – size) and extracted with an equal volume of chloroform. The emulsion at the chloroform-

aqueous interface was collected by centrifugation at room temperature for 2 min at 10.000 g. The pellet was washed once with double-distilled H_2O.

The water-insoluble material (crude extract) was dissolved in 1 mL of PBS (phosphate-buffered saline) by heating at 60°C for 10 min [Nicolas *et al.*, 2004]. Water-insoluble fractions were tested for antimicrobial activity in liquid preparation after serial dilutions with PBS.

The microdilution technique in 96 well-plate ELISA [Parrot *et al.*, 1990] was used to detect the antimicrobial activity of mutacins in liquid preparations. Briefly, a volume of 100 μL of an overnight culture of the indicator strain in BHI (*S. sanguinis* CR311), adjusted to an OD of 0.1 at 550 nm (approximately 10^7 CFU/mL) was added to 100 μL of serial dilutions (1:1 PBS) of the antibacterial preparation. The control group containing only culture diluted 1:1 in vehicle PBS was added in all experiment. The absorbance was read at 550 nm in ELISA Reader (Versa Max Program) before and after incubation at 37°C, for 18 h and 10% CO_2. The titre of preparation corresponded to the reciprocal of the dilution that inhibited the growth of the indicator strain by 100% as compared with the control. All experiments were performed in quadruplicate.

8. Determination of concentration of inhibitory substances in the culture medium (THB with or without sucrose)

In order to determine the importance of mutacin production by sessile cells (biofilm) we quantified the concentration of these substances in THB (DIFCO) and THB sucrose in the respective final phase log of these media (12 and 17 h). There was no biofilm formation in THB, although this media contains trace of dextran in its composition (DIFCO).

The semi quantitative critical dilution method was used to determine the titres of the mutacins produced [Parrot *et al.*, 1990]. Ten microliters of each serial dilution of partially purified mutacin was deposited on plates of Tryptical Soy Agar 1,5% (TSA - DIFCO) and overlaid with 5 ml of Tryptical Soy Broth (TSB – DIFCO) containing soft agar (0,8%) and a standardized suspension 0,5 mL of an exponentially growing culture of *S. sanguinis* CR311 at an A550 of 0,1. The concentrations of these substances in the respective supernatants was defined as the reciprocal of the highest dilution that exhibited a detectable inhibition zone against *S. sanguinis,* after 24 h of incubation at 37° C and CO_2 10% [Qi *et al.*,1999b; Nicolas *et al.*, 2004].

RESULTS

Among 23 mutacin-like producing *S. mutans* in solid media (TSA), only 5 genotypes were carrier of the biosynthesis genes of mutacins previously characterized. No genotype inhibited *S. cricetus*. Table 3 showed the inhibitory spectrum against 29 indicator strains (with exception of the *S. cricetus*) and the frequency of screened structural genes of mutacins.

Only 5 genotypes presented the homologues structural genes to mutacins I (3 genotypes – Sm 7, Sm 12 and Sm 23), II (2 genotype – Sm 7 and Sm 17) and IV (1 genotype – Sm 11) and one genotype (Sm 7) was carrier of *mut*A coding mutacins types I (*mut*AI) and II (*mut*AII) (table 3). No genotype carried of genes homologues to mutacins III, 1140, BNy-266, N or homologues to bacteriocins non-lantibiotics. These data reinforce the existence of high genetic diversity of the mutacins in *S. mutans.* The pair of the primers for mutacins 1140 and BNy-266 can also identify mutacins types I and III due to the high homology between these proteins [Qi *et al.*, 1999b]. In our study, only 2 genotypes (Sm7 and Sm23) revelead specific amplicons using these primers, later these genotypes were identified as carrier of *mut*AI and no type III, 1140 and BNy-266, using a set of primers based on variable amino acid sequence of these mutacins.

Among the 5 strains that carried mutacins biosynthesis genes, only two producer strains expressed positively the structural genes *mut*AI and II. Genotype Sm7 was able to produced both mutacins I and II (table 3).

Mutacins I and II (crude extract) obtained from cultures of producing strains in THB sucrose showed inhibitory activities in liquid media against the indicator strain *S. sanguinis*. The crude extract obtained from cultures of Sm7 strain, producer of both mutacins I and II, and of Sm17 strain, mutacin II producing, inhibited totally the growth indicator strain up to dilutions 1:16 and 1:4, respectively (figure 2).

Figure 3 showed the quantification of the transcript levels of *mut*AI and *mut*AII genes in planktonic and sessile cells grown in THB sucrose. The Student´s t test showed significant difference between the transcription levels of *mut*AI in planktonic and biofilm cells grown in THB sucrose ($p = 0,02$). The mRNA levels of *mut*AI gene increased 1,48-fold in sessile cells. This result was not observed for *mut*AII gene.

The THB sucrose presented higher yields of inhibitory substances in relation to THB without sucrose (about 2 to 8 fold increase) (figure 4). The MIC (production levels) of these mutacins in THB varied between 1:8-1:8 dilutions and in the THB sucrose varied between 1:16-1:64, for Sm17 and Sm7 strains, respectively.

The partially purified mutacins from culture supernatant of producer strains grown in THB sucrose presented higher inhibitory activity in solid medium than in liquid medium. The MIC of these substances in liquid and solid medium varied between the dilutions 1:16 and 1:64 for the Sm 7 strain, and between 1:4 and 1:32 for Sm 17 strain, respectively (figures 2 and 4).

In addition, the antimicrobial spectra of Sm7 and Sm17 strains, by modified antagonism method, were against 22 (74%) and 8 (27%) indicator strains of total of 30 used, respectively.

Strains Sm11, Sm12 and Sm23 were carrier of the structural genes of mutacins I or IV but did not produce mutacins in THB sucrose in detectable concentrations and the structural genes were not transcript in these conditions. On the other hand, the expressions of *nlm*AB genes of mutacin IV were detected in the *S. mutans* UA159 growth in the same conditions (data not showed).

DISCUSSION

The low frequency of mutacins structural genes detected in this study is according to previous studies [Longo *et al.*, 2003; Kamiya *et al.*, 2005b; Li *et al.*, 2005] and suggests the existence of high diversity and polymorphism in production genetic determinants of the mutacins-like substances in *S. mutans* isolates.

According to van der Ploeg [2005] and Hale *et al.* [2005] there are 12 small open reading frames similar to leader peptides of NlmA and NlmB (mutacin IV) in *S. mutans* UA159 genome, which may codify inhibitory substances. Such substances would represent a large repertoire of combination between two synergic peptides in the specie [van der Ploeg, 2005; Hale *et al.*, 2005], and they may be one possible explanation for the great diversity of mutacins not yet identified.

Most studies detected mutacins inhibitory activity in solid media by the antagonism method, this condition is reminiscent of a biofilm on the tooth surface, suggesting that

mutacins production may be triggered by dense colonization of the surface by oral bacterial [Qi *et al.*, 2001; Kreth *et al.*, 2004].

In the present study, 5 genotypes, carrier of mutacins structural genes, were tested as the mutacin production in planktonic and sessile cells grown in the THB sucrose. Only 2 strains, carrier *mut*A coding mutacins I and II produced them in the tested conditions and one strain produced both mutacins, presenting higher inhibitory spectrum. These data may suggest that the production of more than one type of mutacin increase the number of susceptible bacteria and may be ecological advantageous to the producing strain.

The analysis of inhibitory activity and the expression of structural genes of mutacins I and II suggest that mutacin I have higher inhibitory spectrum than mutacin II; however it was less produced/expressed in liquid cultures. According to Qi *et al.* [2001] the mutacin I production appears to be dependent on the culture conditions, since they could be detected only after growth on agar or agarose-containing medium. This study demonstrated that mutacin I was produced by cells grown in a biofilm or in large aggregates, but not by planktonic cells grown in liquid medium.

According to our studies, KRETH *et al.* [2004], using fluorescent protein and glucuronidase reporters system showed that *mut*AI is expressed in both planktonic and biofilm cells, even though mutacin activities are normally detected only in biofilm cells. In our studies we detected inhibitory activity of mutacin I partially purified from supernatant of liquid culture against planktonic cells of one indicator strain. Our results suggest possible inhibitory activity of these antimicrobial peptides in saliva. The mutacins production in saliva may have an important role in the inhibition of early colonizers and may be crucial to initial colonization *S. mutans,* before their adherence in dental biofilm [Qi *et al.*, 2001].

The analysis of the expression showed that *mut*AI was more expressed in *S. mutans* cells that are immobilized on solid surface or biofilm cells *in vitro*. During the biofilm formation, the bacteria undergo a developmental program in response to environmental signals that leads to the expression of new phenotypes that distinguish these sessile cells from planktonic cells [Costerton *et al.*, 1995; O´Toole *et al.*, 2000]. The physiological processes cell density-dependent or controlled by *quorum-sensing* system may be essential for the survival of the cell, mainly in oral biofilm where there is high-density cell, great

biodiversity, limited food supply and high competition among community members [Fabret & Hoch, 1998; Martín *et al.*, 1999].

Previous studies demonstrated that the production of mutacin I greatly increased with cell density suggesting regulation of gene transcription by *quorum-sensing* mechanisms [Qi *et al.*, 2001; Qi *et al.*, 2004]. In the present study, we demonstrated that the expression of *mut*AI was higher in sessile cells than in planktonic cells, the mutacin I production was dependent on growth conditions and may be part of the stress responses that *S. mutans* displays under adverse conditions, as the dental biofilm.

The mutacin II expression/production in Sm7 strain regarding Sm17 was statistically decreased ($p < 0,05$) probably due to the energetic loss to produce both mutacins I and II. For synthesis of mutacins I and II it is necessary the expression at least of 11 and 7 different proteins, respectively, involved in regulation, transport, modification and immunity to these substances [Qi *et al.*, 1999a; Qi *et al.*, 2000]. According to Riley and Wertz [2002] a small population of producers cannot invade an established population of sensitive cells, this failure occurs because the producer pay a price for bacteriocin production and the energetic costs can lead to lethality of producers cells.

There was no difference in the expression level of *mut*AII in planktonic and biofilm cells. This result suggests that mutacin II production is not regulated by *quorum-sensing* mechanisms. This data is in agreement with the results of QI *et al.* [1999a], which demonstrated that the production of mutacin II is not dependent on the growth stage, the density cell and other regulator mechanisms.

However, other studies demonstrate that the truncation of *dgk* gene encoding protein involved with adaptation to environmental stress [Yamashita *et al.*, 1993] affected the transcription of the structural gene *mut*AII [Chen *et al.*, 1998] and reduced the *S. mutans* resistance to low pH, elevated temperatures, high osmolarity and bacitracin [Lis & Kuramitsu, 2003].

Our results demonstrated higher concentration of mutacins in THB containing sucrose in relation to THB (about 8-fold increase). These data suggest that the increased yield of mutacins in the supernatant of culture in THB sucrose be due to mutacins production by sessile cells, suggesting the importance of the synthesis of these substances by biofilm cells and possible extrinsic biologic activity of mutacins in the saliva.

The action mode of bacteriocins occurs with their bound to cell surface of sensible and/or producer strains through interaction in specific or nonspecific receptors, respectively [Yang *et al.*, 1992]. This adsorption ability is proportional to increase of mass cell and dependent on elevated pH [Wu *et al.*, 2004]. Based on the characteristics of adsorption-desorption of bacteriocins, our data suggests that the secretion of mutacins I and II in supernatant by biofilm cells have been influenced by decreased pH in the medium containing sucrose or these mutacins act through the linking with specific receptors present only in sensible cells. Then in the absence or reduced number of sensible cells in the oral biofilm, mutacins I and II may be released from biofilm acting extrinsically in saliva, for example.

In addition, 3 genotypes were carrier of mutacin I or IV production genes, and did not present detectable levels of mutacins in liquid medium and the planktonic cells did not express these genes in tested conditions either. No expression of these genes in liquid medium was probably due to genetic mutation and/or loss of regulatory system [Chen *et al.*, 1998; Chen *et al.*, 2001; Qi *et al.*, 2004; van der Ploeg, 2005].

In conclusion, there is a high diversity in the genetic determinants needed for the synthesis of mutacins in *S. mutans* isolates. Mutacins I and II could be produced in planktonic and biofilm cells and showed inhibitory activity in liquid cultures, suggesting their importance in inhibiting competitor microorganisms in the saliva, making possible the subsequent colonization of *S. mutans* in dental biofilm. The mutacin I production was dependent on growth conditions and may be part of the stress response that *S. mutans* displays under adverse conditions, as the dental biofilm.

In addition, the intrinsic or extrinsic production/activities of mutacins in dental biofilm may play significant roles in primary colonization of *S. mutans* and give a competitive advantage to producer strains occupying critical ecological niches as the dental biofilm.

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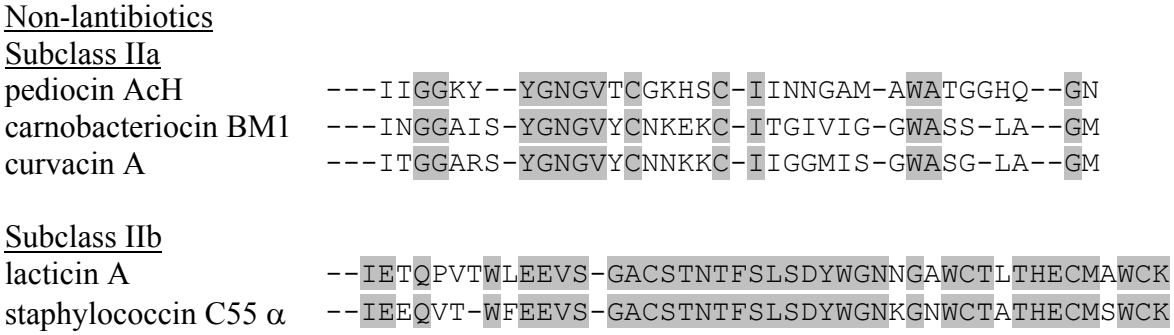
Table 1. PCR specific primers to structural genes *mut*A (mutacins I, II and III) and *nlm*A and *nlm*B genes (mutacin IV)

Primers	Sequences	Mutacin	GenBank	Amplicon
*mut*AI_Forward	TAGAAGTCCTTGGTACTGAA	I	AF238860	163bp
*mut*AI_Reverse	TTGAAACTAGGATTTTTCAC	I		
*mut*AII_Forward	CAGTAACGCAGTAGTTCCTT	II	U40620	162bp
*mut*AII_Reverse	TTAACAGCAAGTGAAAACAT	II		
*mut*AIII_Forward	CAATTATTAGAAGTCCTTGG	III	AF154675	194bp
*mut*AIII_Reverse	TATTGAAACTACCTGTCCTT	III		
*nlm*AIV_Forward	CAATTTGATGTAATGGACAG	IV	based on *S. mutans* genome (NC004350)	154bp
*nlm*AIV_Reverse	CTACACAATATGGGGTAACA	IV		
*nlm*BIV_Forward	AGTTTTGGTGGAGATAAAC	IV		156pb
*nlm*BIV_Reverse	GGAAAAACTACAGATCCAA	IV		

Figure 1. Alignment of bacteriocins non-lantibiotics subclass IIa and IIb



Alignment of bacteriocins non-lantibiotics (Class II) with Subclass IIa include pediocin-like substances active against *Listeria monocytogenes,* as pediocin AcH of *Pediococcus acidilactici*, carnobacteriocin BM1 of *Carnobacterium piscicola* and curvacin A of *Lactobacillus lactis*. Subclass IIb includes bacteriocins homologues to lacticin of *Lactococcus lactis* and staphylococcin C55 α of *Staphylococcus aureus*. Shaded residues indicate conserved amino acids.

Table 2. PCR degenerate primers based on consensus of bacteriocins non-lantibiotics (subclass IIa and IIb).

	Primers	Sequences	consensus	Amplicon
Non-lantibiotics				
Subclass IIa	*sub*IIa_Forward	TAIGGTAATGGIGTT	YGNGV	102 bp
	*sub*IIa_Reverse	ACCATIAGCCCATIC	GWASG	
Subclass IIb	*sub*IIb_Forward	TGGTTGGAIGAAGTI	WLEEV	131 bp
	*sub*IIb_Reverse	AGICATACATTCIGT	THECM	

Table 3. Mutacin inhibitory spectrum against 29 indicator strains and frequency of production genes of mutacins I, II, III and IV in 23 mutacin producing strains.

S. mutans isolates	Number of inhibited strains of different bacterial species (mean; standard deviation of inhibition zone in mm)		Detection of production genes by PCR
	mutans group (n=21)	mitis group (n=8)	
Sm1	0	2 (4.0;0)	-
Sm2	0	1 (5.0; 0)	-
Sm3	0	1(5.0; 0)	-
Sm4	0	1(4.0; 0)	-
Sm5	0	2 (4.0; 0)	-
Sm6	0	2 (4.0; 0)	-
Sm7	15 (5.73; 2.98)	7 (11.28; 6.10)	*mut*A I* and II*
Sm8	0	1 (5.0; 0)	-
Sm9	0	3 (4.33; 0.57)	-
Sm10	0	3 (4.0; 0)	-
Sm11	0	3 (4.0; 0)	*nlm*AB
Sm12	0	1 (4.0; 0)	*mut*A I
Sm13	0	2 (4.0; 0)	-
Sm14	0	2 (4.0; 0)	-
Sm15	13 (5.61; 2.43)	0	-
Sm16	0	1 (4.0; 0)	-
Sm17	1 (14.0; 0)	7 (14.28; 7.40)	*mut*A II*
Sm18	0	1 (4.0; 0)	-
Sm19	0	4 (4.75; 0.95)	-
Sm20	0	3 (4.33; 0.57)	-
Sm21	21 (13.82; 5.96)	8 (12.75; 7.27)	-
Sm22	20 (11.2; 3.18)	8 (11.75; 6.18)	-
Sm23	21 (10.76; 4.54)	8 (10.55; 5.31)	*mut*A I

* Positive expression by RT-PCR

Figure 2. Detection of inhibitory activity against *S. sanguinis* in liquid preparation and different dilutions of Sm7 and Sm17 strains crude extract. The data are expressed as means and standard deviations of quadruplicate experiments.

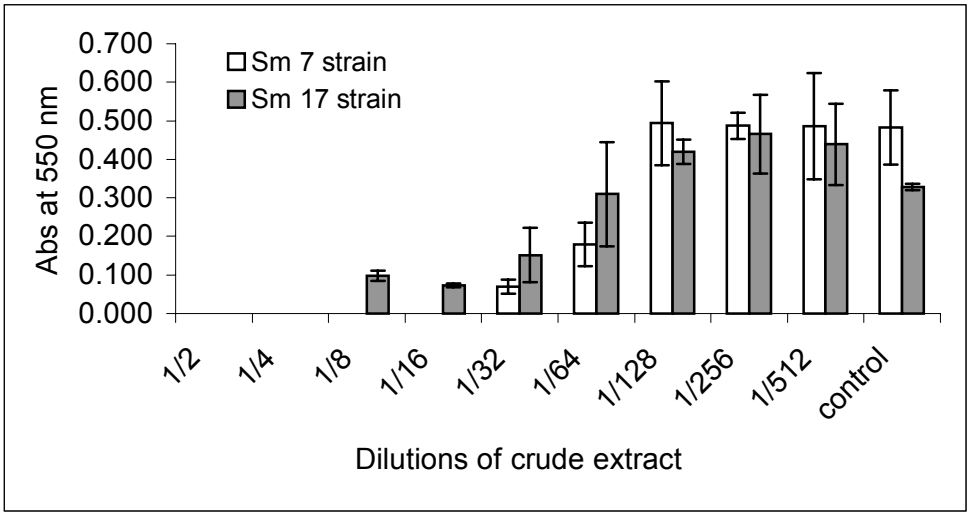
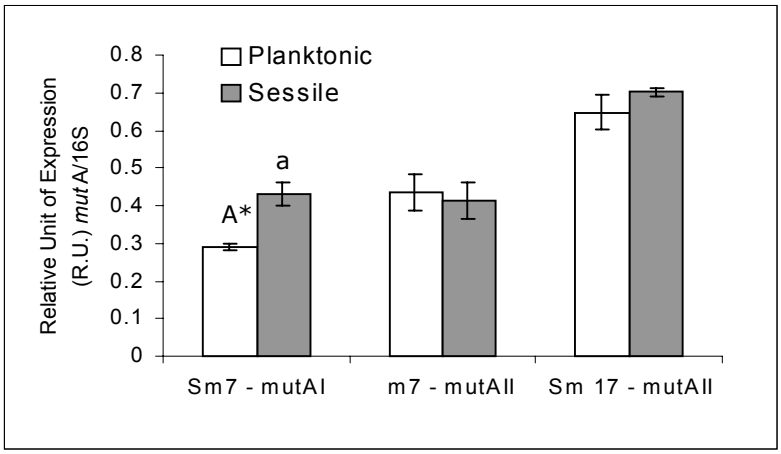


Figure 3. Relative expression of *mut*AI and *mut*AII genes in planktonic and sessile cells grown in the THB sucrose (0,5%).



Mutacins I and II producing strains were used to study the expression of the *mut*AI and *mut*AII in planktonic and sessile cells by quantitative RT-PCR. For each strain/conditions, cDNA samples derived from three independent experiments were submitted to PCR with *mut*A specific primers and 16S normalizing gene. * significant difference ($p < 0,05$).

Figure 4. Concentration of inhibitory substances in the culture supernatant of the mutacins producer strains grown in THB and THB sucrose (0,5%) at final log phase. The data are expressed as means and standard deviations of quadruplicate experiments.

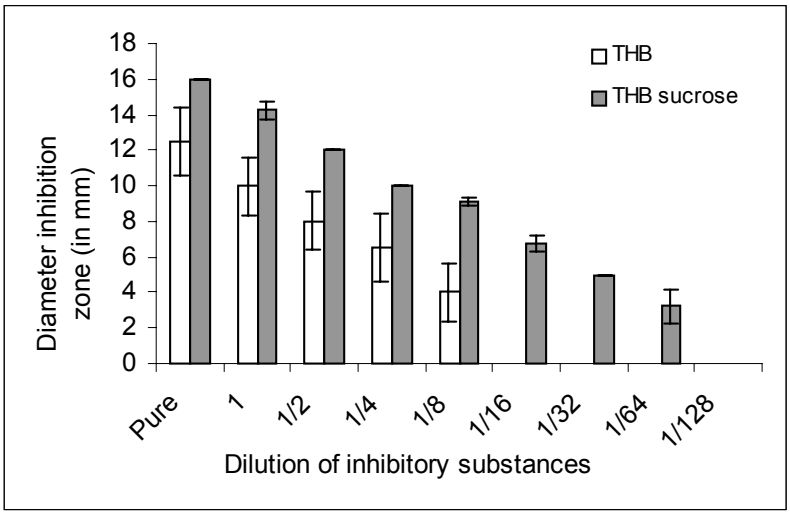


Figure 4a. Inhibitory substances concentration in the culture supernatant of strain Sm 7 in THB and THB sucrose.

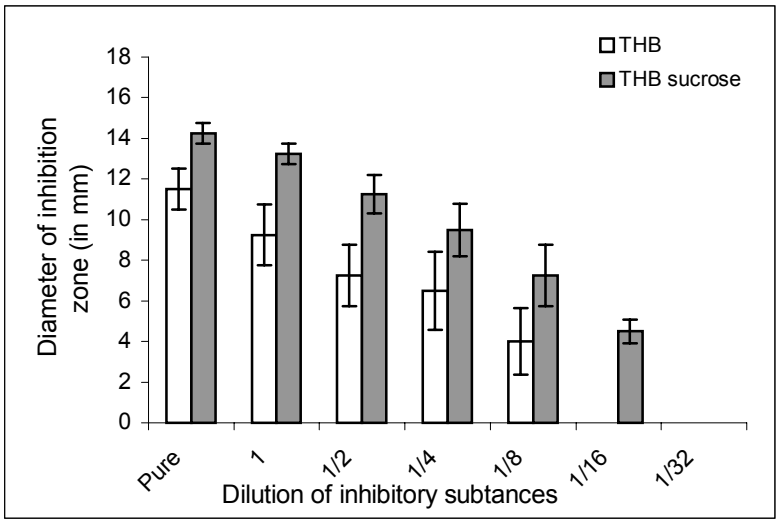


Figure 4b. Inhibitory substances concentration in the culture supernatant of strain Sm 17 in THB and THB sucrose.

CAPÍTULO 3

EXPRESSION ANALYSIS OF MUTACINS BIOSYNTHESIS AND PROTEINS KINASE GENES IN *Streptococcus mutans*

Esse artigo será submetido à publicação no periódico **Microbiology.**

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Short title: Mutacin Biosynthesis and Proteins Kinase Genes

Key words: *Streptococcus mutans*, mutacins, proteins kinases, *quorum-sensing*

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ABSTRACT

In the previous studies, histine kinase proteins involved in *quorum-sensing* system for mutacins I and II production were identified. The aims of this study were to verify if the mutacins I and II synthesis have the same genetic expression profile observed in some proteins of *S. mutans quorum-sensing*. In addition, we analyzed the role or activities of mutacins I, II and some kinase proteins during planktonic and sessile growth (sucrose-dependent biofilm formation) by semi quantitative RT-PCR technique.

In summary, the expression profiles of mutacins I and II structural genes, during the planktonic and sessile growth were variable and similar to transcription profiles of kinase proteins CiaH, Dgk and ComD, suggesting regulation of production of these lantibiotics by *quorum-sensing* mechanisms. Based on the genetic expression patterns of mutacin I (*mut*AI), mutacin II (*mut*AII) and kinase proteins (*cia*H, *dgk* and *com*D), the *S. mutans* cells developed phenotypes that distinguish planktonic and sessile conditions. The up expression of the studied kinase proteins did not influence directly the increase of mutacins I and II production, however these coadjutant or secondary mechanisms may represent an important signalization for virulence factors related to survival of producer strains mainly in complex environment.

In addition, more studies will be necessary to highlight the mechanisms and pathways that regulate the synthesis of these lantibiotics in the free and/or biofilm cells. The *S. mutans* ability to maintain the mutacins production during sessile growth may be an important virulence trait related to survival and competition of the species in the dental biofilm.

INTRODUCTION

Streptococcus mutans has been implicated as the principal etiological agent in dental caries. One of the most important virulence properties of these organisms is their ability to form biofilms, survival in low pH and mutacins production [Bowden & Hamilton, 1998; Kamiya *et al.*, 2005a; Napimoga *et al.*, 2005].

Biofilms are sessile bacterial communities adherent to a surface, and their formation occurs in response to a variety of environmental variations [O´Toole *et al.*, 1998; O´Toole *et al.*, 2000]. Biofilm bacteria undergo a developmental program in response to environment signals that leads to the expression of new phenotypes that distinguish these sessile cells from planktonic cells [Costerton *et al.*, 1995; O´Toole *et al.*, 2000]. The environmental encountered by *S. mutans* in dental biofilms is highly variable, including low pH, high cell density, varied oxidation potential, signal molecules concentrations and nutritional limitations [Li *et al.*, 2001 a b; Yoshida & Kuramitsu, 2002; Aspiras *et al.*, 2004]. In view of stress conditions and high cell density encountered in biofilm, the mutacins production may likely confer ecological advantage to *S. mutans* through of the inhibition of competitor and sensible species [Hillman *et al.*, 1987; Balakrishnan *et al.*, 2002; kamiya *et al.*, 2005b].

Interestingly, these physiological characteristics related to survival and resistance of *S. mutans* under environmental stress seems to be controlled by *quorum-sensing* mechanisms. Among these mechanisms, two-component signal transduction system (TCSTSs) has been well studied in *S. mutans*.

The TCSTS system consists of a sensor, or histidine protein kinase, which is anchored to the cytoplasmic membrane, and an effector, or response regulator, which is a DNA binding protein that modulates the expression of certain target genes. Changes in the environment and specific signal molecules are sensed by histidine kinase, the signal is transmitted to the regulatory protein in the form of phosphorylation, which activates or represses transcription of specific genes to bring about the adaptation response [Appleby et al, 1996; Fabret & Hoch, 1999].

Recently, genome analyses have revealed 13 separate putative TCSTSs in *S. mutans* [Li *et al.*, 2002]. Mutations or inactivations in some of these systems as *cia*RH, DGCs and *com*DE created individual mutants strains whose the production of mutacins as types I, II,

IV and Smb was altered or abolished, respectively [Chen *et al.*, 1998; Qi *et al.*, 2004; van der Ploeg et al, 2005; Kreth *et al.*, 2005; Yonezawa & Kuramitsu, 2005]. Interestingly, alterations in the mutacins production in the mutant strains occur concomitantly with other phenotypes as reduction of acid stress tolerance, competence development and/or biofilm formation.

The regulatory mechanisms and pathways that modulate the lantibiotics mutacins I and II production is poorly understood. In the mutacins I and II biosynthetic gene clusters were identified the *mut*R gene that codes response regulators [Qi *et al.*, 1999b; Qi *et al.*, 2000], although the gene coding its cognate histine kinase protein as well as the signal molecule that trigged the mutacin production not been identified yet. In the case of nisin lantibiotic, it was demonstrated that it acts as a signal molecule inducing its own synthesis [Kuipers *et al.*, 1995], however for the mutacins this is not clear.

Previous studies verified that mutations in *cia*H and *dgk* genes coding kinase proteins abolished mutacins I and II production, respectively, suggesting that the production of these substances may be controlled by *quorum-sensing*-like mechanisms [Chen *et al.*, 1998; Qi *et al.*, 2004]. The mutacin I production cell density-dependent and growth phase state reinforce this hypothesis [Qi *et al.*, 2001; Merritt *et al.*, 2005; Kamiya *et al.*, submitted – chapter 2], although such data was not observed during the mutacin II synthesis [Qi *et al.*, 1999a; Kamiya *et al.*, submitted].

In this study, the expression of mutacins I and II structural genes was analyzed by semi-quatitaive RT-PCR method, as well as kinase proteins genes (*cia*H, *dgk* and *com*D), during different phases of the planktonic and sessile growth.

MATERIALS AND METHODS

1. *S. mutans* isolate: We selected for this study 1 *S. mutans* strain (Sm 1), isolated from child in the previous studies [Flório *et al.*, 2004; Klein *et al.*, 2004]. The Sm1 strain was previously characterized as producer of mutacins I and II by semi-quantitative RT-PCR method [Kamiya *et al.*, submitted – chapter 2].

2. Quantification of Planktonic and Sessile Growth

Cells suspension of a subculture of Sm 1 strain (OD = 0,1 at 550nm) was inoculated into 40 mL of Todd-Hewitt Broth (THB, DIFCO Laboratories, Sparks, MD) supplemented with sucrose (0,5%) at 37^{o}C and CO_2 (10%). The planktonic growth was monitored at 550

nm, using a spectrophotometer. The experiments were realized in triplicate and the optical densities averages were used to build the planktonic growth curve of the strain.

For quantification of sessile growth in the tube surface (biofilm formation), the tubes of polyethylene (Greiner Labortechnik Gmbh) were dried three times with saline and adhered firmly cells, correspondent to different planktonic growth phases, were removed mechanically by scratching. Each tube was completed with sterile saline (40 mL) and the optical density was read in a spectrophotometer at 550 nm.

When the cultures reached variable optical densities of 0.4 to 1.0 at 550 nm (exponential and stationary phases), the final pH of supernatant was measured, using pH meter and the planktonic and sessile cells, correspondent to different periods, were colleted by centrifugation and submitted to RNA extraction.

3. RNA extraction and RT-PCR:

The total RNA was extracted from planktonic and sessile cells by phenol-chloroform method as described previously by Qi *et al.* [1999b]. The residual DNA of extracted RNA was purified with Deoxyribonuclease I (Life Technologies – Gibco, Grand Island, NY), according to the manufacturer's instructions. The cDNA synthesis was realized with 24 ng of total RNA using random primers Mix (20 μM) Ea1, Ea7, Es1, Es3 and Es8 [Chia *et al.*, 2001] and the enzyme Super Transcrispt RT III (Gibco), following the manufacturer's protocol, with some modifications.

In the first phase, primers random Mix (20 μM), dNTPs Mix (10 mM) and DEPC to 10 μL were added to the total RNA. The solution was heated at 65^{o}C for 5 min and cooled at 4^{o}C for 1 min. Subsequently, it was added to the reaction mixture: 10x RT buffer, $MgCl_2$ (25 mM), DTT (0,1 M), RNase OUT (40 U) and Super Transcript RT III (200 U). The total solution containing cDNA (20 μL) was submitted to the following thermal cycle: 25^{o}C/10min; 50^{o}C/50min; 85^{o}C/5min and 4^{o}C/1min. For each RNA sample, the cDNA synthesis reaction was also carried out without reverse transcriptase in order to identify contamination by residual genomic DNA. For the maximum efficiency, RT-PCR primers were designed to generate amplicons ranging from 150 to 200 bp in size (table 1).

A 1 μL volume of cDNA was used as template in the PCR (25 μL) with 50 mM of $MgCl_2$, 0.3 mM of each specific primer (table 1), 200 μM of dNTPs, 1.25 U of Taq polymerase (Gibco) and 10x PCR buffer. All samples of cDNA were tested with primers to

the house-keeping gene 16S, a constitutively transcribed control gene whose expression was invariant under the experimental conditions used. The primer set *16S*/forward (5'-cggcaagctaatctctgaaa-3') and *16S*/reverse (5'-gcccctaaaaggttacctca-3'), designed based on *S. mutans* UA159 genome (NC004350), was used to normalize the expression mutacin production genes (*mut*A) and proteins kinase genes (*cia*H, *com*D and *dgk*). Controls for RT-PCR included reaction mixtures without template cDNA, to effectively rule out the presence of contaminating DNA and/or the formation of primers dimmers.

The synthesized cDNA was used to generate critical threshold (Ct) values for specific primer sets. The Ct values were determined as the cycle at which density was intermediary between the first cycle that gave rise to a detectable PCR product above the background and the first cycle in which there is saturation of PCR reaction.

The PCR conditions included 25 cycles for 16S gene and 37 for other genes, as followed: initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 45 sec, annealing at 50ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 7 min. A 100 bp DNA ladder was included in each gel. The amplicons were visualized in agarose gel (2%) with ethidium bromide (0,3 mg/mL) (Gibco), and the densitometry was measured by KODAK Program (Gel logic 100 Imaging System, Eastman Kodak Co., USA).

4. Determination of inhibitory substances concentration in the supernatant

The mutacins were partially isolated (crude extract) from culture supernatants of respective periods of the planktonic growth analyzed. The isolation of mutacins was realized according to previous studies [Qi *et al.*, 1999a b; Hillman *et al.*, 1998; Novák *et al.*, 1994], with some modifications.

Briefly, the cultures of mutacins producing strains were centrifuged for 10 min at 10.000 g and 4^{o}C. The supernatant was filtrated in milli-pore membrane (0,45 μm pore – size) and extracted with an equal volume of chloroform. The emulsion at the chloroform-aqueous interface was collected by centrifugation at room temperature for 2 min at 10.000 g. The pellet was washed once with double-distilled H2O.

The water-insoluble material (crude extract) was dissolved in 1 mL of PBS (phosphate-buffered saline) by heating at 60^{o}C for 10 min [Nicolas *et al.*, 2004]. Water-insoluble fractions were serial diluted 1:1 with PBS and the semi quantitative critical dilution method was used to determine the titres of the mutacins produced [Parrot *et al.*,

1990]. Ten microliters of each serial dilution of partially purified mutacin were deposited on Trypiticase Soy Agar (TSA, OXOID - 1,5%) plates and overlaid with 5 ml of Trypiticase Soy Broth (TSB, OXOID) soft agar (0,8% agar) containing a standardized suspension 0,5 mL of an exponentially growing culture of *S. sanguinis* CR311 at an A550 of 0,1. The concentrations of these substances were expressed in arbitrary units per mL (AU/mL), which corresponds to the reciprocal of the last dilution showing a detectable inhibition zone against *S. sanguinis,* after 24 h of incubation at 37^{o} C and CO_2 10% [Qi *et al.*, 1999a; Nicolas *et al.*, 2004].

5. Statistical Analysis

The differential analyses of genetic expression inter-periods were evaluated by the values of the averages and standard deviations of independent experiments realized in triplicate. The Test t estimated the statistical differences inter-groups or inter-periods for each gene.

RESULTS

The planktonic growth rate and the biofilm formation of Sm 1 strain were assessed in independent growth curves experiments as shown in Fig. 1. Interestingly during the stationary phase there was no apparent reduction of pH in the supernatant and increase in the number of the planktonic cells or optical density.

The expression analyzes of proteins kinase genes in different planktonic growth periods revelead picks or up expression in the exponential and stationary phases ($p < 0,05$). These genes showed oscillatory expression profiles during the *S. mutans* growth (figure 2).

Differently from the house-keeping 16S, used as the reference gene in this study, that was invariably transcribed under the experimental conditions used, the structural genes (*mut*AI and *mut*AII) presented an oscillatory and similar expression profile to regulatory genes (*cia*H, *com*D and *dgk*), suggesting that the mutacin I and II production may be regulated by *quorum-sensing* mechanisms. In addition, the pH did not influence in the increase of *mut*AI and II transcription during the stationary phase of planktonic growth. Since the pH has been maintained stable there were picks of expression of mutacins structural genes during this phase.

In addition, this study evaluated the expression of these genes during sessile growth correspondent to the same period of planktonic growth (figure 2). The results showed that

there was not any synchronism between genetic expression of the planktonic and biofilm cells in the respective periods analyzed, suggesting modifications of genetic expression due to transition from the planktonic to the biofilm states, in which the cells were exposed to different environmental conditions.

The Sm 1 strain presented two significant up expressions of the regulatory genes during sessile growth (12 and 24 h, $p < 0,05$). Although the *mut*AI and II genes were more expressed in the early stages of the biofilm formation (12h, $p < 0,05$). The mutacins I and II production was lower however stable during the latter phases of sessile growth (17 to 24 h, with no significant differences among the periods) (figure 2).

The increase of *cia*H and *dgk* transcription during planktonic growth and biofilm development did not induce necessarily the up expression of *mut*AI and *mut*AII, respectively (fig. 2).

Figure 3 showed the quantification of inhibitory substances titres in different periods of the planktonic growth. There was no correlation between the transcription levels of *mut*AI and II genes and the concentration of these inhibitory substances in the supernatant in the respective analyzed periods. Mutacins I and II saturating conditions were observed in the latter stationary phase, probably due to the high half-life of these lantibiotics.

DISCUSSION

It is possible that the biofilm-forming potential of a strain would depend on the extent of its planktonic growth [Bhagwat *et al.*, 2001]. Our results showed the sessile growth during stationary phase, suggesting higher cell metabolism and resistance in adhered cells in relation to planktonic cells, even under stress conditions as nutritional restriction and low pH in the supernatant. In fact, in agreement with our results, Li *et al.* [2001b] showed that *S. mutans* cells grown in biofilms not only survived better than planktonic cells but also were capable of growth at the lower pH.

The differences in the cell metabolism and in the genetic expression profiles observed between free and sessile cells in this study may be due to distinct environmental characteristics as limitations or excess of the nutrients, the pH variations, osmolarity differences and oxidation potential in which cells were exposed as previously suggested by other studies [Li *et al.*, 2001a, b; Yoshida & Kuramitsu, 2002; Aspiras *et al.*, 2004]. In

biofilms it was observed the existence of low pH micro-environments and the presence of extra cellular storage polysaccharides that increase the amount of carbohydrates available to subpopulations of cells in division within the biofilm and might account for the apparent stimulation of expression of some genes involved with the *S. mutans* adherence [Li & Burne, 2001].

There are evidences that extra-cellular signal molecules releases during cells growth will be able to enhance the induction of genes expression that coding phenotypes related with the *S. mutans* resistance and survival as acid adaptation, bacteriocins production, competence development and organization in biofilm [Li *et al.*, 2001a, b; Qi *et al.*, 2004; van der Ploeg, 2005; Petersen *et al.*, 2005]. The planktonic cells need to reach a critical concentration of signal molecules to trigger target genes, unlike the organized biofilms where these signal molecules may accumulate rapidly. The higher concentration of signal molecules and population density in biofilm allow a better adaptation to stress in comparison to planktonic cells [Li *et al.*, 2001b].

The oscillatory expression profile of proteins kinase and mutacins I and II during planktonic and sessile growth reflects in a brief period of gene transcriptional activity and in a cell energy economy. This transcriptional activity may be a minimal requirement for activation of the target genes and for conservation of the phenotypes resistance to different environmental stresses and stimulus.

The stress resistance mechanism in the stationary phase is likely very important for the survival of *S. mutans* in biofilms since it is believed that the biofilm phenotype is akin to the physiological state that cells exist in the referred phase [Zhu *et al.*, 2001]. In this phase there is extra-cellular signal molecules or pheromones peptides saturation as CSP (Competence Stimulating Peptide) and some bacteriocins as nisin [Kuipers *et al.*, 1995; Kreth *et al.*, 2005]. In the same way, we detected a higher inhibitory substances concentration (mutacins I and II) in the latter stationary-phase of the static cultures.

During the planktonic growth, the picks of genes expression in the exponential phase may suggest a cell density-dependent regulation, however the up expression of these genes in the stationary phase is suggestive of regulation dependent on signal molecule(s) saturation in the supernatant. Probably, the saturation of these proteins or signal molecules acts as a secondary signal to amplify the induction of genetic expression [Li *et al.*, 2001a].

The genetic expression of the sessile and planktonic phases was not synchronized within them in the respective analyzed periods. These data reinforce the hypothesis that the adhered cells or in suspension were exposed to different environmental conditions, although, physiological states of bacterial cells living in biofilm, in terms of growth rate, growth phase or metabolic activities are heterogeneous which allows that cells respond to stress in different ways [Li *et al.* 2001b].

The biofilm model used in this present study was similar to the one used by Qi *et al.* [2004], who employed *S. mutans* static culture in THB sucrose. In this system, was observed by confocal laser scanning microscopy the formation of the multilayered sheet that was loosely attached to the surface. A similar structure was observed in the scanning electron micrographs corresponding to 24 h-old mature biofilms grown in a chemostat-based fermentator [Li *et al.*, 2001a]. Second this study, the cells aggregates obviously provided an environment conductive to the secretion and detection of the natural signal peptide molecule capable of initiating the cascade needed for competence development.

Previous studies demonstrated that the disruption of the *cia*H and *dgk* genes reduced the biosynthesis of mutacins I and II, respectively. Interestingly, the inactivation of these regulatory genes also diminished competence development, acid stress tolerance and bactiracin resitance [Qi *et al.*, 2004; Chen *et al.*, 1998; Lis & Kuramitsu, 2003]. In our study, the production of these lantibiotics during the cell growth (in living or sessile state) was independent of the genetic transcription levels of *cia*H and *dgk*. These results suggest that the synthesis mechanisms of mutacins I and II may be dependent on the other regulatory process that is still unclear.

Isolation and characterization of genes defective in biofilm formation may contribute to the identification of the genes necessary for biofilm development and to understand how *S. mutans* responds to environmental signals in the oral biofilm. However, the technique that creates mutants through genetic disruption may also generate varied phenotypes that secondarily modify this biofilm formation. For example, the *lux*S mutation plays critical roles in modulating key virulence properties of *S. mutans*, as well as acid and oxidative stress tolerance and biofilm formation [Wen & Burne, 2004]. Recently, it was demonstrated that *lux*S mutant *S. mutans* strain had also an impaired mutacin I production ability [Merritt *et al.*, 2005]. In this same study, through microarrays analyses, nine genes

were found to be strongly induced in *lux*S mutant, which a putative transcription repressor (Smu 1274), named *irv*A gene (inducible repressor of virulence), was identified as a possible inducible repressor to suppress mutacin I gene expression.

Therefore, genetic mutations may create different phenotypes through of the distinct pathways, many of which remain unknown yet. Based on our results, there are pathways different from *cia*H *and dgk* activities that may regulate the mutacins I and II synthesis, respectively. Even though the idea that the mutacins I and II production mechanisms are regulated secondarily by these proteins kinase, it cannot yet be excluded.

Recently, Tsang *et al.* [2006], using a random insertional mutagenesis approach, identified 25 genes associated with the mutacin I production defective, among them genes from two-component sensory systems, stress responses, energy metabolism and central cellular process, suggesting that mutacin I production is stringetly controlled by diverse and complex regulatory pathways.

The nisin lantibiotic produced by *Lactococcus lactis* acts as signal molecule inducing its own synthesis. The nisin and subtilin biosynthetic gene clusters include genes encoding a sensor protein kinase (*nis*K and *spa*K, respectively) and a response regulator (*nis*R and *spa*R) that have been shown to be essential for production these lantibiotics [Siezen *et al.*, 1996; Kuipers *et al.*, 1995]. Interestingly, in the mutacins I and II biosynthetic gene locus there are genes coding a regulatory proteins (*mut*R), although the gene coding sensor proteins kinase for respective lantibiotics has been not identified yet [Qi *et al.*, 1999a; Qi *et al.*, 2001]. Is not clear if these proteins act as signal molecules inducing its own synthesis, but it is suggestive that the CiaH and Dgk, as well as other possible kinase proteins not identified yet, may act as a sensor receptor for own mutacins or other signal molecule(s) that trigged the production of these inhibitory substances.

Based on our results, the picks of the *mut*AI and II expression during the growth in the stationary phase suggest regulation of production due to saturation of these inhibitory substances in the supernatant. It is a circumstantial evidence that the mutacins may act as signal molecule inducing its own synthesis.

Supporting this hypothesis, only one up expression of *mut*AI and II was observed during the biofilm development of strain, suggesting that the majority of produced mutacins by sessile cells probably were released to supernatant, acting in the increase of structural

genes expression in the planktonic phase, where the up expression was more frequent. In our *in vitro* previous study [Kamiya *et al.*, submitted], we suggest that the liberation of mutacins synthesized by biofilm cells in supernatant could increase the titre of these inhibitory substances outside of the biofilm besides act in the inhibition of sensible microorganisms in the saliva. Although the concentration of mutacins or signal pheromones peptides within organized biofilm was a minimal to induce the up expression of proteins kinase genes or maintain the *mut*AI and II transcription levels during the sessile growth.

In summary, the mutacins I and II production is dependent on the planktonic or sessile conditions and may be controlled by *quorum-sensing* system. However more studies will be necessary to highlight the mechanisms and pathways that regulate the synthesis of these lantibiotics in the free and/or biofilm cells. The *S. mutans* ability to maintain the mutacins production during sessile growth may be an important virulence trait related to survival and competition of the species in the complex oral biofilm.

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Table 1. PCR specific primers to structural genes *mut*A (mutacins I and II) and proteins kinase genes (*cia*H, *com*D and *dgk*).

Primers	Sequences	GenBank	Amplicon
*mut*AI_Forward	TAGAAGTCCTTGGTACTGAA	AF238860	163bp
*mut*AI_Reverse	TTGAAACTAGGATTTTTCAC		
*mut*AII_Forward	CAGTAACGCAGTAGTTCCTT	U40620	162bp
*mut*AII_Reverse	TTAACAGCAAGTGAAAACAT		
*cia*H_Forward	TACCTTAACTGTCACTGTCC	based on *S. mutans* UA159 genome (NC004350)	183pb
*cia*H_Reverse	ATGGCTGCTAATACAGATAT		
*com*D_Forward	GTATGGTAATGAAATCAAGC		162bp
*com*D _Reverse	GTTATAATATCGGCCATCTA		
*dgk*_Forward	AGAAGAGCGTAATATGAAAA		192bp
*dgk*_Reverse	AACATGGAAAAATGATAGTC		

Figure 1. Planktonic and Sessile Growth Curves and pH measure of supernatant in different periods. The data are expressed as means and standard deviations of triplicate experiments.

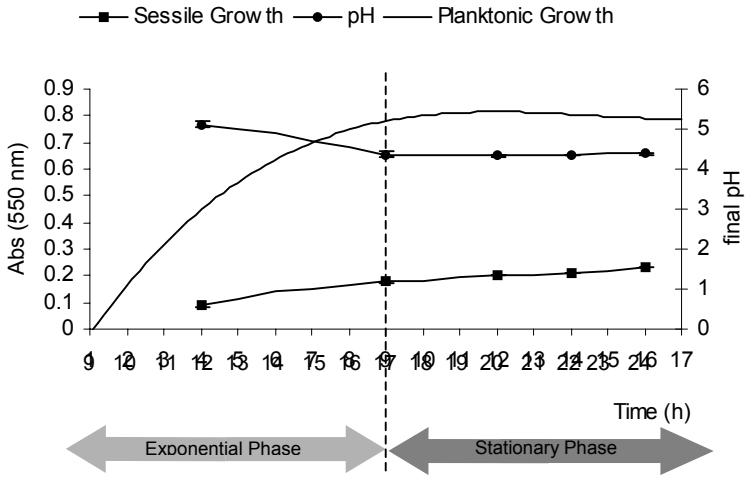
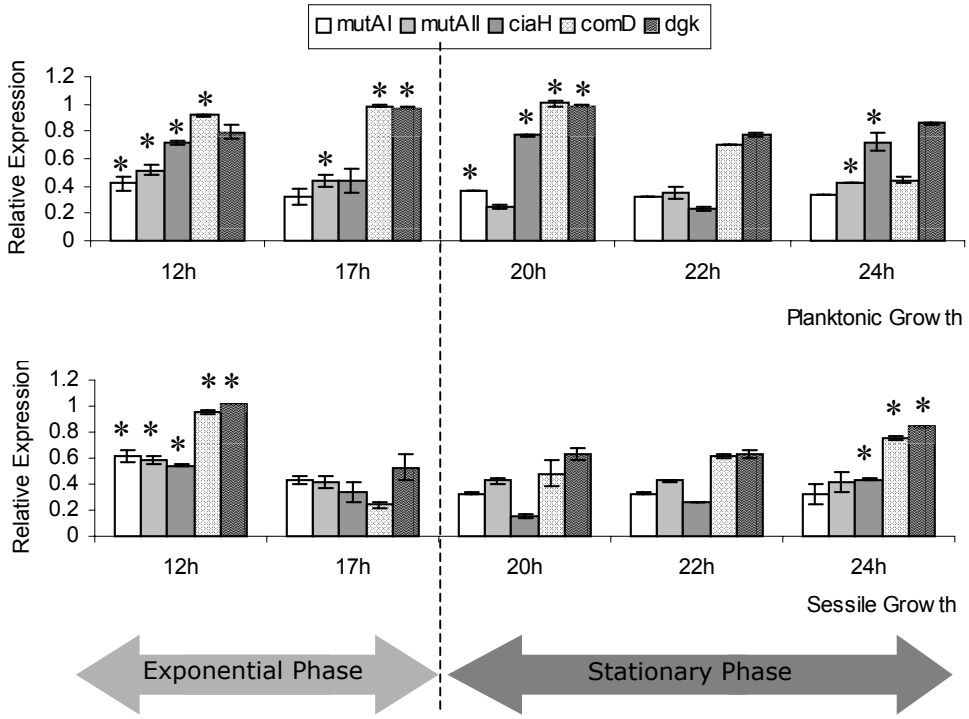


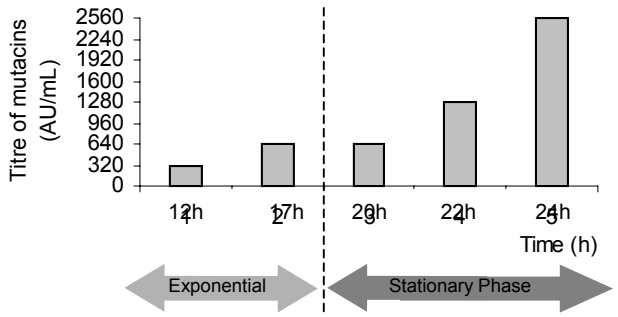
Fig. 1. Planktonic and sessile growt. Bacteria grown in THB sucrose (0,5%) medium, the absorbance of the cultures was monitored at 550 nm and the pH was measured periodicaly. The tendency line of planktonic growth was traced and the final-exponential phase was determined (17h).

Figure 2. Expression profiles of *mut*A, *cia*H, *com*D and *dgk* genes in different phases of planktonic and sessile growth of Sm 1 strain. The data are expressed as means and standard deviations of triplicate experiments.



* Up expression ($p < 0,05$), however without difference among them.

Figure 3. Determination of mutacins titre (AU/mL) in the culture supernatant in different periods of the planktonic growth.



VI – DISCUSSÃO GERAL

A cárie é uma doença multi-fatorial, que envolve vários fatores de virulência bacterianos, dentre os quais os produtos ácidos e antimicrobianos (bacteriocinas), sintetizados por microrganismos cariogênicos, como *S. mutans*. A aciduricidade e a síntese de bacteriocinas poderiam representar vantagens seletivas e ecológicas ao *S. mutans,* em ambientes competitivos, como o biofilme e lesões de cárie [Li *et al.*, 2001b; Balakrishnan *et al.*, 2002].

Vários estudos sugeriram que o potencial de produção de mutacinas por *S. mutans* poderia estar correlacionada com a prevalência e estabilidade da espécie, em diferentes nichos da cavidade oral como a saliva, o biofilme dental e lesões de cárie, representando assim, um importante fator de virulência para o desenvolvimento e progressão da doença cárie [Fabio et al, 1987; Hillman *et al.*, 1987; Gronröos *et al.*, 1998; Balakrishnan *et al.*, 2002].

Baseando-se em nossos estudos [Kamiya *et al.*, 2005a – anexo 3], o perfil de produção das mutacinas (espectro inibitório), em genótipos de *S. mutans* isolados de indivíduos cárie-ativos e livres de cárie, foi correlacionado com os padrões de colonização e biodiversidade microbiana, previamente descritos nos respectivos grupos [Nyvad & Kilian, 1990; Napimoga *et al.*, 2004]. Fenótipos, produtores de mutacinas de amplo espectro, foram mais freqüentes entre os isolados de indivíduos cárie-ativos [Kamiya *et al.*, 2005a], que apresentam uma microbiota mais complexa e de alta diversidade genética, em relação aos voluntários livres de cárie [Nyvad & Kilian, 1990; Napimoga *et al.*, 2004]. Estes resultados evidenciaram o possível papel ecológico das mutacinas, abrindo futuras perspectivas, sobre a necessidade de se identificar os determinantes genéticos, relacionados com os diferentes fenótipos produtores de mutacinas, identificados entre os *S. mutans* isolados dos grupos cárie-ativo e livre de cárie.

O rastreamento e a identificação destes determinantes genéticos foi realizado em estudos posteriores [Kamiya *et al.*, 2005b – capítulo 1]. Diante das seqüências nucleotídicas, até então disponíveis no GenBank, foi realizado o rastreamento dos genes de produção das mutacinas I, II, III e IV, em isolados de *S. mutans* dos grupos cárie-ativo e livre de cárie, utilizando a técnica de PCR e *primers* específicos para os respectivos genes.

Determinantes genéticos de mutacinas de amplo espectro, como as dos tipos I e III, foram identificados entre os isolados de *S. mutans* do grupo cárie-ativo, concluindo que a produção de mutacinas de amplo espectro pode exercer um importante papel na colonização do *S. mutans,* principalmente em nichos de alta complexidade microbiana, comumente descritos, em indivíduos com atividade de cárie.

Entretanto, a baixa freqüência dos genes rastreados, impossibilitou a identificação dos genes responsáveis pela maioria dos fenótipos identificados em estudos prévios [Kamiya *et al.*, 2005a]. Tais resultados sugeriram a existência de alta diversidade e polimorfismo nos determinantes genéticos, necessários para a produção de substâncias semelhantes às mutacinas, em isolados clínicos de *S. mutans,* concordando com os dados obtidos na literatura [Longo *et al.*, 2003; Li *et al.*, 2005].

O estudo, descrito no capítulo 2, confirmou a hipótese de alta diversidade de substâncias semelhantes às mutacinas, ainda não identificadas. Neste estudo, objetivando identificar novas substâncias inibitórias, em *S. mutans* isolados de crianças, foi realizado o rastreamento adicional de genes homólogos às bacteriocinas, identificadas em outras espécies bacterianas, baseando-se na hipótese da transmissão horizontal. A transmissão horizontal dos determinantes genéticos, necessários para a síntese de antibióticos naturais, fundamenta-se nas seqüências nucleotídicas e/ou peptídicas de bacteriocinas, altamente conservadas entre as diferentes espécies e na presença de genes transposase (*tra*) próximos ao operon de diferentes bacteriocinas [Buchman *et al.*, 1988; McLaughlin *et al.*, 1999; Chen *et al.*, 1999]. A inexistência de genótipos de *S. mutans,* portadores de genes homólogos às bacteriocinas rastreadas, reforçou a hipótese de alta biodiversidade de peptídeos inibitórios semelhantes às mutacinas, dentre os quais muitos ainda não foram identificados, na espécie *S. mutans.*

Em adição, neste estudo [capítulo 2], foi verificado que a presença do gene de produção das mutacinas rastreadas, não necessariamente, resultou em expressão gênica positiva do mesmo. Segundo alguns estudos, a perda da atividade inibitória ocorre por mutação genética ou perda de mecanismos regulatórios coadjuvantes, envolvidos direta ou indiretamente com a síntese destas substâncias, como por exemplo elementos regulatórios

do sistema *quorum-sensing* [Chen *et al.*, 1998; Chen *et al.*, 2001; Qi *et al.*, 2004; van der Ploeg, 2005].

Recentemente, a síntese das mutacinas I, II, IV e Smb, têm sido relacionadas com mecanismos regulatórios do *quorum-sensing* [Chen *et al.*, 1998; Qi *et al.*, 2004; van der Ploeg, 2005; Yonezawa & Kuramitsu, 2005]. Condições de estresse ambiental, comumente encontradas em biofilme dental, tais como restrição nutricional, alta densidade celular, baixo pH, potencial de óxido-redução alterado, e saturação de moléculas sinais, podem ativar o mecanismo *quorum-sensing*, gerando fenótipos, em biofilme, diferenciados e mais resistentes às condições ambientais extremas, em relação aos fenótipos desenvolvidos por células planctônicas [Li *et al.*, 2001a b; Yoshida & Kuramitsu, 2002; Aspiras *et al.*, 2004]. A produção de mutacinas, neste ambiente complexo, representaria uma vantagem seletiva e ecológica à cepa produtora, com a inibição de microrganismos competidores [Balakrishnan *et al.*, 2002].

Visando elucidar os mecanismos regulatórios envolvidos com a síntese das mutacinas e o possível papel destas substâncias, no biofilme ou na saliva *in vivo*, alguns estudos que analisam a expressão dos genes de produção das mutacinas, *in vitro*, têm sido realizados, dentre eles os descritos nos capítulos 2 e 3 [Qi *et al.*, 1999b; Qi *et al.*, 2000; Kreth *et al.*, 2004].

No capítulo 2, utilizando o modelo de biofilme em culturas estáticas, foi observada a maior expressão do gene *mut*AI, em células crescidas na condição séssil em relação àquelas submetidas ao crescimento planctônico, o que poderia sugerir que a síntese da mutacina tipo I, diferentemente da mutacina II, poderia ser controlada por mecanismos *quorum-sensing*. Estudos anteriores comprovaram esta hipótese, demonstrando que a síntese da mutacina I foi dependente da densidade celular e da condição séssil de crescimento, diferentemente da mutacina tipo II [Qi *et al.*, 1999b; Qi *et al.*, 2001; Kreth *et al.*, 2004]. Os fenótipos de produção de mutacina I foram diferenciados nos estados séssil e plantônico, provavelmente devido às diferenças ambientais aos quais as células foram expostas [Kamiya *et al.*, submetido – capítulo 2].

Estudos baseados em mutações genéticas, têm identificado genes do mecanismo *quorum-sensing*, responsáveis pelos fenótipos relacionados com a virulência de *S. mutans,* dentre os quais a produção de mutacinas. A disruptura dos genes *cia*H, *dgk* que *com*D, que codificam proteínas histina kinases sensoras, alterou ou aboliu a síntese das mutacinas I, II e IV, respectivamente [Chen *et al.*, 1998; Qi *et al.*, 2004; van der Ploeg, 2005].

Visando esclarecer o papel regulatório das proteínas kinases CiaH e Dgk na síntese de mutacinas tipos I e II, respectivamente, foi desenvolvido o terceiro trabalho [capítulo3]. A análise comportamental dos referidos genes, em diferentes fases do crescimento planctônico e séssil, demonstrou padrões de expressão variáveis. O mesmo perfil foi observado para os genes de produção das mutacinas I e II, sugerindo que a síntese destas substâncias pode ser regulada por mecanismos *quorum-sensing*.

A produção destes lantibióticos foi independente dos níveis de expressão dos genes *cia*H e *dgk,* sugerindo que outros mecanismos ou proteínas kinases, ainda desconhecidos, poderiam estar mais relacionados com a produção destas substâncias inibitórias em estados séssil ou planctônico. Estes dados não podem excluir o papel secundário dos genes *cia*H e *dgk* na regulação da produção das mutacinas I e II, respectivamente.

Mutações nos genes, que codificam proteínas histina kinase CiaH e Dgk,, produziram outros fenótipos relacionados com a resistência ao pH ácido e aos antibióticos, com o desenvolvimento da competência e com a formação de biofilme em *S. mutans* [Lis & Kuramitsu, 2003; QI *et al.*, 2004]. Em estudos recentes, foi demonstrado que em cepas mutantes, o padrão de expressão gênica é diferenciado em relação à cepa parente; a indução ou repressão de genes que codificam proteínas regulatórias (sejam indutoras ou repressoras) pode modificar a transcrição de diferentes genes alvos, originando, desta forma, os diferentes fenótipos, através de vias similares ou distintas [Merritt *et al.*,2005]. Neste estudo, utilizando a técnica de *microarrays*, foram identificados nove genes, altamente induzidos na cepa mutante *lux*S, entre eles, um possível repressor de virulência, que inibiu a expressão do gene *mut*AI. Mutações no *lux*S também reduziu os fenótipos relacionados com a tolerância ao estresse ácido e oxidativo, bem como inibiu a formação de biofilme [Wen & Burne, 2004]. Dentro do mesmo raciocínio, sugere-se que mutações nos genes

*cia*H e *dgk* poderiam ter induzido a redução da síntese das mutacinas I e II, respectivamente, por vias ainda desconhecidas.

Os picos de expressão das mutacinas e proteínas kinases CiaH, Dgk e ComD, durante a fase estacionária, foram independentes do aumento da densidade celular e da queda de pH (fatores constantes, durante esta fase de crescimento). Tais dados sugerem a possível ativação da transcrição gênica devido à saturação de moléculas sinais, produzidas durante o crescimento celular. Através da quantificação das mutacinas no sobrenadante, em diferentes períodos do crescimento planctônico, verificou-se a saturação destas substâncias inibitórias, ao final da fase estacionária, bem como aumento da expressão de *mut*AI e II em células planctônicas, sugerindo que as mutacinas I e II poderiam agir como molécula sinal para sua própria síntese, assim como o observado no sistema regulatório de produção do lantibiótico Nisina [Kuipers *et al.*, 1995].

Os perfis de expressão gênica das mutacinas e proteínas kinases não foram sincronizados durante crescimento planctônico e séssil, nos respectivos períodos analisados. O modelo de biofilme utilizado, obtido a partir de culturas estáticas, foi suficiente para detectar a expressão gênica diferencial entre os estados séssil e planctônico. Os resultados sugerem que a regulação da expressão dos genes estudados é dependente da condição ambiental ao qual as células são expostas. De acordo com estudos prévios, fenótipos distintos podem ser identificados na condição de crescimento planctônica e de biofilme, devido às diferenças dos estímulos e das condições ambientais aos quais as células se encontram [Costerton *et al.*, 1995; Burne *et al.*, 1997; O´Toole *et al.*, 2000; Kamiya *et al.*, submetido – capítulo 2].

A constante síntese de mutacinas I e II durante o crescimento séssil representaria um importante fator de virulência de *S. mutans,* relacionado com a sobrevivência e competitividade da espécie cariogênica, em biofilme dental. A manutenção da expressão do *mut*AI e *mut*AII, durante a formação do biofilme *in vitro*, sugere atividade inibitória das mutacinas intrínseca a este ambiente complexo. A constante produção de mutacinas, durante o desenvolvimento do biofilme, poderia contribuir para o aumento ou saturação da concentração destes peptídeos, no sobrenadante de cultura, ao final da fase estacionária. Extrapolando para um sistema *in vivo,* parte das mutacinas produzidas por células aderidas

à superfície dental, poderia ser liberada para o ambiente externo, aumentando a concentração destes peptídeos antimicrobianos na saliva, onde a atividade biológica das mutacinas seria extrínseca ao biofilme bacteriano.

Os estudos, apresentados nesta tese, demonstraram o papel das mutacinas como um fator de virulência, altamente diversificado entre a espécie *S. mutans*, e particularmente, relacionado com o risco de cárie. Este fator de virulência, pode ser regulado por mecanismos *quorum-sensing*, sendo assim, dependente da condição de crescimento séssil ou planctônica. A regulação da produção de mutacinas, por mecanismos *quorum-sensing*, pode representar uma vantagem seletiva à espécie produtora, principalmente em ambiente complexo, como o biofilme dental e lesões de cárie.

Como perspectivas futuras, mais estudos serão necessários para caracterizar novos determinantes genéticos, relacionados com a produção de substâncias semelhantes às mutacinas, bem como, identificar os mecanismos e componentes do *quorum-sensing*, que possam modular a expressão destas substâncias inibitórias em *S. mutans.*

V – CONCLUSÃO GERAL

Diante do trabalho exposto, pode-se concluir:

- ✓ as mutacinas produzidas por *S. mutans* representam um importante fator de virulência, relacionado com o risco de cárie dental;
- ✓ existe uma grande diversidade nos determinantes genéticos, necessários para a produção de sustâncias semelhantes às mutacinas, entre os isolados de *S. mutans* analisados;
- ✓ a presença destes determinantes não implica em expressão genética positiva;
- ✓ a produção de mutacinas I e II por *S. mutans* é dependente das condições de crescimento séssil ou planctônica;
- ✓ a produção de mutacinas I e II pode ser controlada por mecanismos e vias do sistema *quorum-sensing*, dos quais ainda são pobremente conhecidos ou não identificados.

VI – REFERÊNCIAS

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CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Análise fenotípica e genética da produção e imunidade às mutacinas produzidas por Streptococcus mutans isolados de crianças em estudo longitudinal", sob o protocolo nº **125/2003**, da Pesquisadora ***Regianne Umeko Kamiya***, sob a responsabilidade do Prof. Dr. **Reginaldo Bruno Gonçalves**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 01 de outubro de 2003

We certify that the research project with title "Phenotypic and genetic analysis of the production and immunity to the mutacinas produced for isolated Streptococcus mutans of children in longitudinal study", protocol nº **125/2003**, by Researcher ***Regianne Umeko Kamiya***, responsibility by Prof. Dr. **Reginaldo Bruno Gonçalves**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, October 01 2003

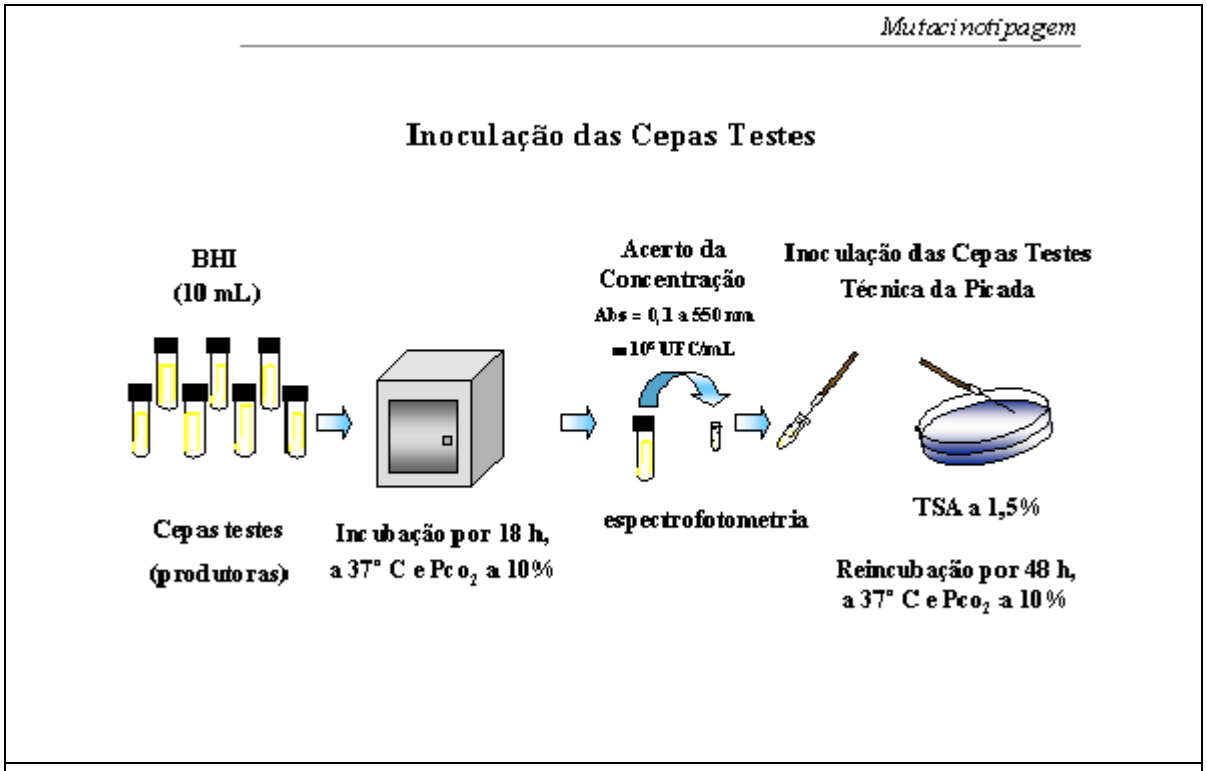
Prof. Dr. Antonio Fernando Martorelli de Lima
Secretário
CEP/FOP/UNICAMP

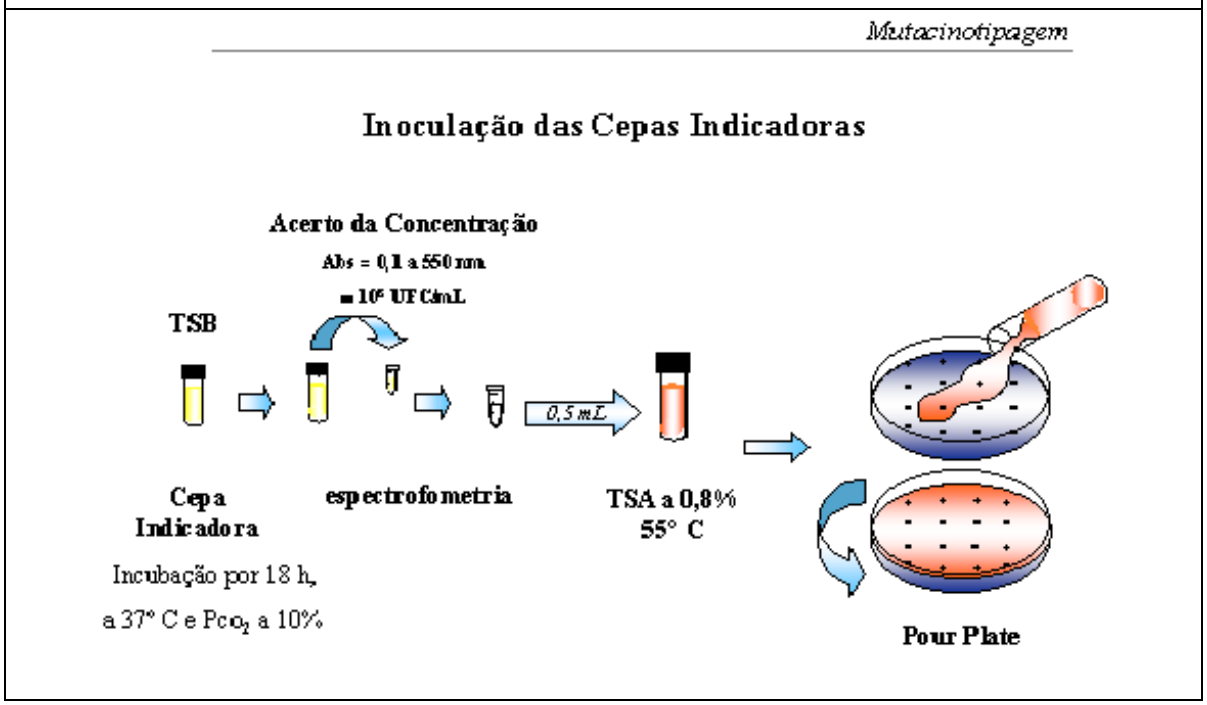
Prof. Dr. Antonio Bento Alves de Moraes
Coordenador
CEP/FOP/UNICAMP

ANEXO 2

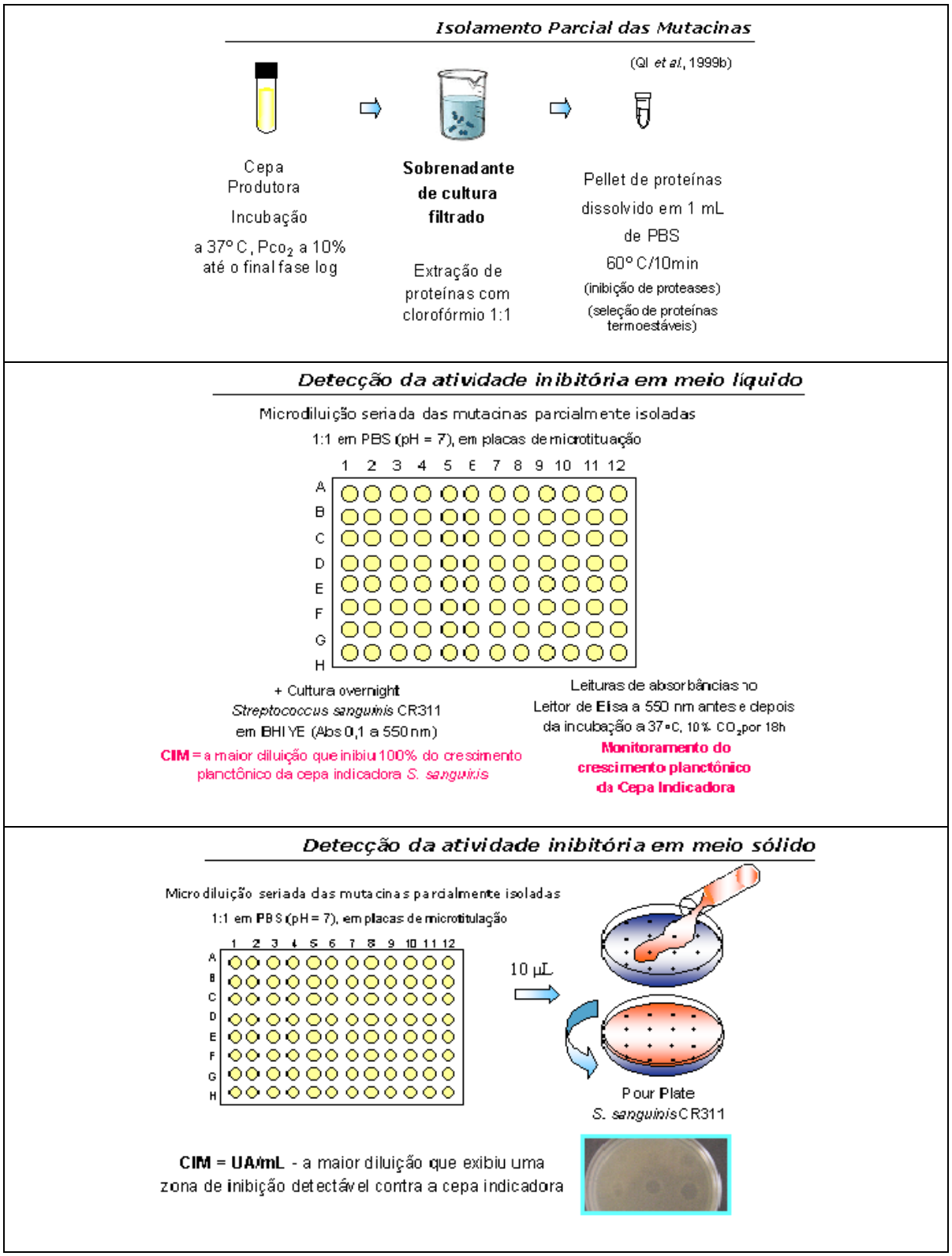
Material & Métodos

4.1. Mutacinotipagem

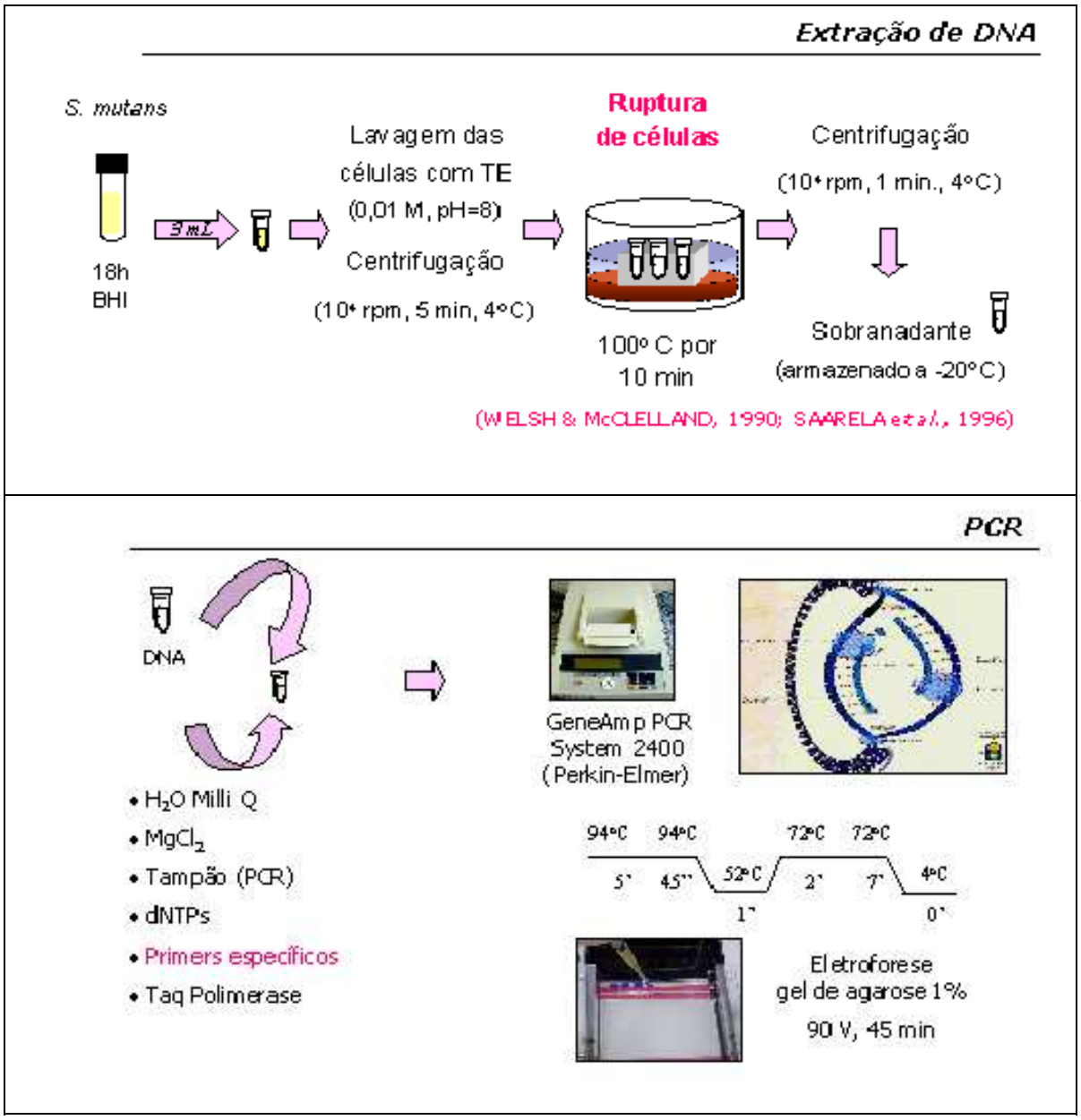




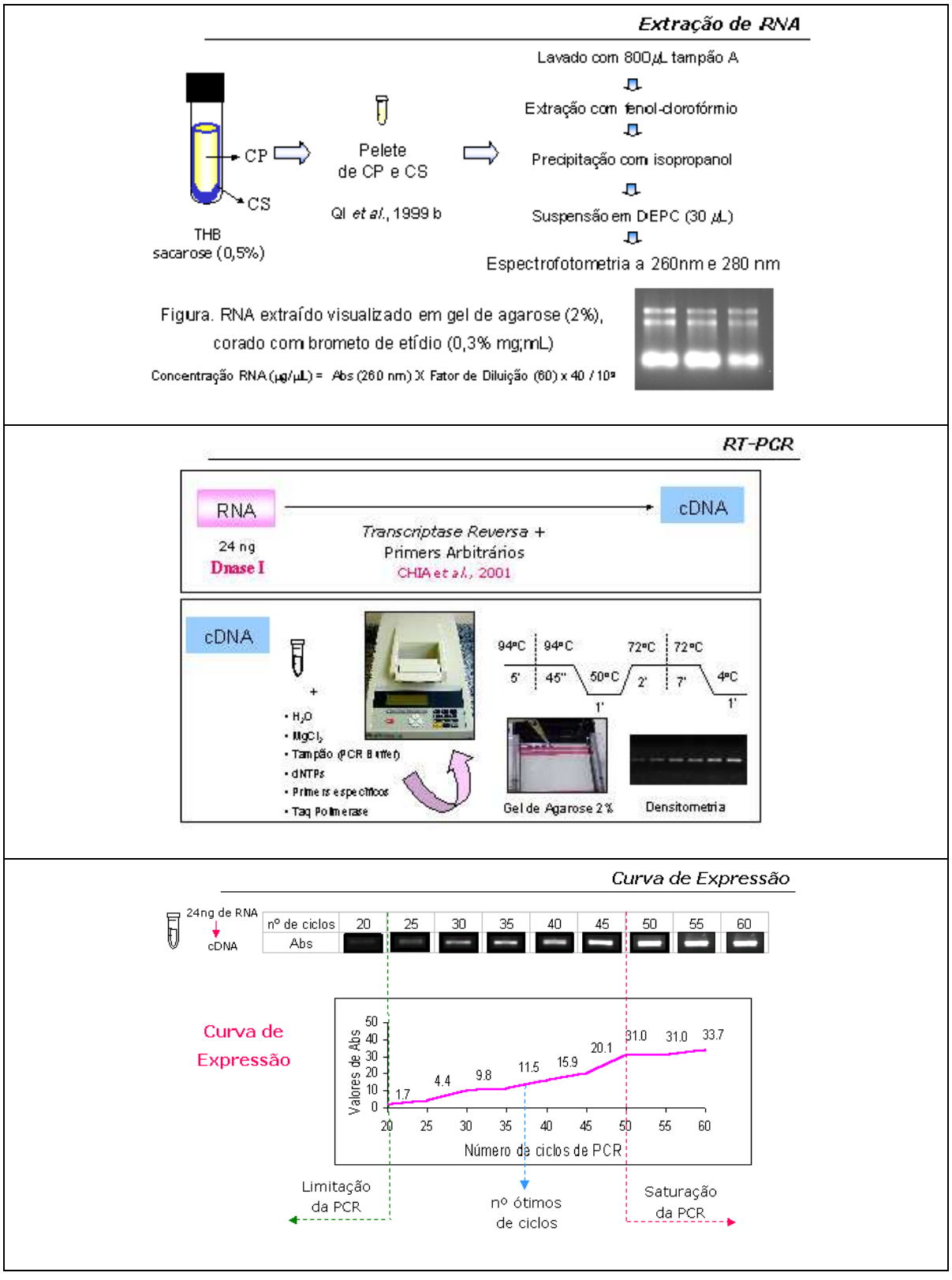
4.2. Isolamento Parcial das Mutacinas e Detecção da atividade inibitória em meio líquido e sólido



4.3. Extração de DNA e PCR



4.3. Extração de RNA , RT-PCR e Curva de Expressão



ANEXO 3

Este artigo intitulado: “Frequency of four different mutacin genes in *Streptococcus mutans* genotypes isolated from caries-active and caries-free individuals”, foi puiblicado no ***Journal of Medical Microbiology*****,** e é referente ao capítulo 1 desta tese.

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Frequency of four different mutacin genes in *Streptococcus mutans* genotypes isolated from caries-free and caries-active individuals

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The ability of *Streptococcus mutans* to produce mutacins, combined with the production of other virulence factors such as lactic acid, may contribute to the pathogenesis of this bacterium. In the present study, the detection of genes encoding mutacin types I/III, II and IV was performed by PCR with specific primers to each type in a total of 63 *S. mutans* genotypes isolated from caries-active and caries-free individuals. In the caries-free group, PCR screening for mutacin IV revealed that 31·8 % of strains were positive for this mutacin. PCR for the other three mutacins tested (I/III and II) did not yield amplicons in any *S. mutans* strains in this group. The PCR with primers of mutacin IV showed 68·3 % positive genotypes in the caries-active group, on the other hand, the amplicons of mutacins I/III revealed 41·5 % positive strains that carried these genes. The chi square test showed significant differences in the number of positive strains to mutacin IV when comparing the caries-free and caries-active genotypes of *S. mutans* ($P = 0{\cdot}01$). All tested *S. mutans* strains were negative by PCR for mutacin II. The low frequencies of detection of some mutacin genes suggest the existence of high diversity and polymorphism in the production of genetic determinants of mutacin-like substances. In addition, the production of a wide spectrum of mutacins can play an important biological role in colonization by S. *mutans* strains, mainly in the niche of high-complexity microbial communities.

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INTRODUCTION

Mutans streptococci are generally accepted as one of the principal aetiological agents of dental caries (Loesche, 1986; Becker *et al.*, 2002). The dental biofilm consists of a complex bacterial community, and the ability of specific strains of *Streptococcus mutans* to compete with other strains may be essential for colonization. Alaluusua *et al.* (1996) suggested that some strains of *S. mutans* might be able to colonize the host and induce dental caries better than other strains. Alternatively, dietary patterns of the host may be an important factor, since a high salivary mutans streptococci count does not necessarily exert a cariogenic challenge (van Palenstein Helderman *et al.*, 1996).

Numerous factors affect the equilibrium among oral populations of micro-organisms, and several inhibitory substances have been identified, including mutacins (Fukushima *et al.*, 1985; Caufield *et al.*, 1985; Delisle, 1976). Mutacins are peptide or protein antibiotics that are mainly bactericidal for other bacteria of the same or closely related species, as well as for other Gram-positive micro-organisms, and are likely to confer an ecological advantage in diverse bacterial communities such as the dental biofilm (Parrot *et al.*, 1990; Balakrishnan *et al.*, 2002). Some studies have demonstrated that the mutacin activity of *S. mutans* could be related to the prevalence of this species in the dental biofilm, saliva and dental caries (Berkowitz and Jordan, 1975; Hillman *et al.*, 1987).

Classification of mutacin-producer strains based on their bactericidal activity divides mutacins into four types, I, II, III and IV. The antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci, while that of mutacins I, II and III is broader (Qi *et al.*, 1999a, b, 2001).

The relationship between caries activity and the higher synthesis of some virulence factors by different strains of *S. mutans* has been demonstrated in the literature (Mattos-Graner *et al.*, 2000). In a previous study, we observed a statistically significant positive association between the level of synthesis of water-insoluble glucan by *S. mutans* clinical isolates and the frequency of adherent cells in the presence of sucrose in caries-active subjects, but not in caries-free subjects (Napimoga *et al.*, 2004). In addition, the strains of *S. mutans* isolated from caries-active individuals produced a wide spectrum of mutacins in comparison with those from

caries-free individuals (Kamiya *et al.*, 2005), suggesting that isolates from subjects with high caries activity were better at colonizing and accumulating on teeth, and, consequently, inducing caries.

In the present study, we analysed the relationships between the frequencies of detection of four different mutacins (mutacins I, II, III and IV) from *S. mutans* genotypes isolated from caries-free and caries-active individuals.

METHODS

Bacterial strains. A total of 63 clinical isolates previously genotyped (Napimoga *et al.*, 2004) and 3 reference strains, as positive controls, of *S. mutans* were used. The clinical *S. mutans* were previously isolated from individuals aged 18–29 years (mean ± SD, 23·5 ± 3·9). The clinical strains were identified by PCR and genotyped by arbitrarily primed PCR (Napimoga *et al.*, 2004), and included 41 strains from eight caries-active individuals and 22 strains from eight caries-free individuals.

The isolates were previously evaluated for the production of mutacin-like substances (Kamiya *et al.*, 2005) by a modification of the deferred antagonism method (Hamada & Ooshima, 1975). For mutacin-activity testing, the following 12 *Streptococcus* species were used as indicator strains: *S. mutans* CCT3440, *S. mutans* 32K, *Streptococcus sobrinus* ATCC 27607, *S. sobrinus* 6715, *Streptococcus mitis* A, *S. mitis* ATCC 903, *Streptococcus salivarius* ATCC 25975, *S. salivarius* 66·4, *Streptococcus sanguinis* CR311, *S. sanguinis* M5, *S. sanguinis* ATCC 10556 and *Streptococcus oralis* PB182. These strains were acquired from the respective collections of bacterial strains.

Extraction of chromosomal DNA. The strains were grown planktonically in brain heart infusion broth (Difco). DNA from all strains was extracted using a simple DNA preparation in which the cells were washed and boiled for 10 min with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0) modified from Welsh & McClelland (1990) and Saarela *et al.* (1996), the debris pelleted and the supernatant used for detection of mutacin I/III, II and IV genes by PCR.

PCR screening of mutacin genes. The detection of genes encoding mutacin types I, II, III and IV (Qi *et al.*, 1999a, b, 2001) was performed by PCR using specific primers to each type. Only one pair of primers was used to detect genes encoding mutacin types I and III due to high homology between them (Qi *et al.*, 1999b). Primers to the genes encoding mutacin types II and IV were designed based on sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). The homologous genes to mutacin types I and III were detected by a pair of primers based on conserved amino acid sequences from mutacin 1140, mutacin NY266, epidermin and gallidermin (Qi *et al.*, 1999b).

Amplification by PCR was performed in the GeneAmp PCR System 2400 (Perkin Elmer). The 50 µl PCR consisted of 1× PCR buffer containing 2·5 mM $MgCl_2$, 200 µM of each deoxynucleotide, 0·3 µM of each oligonucleotide primer, 1·25 U of Taq DNA polymerase (GIBCO) and 50 ng of template DNA. Besides the strains tested, positive and negative controls were used in each PCR reaction: purified genomic DNA from *S. mutans* UA159 was used as a positive control for mutacin type IV genes and two genotypes previously isolated from a mother/child pair (Klein *et al.*, 2004) were used as positive controls to mutacin types I/III and II genes, respectively (unpublished results). Distilled water was used as a negative control.

The PCR conditions were optimized for the control strains. The PCR conditions included initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. The PCR products were analysed by electrophoresis in 1·0 % agarose gel using Tris/borate/EDTA buffer (pH 8·0). A 250 bp DNA ladder was included in each gel. The DNA was stained with 0·5 µg ml^{-1} ethidium bromide and visualized under UV illumination.

Statistical analysis. The chi-square test was applied to detect differences in the frequency of mutacin genes.

RESULTS

In the caries-free group, among the 22 *S. mutans* isolates analysed, four (18·2 %) showed inhibitory activity against mutans streptococci, 20 (90·9 %) showed inhibitory activity against mitis streptococci and five (22·7 %) against *S. salivarius* (Table 2). In the caries-active group, among 41 isolates, 13 (31·7 %) showed inhibitory activity against mutans streptococci, 33 (80·5 %) against mitis streptococci and seven (17·1 %) showed inhibitory activity against *S. salivarius* (Table 3). Two *S. mutans* strains from the caries-free and four from the caries-active group showed no inhibitory activity against any of the 12 indicator-strains.

In the caries-free group, PCR screening with primers of mutacin IV revealed that seven out of 22 (31·8 %) strains were positive for this mutacin. PCR for the other three mutacins tested (I/III and II) did not yield amplicons in any *S. mutans* strains in this group (Table 2).

The PCR with primers of mutacin IV showed that 28 out of 41 (68·3 %) strains were positive in the caries-active group; on the other hand, the amplicons of the mutacin I/III genes revealed that 17 out of 41 (41·5 %) strains carried these genes (Fig. 1). Significant differences were found in the number of positive strains that carried the mutacin IV gene when

Table 1. PCR primers

Primers	Primer sequence	GenBank	Reference
mut I/III forward	5′-AGTTTCAATAGTTACTGTTGC-3′	AF 267498 and AF 154675	Qi *et al.* (1999b)
mut I/III reverse	5′-GCCAAACGGAGTTGATCTCGT-3′		
mut II forward	5′-AACGCAGTAGTTTCTTTGAA-3′	U40620	Novak *et al.* (1994)
mut II reverse	5′-TTCCGGTAAGTACATAGTGC-3′		
mut IV forward	5′-ATGGGATATTTAAAGGGAAA-3′	NC 004350	Qi *et al.* (2001)
mut II reverse	5′-TCAGAGCAGCTACAAAAACT-3′		

Table 2. Mutacin inhibitory activity against 12 indicator strains, and detection of mutacin IV by PCR in 22 *S. mutans* strains from caries-free individuals

Values indicate the number of test strains inhibited. No positive PCR results were obtained for the mutacin I/III and II genes in the tested strains.

S. mutans isolate	Inhibitory activity against			PCR screening for mutacin IV
	Mutans group ($n = 4$)	Mitis group ($n = 6$)	*S. salivarius* ($n = 2$)	
AD13	2	6	2	−
AD8	0	6	2	−
AS4	0	6	2	−
AS38	0	5	1	−
CD35	0	0	0	−
CS2	1	5	1	−
CD34	0	3	0	−
DD31	0	1	0	−
DS37	0	3	0	−
DP38	0	3	0	−
ES31	0	1	0	−
ED33	0	2	0	+
GD2	0	2	0	+
GS33	0	0	0	−
GS43	0	3	0	−
IP35	0	3	0	+
OS31	0	3	0	+
OS40	0	2	0	+
OP35	0	1	0	+
OD32	0	4	0	+
RP2	1	4	0	−
RS7	2	4	0	−
Total ($n = 22$)	4	20	5	7

comparing the caries-free and caries-active genotypes of *S. mutans* ($P = 0{\cdot}01$). PCR with mutacin II did not yield amplicons in any *S. mutans* strains (Table 3).

In both groups, there were some strains that produced mutacin and showed *in vitro* inhibitory activity against at least one indicator strain but did not yield amplicons to mutacin genes. On the other hand, in the caries-active group, some genotypes that showed amplicons to mutacin IV and to mutacins I/III did not reveal inhibitory activity against any of the indicator strains tested (Table 3).

DISCUSSION

Mutacins have been implicated as virulence factors in dental caries (Hillman *et al.*, 1987; Novak *et al.*, 1994). In this study, we investigated the detection frequency of mutacin genes in *S. mutans* strains isolated from caries-free and caries-active individuals. We found that *S. mutans* strains recovered from caries-active individuals showed a higher frequency of detection of mutacin IV than the *S. mutans* strains recovered from caries-free individuals. In addition, only *S. mutans* in the caries-active group showed amplicons corresponding to mutacin I/III genes.

The oral biofilm is subjected to variable environmental stress, including the availability of nutrients, acidic pH (Carlsson, 1989) and mutacin activity (Qi *et al.*, 2001). Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental biofilms: the mutacin-producing strains might colonize more easily and suppress non-producing or susceptible strains (Hillman *et al.*, 1987).

Longo *et al.* (2003) analysed 19 strains isolated from children and found only one strain that showed an amplicon homologous to the gene for mutacin II. Qi *et al.* (2001) searched clinical isolates of *S. mutans* for the presence of mutacin IV genes by PCR and found >50 % positive results. According to Qi *et al.* (2001), mutacin IV is produced by planktonic cells while mutacin I is produced by biofilm-like cells. Different mutacins may serve different purposes during the process of colonization by *S. mutans*. For instance, production of mutacin IV by planktonic cells in saliva may help *S. mutans*

Table 3. Mutacin inhibitory activity against 12 indicator strains, and detection of mutacins I/III and IV by PCR in 41 *S. mutans* strains from caries-active individuals

Values indicate the number of test strains inhibited. Mutacin II showed no positive amplicons in the tested strains.

S. mutans isolate	Inhibitory activity against			PCR screening	
	Mutans group ($n = 4$)	Mitis group ($n = 6$)	*S. salivarius* ($n = 2$)	Mutacin IV	Mutacin I/III
C1S2	0	3	0	+	+
C1D3	0	3	0	+	+
C2D2	0	4	0	+	–
C2D12	0	4	0	+	–
C2S13	0	0	0	+	–
C2S4	4	6	2	–	+
C2D8	0	0	0	+	–
C2D6	0	0	0	+	–
C2D13	0	3	0	+	–
C2D7	0	4	0	–	–
C3D15	1	1	0	+	–
C3D14	1	1	0	+	–
C3D9	2	0	0	+	–
C3P13	1	1	0	+	–
C3S13	1	0	0	+	–
C4P4	0	1	0	+	–
C4P5	0	1	0	+	–
C4D3	0	0	0	–	–
C4S3	0	1	0	–	–
C4D6	1	0	0	–	–
C5P5	0	2	0	+	–
C5D5	0	2	0	+	–
C5D15	0	2	0	+	–
C5S2	4	6	2	+	+
C7D10	0	1	0	–	+
C7D1	0	2	0	–	+
C7P3	0	3	0	+	–
C7D11	0	1	0	+	–
C7D9	0	1	0	+	–
C7S4	0	1	0	–	+
C7S5	0	3	0	+	+
C8D7	4	6	2	–	+
C8P12	2	2	2	–	+
C8S4	0	0	0	+	–
C8D10	4	6	2	–	+
C9D3	4	6	1	–	+
C9S14	4	6	1	–	+
C9S15	0	1	0	+	+
C9S10	0	3	0	+	+
C9S2	0	3	0	+	+
C9S9	0	3	0	+	+
Total ($n = 41$)	13	33	7	28	17

kill the primary colonizers on the tooth surface to make room for its own population. Supporting this hypothesis, the antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci (Qi *et al.*, 2001). Nevertheless, our study suggests that given the increasing complexity of the oral microbiota, as found in caries-active individuals (Napimoga *et al.*, 2004), the *S. mutans* strains producing a wide spectrum of mutacins, including mutacins I, II and III, could become prevalent in most oral sites.

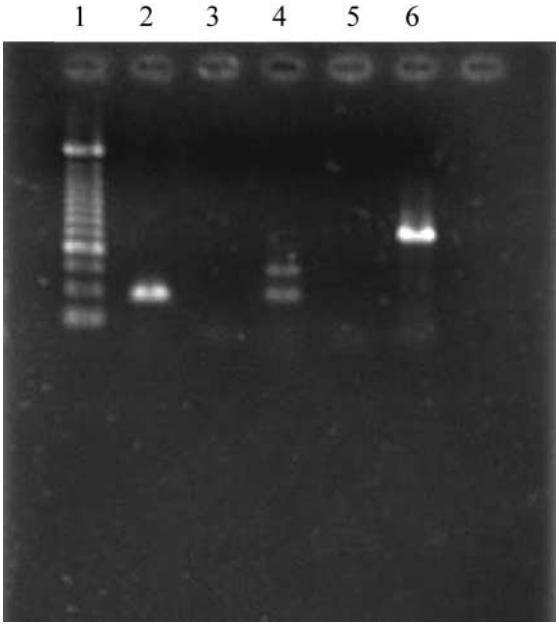

Fig. 1. PCR screening of four different mutacin genes in *S. mutans*. Lanes: 1, 250 bp marker ladder; 3 and 5, negative control (distilled water); 2, mutacin II (444 bp); 4, mutacin I/III (750/450 bp); 6, mutacin IV (1344 bp).

Longo *et al.* (2003) related no association between mutacin inhibitory spectrum and infecting levels of mutans streptococci or caries incidence in the host, suggesting that the mutacin production may not be relevant to the ability of the strain to colonize the host and induce disease. In a previous study we showed distinct mutacin production profiles between *S. mutans* isolated from caries-active and *S. mutans* isolates from caries-free individuals, which can be related to the different colonization profiles described in these individuals (Kamiya *et al.*, 2005).

In the caries-active individuals the sites from which *S. mutans* were recovered were more diverse, probably because production of organic acids and mutacins within the biofilm resulted in a more complex community compared to caries-free individuals (Paddick *et al.*, 2003). Probably due to this complexity, *S. mutans* genotypes recovered from caries-active individuals presented higher frequencies of mutacin IV and a wide spectrum of mutacins, such as I/III, and presented greater mutacin activity *in vitro* compared to *S. mutans* recovered from caries-free individuals (Kamiya *et al.*, 2005).

We also found some mutacin-producing strains in both groups that showed *in vitro* inhibitory activity against at least one indicator strain but did not yield amplicons to mutacin genes. These data suggest a high genetic diversity at the mutacin locus or absence of the structural genes encoding these genes. One possible explanation is that the polymorphism at the mutacins locus may have compromised primer annealing in the PCR, which suggests that there are different mutacin-coding genes that have a similar phenotype.

On the other hand, in the caries-active group, some genotypes showed amplicons to mutacin IV and to mutacin I/III but did not reveal inhibitory activity against any of the indicator strains. One possible explanation is the modification of only one amino acid, which has already been shown to alter or prevent the activity of certain mutacins (Mulders *et al.*, 1991; Rollema *et al.*, 1995; Chan *et al.*, 1996). The inhibition assay previously performed (Kamiya *et al.*, 2005) could also result from the production of more than one inhibitory substance, showing that the understanding of the genetic determinants of several mutacins still needs to be improved.

This study evaluated the frequency of mutacins I, II, III and IV in *S. mutans* strains recovered from caries-active and caries-free individuals, and compared them with the phenotypic profiles of these substances *in vitro*. Our results suggest high diversity and polymorphism in the production of genetic determinants of mutacin-like substances. In addition, the production of a wide spectrum of mutacins can play an important biological role in colonization by *S. mutans* strains, mainly in the niche of high-complexity microbial communities.

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