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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

MARLISE INÊZ KLEIN
Cirurgiã Dentista

Caracterização fisiológica e genética de isolados clínicos 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 Concentração em Microbiologia e Imunologia.

PIRACICABA
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Orientador: Prof. Dr. Reginaldo Bruno Gonçalves
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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 25 de Setembro de 2006, considerou a candidata MARLISE INÊZ KLEIN aprovada.

PROF. DR. REGINALDO BRUNO GONCALVES

PROF. DR. EDVALDO ANTÔNIO RIBEIRO ROSA

PROF. DR. WANDERLEY DIAS DA SILVEIRA

PROF. DR. JOSÉ FRANCISCO HOFLING

PROF. DR. JAIME APARECIDO CURY

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Sua Santidade Dalai Lama (Tenzin Gyatno)

SUMÁRIO

RESUMO

Streptococcus mutans são os principais patógenos da cárie dentária, um importante problema de saúde pública no país. Para um melhor entendimento da biologia desta espécie bacteriana, o objetivo desta tese de doutorado foi avaliar características genéticas e fisiológicas relacionadas à virulência de isolados clínicos de *S. mutans*. Neste microrganismo, o sistema de indução da competência, "*quorum-sensing*", é dependente da densidade celular e está envolvido na capacidade de crescimento em biofilme. Foram pesquisados, os genes de sistema "*quorum-sensing*" e os genes envolvidos no processo de transformação da espécie *S. mutans*. Foi demonstrada a presença de todos os genes avaliados em *S. mutans*, bem como a presença de polimorfismo em alguns genes pesquisados, dados ainda inéditos na literatura. Posteriormente, genótipos clínicos de *S. mutans* foram submetidos a uma análise genética e fisiológica em que foram avaliadas a tolerância e adaptação ao pH ácido, a capacidade de formação de biofilme e curvas de crescimento sob diferentes condições (pHs e diferentes fontes de carbono). Os dados de expressão gênica confirmaram os resultados obtidos nas análises fisiológicas. As cepas clínicas apresentaram um comportamento heterogêneo frente aos mesmos desafios ambientais, o que pode favorecer a sobrevivência das mesmas na cavidade bucal, um ambiente com diversas condições de estresse. Os dados obtidos, comparados aos existentes na literatura, sugerem que além da atividade ATPase, os sistemas de transporte de açúcares fosfotransferase fosfoenolpiruvato:açúcar dependente (pep-PTS) também estariam envolvidos na tolerância de *S. mutans* ao estresse ácido. Em conjunto, as observações decorrentes das análises efetuadas, nesta tese, podem contribuir para a compreensão dos processos biológicos de *S. mutans*.

ABSTRACT

Streptococcus mutans is considered the primary etiological agent of human dental caries, an important public health problem in Brazil. The purpose of this thesis was to evaluate genetic and physiologic properties related with virulence in clinical isolates of *Streptococcus mutans.* The quorum-sensing system, that induces and regulates genetic competence, depends on cellular density and also may play a role in biofilm growth and structure in *S. mutans* species. A screening of genes of the quorum-sensing system and genes involved with genetic transformation was performed. The analysis revealed that all genes are widespread within the *S. mutans* species, and some genes presented polymorphisms. These data are new in the scientific literature. Furthermore, clinical genotypes of *S. mutans* were subjected to genetic and physiological analysis, including tolerance and acid adaptation to low pH, ability to form stable biofilm and growth kinetic under different conditions (pH and carbon source). The profile of gene expression confirms the data found in the physiologic assays. The studied strains shown heterogenic behavior at the same environmental challenge, that could favor these strains survive in the oral cavity, an environmental with several stress conditions. The data obtained here and supported by scientific literature, suggest that besides ATPase activity the sugar:phosphotransferase systems (PTS) could help to mount an adaptive acid tolerance response in *S. mutans.* Taken together all data obtained in this thesis may help to understand *S. mutans* biological processes.

I - INTRODUÇÃO GERAL

Os estreptococos grupo mutans, em destaque a espécie *Streptococcus mutans*, são os principais agentes etiológicos da cárie dental (Hamada & Slade, 1980; Loesche, 1986). Estas bactérias têm a capacidade de colonizar e se acumular no biofilme dental na presença de sacarose através da produção de uma matriz de polissacarídeos extracelulares, produzindo e tolerando os ácidos resultantes do seu metabolismo. O processo de adaptação fisiológica às condições de estresse favorece a manutenção deste microrganismo na cavidade bucal. E, a secreção de ácidos pode resultar na desmineralização dos tecidos dentários com conseqüente desenvolvimento de lesões de cárie. Embora os mecanismos de produção de glicanos extracelulares a partir da sacarose tenham sido extensamente estudados, e determinados como importantes no processo de formação de biofilme por *S. mutans*, pouco se conhece, ainda, sobre os mecanismos moleculares independentes de glicano envolvidos no crescimento desta espécie na forma de biofilmes.

Em 2002, foi concluído o seqüenciamento do genoma da cepa *S. mutans* UA159, e cerca de 84% dos genes identificados no cromossomo não possuem função conhecida (Adjic *et al.*, 2002), abrindo um vasto campo de pesquisa no que concerne aos mecanismos de virulência ainda desconhecidos. Vários processos importantes na biologia de *S. mutans* envolvem os sistemas de transdução de sinais de dois componentes (TCS, do inglês *two component signal transduction systems*). Um total de 14 TCS foi identificado no genoma da cepa UA159, mas o papel da maioria desses sistemas ainda precisa ser elucidado.

O TCS mais estudado em *S. mutans* é o sistema *quorum-sensing* (QS) *comCDE*, que regula o processo de competência (captação de DNA extracelular) nesta espécie (Li *et al.*, 2002b). Os genes que constituem este sistema de comunicação intercelular

envolvem a produção e translocação de pequenos peptídeos, que ao atingir concentrações ótimas no meio extracelular, o que é obtida com o aumento da densidade celular, ativam histidina-quinases da superfície bacteriana, transmitindo um sinal intracelular que ativa a expressão de diversos genes, não somente os envolvidos na captação de DNA extracelular mas também aqueles envolvidos na capacidade das espécies de responderem aos estímulos como pressão osmótica, diminuição de pH e outras condições de estresse (Shapiro, 1998; de Kievit & Iglewski, 2000). Possivelmente, outros genes com funções desconhecidas também são regulados por este mesmo mecanismo (de Kievit & Iglewski, 2000). Este sistema de sinalização intercelular envolve pelo menos 5 produtos gênicos codificados por *cslAB* (*comAB*) (Petersen & Sheie, 2000) e *comCDE* (Li *et al.*, 2001b). Os genes *comC, comD* e *comE* codificam, respectivamente, um peptídeo precursor de estimulação de competência (CSP), seu sensor para histidina quinase e um regulador de resposta intracelular.

Foi demonstrado que o QS *comCDE* é importante na formação de biofilme e na tolerância ao ácido por *S. mutans* (Li *et al.*, 2001a,b; 2002a); pois, este sistema ativa um conjunto de genes importantes para o fenótipo na formação de biofilme e permite lidar melhor com o estresse envolvido neste modo de crescimento. Alguns desses genes são regulados por um fator sigma alternativo, o ComX, que parece ser também induzido por ComE, como estabelecido previamente para *S. pneumoniae* (Peterson *et al.*, 2004), e regular a expressão de diversos genes envolvidos em eventos iniciais e tardios de competência, incluindo captação de DNA, transporte, processamento e recombinação do mesmo (Morrison & Lee, 2000). Genes ortólogos aos genes envolvidos nos eventos tardios (*comYA, comYB, comYC, comYD, cinA*, coiA, *comEA, comEC, comFa* e *comFc*), e o regulador negativo de competência (*mecA*) foram todos identificados no genoma da cepa naturalmente competente *S. mutans* UA159 (Adjic *et al.*, 2002). Além do *locus comCDE*, a cepa UA159 possui pelo menos mais dois sistemas TCS de "*quorum-sensing*"

relacionados à competência (Merritt *et al.*, 2005), o LuxS, que envolve o autoindutor AI-2 codificado pelo *luxS*, e o TCS CiaH/R. Ambos parecem possuir alguma função no crescimento e estrutura do biofilme (Wen & Burne, 2002; Qi *et al.*, 2004; Merritt *et al.*, 2005; Yoshida *et al.*, 2005; Ahn *et al.*, 2006).

A diversidade genética dos componentes do processo de competência é desconhecida. A freqüência de transformação de *S. mutans* é baixa, e a maioria dos isolados clínicos da espécie *S. mutans* parece não ser transformável *in vitro* (Westergren & Emilson, 1983; Murchison *et al.*, 1986), ao contrário de *S. pneumoniae* e outras espécies de estreptococos comensais da cavidade bucal, por exemplo, *Streptococcus gordonii*. Deste modo, a análise da freqüência de genes envolvidos no sistema "*quorum-sensing*", que regulam a competência natural em *S. mutans*, e de genes responsáveis pela transformação, é importante para o entendimento da biologia e evolução das populações desta espécie.

Análises funcionais do genoma de *S. mutans* mostram que os requisitos primários para a formação de biofilme envolvem a integração de circuitos regulatórios genéticos com captação de sinais ambientais para alterar a expressão de fatores requisitados para adesão, interações intercelulares, e crescimento em resposta às condições ambientais durante a formação do biofilme (Len *et al.*, 2004a,b; Ahn *et al.*, 2006; Abranches *et al.*, 2006). Além disso, recentemente, alguns trabalhos de comparação das seqüências de genomas disponíveis de espécies de *Streptococcus* têm mostrado que diferentes cepas possuem seqüências peculiares para determinados genes, o que explicaria diferenças nas manifestações de virulência (Tettelin *et al.*, 2005; Silva *et al.*, 2006). Portanto, a análise de fatores relacionados ao sucesso de colonização e sobrevivência de *S. mutans* na cavidade bucal pode ajudar a elucidar os processos biológicos deste patógeno.

Os estreptococos cariogênicos dependem do fenótipo de biofilme para sobreviver e persistir na cavidade bucal e têm desenvolvido mecanismos sofisticados para lidar com

os estresses ambientais. As principais condições de estresse ambiental no biofilme dental são o pH baixo e as alternâncias de fonte e disponibilidade de carbono, que conferem impacto na ecologia do biofilme supragengival e o desenvolvimento de cáries. O estilo de vida denominado "*feast or famine*" (Carlsson, 1997) exige que os microrganismos, tais como o *S. mutans*, aproveitem as vantagens dos períodos de excesso de carboidratos e pH baixo para aumentar numericamente os membros no biofilme dental. Ainda, esse estilo de vida permite a estes microrganismos competirem de modo efetivo quando as condições ambientais são menos favoráveis (Lemos *et al.*, 2005). O estudo de fatores fundamentais que capacitam a formação de biofilme por *S. mutans* tem mostrado que cepas com mutações em diferentes genes que respondem ao estresse apresentam fenótipo com menor formação de biofilme, ou biofilme "defeituoso" (com arquitetura e biomassa distintas, em relação ao biofilme formado pela cepa selvagem). Tais genes incluem os genes *comABCDE*, associados à competência genética (Li *et al.*, 2002b; Yoshida & Kuramitsu, 2002), genes com funções regulatórias globais – *ccpA*, *luxS* e *relA* (Merritt *et al.*, 2003; Wen & Burne, 2002; 2004; Lemos *et al.*, 2004), e os TCS (Bhagwat *et al.*, 2001; Li *et al.*, 2002a; Ahn *et al.*, 2006). Esses estudos foram realizados com biofilmes monoespécie de *S. mutans* (mutantes e selvagens), e os dados em relação à formação de biofilmes multiespécies ainda são escassos na literatura; embora, esses dados sejam fundamentais para a compreensão dos processos de colonização da cavidade bucal.

Os microrganismos possuem capacidade de versatilidade metabólica (Postma *et al.*, 1993). Na maioria das bactérias observa-se a utilização de diversas fontes de carbono, bem como a adaptação as mudanças contínuas do meio, e isto lhes confere a capacidade de competirem efetivamente com outros organismos por nutrientes escassos. Como mencionado anteriormente, as bactérias albergam dispositivos para "sentir" e monitorar o ambiente, o que favorece sua capacidade de: 1) ativar ou desativar a utilização de uma grande variedade de fontes de carbono; 2) adaptar-se às mudanças

osmóticas, às condições de estresse, ao oxigênio (presença ou ausência) e à escassez de nutrientes. A captação de sinais externos à célula, que são convertidos em resposta, envolve a mudança na síntese de proteínas, a regulação da atividade enzimática, mudança no comportamento, ou outros processos. Um destes sistemas sensoriais importantes é o sistema de transporte de açúcares fosfotransferase fosfoenolpiruvato:açúcar dependente (pep-PTS). Este sistema envolve o transporte e fosforilação de grandes quantidades de carboidratos, a movimentação na direção da fonte de carboidrato (quimiotaxia), e a regulação de vias metabólicas (Postma *et al.*, 1993).

A quantidade e o tipo de carboidrato no meio em que os organismos cariogênicos estão crescendo influenciam diversos fatores, tais como, a síntese e o catabolismo de polissacarídeos extracelulares, a produção de ácido, os níveis de resistência ao estresse ambiental, e a atividade PTS (Vadeboncoeur *et al.*, 1987; Belli & Marquis, 1991; Belli & Marquis, 1994; Vadeboncoeur & Pelletier, 1997; Burne, 1998; Bruckner & Titgemeyer, 2002; Li & Burne, 2001). Os produtos finais decorrentes do consumo e da fermentação de carboidratos levam ao acúmulo de íons H^+ no ambiente, e no interior das células bacterianas, e para que a acidificação do citoplasma não leve estas células à morte, elas possuem mecanismos para driblar esta situação.

A espécie *S. mutans* possui propriedades constitutivas de tolerância ao ácido e também é capaz de montar uma resposta adaptativa de tolerância ao ácido. Esta tolerância ao ácido é devido primariamente à atividade de uma F_1F_0-ATPase ligada à membrana, que é o mecanismo primário para a manutenção da homeostase do pH, através da extrusão de prótons (Quivey *et al.*, 2001). Esta característica é importante para a proteção das enzimas glicolíticas sensíveis ao ácido, e para a manutenção do equilíbrio de pH para processos bioenergéticos.

A tolerância ao ácido esta relacionada ao pH ótimo da enzima F_1F_0-ATPase (Sturr & Marquis, 1992). A resposta de tolerância ao ácido, ou adaptação ao ácido, é

caracterizada por aumento à resistência à morte em ambiente ácido por células cultivadas em pH ácido quando comparadas com células cultivadas em pH neutro. Assim, o crescimento em ambiente com condições ácidas ajuda o microrganismo menos vulnerável à acidificação letal a se adaptar e sobreviver, e esta adaptação está associada ao aumento na capacidade glicolítica e aumento na atividade de translocação de prótons via F-ATPase (Belli & Marquis, 1991, Hamilton & Buckley, 1991). Tem sido demonstrado que um aumento na atividade F-ATPase é correlacionada com aumento na transcrição do operon F-ATPase (Quivey *et al.*, 2000b), e o operon *atp* pode ser regulado, em nível de transcrito, em resposta ao pH de crescimento (Smith *et al.*, 1996). É importante não se excluir que a regulação alostérica da atividade da enzima em baixo pH é responsável pela atividade ATPase em células que possuem adaptação ao crescerem sob condições ácidas. Ainda, existem mecanismos adicionais que também podem ter um papel na adaptação ao ácido por *S. mutans,* como a ativação do operon sat (do inglês *secretion and acid tolerance*), que contêm 5 genes - *ylxM, ffh, satC, satD, satE* - (Gutierrez *et al.*, 1996; Gutierrez *et al.*, 1999; Kremer *et al.*, 2001), a indução da expressão de proteínas relacionadas ao estresse – chaperonas (Jayaraman *et al.*, 1997; Svensäter *et al.*, 2000; Lemos *et al.*, 2001, Len *et al.*, 2004), de enzimas de reparo do DNA (Hahn *et al.*, 1999, Hanna *et al.*, 2001), mudanças na composição da membrana celular (Quivey Jr *et al.*, 2000; Fozo & Quivey Jr, 2004), um sensor de H^+-glicose que funciona em pH baixo - pH 5.0 (Cvitkovitch *et al.*, 1995), indução de reguladores de resposta e de genes do sistema "*quorum-sensing*" (Li *et al.*, 2002b; Wen & Burne, 2004; Ahn *et al.*, 2006), e um sistema agmatina diaminase (AgDS) (Griswold *et al.*, 2004), que envolve o catabolismo de agmatina, um derivado descarboxilizado da arginina (Simon & Stalon, 1982). Esses mecanismos em conjunto podem favorer a manutenção do *S. mutans* no biofilme dental.

As células bacterianas carregam mais informações genéticas que aquelas utilizadas ou expressas sob determinada condição ambiental. Desta forma, cada célula possui um mecanismo regulador que, dependendo do ambiente atual, permite a transcrição de alguns genes, enquanto previne a transcrição de outros. Técnicas moleculares de análise de RNA têm sido utilizadas para a identificação de genes expressos de modo diferenciado em bactérias. A análise conjunta de dados de expressão gênica e de propriedades fisiológicas pode fornecer um panorama geral dos processos biológicos de uma cepa bacteriana frente aos desafios ambientais aos quais a mesma é submetida.

As diferenças nas condições de crescimento podem influenciar a comparação de resultados de diferentes estudos (Len *et al.*, 2004b; Wilkins *et al.*, 2002), o que configura-se em um problema para os pesquisadores que utilizam tecnologias como o "*microarrays*" e análises proteômicas. Portanto, o uso de cultura de fluxo contínuo (quimiostática) é particularmente vantajoso para estudos fisiológicos, pois as células são crescidas até a fase "*steady-state*". No crescimento até esta fase, as células são cultivadas sob diferentes condições, com diferenças apenas em uma variável examinada inicialmente, e não apresentam diferenças na taxa de crescimento, disponibilidade de nutrientes e assim por diante. Logo, o uso de quimiostato permite o controle cuidadoso da fisiologia dos microrganismos, com possibilidade de definir regras adicionais ao pH que influenciariam nas propriedades fisiológicas, como por exemplo, a fonte de carbono (Lemos *et al.*, 2005).

Vista a exposição acima, esta tese de doutorado inclui o detecção de genes do sistema "*quorum-sensing*" e relacionados à competência bacteriana em genótipos clínicos de *S. mutans*; a caracterização de resposta fisiológica de genótipos clínicos de *S. mutans* frente a condições ambientais e a análise da expressão de genes envolvidos no metabolismo de açúcares, adaptação ao ambiente ácido e resposta ao estresse.

II - PROPOSIÇÃO

Os objetivos deste estudo foram:

1. Detectar a freqüência dos genes do sistema *"quorum-sensing"* e dos genes envolvidos no processo de transformação da espécie *S. mutans*, e definir a extensão da conservação destes genes entre isolados clínicos de *S. mutans*.

2. Caracterizar a resposta fisiológica de genótipos clínicos de *S. mutans* frente as condições ambientais e a analisar a expressão gênica de genes envolvidos no metabolismo de açúcares, adaptação ao ambiente ácido e resposta ao estresse nas mesmas.

III – CAPÍTULOS

O trabalho desenvolvido foi aprovado pelo Comitê de Ética em Pesquisa da FOP – UNICAMP parecer nº 017/2004 (anexo 1). Esta tese foi realizada no formato alternativo para teses conforme a deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 002/06. Esta regulamentação permite a inserção de artigos científicos de autoria do candidato para a confecção de tese. Deste modo, a presente tese de doutorado é composta por 2 capítulos contendo um artigo publicado e outro em fase de submissão, em periódicos científicos, conforme descrito abaixo:

- Capítulo 1

"Genetic diversity of competence gene loci in clinical genotypes of *Streptococcus mutans*". Klein MI, Bang S, Flório FM, Höfling JF, Gonçalves RB, Smith DJ, Mattos-Graner RO. Este artigo foi publicado no periódico ***Journal of Clinical Microbiology.*** 2006; 44(8):3015-20.

- Capítulo 2

"Physiologic and genetic characterization of clinical isolates of *Streptococcus mutans* submitted to acid challenge". Klein MI, Abranches J, Lemos J, Gonçalves RB, Burne RA. Este artigo está em fase de submissão ao periódico ***Journal of Clinical Microbiology.***

CAPÍTULO 1

"Genetic diversity of competence gene loci in clinical genotypes of *Streptococcus mutans*" ♦

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Running title: Diversity of competence genes in *S. mutans*

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The frequencies of 21 competence genes were analyzed in 94 genotypes of *Streptococcus mutans*. These include those of a main regulatory system (*comCDE*), structural, and other regulatory orthologues identified in the genome of strain UA159. PCR and Southern blot analysis revealed that all genes are widespread within the species.

Streptococcus mutans are the major pathogens of dental caries, a biofilm-dependent infectious disease. These organisms are able to prevail in the complex microbial community of the oral biofilm in the presence of sucrose, under extremely low pHs responsible for tooth demineralization, and can physiologically adapt to the stressful conditions to which the cariogenic biofilm is exposed. Several of these processes involve two component signal transduction systems (TCS). The most studied TCS of *S. mutans* is the quorum-sensing system *comCDE* that regulates genetic competence (12), and also has been shown to play a role in biofilm formation and acid tolerance (10-12). Other regulatory (*comX1*, *mecA*, *ciaH/R*, *LuxS*) genes appear to be involved in competence via a complex net of signals that may regulate structural genes involved in the early and late events of competence, including DNA binding, transport, processing and recombination (16). These genes also may play a role in biofilm growth and structure (14,20,23,27). The genetic diversity of the components of competence is unknown. In contrast to *S. pneumoniae* and many commensal streptococcal species of the oral cavity, e.g. *Streptococcus gordonii*, the frequency of *S. mutans* transformation is low, and the majority of isolates appear not to be transformable *in vitro* (17,25). In this study, we characterized the genetic organization of eleven chromosomal loci of regulatory and structural genes with known or putative roles in competence, in a collection of *S. mutans* genotypes isolated from children during the initial phase of colonization.

A total of 94 *S. mutans* genotypes were analyzed. Fifty genotypes were isolated from fourteen 6-24 month-old children, two of whom presented with initial caries lesions (8). The other 44 genotypes were isolated from 35 12-30-month-old children in a separate study of the same population (13). Carious lesions were detected in 15 of these children. The genotypic identities were determined by arbitrarily primed polymerase chain reaction (AP-PCR) as described in previous studies (8,13). Strains were grown from frozen stocks in Todd Hewitt (THB) or Brain Heart Infusion broth (BHI), at 37°C at an atmosphere of 10% CO_2, 90% O_2.

Genomic DNAs were purified from 1.5 ml of culture, using the MasterPure DNA purification kit (Epicentre Technologies, Madison, Wis.) as recommended by the manufacturer. Twenty one genes from 11 loci within the genome of *S. mutans* strain UA159 (http://www.genome.ou.edu), were analyzed by PCR (Table 1). Gene organization within the UA159 chromosome and the position of respective primer sets used for screening are shown in Figure 1. Table 2 shows sequences of primers specific for each locus. PCR reactions were performed in volumes of 50 µl (200 µM of dNTPs, 2.5mM of $MgCl_2$, 0.3 µM of each upper and lower primer and 1.25 units of Taq DNA polymerase) (Invitrogen). Thermal conditions varied slightly for each locus analyzed and included 35 cycles of denaturing at 95°C for 45s, annealing from 50-52°C for 1 min (Table 2), and extension at 72°C for 2 min. Genomic DNA of strain UA159 was used as a positive control in all PCR baths. *S. sobrinus* strain 15JP1 was used as a negative control. PCR products were resolved (8V/cm in Tris-borate-EDTA) in 0.8% agarose gels, and stained with ethidium bromide. To confirm gene absence in the PCR-negative genotypes, Southern blot assays were performed using the amplicons obtained from the control UA159 as probes. Restriction maps are shown in Figure 1. After digestion of 3 µg of genomic DNA at the appropriated conditions, fragments were electrophoretically resolved (3V/cm in 0.8%

agarose gels) and transferred to Hybond+ membranes (Amersham Biosciences, Little Chalfont, Burckinghamshire, UK), as described elsewhere (22). Membranes were then probed and developed using the ECL system (Amersham Biosciences, Little Chalfont, Burckinghamshire, UK), as recommended by the manufacturer.

Several strains did not yield amplicons for one or more loci; however, Southern blot analysis revealed that all 11 gene loci were present in each of the tested strains (Table 3), although atypical restriction patterns were frequently observed (Table 3). Ten distinct classes of restriction (Figure 2) were identified among the 19 strains showing a Southern blot pattern distinct from UA159 at the comCD locus (Table 3). Among the 26 genotypes that were PCR-negative for *comCD*, ten were also PCR-negative for *comDE*, suggesting that polymorphism might be involved in at least *comD* or, perhaps, the whole *comCDE* locus. Southern blot analysis of three randomly selected clinical isolates that were PCR-positive for *comCD* revealed a UA159-type pattern (data not shown). The lower number of atypical patterns in assays with comDE probe, when compared with the comCD probe (Table 3), might be due to differences in conservation of the restriction sites used. All the strains yielded amplicons for ciaHR. It is possible that the primer set designed for these genes included highly conserved sequences, because ciaHR primers have also generated amplicons for *S. sobrinus* strains, a species closely related to *S. mutans* that is also implicated in dental caries pathogenesis. Blast analysis of ciaHR primer sequences against the unfinished genome of the strain *S. sobrinus* 6715 (http://www.tigr.org/tdb/mdb/mdbinprogress.html) did not reveal regions of homology that might account for amplification (data not shown). Except for ciaHR primers, all the others appeared to be *S. mutans*-specific, since they did not yield amplicons for the *S. sobrinus* strain 15JP1. Additionally, during the course of this study, a total of 11 strains previously defined as *S. mutans* species, were negative in PCR for most other *com* genes analyzed (data not shown). Sequencing analysis of the 16S rRNA gene, revealed that those 11

strains were *S. sobrinus* (data not shown) and were thus excluded from the analysis. The reason(s) for significant differences in the capacities for genetic transformation between *Streptococcus* species, and between strains within the same species, are not understood. The ability to achieve competence in virulence is unclear. It is hypothesized that incorporation of foreign DNA may improve fitness to environmental stresses, providing competitive advantages (3,7). There is evidence that some TCS which are involved in competence may regulate multiple virulence factors (10,12,24,27,28), and their components could be important targets for antibacterial therapy. Thus it is important to establish the conservation of these systems within the *S. mutans* species.

Sequence comparisons of *comCDE* genes between several species of the genus streptococcus have indicated that interspecies gene replacements may frequently occur between naturally competent streptococci within the *Streptococcus mitis* group (6). Differences in GC content within the *comCDE* locus in comparison with the whole streptococcal genome concur with this idea (7). A locus with homology to *comCDE* (named *silCDE*) was identified in the screening of virulence genes in the *S. pyogenes* genotype JS95 which was isolated from a subject with invasive infection. *S. pyogenes* is a species with a low rate of transformation and causes infections having a wide range of severity (7). Similar to the organization of *comCDE* genes in the genome of *S. mutans* UA159, the *silC*, *silD* and *silE* genes are flanked by Blp bacteriocin genes, and also by a transposable element IS1562. The *silCDE* locus was shown to confer competence in the virulent genotype (7), a trait that is not observed in the *S. pyogenes* strain M1 whose genome was sequenced, and which does not have *silCDE* genes (7). M1 is defective in transformation although it contains several *com* genes (4). Its inability to be transformed was attributed to the lack of *comAB* genes (4). In screening 214 strains of *S. pneumoniae*, *comC* and *comA* genes were shown to be widespread (21), and these genes were detected in all the *S. mutans* strains (Table 3). The upper primer utilized for screening of

the *comCD* sequence targeted a small intergenic space between ORFs SMU1914c and SMU1913c (Figure 1), which encodes homologues of the immunity protein BlpI and a bacteriocin peptide BlpJ, also identified in the *S. pneumoniae* strain TIGR4.

Because two distinct alleles of *comC* were detected among 42 strains of *S. pneumoniae*, the hypothesis that the combination of incompatible alleles of *comC* (encoding the CSP precursor) and *comD* (the respective receptor) was raised to explain deficiency in *in vitro* transformation observed among 50% of the *S. pneumoniae* isolates (19). However, 93% of 60 strains analyzed have shown only two distinct *comC* alleles matched with the respective *comD* alleles (26), arguing against the incompatible allele hypothesis. Thus other factors may be involved in variations in the competence phenotype. It has been shown in *S. pneumoniae* that the effect of gene inactivation of components of TCS which are involved in virulence is dependent on the strain background (2). Since few *S. mutans* strains were identified as suitably transformable *in vitro*, most of the genetic studies on molecular mechanisms of virulence have been limited to a few strains, mainly GS5, NG8, UA159 and its variant LT11. The distribution and/or diversity of regulatory and structural genes implicated in competence might help to explain differences in competence. However, to our knowledge, there is no such information describing *S. pneumoniae* or other streptococcal species.

It was estimated that transformation occurs in only 28% of clinical isolates of *S. mutans in vitro* (25), and it is not known whether this low frequency of transformation has a genetic basis or might be simply due to unsuitable lab conditions. The frequency of transformation is significantly variable among naturally competent strains (e.g, GS5, NG8, UA159, LT11 and UA140), and it has been suggested that variation in the genetic background (17) and/or in the expression of late competence genes may be associated with these variations (15). The alternate sigma factor *comX1* that appears to regulate several genes involved in late events of competence was detected in all the strains (Table

3). In contrast to *S. pneumoniae*, in which at least 2 alleles of *comX*1 were identified in the genome (9), our analysis indicated that *S. mutans* genotypes contain only a single copy of *comX*1, as verified for the strain UA159 (1). All other structural and regulatory loci studied herein have shown some degree of genetic diversity, although lower in frequency when compared with the *comCDE* locus (Table 3). However, a more detailed analysis of these genes should be performed to allow comparisons in degree of conservation, since Southern blot assays were performed only with PCR-negative strains, and a PCR-positive result does not imply absence of polymorphisms. Apart from the modest diversity identified, the results indicated that all 11 loci which contain genes with a regulatory or structural role in competence were present in all *S. mutans* genotypes analyzed, suggesting that these genes each play a fundamental role in *S. mutans* biology. This knowledge may increase the interest in these genes as therapeutic targets. Furthermore, sequencing analysis of those polymorphic strains identified in the present study, along with gene expression studies might help to explain additional variation in the competence phenotype previously reported within the *S. mutans* species.

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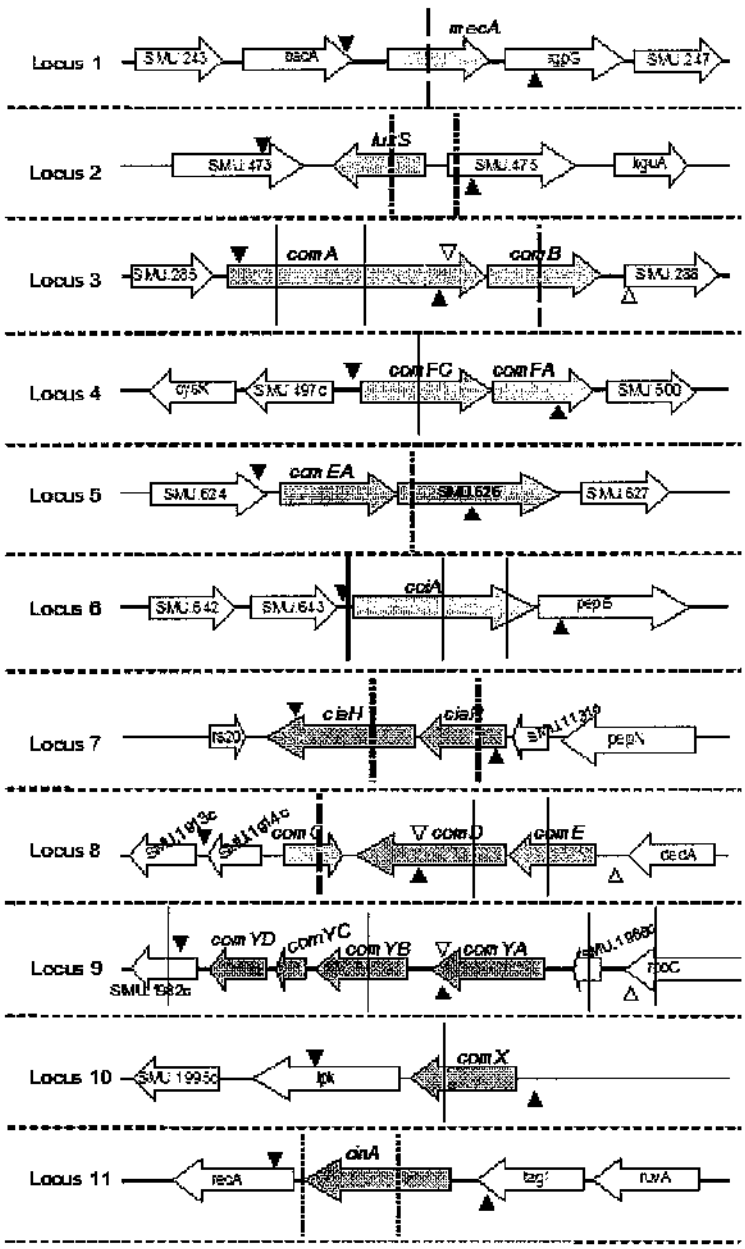

Figure 1. Genomic organization of the eleven loci identified in the genome of the strain UA159 which contains 21 competence-related genes, as analyzed by PCR and Southern blot. Shaded arrows indicate the ORF of the *com* genes and direction of transcription. Locations of primers designed for PCR screening are indicated by bullets. Sets of primers designed for each amplicon are differentiated by color (black or white). Vertical lines indicate the restriction sites of the endonucleases selected for the Southern blot analysis within each amplicon: (·····) *Hind*III, (——) *Hae*III and (— — -) *Hha*I. Purified amplicons identified in PCR reactions with chromosomal DNA of the strain UA159 were applied as probes for Southern blot analysis.

Table 1.Competence genes identified in the genome of the strain UA159 that were analyzed in this study.

Gene	Acc. n°.*	Assigned function
mecA	24378754	Negative regulator of genetic competence
luxS	24378961	Putative autoinducer-2 production protein LuxS
comA	24378790	ABC transporter, ATP-binding protein comA
comB	24378791	ComB, accessory factor for ComA
comF	24378982	Late competence protein, required for DNA uptake
comFa	24378983	Late competence protein
comEA	24378983	DNA uptake protein and related DNA-binding proteins
comEC	24379099	DNA internalization-related competence protein ComEC/Rec2
coiA	24379117	Putative competence protein, transcription factor
ciaH	24379560	Putative histidine kinase sensor CiaH
ciaR	24379561	Putative response regulator CiaR
comC	24380265	Competence stimulating peptide, precursor
comD	24380266	Putative histidine kinase of the competence regulon, comD
comE	24380267	Putative response regulator of the competence regulon, ComE
comYD	24380327	Putative late competence protein comYD, exogenous DNA-binding protein
comYC	24380328	Late competence protein, exogenous DNA-binding protein
comYB	24380329	Putative ABC transporter subunit ComYB; part of the DNA transport machinery
comYA	24380330	Putative ABC transporter, ATP-binding protein comYA; late competence gene
SMU1988c		Putative DNA binding protein
comX	24380340	Transcriptional regulator of competence-specific gene
cinA	24380420	Putative competence and damage inducible protein CinA

* GenBank: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

Table 2. Oligonucleotides used for PCR-screening of genes identified in the 11 *loci* of the *S. mutans* strain UA159 with known or putative role in the phenotype of competence in *S. mutans*.

Locus	Amplicon name (expected size)	Primer sequence (Forward/Reverse)	Annealing temperature (°C)	Competence genes within the amplicon
1	MecA (1083 bp)	5'- GCACGATTCTAACTGGTAGT -3' 5`- AGGAGTCAAAACAAGTGAAG-3´	52	*mecA*
2	LuxS (1496 bp)	5'- GATCGTAGTCGAGTTCCTTA -3' 5´- GCTCATCAATATGCCTAGAT -3´	52	*luxS*
3	ComA (2900 bp)	5'- GTCATAGCCGTTAACATTCT -3' 5'- GAGAAATAAGACAGCAAAGC -3'	52	*comA*
	ComAB (1425 bp)	5'- TTTTAGCTAAGCAAGGTTTC -3' 5'- CTTTACGTCTGGACTGATTT -3'	52	*comA, comB*
4	ComFFa (1776 bp)	5'- GTGATGGAGAATTTAGAGA –3' 5'- GTTTTACACTACCATCTTCT –3'	50	*comF, comFa*
5	ComEA (1670 bp)	5'- CTGGAACCCAGAAAAGCATC –3' 5'- AAAAGGGCAGCTGAATAGCA –3'	50	*comEA, SMU.626*
6	CoiA (1367 bp)	5'- TGAAATCTACTTTAGTCCTT –3' 5´-AGCTTGTAATTCTTGATAGT-3´	50	*coiA*
7	CiaHR (1385 bp)	5-CGAGGTTGAATTTCTGTTAT-3' 5'-AAATTTTTGATCGTATCTGG-3'	52	*ciaH, ciaR*
8	ComCD (1771 bp)	5'-TATCAGATGAGTTTGTCC-3' 5'-ATATCACCCAGTATAGTCAG -3'	52	*comC, comD*
	ComDE (1862 bp)	5'- AGAGATTCTATTTGCTGACT-3' 5'-TATGTAGGAAGAGTTGAACA -3'	50	*comD, comE*
9	ComYDA (1978 bp)	5'- TGACTCAACTGAGTAAGAAT- 3' 5'- GTTTATGCTAGGATGTTAGA –3'	52	*comYD, comYC, comYB, comYA*
	ComYA1988 (1399 pb)	5'- TCTAACATCCTAGCATAAAC - 3' 5'-AGAGGACACAGTAGAAGAGT–3'	50	*comYA, SMU1988c*
10	ComX (1437 bp)	5'- GACAAAGTAGTCGCTAAAGG -3' 5'- ACATACCCTGCTTTATCTTG -3'	52	*comX*
11	CinA (1789 bp)	5'- CAATATCAAGAGCCAGACTT -3' 5´-GTATACCTGCTCAAACGAAT-3´	52	*cinA*

Table 3. Structural analysis of 21 competence-related genes organized in 11 *loci* identified in the chromosome of the strain UA159 in 94 clinical genotypes of *S. mutans*.

Amplicon	N° (%) of clinical genotypes			
	PCR screening in 94 genotypes		Southern blot analysis of PCR-negative genotypes	
	PCR-positive	PCR-negative	Polymorphic pattern	UA159 pattern
MecA	93 (98.9)	1 (1.1)	1 (1.1)	0
LuxS	82 (87.2)	12 (12.8)	8 (8.5)	4 (4.2)
ComA	93 (98.9)	1 (9.3)	0	1 (1.1)
ComAB	88 (93.6)	6 (6.4)	3 (3.2)	3 (3.2)
ComFFa	71 (75.5)	23 (24.4)	12 (12.8)	11 (11.7)
ComEA	89 (94.7)	5 (5.3)	5 (5.3)	0
CoiA	92 (97.9)	2 (2.1)	0	2 (2.1)
CiaHR	94 (100,0)	-	-	-
ComCD	68 (72.3)	26 (27.7)	19 (20.2)	7 (7.4)
ComDE	73 (88.3)	11 (11.7)	2 (2.1)	9 (9.6)
ComYDB	90 (95.7)	4 (4.3)	1 (1.1)	3 (3.2)
ComYA1988	80 (85.1)	14 (14.9)	0	14 (14.9)
ComX	84 (89.4)	10 (10.6)	0	10 (10.6)
CinA	90 (95.7)	4 (4.3)	1 (1.1)	3 (3.2)

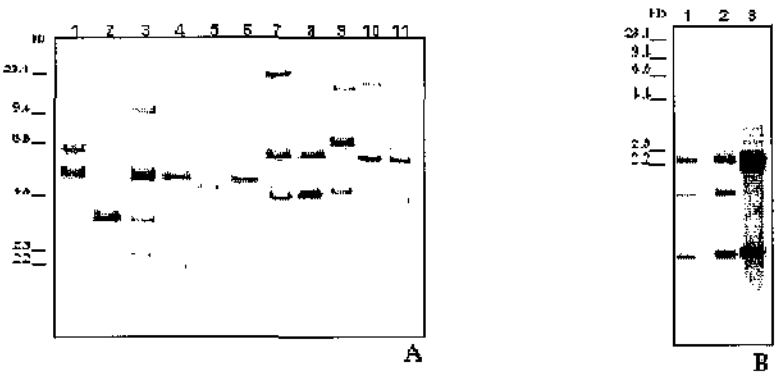

Figure 2. Southern blot analysis of the *comCDE* locus in UA159 and clinical genotypes that were PCR-negative for comCD (A) and comDE (B) sequences. Lanes number 1 corresponds to the predicted Southern blot pattern obtained for the control strain UA159. A) Lanes 2 to 11 correspond to strains representative of each one of the 10 distinct classes of Southern blot identified in 19 strains with atypical Southern blots probed with the comCD amplicon. B) Lanes 2 and 3 are representative patterns of Southern blots probed with comDE amplicon in the comDE PCR-negative strains. Note that some PCR-negative strains, represented in lane 2, showed the same pattern of strain UA159.

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CAPÍTULO 2

"Physiologic and genetic characterization of clinical isolates of *Streptococcus mutans* submitted to acid challenge"♦

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Running Title: PTS and acid tolerance.

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ABSTRACT

Streptococcus mutans is considered the primary etiological agent of human dental caries, a biofilm-dependent infectious disease. This bacterium is capable of surviving at acidic pHs by mounting an acid tolerance response (ATR). However, most of *S. mutans* ATR studies focused on few laboratory strains. The aim of this study was to evaluate physiologic and genetic traits of three clinical isolates of *S. mutans*. We used strain UA159 as control and 3 clinical isolates of *S. mutans*; strains C2(3)5, C5(5)6 and C32(5)1. Cells grown to steady state in continuous cultures were assessed for glycolitic profile, acid tolerance, ATPase and sugar:phosphotransferase system (PTS) activities. Real Time quantitative RT-PCR was used to investigate the transcriptional levels of DnaK, F-ATPase and PTS related enzymes in chemostat grown cells. Biofilm formation was assayed by growing the cells in microtitre plates with medium BM supplemented with sucrose or glucose. Chemostat cells grown at pH 5.0 were able to decrease the pH through glycolysis to higher extent than pH 7.0 grown cells. When the cells were subjected to acid killings, 3 strains, UA159 and C5(5)6 and C32(5)1 became acid adapted, whereas strain C2(3)5 was not able to resist and survive under acid conditions as well as strain UA159. The PTS activity for glucose, fructose and mannose was higher in cells grown at pH 5.0 for isolate C2(3)5, and with this strain also presenting the highest PTS activity among all studied strains. Strains UA159, C5(5)6 and C32(5)1 presented the same pattern of ATPase activity with optimal pH 6.0. Strain C2(3)5 had the highest ATPase activity among the tested strains. The profile of gene expression of DnaK, F-ATPase and PTS related enzymes confirm the data found in the physiologic assays. The ability to form biofilm in BM with sucrose was similar for all strains. However, cells grown in BM with glucose showed different patterns, with strain C2(3)5 showing lower ability to form biofilm. Although the studied strains shared physiological properties, each strain behaved as unique, presenting

peculiar characteristics and genetic expression. Collectively, the data suggest that besides ATPase activity the PTS systems could help to mount an ART in *S. mutans.* This work has revealed new information that challenges knowledge about the pathogenic properties of important human pathogens.

INTRODUCTION

Streptococcus mutans is considered the primary etiological agent of human dental caries (Loesche, 1986), a biofilm-dependent infectious disease. This bacterium is able to accumulate in the dental biofilm and succeed under extremely low pHs, being responsible for tooth demineralization, and can physiologically adapt to the stressful conditions, such as rapid changes in pH, nutrient availability, carbohydrate source and oxygen tension (Marsh, 2006).

Carbohydrate metabolism is an essential survival strategy for *S. mutans*, a bacterium capable to metabolize a wide variety of carbohydrates, especially at low pH (Adjic *et al.* 2002). The uptake of sugars from the environment is a key component of *S. mutans* carbohydrate metabolism. Although at least five sugar ABC transport systems exist, including the well described multiple sugar metabolism system (Russel *et al.*, 1992), most sugars are transported by sugar:phosphotransferase system (PTS) (Vadeboncoeur & Peltier, 1997). The PTS is the primary sugar transport system in oral streptococci, especially under carbohydrate-limiting conditions, and plays important roles in global control of gene expression (Postma *et al.*, 1993; Saier *et al.*, 1996; Vadeboncoeur & Peltier, 1997; Stulke & Hillen, 1998; Abranches *et al.*, 2006). This system consist of two nonspecific energy coupling components, enzyme I (EI) and a heat-stable phosphocarrier protein (HPr), and a variety sugar-specific permeases known as enzyme II complexes

(EII), that catalyze the transport and concomitant phosphorylation of their cognate substrates (Postma *et al.*,1993). A complete glycolytic pathway is present in *S. mutans*, leading to the production of pyruvate that is then reduced to various fermentation products, such as lactic acid that can lower the pH in oral biofilm (Vadeboncoeur & Peltier, 1997).

The acidification of the local environment by the end products of metabolism inhibits many competing bacterial species, enabling *S. mutans* to maintain its niche. This bacterium possesses constitutive acid tolerance properties and is also capable of mounting an adaptive acid tolerance response (ATR). The acid tolerance of *S. mutans* is based primarily on the presence of a membrane-bound, acid-stable, proton-translocating F0F1 ATPase that can maintain the intracellular pH at 7.5 (Quivey *et al.*, 2001). Adaptation to growth in moderately acidic conditions helps the organisms less vulnerable to lethal acidification and is associated with enhanced glycolytic capacities and increased activity of the proton-translocating F-ATPase (Belli & Marquis, 1991, Hamilton & Buckley, 1991). In addition, some other mechanisms have also shown to play a role in acid adaptation by *S. mutans* including the activation of sat operon (Gutierrez *et al.*, 1996; Gutierrez *et al.*, 1999; Kremer *et al.*, 2001), the induction of stress proteins (Jayaraman *et al.*, 1997; Svensäter *et al.*, 2000; Lemos *et al.*, 2001; Len *et al.*, 2004a), DNA repair enzymes (Hahn *et al.*, 1999; Hanna *et al.*, 2001), changes in the cell membrane composition (Fozo & Quivey Jr, 2004; Quivey Jr *et al.*, 2000), an H^+-glucose symporter that operates at pH 5.0 (Cvitkovitch *et al.*, 1995) induction of response regulators and quorum sensing genes (Li *et al.*, 2002; Wen & Burne, 2004; Ahn *et al.*, 2006), and an ammonia/polyamine generating pathway (Griswold *et al.*, 2004).

Most studies of ATR by *S. mutans* were performed using laboratory strains, and established that this organism possesses an inherent acid resistance (Belli & Marquis, 1991, Hamilton & Buckley, 1991). However little is known about the ability of fresh clinical

isolates of *S. mutans* to mount an ATR. Thus, a comparative analysis of the behavior of clinical strains submitted to stressful conditions would help us to understand the distinct capacity of this organism to colonize the oral cavity of children. The purpose of this report was to conduct a more detailed analysis of the responses of clinical isolates of *S. mutans* to environmental acidification.

MATERIAL AND METHODS

Bacterial strains and growth conditions: *S. mutans* strain UA159 and 3 clinical isolates of *S. mutans* (C2(5)3, C5(5)6 and C32(5)1) were routinely grown in brain heart infusion (BHI) medium in a 5% CO_2 atmosphere at 37°C. These clinical genotypes were isolated previously (Klein *et al.*, 2004) and had their identity confirmed by sequencing analysis of the 16S rDNA gene (data not shown). To investigate the ATR of these strains, cells were grown in a Biostat®i chemostat (Sartorius BBI Systems Inc., Bethlehem, PA) with a working volume of 700 ml in TY base medium (3% tryptone, 0.5% yeast extract) containing 25 mM glucose, as previously described (Jayaraman *et al.*, 1997). The pH of the culture was controlled at pH 5.0 or pH 7.0 by automated addition of 2.0 N KOH. Cells were grown for a minimum of 10 generations at a dilution rate of D = 0.3 h^{-1} to achieve a steady state for each strain evaluated. Samples were obtained from the chemostat by aspiration from the vessel into tubes at selected time points. Cells were collected immediately by centrifugation at 4°C and subjected to acid killing and pH drop experiments, or stored at -80°C for further analysis.

Growth kinetics. Growth of all strains in tryptone-vitamin base (TV) medium supplemented with various carbohydrate sources (0.5% glucose, 0.5% fructose, 0.5%

galactose, 0.5% lactose, 0.5% mannose, 0.5% inulin + 0.05% glucose, 0.5% cellobiose + 0.05%glucose, 0.5% rafinnose + 0.05% glucose and 0.5% lactose + 0.05% glucose), as well as in buffered TY (0.5% glucose) pH 7, 6 and 5 was assessed using a Bioscreen C lab system (Helsinki, Finland) Microbiology Reader with multiwell disposable microtiter plates. The Bioscreen reader was equipped with Biolink software that allowed automatic recording and conversion of optical density readings into growth curves. The cultures were diluted 1:100 in fresh desired medium, and 300μl aliquots of the cell suspension were inoculated into the wells of the microtiter plates. Inocula were adjusted to the same OD600 before dilution. Each sample was assayed in triplicate and wells containing uninoculated growth medium were used as negative controls.

Biofilm assay. The ability to form stable biofilms was assessed by growing the cells in 96-well, flat-bottom microtiter plates (Costar 3595; Corning, Inc., Corning, N.Y.) as previously described (Ahn *et al.*, 2005). Briefly, overnight cultures of *S. mutans* strain UA159 and clinical isolates were transferred to pre-warmed BHI medium and grown at 37°C in a 5% CO_2, aerobic atmosphere to an OD600 of 0.5. The cultures were diluted 1:100 in fresh BM supplemented with glucose or sucrose, and 200 μl aliquots of the cell suspension were inoculated into the wells of the microtiter plates. Wells containing uninoculated growth medium were used as negative controls. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. For biofilm quantification, the microtiter plates were carefully washed with water twice to remove the remaining planktonic and loosely bound cells. The plates were then blotted on paper towels and air dried. The adherent bacteria were stained with 50 μl of 0.1% crystal violet for 15 min at room temperature and then the plates were slowly immersed in water twice to rinse the wells. After, the plates were blotted on paper towels and air dried. The bound dye was extracted from the stained cells by addition of 200 μl of ethanol-acetone (8:2), for 15 minutes. Biofilm formation was quantified by measuring the

absorbance of the solution at 575 nm. To quantify cell density, before stain the biofilm, the cells from one well were removed from it and diluted in water for reading at 600nm.

Acid killing and pH drop experiments. For acid killing experiments, steady-state chemostat cells were washed once with 0.1 M glycine (pH 7.0) and resuspended in 0.1 M glycine (pH 2.8). An aliquot of cells was removed at time 0 min and after that, the samples were stirred continuously at room temperature and aliquots of cells were removed at 30 and 60 min. Cells were serially diluted, plated on BHI-agar plates, and incubated at 37°C for 48 h. The colonies were counted and cell viability at each time point was expressed as the ratio of viable cells at time zero (log of CFU ml^{-1}).

The ability of *S. mutans* strains to lower the pH through glycolysis was monitored as previously described (Belli & Marquis, 1991). Briefly, cells from steady-state chemostat cultures were harvested, washed with one culture volume of cold distilled sterilized water, and resuspended in a solution of 50 mM KCl and 1 mM $MgCl_2$ in 1/10 of the original culture volume. The suspension was titrated twice with 0.1 M KOH to a pH of 7.2, pH drops were initiated by addition of 55.6 mM glucose, and the pH was recorded over time.

Biochemical assays. For F-ATPase assays, cells were permeabilized with toluene and incubated with 5 mM ATP in ATPase buffer, as described by Belli & Marquis (1991). The optimum pH of the ATPase buffer was assayed using a pH range of 4.0 to 8.0. Samples were removed at various intervals and assayed for inorganic phosphate released from ATP using the Quantichrom TM Phosphatase Assay Kit (DIPI-500, BioAssay Systems, Hayward, CA).

To measure sugar transport by the PTS, cells obtained from the chemostat were washed twice with 0.1 M sodium-potassium phosphate buffer (pH 7.2) and suspended in 10% of the original volume using the same buffer. The cell suspension was then

permeabilized with 50 µl of toluene-acetone (1:9) per ml of cells. Permeabilized cells (10 to 50 µl) were assayed using the method of LeBlanc *et al.* (1979). Glucose, fructose and mannose were the sugars used in the experiments. Protein concentrations were determined by using the bicinchoninic acid assay (Sigma, Saint Louis, MO).

RNA isolation and Real Time quantitative RT-PCR. RNA was extracted from *S. mutans* chemostat-grown cells (an aliquot of 50 ml) as described elsewhere (Abranches *et al.*, 2006). Real Time quantitative reverse transcription (RT)-PCR was used to investigate the transcriptional levels of DnaK, F-ATPase and PTS related enzymes. RNA samples obtained from *S. mutans* chemostat-grown cells (total of 3 chemostat runs) were isolated. One microgram of RNA and the iScriptTMcDNA Synthesis Kit containing random primers (Bio-Rad, Hercules, CA) were used to generate cDNA. Real-time PCRs were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad) using iQSYBR green supermix (Bio-Rad) and gene specific primers. The oligonucleotides (Table 1) used in all real-time PCR experiments were designed using DNA mfold (http://www.bioinfo.rpi.edu/applicatons/mfold/old/dna) and Beacon Designer 2.0 software (PREMIER Biosoft International, Palo Alto, CA). Standard curves for each gene were prepared as described previously (Yin *et al.*, 2001).

Table 1: Oligonucleotides used for Real Time quantitative RT-PCR for to investigate the transcriptional levels of DnaK, F-ATPase and PTS related enzymes. All primers have the same annealing temperature (60°C). Amplicom: gene designation and NCBI number.

Amplicon (expected size; bp)	Primer sequence (*Forward/Reverse*)	Reference
atpB - 1028766 (85 bp)	5'- CGTGCTCTCTCGCCTGAAATAG -3' 5`- ACTCACGATAACGCTGCAAGAC -3´	*This study*
dnaK - 1029666 (124 bp)	5'- TTCGTCAAGCCCTTTCAGAT -3' 5´- GGTTCTTTGCCAGTTTCAGC -3´	Ahn *et al.*, 2006
manL- 1029081 (79 bp)	5'- GCATCTGACACAGTTGCTAAGG -3' 5'- CATTAGCTTTAACACCGCCAGG -3'	Abranches *et al., 2006*
ptsG - 1029230 (109 bp)	5'- GGAGTGGACATTCCTAAGACAG -3' 5'- CTTGACCATCTGCCACAGTTG -3'	Abranches *et al., 2006*
SMu0102 - 1029691 (97 bp)	5'- GGCTAGGTGCTTTTGAAAATGC –3' 5'- GCCGCTTTATTAACAGGACCAC –3'	*This study*
Smu0795 (fruA, fxpC) - 1028238 (135 bp)	5'- TTCAGACTTCAACTGGCCTTGG –3' 5´- CAACAGGTTCACCATCAAGAGC - 3´	*This study*
levD - 1029151 (90 bp)	5'- GCCCTTTGACAACAGCTTGC -3' 5'- CTGCCATTGGTAAGTTCATCCC -3'	*This study*

RESULTS

Growth kinetics. First, we performed the analysis of growth curves that provide the doubling time for each strain assayed and shown the ability of these strains to growth at distinct pH (Table 2) and to acquire different sugars. After obtain these data, we growth the strains in continuous chemostat cultures for to investigate the responses of these strains to acid adaptation.

Table 2: Doubling Time of *S. mutans* strains assessed. The mean of 3 experiments and standard errors are shown.

Strain	pH (avarege ± STD)	
	pH 7.0	pH 5.0
UA159	95 ± 5	195,0 ± 0
C2(5)3	160 ± 0	282,5 ± 11.5
C5(5)6	77,8 ± 3.2	168,5 ± 21.4
C32(5)1	83,9 ± 0.4	148 ± 1.5

Biofilm formation by *S. mutans* clinical isolates. The ability to form biofilm in BM with sucrose was similar for all strains (Figure 1). However, cells grown in BM with glucose showed different patterns, with strain C2(3)5 showing a lower ability to form biofilm (Figure 1).

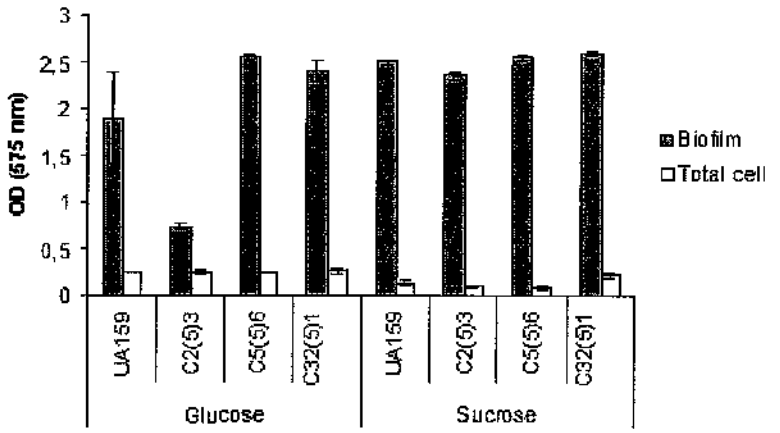

Figure 2: Biofilm formation by *S. mutans* UA159 and clinical isolates. Biofilm in BM with glucose and BM with sucrose. The mean of 3 experiments and standard errors are shown.

Acid adaptive response of clinical isolated of *S. mutans*. To determine if the clinical isolated of *S. mutans* had the ability to mount an ATR, acid killing and pH drop experiments were performed with cells obtained from steady-state chemostat cultures. As a control, chemostat cultures of *S. mutans* UA159 grown under the same conditions were also analyzed. Steady-state pH 7.0 or pH 5.0 cultures of these strains were subjected to acid killing at pH 2.8. After 60 minutes of incubation, 3 strains, UA159, C5(5)6, and C32(5)1, became acid adapted, whereas strain C2(5)3 was not able to resist and to survive under acid conditions as well as strain UA159 (Figure 2).

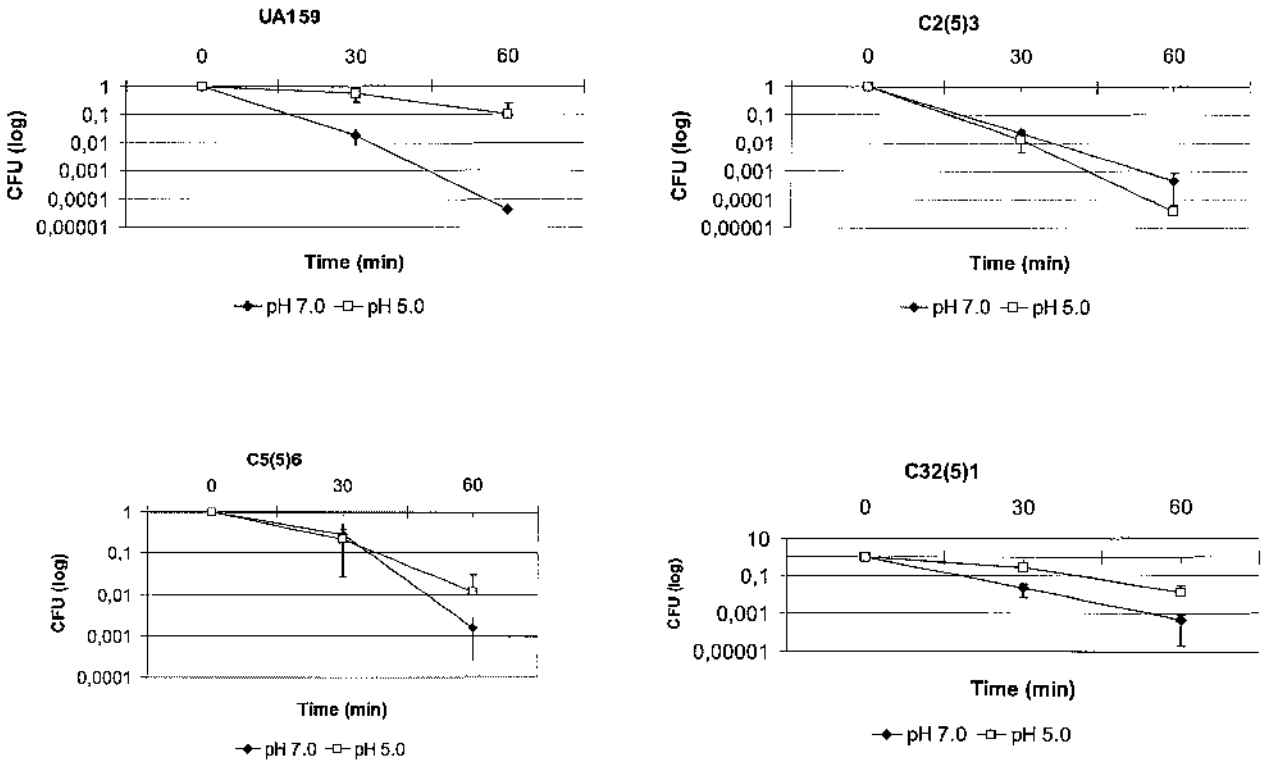

Figure 2: Acid killing results. The mean of 3 experiments and standard errors are shown.

In the pH drop experiments, cells grown at pH 5.0 were able to decrease the pH through glycolysis to higher extent than pH 7.0 grown cells (Figure 3). The necessary time through glycolysis, by cells grown at pH 7.0 and 5.0, to lower the pH to 3.8 was about 30 min (the same time for all strains evaluated).

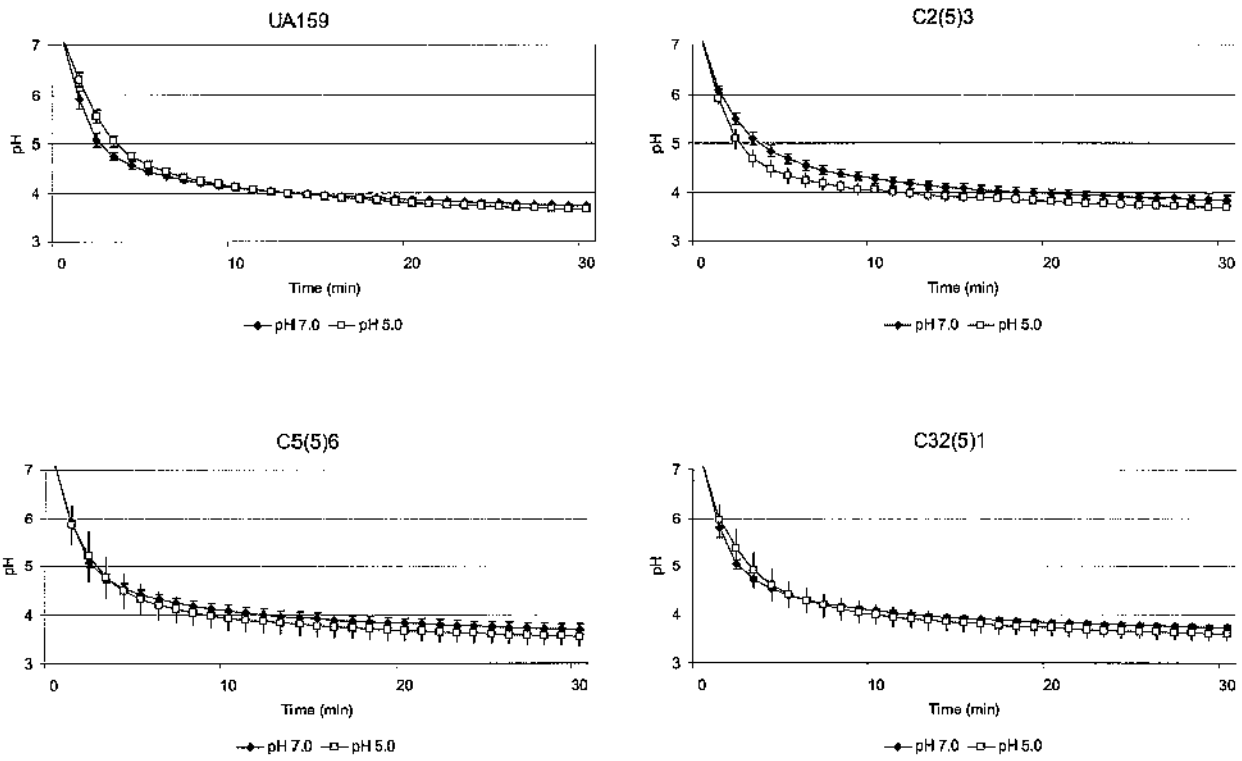

Figure 3: pH drop results. The means of 3 experiments and standard error are shown. No significant differences were detected ($p>0.05$; Student's t-Test) between values for glycolitic profile at pH 7.0 and 5.0.

F-ATPases activity and acid tolerance by clinical isolated of *S. mutans*. The optimal pH for ATPase activity was assessed and found to be the same for all *S. mutans* strains evaluated (pH = 6.0), similar to previous data (Bender *et al.*, 1986; Sturr & Marquis, 1992). The ATPase activity for chemostat grown cells at pH 7.0 and 5.0 presented significant differences in the four strains assayed (Figure 4). Strains UA159, C5(5)6 and C32(5)1 presented almost the same pattern of ATPase activity and strain C2(3)5 had the highest ATPase activity among the tested strains. Real Time quantitative RT-PCR analysis of total mRNA were used to test whether the levels of F-ATPase activity between *S. mutans* UA159 and clinical isolates of *S. mutans* correlated with mRNA for the F-ATPase genes. The real time data for *atpB* gene, that codes for ATP beta subunit of the F-ATPase enzyme, showed up-regulation of this gene when *S. mutans* strains were grown at pH 5.0 (Figure 6). Strain C2(3)5, that did not exhibit a classic ATR, showed a higher number of copies for *atpB* gene in comparison with UA159 and the other 2 clinical isolates of *S. mutans* (data not shown).

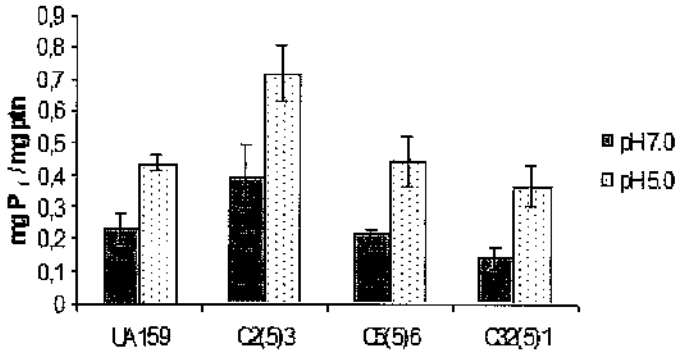

Figure 4: ATPase activity by *S. mutans* strains. Significant differences were detected ($p<0.05$; Student's t-Test) between values for ATPase activity at pH 7.0 and pH 5.0 in all strains assayed. The means of 3 experiments and standard errors are shown.

PTS activity in clinical isolated of *S. mutans*. The glucose, fructose and mannose-PTS activities of chemostat-grown *S. mutans* cells were assessed. In this work, we showed that one *S. mutans* clinical isolate, strain C2(5)3, have a PTS activity for glucose, fructose and mannose induced at low pH, and this strain also presented the highest PTS activity (Figure 5). Strains UA159 and the other two clinical isolates, strain C5(5)6 and strain C32(5)1 presented higher glucose-PTS activity at pH 7.0; fructose-PTS at pH 5.0; and mannose-PTS activity was higher at pH 5.0 for strain UA159 and at pH 7.0 for strains C5(5)6 and C32(5)1 (Figure 5).

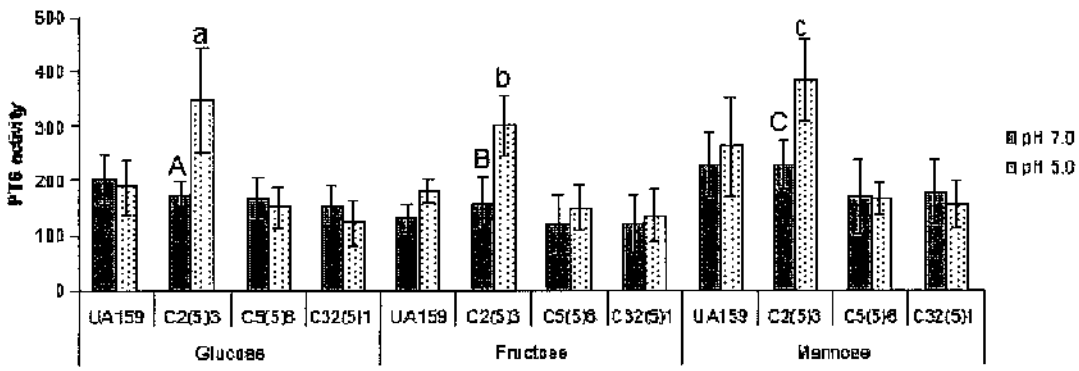

Figure 5: PTS activity. Significant differences were detected on strain C2(5)3 ($p<0.05$; Student's t-Test) between values for glucose, fructose and mannose PTS activities at pH 7.0 and 5.0 (different letters on uppercase and lowercase). There are no statistic differences between PTS activity at pH 7.0 and 5.0 for the others strains evaluated ($p>0.05$). The means of 3 experiments and standard errors are shown.

Real Time quantitative RT-PCR was used to investigate the transcriptional levels of some of the sugar-specific EII enzymes. The data of genetic expression, when comparing cells grown at pH 5.0 and 7.0, detected an induction for the genes *ptsG* (PTS system, enzyme II, A component), *manL* (PTS system, mannose-specific component IIAB),

Smu0795 (fructose-specific PTS system enzyme IIBC component) and *levD* (probable PTS system fructose-specific enzyme IIA component), and repression for the gene *SMu0102* (PTS system, fructose-specific IIBC component). Similar to what happened for genes *dnaK* and *atpB*, the higher fold of induction for sugar-specific EII enzymes was presented by strain C5(5)6.

Gene expression patterns of chemostat-grown cells. The profile of gene expression of DnaK, F-ATPase and PTS related enzymes confirmed the data found in the physiologic assays (Figure 6). In this experiment there was a high standard deviation between different chemostat runs. It could be due to changes after gene transcription.

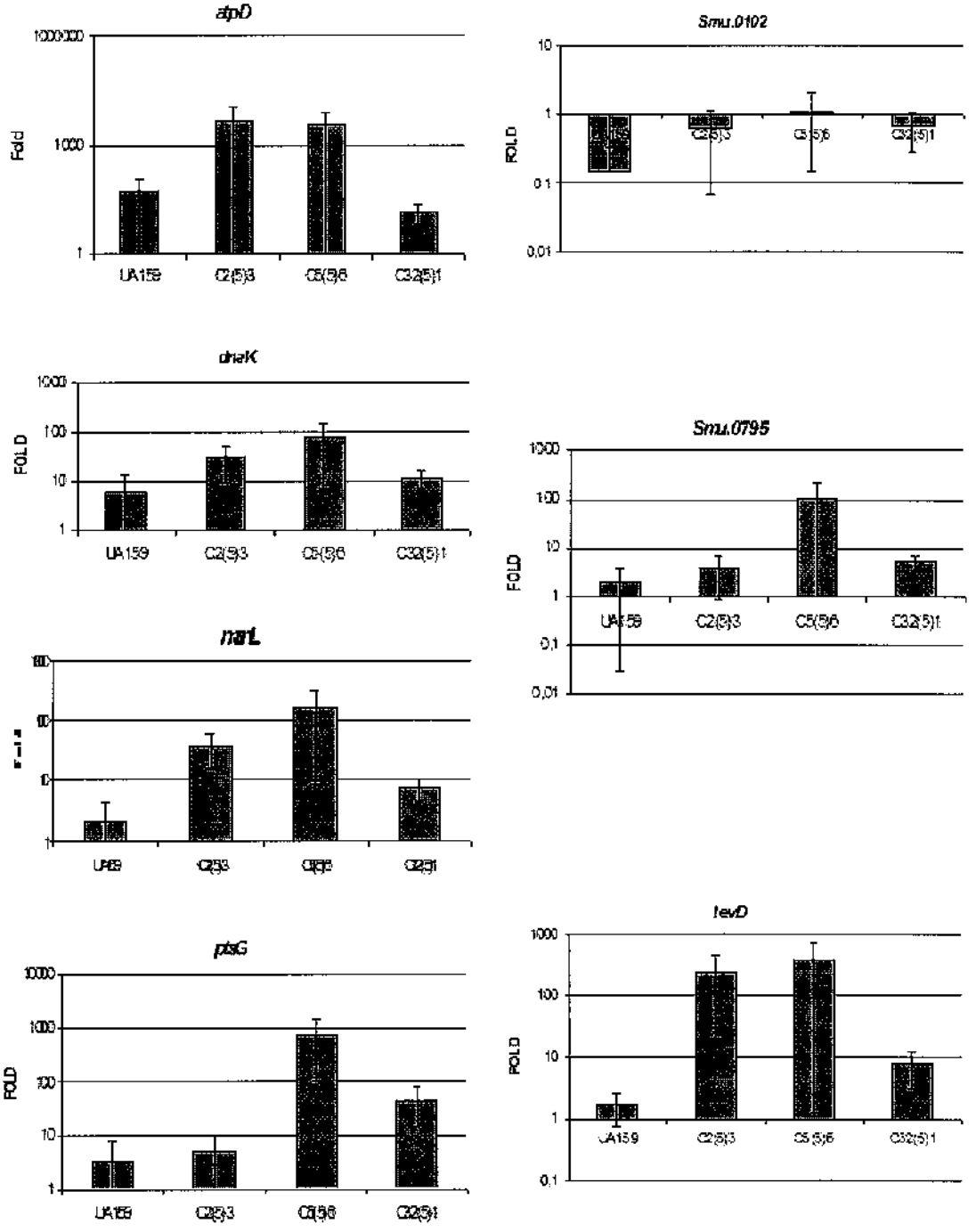

Figure 6: Real time RT-PCR. Ratio of induction (pH 5.0/pH 7.0) for each gene assessed. The mean of 3 experiments and standard errors are shown.

DISCUSSION

S. mutans is able to triumph in the complex microbial community of the oral biofilm, because it can physiologically adapt to the stressful conditions to which the cariogenic biofilm is exposed (Bender *et al.*, 1986; Belli & Marquis, 1991; Hamilton & Buckley, 1991; Nascimento *et al.*, 2004). In the presence of sucrose, under extremely low pHs this bacterium is responsible for tooth demineralization (dental caries development). Our data demonstrate that the ability to form biofilm correlated with the capacity to mount a classical ATR (resistance to acid killing), since the strains that showed higher ability to form biofilm were the same ones that were able to survive in low pH (Li *et al.*, 2001).

Considering the high diversity in the production of virulence factors involved in biofilm formation *in vitro* observed among *S. mutans* clinical isolates (Mattos-Graner et al., 2001; 2004), it seems important to analyze the ART in clinical isolates of *S. mutans* regarding to the success of *S. mutans* initial colonizers. Previous reports showed that strains UA159 and LT11 of *S. mutans* are able to mount an ART (Nascimento *et al.*, 2004; Svensater *et al*, 1997). However, our data demonstrated that not all *S. mutans* strains were able to survive at low pH. The strain C2(5)3, isolated from children with dental caries, was recovered during a follow-up study. Thus, there is other mechanism or interconnected mechanisms that allowed this strain to survive under low pH at dental biofilm. It could be due its high ability to metabolize sugars via PTS systems and its higher ATPase activity, or any other additional traits that were not evaluated here. Perhaps, the acid killing assay is not sensible enough or adequate to evaluate the acid adaptation. Therefore, other analysis must be carried out to assay constitutive acid resistance, such as gene expression of enzymes related to maintain the integrity of a bacterium cell (*dnaK*).

The ATPase activity data confirm earlier studies which report that acid adaptation of *S. mutans* is correlated with an increase in proton-extruding F-ATPase activity (Belli &

Marquis, 1991; Hamilton & Buckley, 1991). Indeed, other study demonstrated about a 2-fold increase in F-ATPase activity in *S. mutans* UA159 growing at pH 5.0 as compared to cells grown at pH 7.0 (Nascimento *et al.*, 2004). It was suggested that increases in F-ATPase activity correlate with increased transcription of the F-ATPase operon (Quivey Jr *et al.*, 2000). In this study, the data from *atpB* gene expression showed up-regulation of this gene when *S. mutans* strains were grown at pH 5.0. Also, the transcriptional levels of *dnaK* gene showed an induction at low pH in all *S. mutans* strains evaluated, in agreement to previous works (Jayaraman *et al.*, 1997; Nascimento *et al.*, 2004).

Our data suggest that glucose, fructose and mannose PTS of *S. mutans* strains can be involved in acid tolerance by this species. The PTS is believed to be critical for high-affinity and high-capacity transport of carbohydrate, especially under carbohydrate-limiting conditions (Hamilton & Martin, 1982; Keevil *et al.*, 1984; 1986; Vandeboncoeur & Pelletier, 1997), and a major contributor to acidogenesis, however, its role in acid tolerance is unclear. Previous research with *S. mutans* strains Ingbritt and UA159 demonstrated that glucose-PTS activity is markedly decreased in cells grown at low pH, compared to those grown at neutral pH values (Hamilton & Ellwood, 1978; Hamilton *et al.*, 1979; Vandeboncoeur *et al.*, 1987; 1991; Nascimento *et al.*, 2004), and it was suggested that repression occurs at the level of EII synthesis (Vandeboncoeur *et al.*, 1987; 1991). Our results demonstrated that the levels of several sugar-specific EII enzymes are up-regulated at low pH in steady state grown cells at glucose limiting conditions. Differences in the results obtained in this study with those of the previously reported are most likely attributable to differences in growth conditions and strains used in the different studies. Furthermore, it was demonstrate that *S. mutans* possesses a glucose transport system that can work independently of the PEP PTS (Cvitkovitch *et al.*, 1995). This system is assumed as the primary transporter for glucose at low pH, working as an H^+-glucose

symport system (Cvitkovitch *et al.*, 1995). Also, PTS plays important roles in the regulation of gene expression (Abranches *et al.*, 2003; 2006; Postma *et al.*, 1993; Saier *et al.*, 1996; Vandeboncoeur & Pelletier, 1997). It was speculated that changes in expression of the glucose-PTS for *S. sobrinus* could modify the acid tolerance by modulating gene expression patterns in a way that enhances protection against environmental acidification (Nascimento *et al.*, 2004). Also, the increases in the glucose-PTS activity by *S. sobrinus* result in higher rates of ATP generation through glycolysis, thus enhancing the ability of the cells to maintain a pH balance (Nascimento *et al.*, 2004).

In summary, the data obtained herein indicate that although the studied *S. mutans* strains share physiological properties, each strain behaves as unique, presenting peculiar characteristics at physiological and genetic expression level. Taken all together the data presented suggest that besides ATPase activity the PTS could help to mount an ART in *S. mutans*. This work has revealed new information that challenges knowledge about the pathogenic properties of important human pathogens.

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IV - DISCUSSÃO GERAL

Os trabalhos apresentados nesta tese de doutorado avaliaram características genéticas e fisiológicas de *S. mutans* relacionadas à virulência desta espécie. O capítulo 1 traz resultados inéditos na literatura científica, onde se demonstrou a conservação dos genes do sitema "*quorum-sensing*" e de genes envolvidos no processo de transformação em *S. mutans*. Já os resultados do capítulo 2 confirmam a capacidade das cepas de *S. mutans* desenvolverem uma resposta de adaptação ao ácido durante o cultivo em pH 5,0 (comumente encontrado no biofilme dental), e, ainda, sugerem a participação dos sistemas PTS na tolerância ao ácido por esta espécie bacteriana. Os resultados encontrados em ambos os trabalhos ilustram a complexidade da biologia da espécie *S. mutans* e podem contribuir para a compreensão da mesma.

Os genes detectados no primeiro capítulo têm sido demonstrados como importantes na adaptação ambiental do *S. mutans*, estando envolvidos na sobrevivência e na virulência desta espécie. Pois, os TCS avaliados (*comCDE*, *luxS* e *ciaHR*), através da captação de sinais ambientais podem levar a uma alteração na expressão de genes ou conjunto de genes específicos. Estes sistemas possuem um sensor histidina-quinase associados à membrana e um regulador de resposta (uma proteína ligante de DNA) que modula a expressão de genes alvo quando fosforilada através da histidina-quinase (Shapiro, 1998; de Kievit & Iglewski, 2000; Li *et al.*, 2001a,b; Wen & Burne, 2004). Muitos genes são requisitados tanto para uma resposta adequada ao estresse ambiental quanto para a formação de biofilme (*hrcA, hk11, comC, comD, clpP, relA, luxS, brpA, ropA, rgg*) estabelecendo uma ligação entre dois atributos críticos à virulência de *S. mutans* (Lemos *et al.*, 2005). Por outro lado, as vias de tolerância ao estresse, que estão intimamente envolvidas com o crescimento e homeostasia dos organismos, influenciam a capacidade das bactérias em formarem biofilmes estáveis (Li *et al.*, 2001a; Ahn *et al.*, 2006,

Abranches *et al.*, 2006). Assim, o entendimento desses processos poderá contribuir para elucidar os mecanismos que favorecem o *S. mutans* durante o processo de colonização da cavidade bucal. No capítulo 2, foi verificado que isolados clínicos distintos possuem capacidade de formação de biofilme diferentes, em meios de cultura contendo sacarose ou glicose, corroborando dados encontrados anteriormente (Mattos-Graner *et al.*, 2004).

Os dados obtidos na análise fisiológica e da expressão gênica de células planctônicas cultivadas em quimiostato fornecem subsídios para novos trabalhos com células crescidas na forma de biofilme. Os resultados obtidos demosntram que as cepas clínicas possuem comportamento heterogêneo quando submetidas as mesmas condições de crescimento, para a avaliação de adaptação e tolerância ao ácido. Os dados obtidos nas análises fisiológicas e de expressão gênica, sugerem que além da atividade ATPase ocorre a participação dos sistemas PTS na tolerância ao ácido por esta espécie bacteriana, o que pode acarretar mudança nos conceitos da biologia de *S. mutans*. Ainda não foi estabelecida a extensão da participação dos sistemas PTS na montagem da resposta ao ambiente ácido. Assim, são necessários trabalhos futuros utilizando ensaios de biofilme, que simulem o ambiente da cavidade bucal, para melhor elucidar a fisiologia dessa espécie bacteriana no seu habitate natural, pois, os gradientes desenvolvidos durante a maturação do biofilme permitem o acúmulo de produtos finais, a concentração de moléculas difusíveis, a geração de heterogeneidade no crescimento dentro da população, e redução ao acesso de nutrientes. Deste modo, a resposta dos microrganismos ao estresse ambiental dentro da maturação dos biofilmes, regulada de forma apropriada, tanto temporal como espacialmente, pode influenciar profundamente na estrutura do biofilme ou nos organismos que estão formando o biofilme. Sob este ponto de vista, a ligação íntima da formação de biofilme e tolerância ao estresse é lógica; assim, se faz necessário um conhecimento maior sobre os mecanismos de homeostase da

população aderida para que se possa controlar os biofilmes orais na saúde e na doença (Lemos *et al.*, 2005).

As vias de resposta ao estresse, que são altamente conservadas nos estreptococos, podem estar envolvidas em funções adicionais (Burne, 1998). Estas bactérias possuem genomas pequenos e não possuem muitos dos circuitos genéticos complexos que outras bactérias possuem para a regulação e "manejo" do estresse, tais como os fatores sigma alternativos σ^S e σ^B, e alguns dos seus genes alvos (Ajdic *et al.*, 2002). Ainda não se sabe se existe, ou não, um regulador principal do estresse em *S. mutans* e se o mesmo poderia governar um vasto conjunto de funções biológicas do mesmo modo que em organismos com genomas mais complexos. É necessário responder de que maneira os estreptococos orais obtém sucesso em um ambiente com mudanças contínuas como é o da cavidade bucal.

O trabalho recente de Ahn *et al.* (2006) sugere que as múltiplas vias que regulam a expressão de diversos genes importantes na fisiologia de *S. mutans* se interconectam; mas, ainda não se sabe o modo exato como ocorreria esta interrelação. Ainda, o mesmo estudo idealiza um modelo de integração de circuitos regulatórios, que ainda precisa ser elucidado. Destaca-se que os genes pesquisados no primeiro capítulo desta tese, presentes em todos os isolados clínicos avaliados, são parte integrante deste modelo que poderia explicar e regular múltiplos fatores de virulência, em especial os genes *comCDE, comX, ciaHR* e *luxS* (Li *et al.*, 2001a; 2002b; Yoshida & Kuramitsu, 2002; Yoshida *et al.*, 2005). Isto ilustra a importância do conhecimento da extensão da conservação desses sistemas na espécie *S. mutans,* verificada nesta tese. Além disso, os componentes TCS envolvidos com a competência, e que poderiam regular múltiplos fatores de virulência, poderiam ser importantes alvos para terapia antibacteriana.

Progressos recentes no conhecimento da genética e da fisiologia dos estreptococos orais aderidos reforçam a teoria de que os organismos possam

desenvolver um "fenótipo de biofilme" (Wen & Burne, 2002; Li *et al.*, 2001a,b; 2002a,b; Abranches *et al.*, 2006). Entretanto, ainda estão disponíveis relativamente poucas informações sobre o comportamento desses organismos em populações complexas tais como o biofilme dental. Em particular, *S. mutans* não vive isolado de outras bactérias, e estes organismos estão envolvidos como membros de comunidades aderentes, altamente dinâmicas e complexas. Ainda não é compreendido como contatos intercelulares com outros organismos e interações antagonistas e sinergísticas dentro do biofime moldam o fenótipo desses organismos (Qi *et al.*, 2004). Por exemplo, a redução na taxa de crescimento geral em biofilmes maduros de *S. mutans,* está associada com a manutenção da transformação sem a concomitante resposta ao estresse observada durante o estágio transitório de competência em culturas bacterianas em batelada (Rathsam *et al.*, 2005). Assim, o conhecimento decorrente desta tese, a constatação da presença de todos os genes envolvidos no processo de transformação em isolados clínicos de *S. mutans*, pode ser utilizado como ferramenta para a análise de fenótipos em biofilmes. Pois, a capacidade de incorporar DNA extracelular pode melhorar a capacidade de adaptação a estresses ambientais, conferindo vantagens competitivas aos microrganismo (Hidalgo-Grass *et al.*, 2002; Dagkessamanskaia *et al.*, 2004).

A formação de biofilme estável, a tolerância ao estresse e a produção eficiente de ácido a partir de carboidratos são características de virulência importantes, e podem contribuir na colonização e na estabilidade de determinados genótipos de *S. mutans* na cavidade bucal. Deste modo, os dados obtidos nesta tese de doutorado poderão ajudar a compreender melhor a biologia do *S. mutans*, e também, fornecem subsídios para o desenvolvimento de trabalhos futuros.

V - CONCLUSÃO GERAL

Os dados obtidos neste trabalho de tese permitem as seguintes conclusões:

1. Os 11 *loci* estudados, que contêm genes de competência com função reguladora ou estrutural, estão presentes em todos os isolados de *S. mutans* avaliados.

2. As respostas fisiológicas de diferentes genótipos clínicos, frente às mesmas condições ambientais, são heterogêneas. Os dados de expressão gênica, de genes envolvidos no metabolismo de açúcares, adaptação ao ambiente ácido e resposta ao estresse, confirmam os dados observadas nos ensaios fisiológicos. Os dados obtidos sugerem que além da atividade ATPase, os sistemas PTS também estariam envolvidos com tolerância ao estresse ácido.

VI - REFERÊNCIAS BIBLIOGRÁFICAS * (INTRODUÇÃO E DISCUSSÃO GERAIS)

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COMITÊ DE ÉTICA EM PESQUISA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Caracterização fisiológica e genética de isolados clínicos de Streptococcus mutans**", protocolo nº **017/2004**, dos pesquisadores **REGINALDO BRUNO GONÇALVES, MARLISE INÊZ KLEIN e RENATA DE OLIVEIRA MATTOS GRANER**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 03/03/2004.

Piracicaba, 27 de julho de 2006

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Physiologic and genetic characterization of clinical isolates of Streptococcus mutans**", register number **017/2004**, of **REGINALDO BRUNO GONÇALVES, MARLISE INÊZ KLEIN and RENATA DE OLIVEIRA MATTOS GRANER**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 03/03/2004. 27/07/2006.

Piracicaba, SP, Brazil, July 27 2006

Profa. Cecília Gatti Guirado
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição
Notice: The title of the project appears as provided by the authors, without editing

ANEXO 2

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