Faculdade de Odontologia de Piracicaba
Universidade Estadual de Campinas

Marcelle Matos Nascimento

Cirurgiã Dentista

Análise de determinantes patogênicos de *Streptococcus sobrinus* submetidos ao estresse ácido

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, como requisito para obtenção do título de Doutor em Odontologia, Área de Concentração em Cariologia.

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Orientador:

Prof. Dr. Reginaldo Bruno Gonçalves

Banca examinadora:

Profª. Drª. Denise Madalena Palomari Spolidorio

Profª. Drª. Marcia Pinto Alves Mayer

Profª. Drª. Maria da Luz Rosário de Sousa

Prof. Dr. Pedro Luiz Rosalen

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À Deus,

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Sumário

Resumo

Microrganismos orais são comumente expostos às alterações dos fatores ambientais, tais como o pH do ambiente bucal. Como resultado, as bactérias desenvolvem estratégias adaptativas com o intuito de minimizar o efeito dos danos causados pelos valores extremos de pH. Alguns dos sistemas bacterianos induzíveis promovem variações do pH interno a membrana celular, como exemplo, a atividade de proteínas ATPases, enquanto outros sistemas envolvem modificações complexas no genoma e proteoma bacteriano, de forma a proteger componentes celulares ácido-sensíveis. Dentre os microrganismos implicados na etiologia da cárie dental, *Streptococcus mutans* vem sendo amplamente caracterizado com relação a sua capacidade de adaptação e resposta aos insultos ambientais, entretanto poucos estudos têm analisado o comportamento e os mecanismos induzidos por *Streptococcus sobrinus* em resposta às freqüentes variações do meio bucal. Desta forma, os objetivos aqui apresentados na forma de tese, com base em dois artigos foram: (1) revisar e discutir os principais aspectos envolvidos na resposta fisiológica e expressão gênica de bactérias orais submetidas aos diferentes tipos de estresse ambiental e (2) realizar uma análise sobre a tolerância de *S. sobrinus* ao estresse ácido e dos possíveis mecanismos utilizados por este microrganismo para desenvolver uma resposta ácido-adaptativa. A revisão descrita no primeiro capítulo indica que as respostas defensivas dos microrganismos orais ao estresse são complexas e relacionadas a processos metabólicos e reguladores, que favorecem a sobrevivência bacteriana e influenciam na patogênese de importantes doenças infecciosas orais. O estudo apresentado no segundo capítulo sugere que *S. sobrinus* é constitutivamente ácido-tolerante e capaz de desenvolver uma resposta ácido-adaptativa durante o cultivo em valores de pH ácido comumente encontrados na placa dental.

Abstract

Oral microorganisms are commonly exposed to environmental challenges, such as the environmental pH. As a result, bacteria have evolved adaptive strategies designed to minimize extreme pH values. Some inducible systems attempt to modify internal pH, such as the ATPases activity, while other systems involve complex changes in the genome and proteome that somehow protect acid-sensitive cellular components. Among the pathogens implicated on dental caries etiology, *Streptococcus mutans* has been widely characterized with respect to its ability to adapt and to respond to environmental insults, however little attention has been given to the behavior and mechanisms induced by *Streptococcus sobrinus* in response to oral environment changes. Thus, the objectives of this thesis based on two papers were: (1) to review and to discuss the main aspects involved in the physiologic response and gene expression of the oral bacteria submitted to different types of environmental stress and (2) to conduct a more detailed analysis of the *S. sobrinus* tolerance to environmental acidification and the possible mechanisms governing the acid-adaptive response. The review described in the first chapter indicate that the defensive responses of oral microorganisms are complex and related to metabolic and regulatory processes allowing the survival and influencing the pathogenesis of oral diseases. The study showed in the second chapter suggest that *S. sobrinus* is constitutively acid tolerant and is capable of developing an adaptive acid response during cultivation at acidic pH values that can readily be achieved in dental plaque.

1. Proposição

Esta tese foi dividida em dois artigos que estão contemplados nos capítulos 1 e 2 *.

- Capítulo 1: a revisão se propôs a apresentar conceitos atuais, bem como estudos considerados de extrema relevância para a melhor compreensão do comportamento bacteriano frente aos processos de estresse presentes no ambiente bucal.

- Capítulo 2: o estudo se propôs a realizar ensaios bioquímicos e moleculares com células de *Streptococcus sobrinus*, 6715 e SL1, crescidas em culturas contínuas de quimiostato e submetidas ao estresse ácido. Com o objetivo de melhor compreender os mecanismos de resposta e tolerância ácida destas cepas bacterianas, foram avaliadas a expressão e atividade de proteínas consideradas importantes nos processos de adaptação às condições de estresse ambiental, tais como a enzima F_1F_0 - ATPase, as proteínas chaperones DnaK e GroEL, e proteínas do sistema de transporte de açúcares fosfotransferase fosfoenolpiruvato:açúcar dependente (pep-PTS).

* Este trabalho foi realizado no formato alternativo e composto por dois artigos submetidos à publicação, conforme a deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 001/98.

2. Introdução Geral

As bactérias orais presentes na placa dental são constantemente submetidas a variações das condições ambientais, ou estresse ambiental, incluindo alteração do pH, temperatura, oxigênio e disponibilidade nutricional. O desenvolvimento do processo carioso ocorre justamente como resultado de uma modificação ecológica ocorrida na placa e induzida por variações no ambiente local (MARSH, 1994). Períodos freqüentes de queda de pH podem resultar em uma placa dental acidúrica e acidogênica, e o conseqüente desequilíbrio nos processos de desmineralização e remineralização, em favor da desmineralização das estruturas dentais (de SOET *et al*., 2000). Desta forma, a tolerância microbiana aos ambientes ácidos pode ser diretamente relacionada a ecologia da placa dental bacteriana e patogênese da cárie dental.

Duas espécies pertencentes aos Estreptococos do grupo mutans, *Streptococcus mutans* e *Streptococcus sobrinus*, são as espécies bacterianas consideradas como os principais patógenos da cárie dental. Enquanto *S. mutans* é detectado em maior frequência na cavidade bucal (LOESCHE, 1986; KOHLER & KRASSE, 1990), a presença de elevados números de *S. sobrinus* é associada a alta prevalência de cárie em alguns estudos epidemiológicos (EMILSON & THORSELIUS, 1988; FURE & ZICKERT, 1990). Esses organismos são capazes de manter o seu metabolismo em ambientes ácidos como a placa dental submetida aos desafios cariogênicos, fator este considerado como uma vantagem seletiva em condições ambientais que freqüentemente excedem os limites para o crescimento de outros organismos (WHILEY & BEIGHTON, 1998). Diferenças comportamentais em relação a produção de ácidos e persistência em ambientes ácidos são também observadas entre as espécies *S. mutans* e *S. sobrinus*, bem como é evidente a existência de variabilidade intra-espécie (de SOET 1989; 1991; KOHLER *et al*., 1995).

de SOET e colaboradores (1989) procuraram investigar diferenças quanto a capacidade de produção de ácidos em valores de pH 5,0 à 7,0, entre cepas de Estreptococos do grupo

mutans e *Streptococcus sanguinis* crescidas em culturas planctônicas. *S. sobrinus* foi considerada a espécie mais acidogênica, sendo capaz de produzir ácido mais rapidamente a partir da glicose, e sustentar a produção de ácidos especialmente em valores de baixo pH. Em uma investigação posterior sobre o potencial cariogênico de isolados clínicos de *S. mutans* e *S. sobrinus* (de SOET *et al.*, 1991), verificou-se que em valores de pH 5,0 e 6,5, *S. sobrinus* produziu uma quantidade significantemente maior de ácido, sendo também considerada a espécie mais cariogênica em ratos submetidos a uma dieta rica em sacarose e glicose.

Estudos sobre o genoma de alguns dos principais patógenos orais (AJDIC *et al.*, 2002; KAPATRAL *et al.*, 2002; NELSON *et al.*, 2003), bem como estudos fisiológicos e bioquímicos dos biofilmes dentais (Li *et al.*, 2002; WEN & BURNE, 2002) e de células crescidas em culturas contínuas de quimiostato (JAYARAMAN *et al.*, 1997; LEMOS *et al.*, 2001) têm propiciado uma melhor compreensão sobre os mecanismos pelos quais as bactérias respondem às alterações de pH na cavidade bucal. *S. sanguinis*, por exemplo, utiliza a via arginina desaminase para sua proteção contra a acidificação do meio, enquanto *Streptococcus salivarius* responde através da degradação da uréia à amônia. Os mecanismos de resposta e tolerância aos ácidos (ATR) em *S. mutans* vêm sendo caracterizados por envolver não somente o aumento da resistência aos ácidos, como também o aumento da capacidade glicolítica (HAMILTON & BUCKLEY, 1991; BELLI & MARQUIS, 1991), da atividade da enzima translocadora de prótons, F_1F_0 -ATPase (BELLI & MARQUIS, 1991), e da produção de enzimas de reparo do DNA (HAMILTON & BUCKLEY, 1991; QUIVEY *et al.*, 2000). A relativa tolerância ácida dos organismos pode ainda ser avaliada pelo valor do pH final de um meio de cultura, onde células bacterianas tornam-se acidificadas como resultado da fermentação de uma grande quantidade de carboidratos (BELLI & MARQUIS, 1991). Considera-se como o organismo mais ácido-tolerante aquele capaz de continuar realizando a glicólise sob os menores valores de pH.

Estudos recentes utilizam géis de eletroforese bidimensionais para monitorar a expressão de proteínas durante a adaptação de microganismos as condições de estresse ambiental (BERNHARDT *et al*., 1997; SVENSATER *et al*., 2000). A comparação da massa molecular proteíca sugere que ambos, proteínas ácido-específicas (componentes da F_1F_0 - ATPase) e proteínas gerais de estresse (*heat-shock* ou Hsp/70) estão presentes em extratos de células submetidas ao choque ácido (SVENSATER *et al*., 2000; WILKINS *et al*., 2002). Cerca de 36 proteínas, sendo 25 associadas ao choque ácido, foram induzidas em *S. mutans* LT11 (HAMILTON & SVENSATER, 1998). Em contraste, a cepa bacteriana *S. sobrinus* CH125/43, previamente considerada como incapaz de induzir a ATR quando crescida em culturas planctônicas (SVENSATER *et al*., 1997), apresentou a indução de apenas 6 proteínas associadas ao estresse ácido. A incapacidade de tolerar ácido por esta cepa de *S. sobrinus* foi atribuída à ausência da síntese de proteínas necessárias para a manutenção da homeostase celular em condições de baixo pH (HAMILTON & SVENSATER, 1998).

A produção de proteínas *heat-shock*, como DnaK e GroEL, é considerada um fator importante relacionado ao crescimento celular sob condições de estresse ambiental. Jayaraman e colaboradores (1997) realizaram o primeiro estudo sobre o papel da proteína DnaK em *S. mutans* crescidos em culturas contínuas de quimiostato. Análises de *Slot Blot* e *Western Blot* foram utilizadas para estabelecer a relação entre a expressão do gene *dna*K e a exposição aos ambientes ácidos de células bacterianas crescidas em pH neutro, células submetidas ao choque ácido e células ácido-adaptadas (crescidas por um longo período em pH 5). Os resultados indicaram que os níveis de expressão de *dna*K RNAm e proteína DnaK são induzidos em resposta ao choque ácido e permancem elevados em células ácido-adaptadas, provavelmente para reparar danos causados às proteínas ácido-sensíveis durante o processo de adaptação.

Assim como observado anteriormente para DnaK, os níveis de *gro*EL RNAm e proteína GroEL foram induzidos em células de *S. mutans* expostas ao choque ácido (LEMOS *et al*., 2001).

Entretanto, após as células terem alcançado a fase *steady-state* em pH 5, fase reconhecida por induzir a ATR, os níveis de *gro*EL RNAm e proteína GroEL permaneceram indistinguíveis àqueles observados em culturas *steady-state* em pH 7. Segundo os autores, as proteínas DnaK e GroEL são induzidas em *S. mutans* durante a resposta ao choque ácido, porém a adaptação ácida envolve a manutenção de níveis elevados de DnaK, mas não de GroEL. Ambos os estudos ressaltam a importância da regulação e da expressão dos genes *dna*K e *gro*EL na fisiologia e virulência deste microrganismo oral.

A atividade da proteína DnaK está associada a um complexo com outras duas proteínas, DnaJ e GrpE. O *operon* no qual está inserido o gene *dna*K foi caracterizado em *S. mutans* como sendo constituído por quatro genes: *hrc*A, *grp*E, *dna*K e *dna*J (LEMOS *et al*., 2001), uma organização única quando comparada ao mesmo *operon* em outros microrganismos orais (SCHULZ & SCHUMANN, 1996; HECKER *et al.*, 1996). É possível que o complexo DnaK-DnaJ-GrpE esteja envolvido na biogênese da enzima F_1F_0 - ATPase, ou mesmo na estabilidade desta em condições de baixo pH. O conceito da interação entre DnaK e F_1F_0 - ATPase baseia-se em estudos onde a depleção do gene *hsp*70, um homólogo do gene *dna*K em eucariotos, afeta a translocação da subunidade β da enzima F_1F_0 - ATPase em *Sacharomyces cerevisae* (BECKER *et al*., 1996).

A aciduricidade de espécies de estreptococos orais é atualmente associada ao pH ótimo para atividade e aumento da atividade de enzimas ATPases de membrana. A função da enzima F_1F_0 - ATPase em *S. mutans* é regulada pelos valores do pH citoplasmático com o propósito de manter estável o pH no interior da célula (BENDER & MARQUIS, 1987; DASPHER & REYNOLDS, 1992; SMITH *et al*., 1996). Quando culturas de *S. mutans* tornam-se acidificadas, esses organismos aumentam os níveis de produção e atividade da enzima F_1F_0 - ATPase, a fim de remover prótons do seu citoplasma e proteger relativamente enzimas glicolíticas e ácido-sensíveis (BELLI & MARQUIS, 1991; HAMILTON & BUCKLEY, 1991; KUHNERT & QUIVEY, 2003). Este

aumento na atividade das ATPases indica que estes microrganismos possuem a capacidade de induzir uma resposta ao crescimento em valores de baixo pH, entretanto os processos de regulação desta resposta não são ainda bem conhecidos.

QUIVEY e colaboradores (2000) demonstraram o aumento de aproximadamente 2 a 3 vezes nos níveis de transcrição dos genes codificadores das subunidades ATPases, estando esta indução de acordo com o aumento da atividade enzimática observada em *S. mutans* expostos ao estresse ácido (BELLI E MARQUIS, 1991). Em *Streptococcus pneumoniae* (MARTIN-GALIANO *et al*., 2001), o aumento da atividade da enzima ATPase e o aumento da produção das subunidades α e β, foi acompanhado pela indução de transcritos atp-específicos em valores de baixo pH, fato este também observado em *Lactobacillus acidophilus* (KULLEN & KLAENHAMMER, 1999). Entretanto, a tolerância ácida das bactérias *S. cerevisiae* e *Enterecocco hirae* (ARIKADO *et al*., 1999) demonstrada pela maior atividade das enzimas ATPases, parece ser relacionada à fase de translocação proteíca e não ao aumento da transcrição dos genes codificadores destas enzimas.

A discussão a respeito da tolerância ácida em estreptococos orais deve também incluir os meios pelos quais estes organismos metabolizam os açúcares em valores de baixo pH. Durante períodos em que os açúcares encontram-se praticamente ausentes na cavidade bucal, o sistema primário de transporte de açúcares fosfotransferase-fosfoenolpiruvato dependente (pep-PTS) age como um mecanismo eficiente para captação de nutrientes específicos e necessários ao crescimento bacteriano (POSTMA *et al*., 1993; SAIER *et al*., 1996; VADEBONCOEUR & PELLETIER, 1997). O PTS consiste de duas proteínas comuns a todos os substratos, enzima I (EI) e a proteína fosfocarreadora HPr, assim como uma variedade de permeases açúcar-específicas, conhecidas como complexo EII, que catalizam o transporte e concomitante fosforilação de um determinado substrato, favorecendo sua metabolização no interior da célula bacteriana.

Grande parte das informações atuais sobre a estrutura dos PTS são provenientes de estudos com *Escherichia coli* e *Salmonella typhimurium*, e particularmente com respeito as enzimas açúcar-específicas, EII (POSTMA *et al*., 1993). Com relação à espécie *S. mutans*, as informações atuais dizem respeito ao arranjo genético da região codificadora de componentes do PTS associados ao transporte de sacarose (SATO *et al*., 1989), manitol (HONEYMAN & CURTIS, 1992) e lactose (ROSEY & STEWART, 1992). Os mecanismos que regulam a atividade da enzima EII estão ainda sendo investigados, entretanto, recentes dados indicam que a proteína também referida como $IIAB^{Man}$ e a proteina HPr, são componentes regulatórios chave para o sistema PTS (VADEBONCOEUR & PELLETIER, 1997), os quais permitem as bactérias selecionar açúcares prontamente metabolizáveis, como a glicose e frutose, frente a carboidratos não prontamente metabolizáveis.

Transporte e fosforilação de açúcares não parecem ser as únicas funções do PTS. O PTS pode também desempenhar um papel em vários aspectos da fisiologia celular bacteriana, como a repressão catabólica (HUECK & HILLEN, 1995; SAIER *et al*., 1996). Quando na presença de um açúcar preferencial, usualmente um açúcar-PTS, o metabolismo de açúcares não preferenciais será provavelmente repremido (VADEBONCOEUR & PELLETIER, 1997; ABRANCHES *et al*., 2003). Muitos exemplos têm sido discutidos na literatura indicando que a glicose e a frutose, dois açúcares do sistema PTS-manose constitutivo, são substratos preferenciais (VADEBONCOEUR & TRAHAN, 1983; LIBERMAN & BLEIWEIS, 1984). A glicose pode ser também transportada pelo PTS-glicose, que reconhece outros açúcares, como a manose, a frutose e o açúcar não prontamente metabolizável 2-dioxiglucose (LIBERMAN & BLEIWEIS, 1984).

Estudos com a cepa *S. mutans* Ingbritt (HAMILTON *et al*., 1989; VADEBONCOEUR *et al*., 1987;1991) demonstraram que em valores de pH próximos a 5, o PTS-glicose é reprimido. Enquanto as proteínas gerais EI e HPr são relativamente imunes às modificações das condições

ambientais, a repressão do PTS-glicose foi associada com a redução da síntese da enzima de membrana para transporte da glicose, EII^{Glc} (CVITKOVITCH *et al*., 1995). Desta forma, o pH parece possuir um forte efeito na transcrição de uma proteína que participa diretamente do metabolismo de açúcares preferenciais. Estudos utilizando culturas contínuas de *S. mutans* têm demonstrado que o PTS-glicose pode também ser reprimido por outras modificações no meio, como exemplo, aumento da taxa de crescimento celular, condições limitantes de sacarose ou mesmo excesso de glicose. A versatilidade dos estreptococos expressa um sistema alternativo não-PTS, que parece funcionar para a fermentação da glicose sob estas condições adversas (ELWOOD & HAMILTON, 1982; CVITKOVITCH *et al*., 1995). A natureza deste sistema e suas características, incluindo circuitos regulatórios e controladores da síntese de proteínas, ainda não foram elucidados. Estudos futuros sobre os efeitos da regulação global na expressão gênica e fisiologia de estreptococos orais durante o crescimento em glicose e outros açúcares são necessários para a compreensão das bases dos diferentes caminhos catabólicos em estreptococos orais.

3. Capítulos

3.1. Capítulo 1

"Oral Bacteria Submitted To Environmental Stress"*

Mini-review

NASCIMENTO, MM[1]; HÖFLING, JF[1]; GONÇALVES, RB [1]

Department of Oral Diagnostics, Dental School of Piracicaba, University of Campinas, Piracicaba, São Paulo, Brazil, 13414.018[1]

Correspondence to:
Reginaldo Bruno Gonçalves
901 Limeira Av., Piracicaba, SP, Brazil
Zip code: 13414-900
Telephone number: +55 19 3412-5379
Facsimile: +55 19 3412-5218
E-mail: rgoncalves@fop.unicamp.br

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ABSTRACT

To colonize and cause disease, oral pathogens must overcome some environmental challenges. These variations and the wide range of micro environments in the oral cavity require great adaptability and strong protective systems. It is now well established that different stressors such as cold, heat, starvation or acid shock initiate stress responses which may share not only common characteristics but also have unique characteristics for each type of stressor. Genomic and proteomic approaches have been able to demonstrate differences in gene and protein expression of significant virulence determinants of the oral pathogens subjected to stress. In the present review, we address the current knowledge regarding the physiology and gene expressions of oral bacteria submitted to environmental insults and discuss their possible contributions to the pathogenesis of oral disease.

INTRODUCTION

Biofilm is the most common mode of growth for bacteria in nature, and organisms on surfaces can differ fundamentally from planktonic cultures in terms of their physiology patterns, gene expression and sensitivity to antimicrobial agents[20, 25, 26, 37]. In the oral cavity, bacteria colonize tooth surfaces and form dental plaque, a typical biofilm, to survive to environmental variations, such as aeration, elevation of temperature following the onset of inflammation, reduced pH after intake of dietary sugars and the availability of essential nutrients [5, 10, 11, 12, 33]. The requirement for adaptation to the oral environment has forced bacteria to develop a series of complex responses to stress. These responses may result in altered expression of some virulence determinants or expression of new properties influencing the progress of the oral diseases.

In general, bacteria respond to adverse environmental conditions by regulating the transcription of stress genes which result in the synthesis of numerous stress proteins, including nucleotide-binding proteins, transport proteins, chaperone proteins and enzymes such as F_1F_0 - ATPases[1, 13, 45]. One class of these proteins is specific to the particular type of stress that has been imposed; the second is a diverse family of general stress proteins that are expressed in response to a wide variety of environmental pressures, characterizing the cross-protection[37]. The cross-protection often induced by the expression of a given adaptive response can be advantageous when cells are exposed to a combination of stresses. However, the cross-protection associated with the adaptive response appears to vary between species, suggesting that the molecular bases of adaptive responses are species specific[33, 38, 39].

ACID STRESS AND DENTAL CARIES

The current knowledge on the environmental stress responses in lactic acid bacteria, such as oral streptococci and lactobacilli, varies between species and depends on the type of stress[5, 19, 22, 38]. The growth of these bacteria is characterized by the generation of acidic end products of fermentation that accumulate in the extra cellular environment, resulting in enamel demineralization and carious lesion formation. The fundamental hypothesis is that dental caries are associated with a succession of specific microbial communities, generated by, and responding to, oral environments. Species may differ in respect to the efficiency of their uptake and catabolism of dietary carbohydrates[15, 40] or in their abilities to survive and grow in low pH environments[11]. The acidogenicity and aciduricity, the abilities to produce acid and to grow under conditions of low pH, respectively, are considered important virulence determinants for bacteria associated with the initiation and progression of caries, such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus* species and certain non-mutans streptococci[3, 42].

Although the effects of acid stress on bacterial physiology are not known in detail, it is possible to enlighten some resistance mechanisms which address negative effects of the acid stress. *S. mutans* is able to mount an acid tolerance response (ATR), and recent works suggest that this organism has both constitutive and acid-inducible mechanisms that act to enhance its ability to tolerate rapid pH drops[9, 18]. The ATR is characterized by increased acid resistance, enhanced glycolitic capacities, and increased activity of the proton-translocating enzyme F_1F_0-ATPase[1, 10, 32]. The ATR has also been found to confer cross-protection against multiple environmental insults, including UV radiation and oxidative stress[32]. All these features together help explain its remarkable competitiveness and ability to survive under different types of environmental stress.

In response to low pH, adaptive changes which are important for maintaining the activity of membrane ATPases also occur in bacterial membranes. Studies demonstrated that the insertion inactivation of *ffh* gene, which plays a role in the localization of membrane proteins, results in acid sensitivity[9]. The adaptation of *S. mutans* to acidification also includes enrichment in mono-unsaturated and longer chain fatty acids in the membrane which could cause the reduction of permeability to protons observed in acid adapted cells[23, 31]. In *L. lactis*, the inactivation of certain penicillin-binding proteins involved in peptidoglycan synthesis increases the acid sensitivity[33]. These data suggest that cell wall composition could also have a role in aciduricity of oral bacteria, possibly through alteration of the cell surface properties.

Other mechanisms for pH homeostasis are the arginine deiminase pathway, ADI[8], and the urease activity[30]. The ADI pathway has been detected in several lactic acid bacteria but its direct involvement in acid tolerance was not always demonstrated. The urease activity was detected both in *S. thermophilus* and in the oral bacteria *S. salivarius* in which it has been mostly studied[32]. The nickel metallo enzyme urease catalyses the hydrolysis of urea to CO_2 and ammonia and it can alkalize the environmental pH. Since urea is present in the saliva, ureolysis is probably

important for the survival of *S. salivarius* under the extreme acidic conditions that can be generated by aciduric bacteria.

The tolerance to environmental insults by cariogenic bacteria can also be characterized by the production of a number of stress proteins called heat shock proteins (HSPs), including the molecular chaperones GroEL and DnaK[14]. Protein folding and the assembly of multimeric structures *in vivo* is not a completely spontaneous process but is facilitated by these proteins. Molecular chaperones bind transiently and noncovalently nascent polypeptides and unfolded or unassembled proteins, aiding in protein biogenesis in two general ways: they block nonproductive protein-protein interactions, and they mediate the folding intermediates, allowing the concerted folding by domains and assembly of oligomers[24]. Additionally, DnaK protein plays a critical role in regulation of the response of bacteria to stress through modulation of the stability of transcriptional factors controlling stress gene expression[13, 16, 35].

Molecular chaperones seem to interact with the glycolitic enzymes at low pH increasing their stability in the presence of an acid challenge[44]. It was previously shown that the rapid acidification of steady-state cultures of *S. mutans* growing at neutral pH resulted in increases in the levels of *dnaK* mRNA and DnaK protein[14, 18], suggesting that DnaK is intimately involved in response to environmental acidification in this oral bacteria. Despite the fact that DnaK chaperone is important for survival under extreme conditions, the role of this protein in acid tolerance has not been defined.

OTHER ORAL ENVIRONMENTAL STRESSES

Among the environmental stress conditions, nutrient starvation is one of the most frequent and bacterial growth itself contributes to nutrient exhaustion and subsequent starvation for one or several compounds. Responses to three types of limiting compounds have largely been studied in bacteria: carbohydrate starvation leading to cell energy depletion, phosphate starvation

which can be deleterious for both energy supply and DNA/RNA synthesis, and nitrogen starvation which primarily results in the limitation of protein synthesis[4]. Clearly the current results indicate a strong regulatory link between the acid stress and carbon/nitrogen starvation responses in *S. mutans*. Earlier results with *S. mutans* H7[36] have demonstrated cross-protection of cells to acid killing by prior exposure of cells to starvation conditions. Carbohydrate-starved cells of *Lactobacillus lactis* IL1403 exhibit enhanced resistance to acid, heat, ethanol, osmotic and oxidative stress with the onset of stationary phase[16].

In order to colonize oral surfaces, the bacteria first have to survive in oral fluids, where they are exposed to oxygen and high oxidation-reduction potentials. Oxygen constitutes a constant challenge for the survival of strict anaerobes in the oral environment. The effect of oxygen have been investigated on the physiology and growth of *Porphyromonas gingivalis*, an anaerobic bacteria strongly implicated in the etiology of adult periodontitis, in a continuous culture system when grown under conditions of hemin limitation and excess. This study might indicate that oxygen stress produces an increased demand for amino acid-derived ATP for the maintenance of internal homeostasis. A certain degree of aerotolerance might be a requirement for all strict anaerobic species present in the oral environment in order to persist as part of the indigenous flora and eventually proliferate in an adequate anaerobic niche. The growth of *P. gingivalis* under oxygenated environments is also accompanied by changes in fermentation end-products, cell morphology, anti-oxidant enzymes and proteinase activity[6].

The ability to tolerate oxidative stress is necessary for growth of bacteria in aerobic environments. In facultative anaerobic bacteria, rapid and efficient oxidative stress response mechanisms are necessary to enable growth and survival under aerobic or reactive oxidant challenges. One of the most important enzymes in oxidative stress tolerance is superoxide dismutase, SOD, and there is now substantial agreement that manganese-SOD acts as a major protection against toxic anions in streptococci[12]. This enzyme is encoded by the *sodA* gene that

was shown to be present in at least 28 species of streptococci[7, 30]. It was suggested that a complex Mn^{+2}-dependent regulatory system operates in the control of the oxidative stress response in *S. gordonii*. These observations would also suitably account for the defects in virulence of Mn^{+2} permease mutants of *Streptococcus mutans*, *S. parasanguis* and *S. pneumoniae*[12].

METHODOLOGIES

Proteomic, genomic and traditional microbiology approaches have been employed to analyze bacteria exposed to environmental stress. To study factors that may cause such deleterious shifts in the oral microflora, a number of laboratory model systems has been developed. Several areas that are being addressed include: the effect of stress on bacterial lipopolysaccharide, cell morphology, membrane integrity, and virulence; the effect of these stresses on gene expression and the role of stress adaptation on cross-protection to subsequent stresses.

The methodologies employed to investigate the response of oral bacteria to environmental stress are diverse, but many rely on conventional biochemical techniques for the measurement of particular enzymatic activities or on molecular approaches. Mixed culture chemostat techniques have proved to be of particular value because specific parameters can be varied independently, allowing cause-and-effect relationships to be determined[13, 14, 27]. Numerous studies have also used the high resolving power of two-dimensional polyacrylamide electrophoresis (2-D electrophoresis) to monitor changes in protein expression during the adaptation of microorganisms to environmental stress[2, 38, 37]. A comprehensive analysis of multiple-stress responses by this methodology confirmed that *S. mutans* responded to environmental stresses such as oxidation, heat, acidity, high salt, by specific or coordinated regulation at protein levels[21, 36].

Dissecting the pathways used to form stable biofilms and to tolerate environmental stress can help to understand the virulence of oral bacteria[26, 28, 41]. Biofilm cell density has been shown to modulate adaptation to acid tolerance at low pH, such that high cell density biofilms were more resistant to lethal pH values than those with lower cell densities[21]. Understanding the mechanisms that bacteria have evolved to withstand stresses found in nature is essential for effectively controlling or eliminating of these pathogens. Since these processes are not well understood, an elucidation of the physiological and genetic factors that induce tolerance of bacterial pathogens to stressful environments and improved methods for detecting stressed or injured bacterial pathogens are needed.

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3.2. Capítulo 2

"THE ADAPTIVE ACID TOLERANCE RESPONSE OF *STREPTOCOCCUS SOBRINUS*"♦

Marcelle M. Nascimento [1,2], José A. C. Lemos[2], Jacqueline Abranches[2], Reginaldo B. Gonçalves[1] and Robert A. Burne[2]*

Department of Oral Diagnostics, Dental School of Piracicaba, University of Campinas, Piracicaba, São Paulo, Brazil, 13414.018[1], Department of Oral Biology, University of Florida College of Dentistry, Gainesville, Florida, USA, 32610[2]

Running Title: Low pH-induced acid resistance

*** Corresponding author:**

Mailing address: Department of Oral Biology, University of Florida, College of Dentistry.
P.O. Box 100424, Gainesville, FL 32610
Phone: (352) 392-4370
Fax: (352) 392-7357
E-mail: rburne@dental.ufl.edu

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ABSTRACT

Streptococcus mutans and *Streptococcus sobrinus* are the bacteria most commonly associated with human dental caries. A major virulence attribute of these and other cariogenic bacteria is acid tolerance. The acid tolerance mechanisms of *S. mutans* have begun to be investigated in detail, including the adaptive acid tolerance response (ATR), but this is not the case for *S. sobrinus*. An analysis of the ATR of two *S. sobrinus* strains was conducted in cells grown to steady-state in continuous chemostat cultures. Compared with cells grown at neutral pH, *S. sobrinus* grown at pH 5.0 showed an increased resistance to acid killing and were able to drive down the pH through glycolysis to lower values. Unlike in *S. mutans*, the enhanced acid tolerance and glycolytic capacities of acid-adapted *S. sobrinus* were not due to increased FATPase activities. Interestingly though, *S. sobrinus* cells grown at pH 5.0 had 2-fold more glucose sugar:phosphotransferase (PTS) activity than cells grown at pH 7.0. In contrast, glucose PTS activity was actually higher in *S. mutans* grown at pH 7.0 than in cells grown at pH 5.0. Silver staining of two-dimensional gels of whole-cell lysates of *S. sobrinus* 6715 revealed that at least 9 proteins were up-regulated and 22 proteins downregulated in pH 5.0-grown cells as compared with cells grown at pH 7.0. Our results demonstrate that *S. sobrinus* is capable of mounting an ATR, but there are critical differences between the mechanisms of acid adaptation used by *S. sobrinus* and *S. mutans*.

INTRODUCTION

The oral cavity is a dynamic environment that undergoes rapid and often substantial changes in pH, nutrient availability, carbohydrate source and oxygen tension. The capacity of oral bacteria to tolerate acidic environments is of major importance in the ecology of dental plaque and is directly related to the pathogenesis of dental caries. Two species of mutans streptococci, *S. mutans* and *S. sobrinus*, have been implicated as etiologic agents of dental caries when elevated

proportions of these organisms were detected in dental plaque from caries active subjects (15, 32). *S. mutans* and *S. sobrinus*, which can grow and continue to carry out glycolysis at low pH, are thought to gain a competitive advantage over other plaque bacteria during the periods of sustained acidification that are conducive to caries development.

Acid tolerance by *S. mutans* has been studied in some detail and it is established that this organism possesses an inherent acid resistance that distinguishes them from organisms not commonly associated with dental caries. *S. mutans* can grow and carry out glycolysis at pH values below 5.0, and can drive the pH to values well below 4.0 (3). The aciduricity of these organisms has been attributed in large part to the possession of a proton-extruding F-ATPase that is expressed at higher levels than many oral bacteria and that functions well at pH values of 5.0 and below – allowing the organisms to maintain adequate ∆pH when the external pH falls to 4.0 and lower. In addition to having constitutive acid tolerance properties, *S. mutans* is also capable of mounting an adaptive acid tolerance response (ATR). Adaptation to growth in moderately acidic conditions renders the organisms less susceptible to lethal acidification and is associated with enhanced glycolytic capacities and increased activity of the protontranslocating F-ATPase (3, 16). Several other mechanisms of resistance to acid stress have been identified in *S. mutans*, including the induction of stress proteins (23, 30), DNA repair enzymes (14, 20), changes in the cell membrane composition (12, 36), an H^+-glucose symporter that operates at pH 5.0 (8), and recently, an ammonia/polyamine generating pathway (13). Moreover, response regulators and quorum sensing genes have also been shown to play a role in acid adaptation by *S. mutans* (31, 48). Although *S. sobrinus* is considered to be the most acidogenic bacteria among the oral streptococci (9, 10, 21), very little is known of the responses of this organism to environmental acidification. While it is known that the organisms produce relatively high levels of F-ATPase, other acid resistance mechanisms have not been explored. In a previous study, mid-exponential phase batch cultures of *S. sobrinus* CH125/43 were unable to induce an ATR after a 2-h incubation in

medium buffered at an acidic, non-lethal pH value (43). In the same study, three different strains of *S. mutans* were shown to induce a strong ATR when grown under the same conditions. From this work, emerged the current concept that *S. mutans* is capable of inducing a strong ATR and that *S. sobrinus*, although relatively acid-tolerant, is unable to mount an ATR. Other than the ATR, the tacit assumption has been that the acid tolerance mechanisms of *S. sobrinus* are the same as those in *S. mutans*.

The ability of oral streptococci to efficiently transport and metabolize a wide variety of sugars, especially at low pH, is another characteristic that allows these bacteria to grow and persist in the mouth and is directly linked to their ability to elicit dental caries. The phosphoenolpyruvate:sugar phosphotransferase system (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 (33, 35, 38, 41, 45). The PTS consists of two proteins that are common to all PTS substrates, Enzyme I (EI) and the heat-stable phosphocarrier protein Hpr, as well as a variety of sugar-specific permeases, known as EII complexes, that catalyze the transport and concomitant phosphorylation of their cognate substrates. Previous research with *S. mutans* strain Ingbritt has demonstrated that glucose-PTS activity is markedly decreased in cells grown at pH 5.5, compared to those grown at neutral pH values, and it was suggested that repression occurs at the level of EII synthesis (46, 47). It is believed that an H^+-glucose symport system may be the primary transporter for glucose at low pH (8). Thus, while the PTS is believed to be critical for high-affinity and high-capacity transport of carbohydrate and a major contributor to acidogenesis, its role in acid tolerance is not established.

Continuous chemostat cultures is considered to be particularly advantageous for studies of acid tolerance and carbohydrate metabolism specifically because steady-state cultures that are grown at different pH values do not differ in growth rate, availability of nutrients, growth phase, and other important physiological parameters. By using chemostats to tightly control the physiology of

the organisms, one can effectively rule out the possibility that variables other than pH are influencing the phenotypic properties. This has been demonstrated repeatedly in studies with *S. mutans*. The purpose of this report was to conduct a more detailed analysis of the responses of *S. sobrinus* to environmental acidification. The results indicate that *S. sobrinus* does indeed possess an ATR, but that there are critical differences in the molecular mechanisms of acid adaptation by *S. sobrinus* and *S. mutans*.

MATERIAL AND METHODS

Bacterial strains and growth conditions. *S. sobrinus* 6715 and SL1 and *S. mutans* UA159 were routinely grown in brain heart infusion (BHI) media in a 5% CO_2 aerobic atmosphere at 37°C. To investigate the responses of *S. sobrinus* and *S. mutans* to acid shock and acid adaptation, cells were grown in a BioFloIII chemostat (New Brunswick Scientific, Edison, NJ) with a working volume of 600 ml in TY base medium (3% tryptone, 0.5% yeast extract) containing 25 mM glucose, as previously described (23). The dilution rate of the culture was 0.3 h^{-1}, corresponding to a generation time of 2.3 hours, and the pH of the vessel was maintained by addition of 1 N KOH. Cells growing at steady-state at pH 7.0 were acid shocked from pH 7.0 to pH 5.0 using 1 N HCl. The total time for titration of the vessel to pH 5 was about 2 minutes. Subsequently, cells were grown for a minimum of 10 generations to achieve steady-state at pH 5.0. Samples were obtained from the chemostat by aspiration from the vessel into ice-cold tubes at selected time points. Cells were collected immediately by centrifugation at 4°C and subjected to acid killing and pH drop experiments, or quick-frozen in a dry ice-ethanol bath and stored at -80°C for further analysis ***(Apêndices)***.

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). Samples were stirred continuously at room temperature and aliquots of cells were removed at 30,

60 and 90 min. Cells were serially diluted, plated on BHI-agar plates, and incubated at 37°C for two days before colonies were counted. Cell viability at each time point was expressed as the percentage of viable cells (CFU ml^{-1}) at time zero. The ability of *S. sobrinus* and *S. mutans* strains to lower the pH through glycolysis was monitored as described by Belli and Marquis (3). Briefly, cells from steady-state chemostat cultures were harvested, washed with 1 culture volume of cold distilled 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 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 a 50 minutes period ***(Apêndices)***.

Biochemical assays. For F-ATPase assays, cells collected from the chemostat were permeabilized with toluene and incubated with 5 mM ATP in ATPase buffer, as previously described (3). Samples were removed at various intervals and assayed for inorganic phosphate released from ATP using the Fiske-Subbarow reagent (Sigma, St. Louis, Mo.). 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.* (28). Protein concentrations were determined using a bicinchoninic acid assay (Sigma) with bovine serum albumin as the standard ***(Apêndices)***.

DNA methods. Chromosomal DNA was prepared from *S. sobrinus* 6715 as previously described (5). Sequences used to create oligonucleotide primers were obtained from the *S. sobrinus* genome sequencing project at The Institute for Genomic Research (TIGR). The following primers were used: *dnaK* sense: 5'- GGTGGTGTCATCACTG -3'; *dnaK* antisense: 5'- GCAGCATCACGTTCAG - 3'; *atpB* sense: 5'- CCTCATCAGAAATCTTGG -3'; *atpB* antisense: 5'- CGAAAGCTGTCGCTG - 3';

ptsG sense: 5'- TGGGTGATGGCTTCGCTGTTC-3'; *ptsG* antisense: 5'- AGGAGCCCATAAGCGTGTTTCG -3'. PCRs were carried out with 100 ng of chromosomal DNA using Taq DNA polymerase, and PCR products were purified using the QIAquick kit (QIAGEN) ***(Apêndices)***.

RNA methods. Cell lysates were obtained by mechanical disruption with a Bead Beater (BioSpec, Bartlesville, Okla.) in the presence of chilled glass beads (0.1 mm diameter) for 3 cycles of 30 s, with colling on ice for 1 min during the intervals. RNA was extracted from *S. sobrinus* and *S. mutans* chemostat-grown cells as described by Chen et al. (7). For quantitative slot blot analysis, equivalent amounts of denatured RNA were transferred to nylon membranes (GeneScreen Plus; NEN Life Science products, Inc., 8 Boston, Mass) by a slot blot apparatus (LTI) as described elsewhere (39). RNAs were UV cross-linked to the membranes, and filter membranes were probed with internal fragments of the genes of interest that had been labeled with psoralen-biotin using the Brightstar labeling kit (Ambion, Inc., Austin, Tex.). Hybridizations and washes were carried out under high-stringency conditions. Signals obtained on autoradiographs were quantified by densitometry using an IS1000 digital imaging system (Alpha Innotech Corp., San Leandro, Calif.) ***(Apêndices)***.

Western blot and two-dimensional gels. Cells were harvested from the chemostat and collected by centrifugation, washed twice with Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 0.9% NaCl), and suspended in SDS boiling buffer (60 mM Tris- HCl, pH 6.8, 5% sodium dodecyl sulfate and 10% glycerol). Cell lysates were obtained by mechanical disruption with a Bead Beater. Protein lysates (10 µg) were subjected to SDS-PAGE, blotted to Immunobilon-P membranes (Millipore, Bedford, Mass.) and analyzed by Western blotting (2). Membranes were incubated with antibodies raised against the *S. pyogenes* DnaK and GroEL proteins (29), or the *S. salivarius* EI or HPr proteins, at dilutions of 1:1000, 1:500, 1:200 or 1:200, respectively, in TBS. Antibodies against

EI and Hpr were a gift from C. Vadenbocoeur, Universite Laval, Quebec, Canada. Immune reactivity was detected by incubation with peroxidase-conjugated goat anti-rabbit IgG followed by detection with 4-chloro-1-naphthol. Two-dimensional (2-D) gel electrophoresis was performed at Kendrick Labs, Inc. (Madison, Wis.) following the protocol described by O'Farrell (34) and the gels were stained with silver. Densitometric analysis was used to compare the intensities of the spots (IS1000 digital imaging system, Alpha Innotech Corp., San Leandro, Calif.) ***(Apêndices)***.

RESULTS

Acid adaptive response of *S. sobrinus*. To determine if *S. sobrinus* had the ability to mount an ATR, acid killing and pH drop experiments were performed with cells obtained from steady-state chemostat cultures. To confirm that the growth conditions were adequate to induce an ATR, 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 *S. sobrinus* 6715 and SL1 and *S. mutans* UA159 were subjected to acid killing at pH 2.8. After 90 minutes of incubation, all of the organisms showed an increase of at least 1-log in the number of survivors (Fig. 1 and Table 1). In pH drop experiments, cells grown at pH 5.0 were able to decrease the pH through glycolysis to values lower than cells grown at pH 7.0 (Fig. 2 and Table 1). In cells growing at pH 7.0 steady state, *S. sobrinus* 6715, *S. sobrinus* SL1 and *S. mutans* UA159 lowered the pH to 3.57 (± 0.09), 3.65 (± 0.02) and 3.52 (± 0.1), respectively, after 50 minutes. When cells cultured at pH 5.0 were used, the final pH values attained in the pH drops by the same strains were 3.21 (± 0.03), 3.15 (± 0.09) and 3.29 (± 0.18), respectively (Table 1).

Contribution of F-ATPases to acid tolerance by *S. sobrinus* and *S. mutans*. Previous data have shown that acid adaptation of *S. mutans* is correlated with an increase in proton-extruding F-ATPase activity (3, 16). Indeed, data from this 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. In contrast, there was no significant increase in the F-ATPase activity of the *S. sobrinus* strains when grown at pH 5.0 (Table 2). It has been suggested that increases in F-ATPase activity correlate with increased transcription of the F-ATPase operon (37). Quantitative slot blots of total RNA were used to test whether the levels of F-ATPase activity between *S. mutans* UA159 and *S. sobrinus* 6715 and SL1 correlated with mRNA for the F-ATPase genes. An internal fragment of the *S. sobrinus atpB* gene, which encodes for the ATP synthase subunit of the F-ATPase enzyme, was used as a probe. In *S. mutans*, there was a 2.4-fold increase of *atpB* mRNA at pH 5.0, whereas in *S. sobrinus* 6715 and SL1 the transcriptional levels of *atpB* were not affected by growth pH (Fig. 3).

PTS activity in *S. sobrinus* and *S. mutans*. The glucose-PTS activities of chemostat-grown *S. sobrinus* and *S. mutans* cells were assessed. In agreement with previous results (17), the *S. mutans* glucose-specific PTS was repressed by 50% at pH 5.0, compared to cells grown at pH 7.0. Interestingly, glucose-specific PTS activity in both *S. sobrinus* strains was higher in cells grown at pH 5.0 compared to pH 7.0, particularly in strain SL1, which showed a 2.6-fold induction at pH 5 (Table 3). It has been demonstrated that the levels of the Hpr and EI proteins are not affected by changes in some environmental conditions, whereas synthesis of the sugar-specific EII enzymes can be downregulated at low pH (47). Western blot analysis using antibodies raised against the Hpr and EI proteins from *S. salivarius* confirmed that the levels of these proteins are not affected by pH in *S. sobrinus* 6715 (data not shown). On the other hand, slot blot analysis using a *ptsG*-specific probe, which encodes the glucose PTS permease EIIGlc, showed that the *S. sobrinus ptsG* was induced after 60 minutes of acid shock (titration of cells from pH 7 to pH 5 with dilute HCl over the course of 2 minutes) and remained elevated in pH 5 steady-state cells (Fig. 4).

Studies with *S. mutans* have also demonstrated that sugar transport by other PTS permeases was repressed by low pH and that this repression was associated with reduced activity of the corresponding sugar-specific EII enzymes (47). To investigate whether the divergent regulation of the glucose-PTS in *S. sobrinus* and *S. mutans* by pH was restricted to glucose

transport, mannose and fructose PTS activity was measured. In *S. mutans*, mannose- and fructose-PTS activities were reduced at low pH, but there were no alterations in the levels transport of these sugars in *S. sobrinus* 6715 or SL1 (data not shown).

Protein expression patterns of chemostat-grown cells. 2-D electrophoresis was used to compare proteins from pH 7.0 and pH 5.0 steady-state cultures of *S. sobrinus* 6715 cells. Using silver staining of the gels, it was found that at least 9 proteins were upregulated and 22 proteins were downregulated in cells grown at pH 5.0 (Fig. 5), compared to cells grown at pH 7.0. In *S. mutans* UA159, the heat shock proteins GroEL and DnaK were upregulated after acid shock (23, 30). Slot blot analysis revealed that the levels of *S. sobrinus dnaK* mRNA were increased about 3-fold when pH 7.0, steady-state cultures were submitted to acid shock, but that there were no significant differences in the *dnaK* mRNA levels in steady-state cells grown at pH 7.0 or pH 5.0 (Fig. 6A). Western blotting confirmed that the levels of DnaK were elevated following acid shock, but returned to basal levels in pH 5.0, steady-state cultures (Fig. 6B). Similar results were obtained in Western blot analyses using an anti-GroEL antibody (Fig. 7).

TABLES

Table 1. Acid-adaptive response of *S. sobrinus* 6715, SL1 and *S. mutans* UA159.

Organism	Growth pH	50 min glycolytic pH (final pH)	% survival after 90 min (± standard deviation)
S. sobrinus 6715	7.0	3.57 (±0.09)	2.5×10^{-3} (1.0×10^{-3})
	5.0	3.21 (±0.03)	4.9×10^{-1} (2.7×10^{-1})
S. sobrinus SL1	7.0	3.65 (±0.02)	3.8×10^{-2} (1.0×10^{-2})
	5.0	3.15 (±0.09)	4.7×10^{-1} (0.9×10^{-1})
S. mutans UA 159	7.0	3.52 (±0.10)	9.0×10^{-3} (1.0×10^{-3})
	5.0	3.29 (±0.18)	3.8×10^{-1} (1.8×10^{-1})

The results are the means and standard deviations of at least three independent experiments

Table 2. F-ATPase activity of *Streptococcus sobrinus* 6715 and SL1 and *Streptococcus mutans* UA 159 grown in chemostat cultures.

Organism	Growth pH	ATPase activity $\mu mol\ min^{-1}$ $(mg\ protein)^{-1}$	Fold induction
S. sobrinus 6715	7.0	21 (±3)	1.0
	5.0	23 (±6.5)	1.0
S. sobrinus SL1	7.0	19 (±1)	1.0
	5.0	23 (±12)	1.2
S. mutans UA 159	7.0	20 (±12)	1.0
	5.0	43 (±3)	2.1

The results are the means and standard deviations of at least three independent experiments

Table 3. Glucose specific PTS activity of *S. sobrinus* 6715 and SL1 and *S. mutans* UA 159 grown in the chemostat.

Organism	Growth pH	PTS-glucose activity	Fold induction
S. sobrinus 6715	7.0	83 (±30)	1.0
	5.0	129 (±44)	1.6
S. sobrinus SL1	7.0	73 (±38)	1.0
	5.0	197(±88)	2.7
S. mutans UA 159	7.0	84 (±35)	1.0
	5.0	42 (±17)	0.5

The results are the means and standard deviations of at least three independent experiments

FIGURES

Figure 1

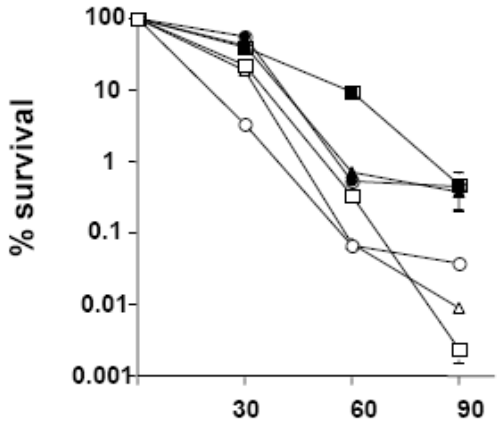

Figure 1. Survival of *S. sobrinus* 6715 (squares), SL1 (circles) and *S. mutans* UA159 (triangles) during acid challenge. Cells were collected from a steady-state chemostat held at pH 7.0 (open symbols) or pH 5.0 (closed symbols) and subjected to acid killing at pH 2.85. Cell viability at each time point is expressed as the percentage of viable cells (CFU ml^{-1} of culture) at time zero.

Figure 2

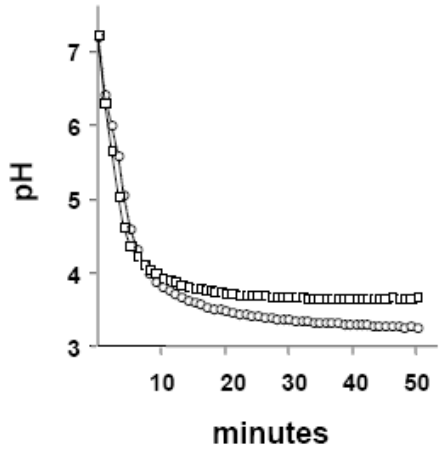

Figure 2. Glycolytic pH profile of *S. sobrinus* 6715. Cells were collected from a steady-state chemostat held at pH 7.0 (squares) or pH 5.0 (circles), washed once with ice-cold water and resupended in a 50 mM KCl/1 mM $MgCl_2$ solution. Glucose was added to the cell suspension to initiate glycolysis, and pH drops were continuously monitored for 50 minutes. Data points were collected every 10 sec, and data for every 2 min and 30 sec are presented. The graph shown is a representative of 5 independent experiments.

Figure 3

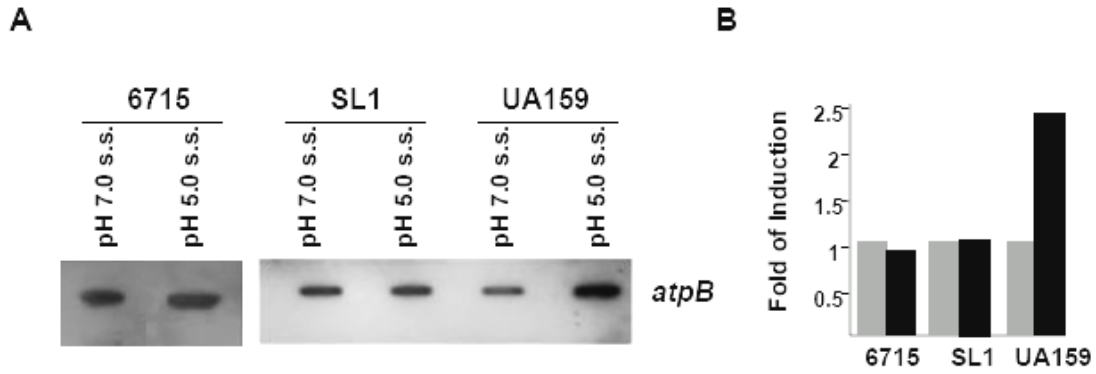

Figure 3. Slot blot analysis of *atpB* mRNA in response to environmental acidification. (A) Total RNA was isolated from *S. sobrinus* 6715, SL1 and *S. mutans* UA159 grown in the chemostat under the conditions indicated in the figure (s.s., steady-state). Hybridization was performed with internal fragments of the *S. sobrinus* and *S. mutans atpB* gene against DNA from the parent organism. RNase-treated samples were used as controls (data not shown). (B) Bar graph of *atpB* mRNA levels as measured by densitometry.

Figure 4

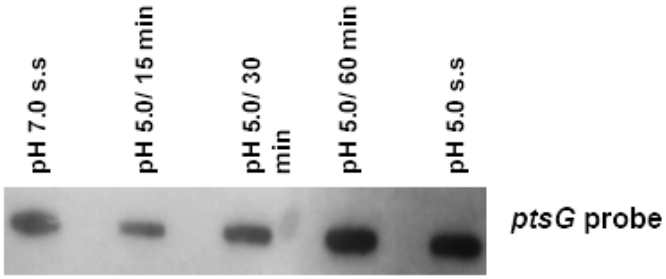

Figure 4. Slot blot analysis of the *S. sobrinus* 6715 *ptsG* gene in response to changes in environmental pH. Total RNA was isolated from *S. sobrinus* 6715 grown in the chemostat under the conditions indicated in the figure (s.s., steady-state). Hybridization was performed with an internal fragment of the *S. sobrinus ptsG* gene. RNase-treated samples were used as controls (data not shown).

Figure 5

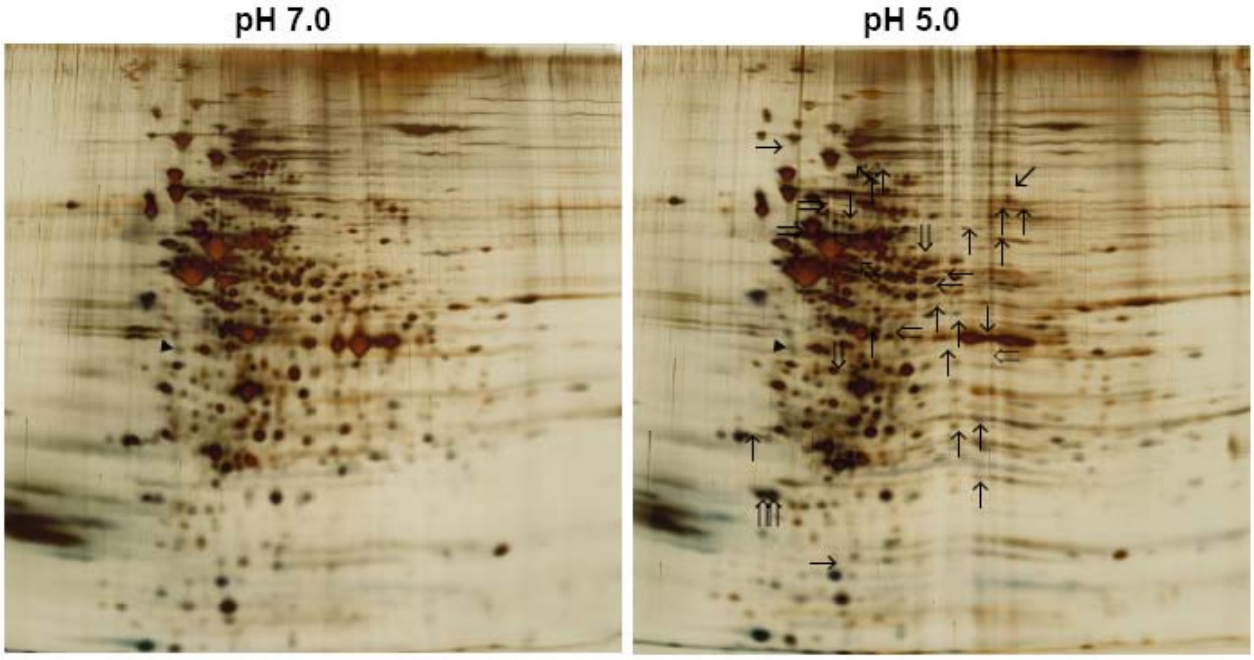

Figure 5. 2D protein patterns of *S. sobrinus* 6715 grown in the chemostat. Proteins with enhance expression at pH 5.0, steady-state are indicated with double-lined arrows and proteins with reduced synthesis are indicated by single-lined arrows. The filled triangle indicates tropomyosin that was loaded on the gel as an internal control.

Figure 6

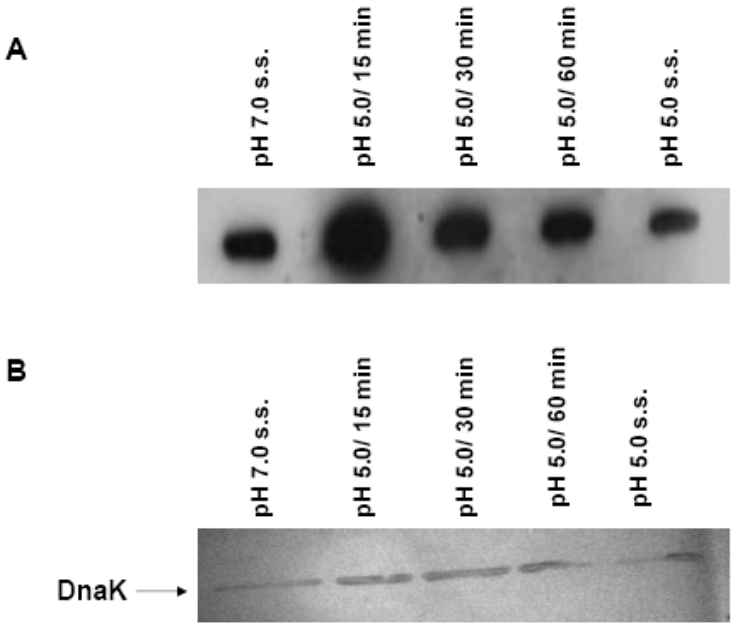

Figure 6. Induction of *S. sobrinus* 6715 *dnaK* mRNA and DnaK in response to environmental acidification. (A) Slot blot of total RNA isolated from cells grown in the chemostat under the conditions indicated in the figure (s.s., steady-state). Hybridization was performed with an internal fragment of the *S. sobrinus dnaK* gene. RNase-treated samples were used as controls (data not shown). (B) Western blot analysis of DnaK with a polyclonal antibody against *S. pyogenes* DnaK (1:1,000).

Figure 7

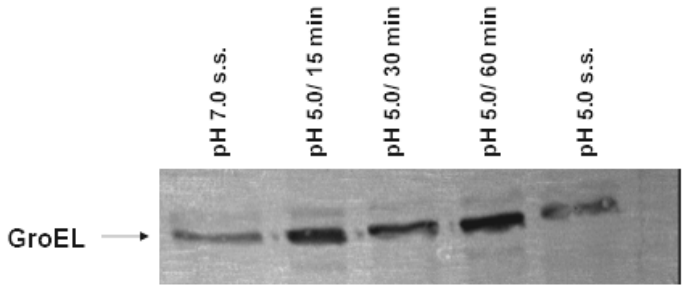

Figure 7. Western blot analysis of *S. sobrinus* 6715 GroEL in response to environmental acidification. Total cell lysates obtained from cells grown in the chemostat under the conditions indicated in the figure (s.s., steady-state) were probed with a polyclonal antibody against *S. pyogenes* GroEL (1:1,000).

DISCUSSION

Tolerance of acidic environments by cariogenic organisms is a critical factor in the ecology of oral biofilms and the pathogenesis of dental caries. *S. mutans* and *S. sobrinus*, along with lactobacilli, are the bacteria most commonly isolated from human dental caries. Some studies have indicated that *S. sobrinus* is more acidogenic and more cariogenic than *S. mutans* (9, 10). Several epidemiological studies found that *S. mutans* is more prevalent than *S. sobrinus* in dental plaque samples (6, 27, 32), although others have shown that high numbers of *S. sobrinus* are more closely associated with caries (10, 11, 22). These apparently contradictory results are likely attributable to heterogeneity among mutans streptococci in their acidogenic and aciduric properties, as well as in study populations, diet and environmental factors. Regardless, *S. mutans* and *S. sobrinus* have consistently been implicated as the primary cariogenic organisms in human dental plaque directly as a result of their ability to tenaciously adhere to the tooth surface and to produce and tolerate copious quantities of organic acids.

The inherent acid tolerance of *S. mutans* and its ability to mount an ATR have been studied in some detail (3, 4, 16), but this is not the case for *S. sobrinus*. A single report classified *S. sobrinus* CH125/43 as a non-acid responder, i.e. unable to induce an ATR, while *S. mutans* Ingbritt and LT11 were considered strong responders (43). The results presented here demonstrate that *S. sobrinus* 6715 and SL1 do indeed have the ability to induce an ATR. The increased resistance to killing at low pH as a result of acid-adaptation during growth at pH 5.0 was of a magnitude comparable to that observed for *S. mutans* UA159. Differences in the results obtained in this study with those of the previously published report (43) are most likely attributable to differences in growth conditions and strains. Most notably, a major difference is that we used steady-state cells grown in continuous chemostat culture instead of batch cultivated cells and buffered media (43). In fact, when we tried to induce an ATR by using a batch culture approach, the enhanced survival of acid-adapted *S. sobrinus* cells was not appreciably different than cells cultivated at neutral pH

(data not shown). We also noted that the ATR induced in *S. mutans* cells in batch culture is not as robust as that from chemostat grown cells. The contrast in the phenotypic properties of steady-state chemostat cells and cells grown batch-wise probably reflect other influences on the physiologic properties of the organisms arising from differences in growth rate, nutrient availability and growth phase in the cells grown in batch culture with buffered media. Another important factor when comparing these studies is that it is generally recognized that significant variations exist in acidogenicity, aciduricity and the ability to elicit caries in experimental animals between different strains of mutans streptococci. *S. sobrinus* 6715 is commonly used in experimental caries models specifically because of its ability to strongly induce both smooth-surface and sulcal caries. Likewise, *S. mutans* UA159 is strongly cariogenic in rats and capable of inducing a potent ATR. The cariogenic properties of *S. sobrinus* CH125/43, a nursing caries isolate, or of strain SL1, have not, to our knowledge, been assessed since the strains were isolated and subjected to laboratory passage. Thus, it is not possible to determine from existing information whether an ATR is a necessary trait for cariogenic microorganisms.

The ability of *S. mutans* to survive environmental acidification appears to be directly related to the membrane-bound, proton-translocating ATPase (F-ATPase) (37). In *S. mutans*, it was demonstrated that a decrease in the external pH causes an increase in F-ATPase activity and that this enhanced activity is correlated with the mounting of an ATR (3, 16, 37). Proton extrusion via the F-ATPase results in an internal pH that is more basic than that of the environment, conferring some protection to acid-sensitive glycolytic enzymes and maintaining ΔpH for bioenergetic processes. Here, the enhanced acid tolerance and glycolytic capacities of pH 5.0-grown *S. sobrinus* could not be attributed to increased F-ATPase activities. Also of note, the enhanced acid resistance of *S. sobrinus* was not correlated with higher constitutive levels of ATPase activity than *S. mutans* or to differences in the optimum pH for enzyme activity. The *S. sobrinus* F-ATPase enzyme was expressed at a level of approximately 20 µmol min^{-1} (mg protein) $^{-}$

[1], essentially identical to the levels found for *S. mutans* UA159. Also, optimal activity at the *S. sobrinus* ATPase was near pH 6.0 (data not shown), similar to what was described for *S. mutans* (42). Collectively, these results reveal a striking and fundamental difference in the mechanisms underlying adaptive acid tolerance in two closely related oral bacteria.

Differences in acid tolerance mechanisms between these two organisms were not restricted to ATPase expression. In *S. mutans* UA159, acid-adapted cells were shown to have increased proportions of monounsaturated membrane fatty acids when compared with unadapted cells grown at pH 7.0 (36). However, in the same study, *S. sobrinus* 6715 showed only minimal changes in its membrane fatty acids composition when grown at low pH. Similarly, a lack of sustained up-regulation of DnaK in *S. sobrinus* growing at pH 5.0, in contrast to what was been observed in *S. mutans* (23), is yet another major difference in the behavior of these organisms during acid adaptation. The lack of up-regulation of the DnaK chaperone also indicates that the cells are not perceiving a stress condition when grown at pH 5.0, indicating that the cells are maintaining an adequate ΔpH to avoid protein denaturation. Thus, *S. sobrinus* must possess other mechanisms to reduce proton permeability or to prevent acid damage to the cells.

Among the more intriguing findings in our results is the linkage of elevated glucose PTS activity with enhanced acid resistance. At low sugar concentrations, which are thought to exist for sustained periods in dental plaque due to fasting by the host, the high-affinity sugar PTS is the primary transport system and plays an important role in carbohydrate uptake (19, 25, 26, 45). Early chemostat studies with *S. mutans* Ingbritt suggested that the glucose-PTS was repressed under a variety of conditions, including growth at low pH, faster growth rates and growth under glucose-excess conditions (17, 18). Subsequent continuous culture studies with *S. mutans* Ingbritt demonstrated that repression of the glucose-PTS was associated with reduced synthesis of the sugar specific permease EII^{Glc} (47). Our results with the *S. mutans* strain UA159 followed the same patterns described above. On the other hand, *S. sobrinus* 6715 and SL1 grown at steady-state at

pH 5.0 had 1.6- and 2.7-fold more glucose-PTS activity, respectively, than cells grown at pH 7.0, which correlated well with expression of the gene for EII^{Glc}. It is also noteworthy that, when compared to *S. mutans* UA159 growing at pH 5.0, overall glucose PTS activity in *S. sobrinus* is some 3- to 5-fold higher than in *S. mutans*. Increases in *S. sobrinus* PTS activity caused by acidification of the growth medium were restricted to the glucose-PTS, since the fructose- and mannose-PTS activities were not affected by pH. Because the PTS also plays important roles in the regulation of gene expression (1, 24, 35, 38, 40, 41, 45), we speculate that changes in expression of the glucose-PTS could modify *S. sobrinus* acid tolerance by modulating gene expression patterns in a way that enhances protection against environmental acidification. In *E. coli*, EII^{glc} was shown to negatively regulate expression of the σ^S (*rpoS*) subunit of RNA polymerase, which controls transcription of a variety of stationary phase and stress survival genes (44). Although, to the best of our knowledge, oral streptococci lack *rpoS*, it is possible that other stress regulators are under EII^{Glc} control. Another possibility is that increases in the glucose-PTS result in higher rates of ATP generation through glycolysis, thus enhancing the ability of the cells to maintain ΔpH. However, in our pH drop experiments with *S. sobrinus* 6715, the rates of glycolysis of pH 7.0- and pH 5.0- grown cells, as measured by the time it took the cells to lower the pH to 3.8, appear to be the same. Therefore, the role of the glucose-PTS in acid tolerance is potential complex and additional studies are needed to better understand the linkage of the PTS enzymes with the ATR.

In summary, the data presented in this study indicate that *S. sobrinus* is constitutively acid tolerant and is capable of developing an adaptive ATR during cultivation at acidic pH values that can readily be achieved in dental plaque. Elucidation of the molecular genetic mechanisms governing adaptation of *S. sobrinus* to acid will lead to a better understanding of the pathogenic mechanisms of this bacterium. The opportunity to contrast the acid-adaptive strategies of *S.*

sobrinus and *S. mutans* should provide much needed insight into the genetic and physiologic control of acid tolerance in important human pathogens.

ACKNOWLEDGMENTS

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4. Considerações Finais

A partir dos estudos descritos no Capítulo 1, se torna evidente que a capacidade de adaptação às condições ambientais é essencial para a sobrevivência dos microrganismos na cavidade bucal. O impacto do estresse ambiental na fisiologia e genética microbiana tem demonstrado sua importância com relação principalmente à expressão acentuada dos fatores de virulência destes microrganismos orais. Algumas das principais doenças infecciosas manifestadas na cavidade bucal, como a cárie dental e a doença periondontal, têm sua etiologia associada a um limitado número de patógenos, e a expressão de seus fatores de virulência vem sendo intensamente estudada. Conforme exposto neste trabalho de revisão, a tolerância central dos microrganismos aos insultos ambientais é baseada na produção de uma variedade de proteínas de estresse, incluindo as moléculas chaperonas GroEL e DnaK, e proteínas específicas, tais como as ATPases e ureases, relacionadas à homeostase do pH celular. Progressos têm sido feitos em termos da identificação de outras proteínas e genes microbianos envolvidos nos processos de resposta aos insultos ambientais, mas ainda não está claro quantos outros genes estão sob o controle de um determinado fator ambiental, tal como o pH, ou sob a regulação generalizada das respostas ao estresse ambiental. Estudos futuros poderão focalizar os mecanismos específicos da expressão de genes regulados por esses insultos, assim como os processos pelos quais os danos causados podem ser prevenidos ou reparados.

As evidências do envolvimento de estreptococos orais no processo de cárie dental têm como base os estudos envolvendo culturas planctônicas e culturas contínuas da espécie *S. mutans*, onde sua patogênese é atribuída, em grande parte, a capacidade de induzir uma resposta e tolerância ácida (ATR). Entretanto, não apenas nosso atual conhecimento sobre a biologia e patogênese de *S. mutans* é incompleto, como também é insatisfatório nosso conhecimento sobre a atuação de outros microrganismos possivelmente envolvidos no processo de cárie. A resistência

e os mecanismos de resposta ao pH ácido da espécie *Streptococcus sobrinus* foram explorados pelo estudo apresentado no Capítulo 2. Os resultados demonstraram que as cepas *S. sobrinus* 6715 e SL1 possuem uma capacidade de adaptação ao estresse ácido comparável àquela presente em *S. mutans* UA159, embora as estratégias utilizadas por essas duas espécies possam diferir em alguns aspectos.

A habilidade de *S. mutans* em resistir à acidificação presente constantemente na placa dental, parece envolver mecanismos adaptativos que incluem a atividade da enzima de membrana translocadora de prótons, F_1F_0 - ATPase. No presente estudo, o aumento da tolerância ácida e capacidade glicolítica de *S. sobrinus*, não pode ser atribuído a participação das ATPases. Desde que não foram observadas diferenças quanto a atividade e/ou indução dessas enzimas entre células de *S. sobrinus* crescidas em pH 7 e 5, outros mecanismos adaptativos poderão esclarecer a tolerância ácida deste estreptococo oral. Outra diferença verificada no estudo quanto aos mecanismos de tolerância ácida entre *S. mutans* e *S. sobrinus* diz respeito à proteína chaperona DnaK. Embora a DnaK seja importante para a sobrevivência bacteriana sob condições ambientais extremas, o papel desta proteína na ATR ainda não está definido. Ao contrário do observado em *S. mutans*, a indução da proteína DnaK em *S. sobrinus*, não foi sustentada em células adaptadas ao crescimento em pH 5, o que possivelmente indica que esta espécie não reconhece este valor de pH como uma situação de estresse.

Neste estudo, foi possível ressaltar a provável relação entre a elevada atividade do PTS-glicose e o aumento da resistência ácida observada com as cepas de *S. sobrinus*. Porque o PTS também desempenha um importante papel na regulação da expressão gênica, foi possível especular que as alterações no PTS-glicose poderiam atuar na modulação dos padrões de expressão gênica, de forma a aumentar a proteção contra a acidificação ambiental. Este estudo pode não apenas contrastar as estratégias de adaptação ácida de duas bactérias cariogênicas dos *Streptococcus* do grupo mutans, como também pode providenciar uma análise mais criteriosa

da espécie *S. sobrinus*. A existência de mecanismos de ATR certamente tem implicações com respeito a patogenicidade deste microrganismo. A elucidação dos mecanismos moleculares envolvidos na adaptação ácida de *S. sobrinus* poderá permitir uma melhor compreensão sobre sua patogenicidade e participação no processo de cárie dental.

5. Conclusões Gerais

O primeiro capítulo relata as informações atuais sobre a fisiologia e expressão gênica de microrganismos orais submetidos aos diferentes insultos ambientais. Esta revisão indica que as respostas ao estresse são de extrema importância para a sobrevivência bacteriana e contribuem na etiologia das principais doenças infecciosas orais. O trabalho enfatiza que uma melhor compreensão sobre os mecanismos de resistência desenvolvidos pelas bactérias ao estresse é essencial para o efetivo controle e/ou eliminação dos patógenos orais.

O estudo referente ao Capítulo 2 demonstra que a bactéria cariogênica, *S. sobrinus*, é constitutivelmente ácido-tolerante e capaz de desenvolver uma resposta ácido-adaptativa durante o cultivo em valor de pH 5,0, comumente encontrado na placa dental.

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7. Apêndices - Materias e Métodos referentes ao Capítulo 2

7.1. Espécies bacterianas e condições de cultivo

As espécies de *S. sobrinus* 6715 e SL1 e *S. mutans* UA159 foram rotineiramente crescidas em meio de cultura BHI a 37°C em atmosfera aeróbica contendo 5% de CO_2. Com o objetivo de investigar a resposta ao choque ácido e o processo de adaptação ácida (ATR) destas cepas de *S. sobrinus* e *S. mutans*, as células bacterianas foram crescidas em quimiostato (BioFlo III chemostat, New Brunswick Scientific, Edison, NJ) em 600 mL de meio TY (3% tryptone, 0,5% yeast extract) contendo glicose a 25 mM glicose (JAYARAMAN *et al*., 1997). O pH do vessel foi mantido pela adição de KOH 1N. Células crescidas em fase *steady-state* pH 7 foram submetidas à choque ácido de pH 7 à pH 5 pela adição de HCl 1N (aproximadamente 2 minutos de titulação), sendo então permitido o crescimento celular em fase *steady-state* pH 5. Alíquotas provenientes das culturas foram aspiradas do vessel, em tempos pré-determinados, e inoculadas diretamente em tubos de ensaio imersos em gelo. As células foram concentradas por centrifugação (4000g/ 12 min/ 4°C) e imediatamente submetidas aos ensaios de acid killing e queda de pH, ou armazenadas a -80°C para a realização das análises futuras.

7.2. Acid killing (aciduricidade) e Queda de pH

A atividade glicolítica das espécies utilizadas foi comparada por experimentos de queda de pH de acordo com protocolo descrito por Belli & Marquis (BELLI & MARQUIS, 1991) e monitorada através de um aparelho medidor de pH. Previamente, células bacterianas coletadas a partir de culturas *steady-state* (pH 7 e pH 5) foram lavadas com o mesmo volume de água destilada gelada, e ressuspensas em 1/10 do volume original da cultura em solução KCl 50 mM e $MgCl_2$ 1 mM. As suspensões foram tituladas com KOH 0,1 M até o valor de pH 7,2, e a queda de pH foi iniciada pela adição de glicose 55,6 mM.

Para se determinar a capacidade de resistência à ácido, as culturas de quimiostato em fase *steady-state* foram lavadas uma vez com glicina 0,1 M (pH 7) e ressuspensas em glicina 0,1 M (pH 2,85). As amostras foram mantidas à temperatura ambiente e alíquotas foram removidas nos intervalos de tempo: 30, 60 e 90 minutos. As amostras foram serialmente diluídas, plaqueadas em BHI-ágar e incubadas a 37°C por dois dias, anteriormente a contagem de colônias. A viabilidade celular em cada tempo foi expressa pela percentagem de células viáveis a partir do tempo zero (CFU mL^{-1}) (BELLI & MARQUIS, 1991).

7.3. Ensaio bioquímico com F_1F_0 - ATPases

Durante os ensaios de atividade das enzimas F-ATPases, as células coletadas do quimiostato foram lavadas uma única vez com tampão Tris 75 mM e $MgSO_4$ 10 mM (pH 7) e permeabilizadas com tolueno. As amostras foram incubadas em tampão ATPase (Tris-maleato 50 mM e $MgSO_4$ 10 mM, pH 6), e a reação foi iniciada pela adição de ATP 0,5 mM (pH 6) em uma concentração final de 5 mM à temperatura ambiente. Amostras foram removidas em diferentes intervalos de tempo, sendo verificada a liberação de fosfato inorgânico a partir do ATP através do reagente Fiske-Subbarow (Sigma, St. Louis, Mo.). A atividade das enzimas ATPases foi expressa em nmol de PO_4 liberado por minuto por cada mg de proteína.

7.4. Ensaio bioquímico com PTS

Células bacterianas obtidas a partir do quimiostato foram lavadas duas vezes com tampão fosfato de sódio-potássio 0,1 M (pH 7,2) contendo $MgCl_2$ 5 mM e ressuspensas em 10 % do volume original usando-se o mesmo tampão. A suspensão celular foi então permeabilizada com 50 μL de tolueno-acetona (1:9) por mL de suspensão celular, e alíquotas de 10 a 50 μL foram submetidas ao ensaio pelo método de LeBlanc. As soluções foram compostas por NADH 0,1mM, 10 U da enzima ácido-lático desidrogenase, NaF 10mM e o açúcar de interesse a 10mM em tampão fosfato de sódio-potássio 0,1 M (pH 7,2). As reações foram iniciadas pela adição de

PEP 5mM e a oxidação das moléculas de NADH foi monitorada durante 3 minutos à 340 nm. As reações controle foram conduzidas na ausência de PEP. A atividade do PTS utilizando-se glicose, manose ou frutose como substrato foi expressa por nmol de moléculas de NADH oxidadas a PEP por minuto por cada mg de proteína.

7.5. Preparo do DNA

O DNA cromossomial de *S. sobrinus* 6715 foi preparado como previamente descrito (BURNE *et al.*, 1987). As seqüências utilizadas na construção dos primers foram obtidas a partir do projeto para análise do genoma de *S. sobrinus* da TIGR (*The Institute for Genomic Research*). Os seguintes primers foram utilizados: *dna*K sense: 5'- GGTGGTGTCATCACTG -3'; *dna*K antisense: 5'- GCAGCATCACGTTCAG -3'; *atp*B sense: 5'- CCTCATCAGAAATCTTGG -3'; *atp*B antisense: 5'- CGAAAGCTGTCGCTG -3'; *pts*G sense: 5'- TGGGTGATGGCTTCGCTG TTC -3'; *pts*G antisense: 5'- AGGAGCCCATAAGCGTGTTTCG -3'. As reações de PCR foram conduzidas com 100 ng do DNA cromossomial e Taq DNA polymerase. Os produtos de PCR foram purificados com o auxílio do kit QIAquick (Qiagen, Inc., USA).

7.6. Preparo do RNA

O RNA total proveniente das culturas de *S. sobrinus* e *S. mutans* crescidas em quimiostato foi isolado de acordo com o método de Chen com algumas modificações. Células bacterianas previamente concentradas foram lavadas com tampão fosfato de sódio 10mM (pH 7) e ressuspensas em uma solução de Tris 50 mM - EDTA 10 mM (pH 8). Em tubos de microcentrífuga (2,0 mL), foram adicionados pérolas de vidro (diâmetro: 0,1 mm), 500 μL da suspensão bacteriana concentrada, 500 μL de fenol-clorofórmio (2:1, pH 4,7) e 100 μL de dodecilsulfato de sódio 10% (SDS), sendo as células submetidas a lise com o auxílio de um Bead Beater (BioSpec, Bartlesville, Okla.), durante 3 ciclos de 30 segundos e resfriamento das amostras entres os intervalos durante 1 minuto. A fase aquosa foi extraída de três a quatro vezes

com igual volume de fenol-clorofórmio, seguida por extração com clorofórmio-isoamílico (24:1). O RNA total foi precipitado com solução de acetato de sódio 3 M (pH 6) e isopropanol durante uma hora a -80°C. Os precipitados foram lavados com etanol 99% e 70%, e ressuspensos em água tratada com DEPC. A purificação do RNA foi realizada com tratamento em coluna por duas vezes e adição de DNAase I livre de RNAase, Kit Rneasy (Qiagen Inc., USA). O RNA purificado foi eluído da coluna com 50 μL de água tratada com DEPC e armazenado a -80°C.

7.7. Slot Blot

Para as análises quantitativas de Slot Blot, quantidades equivalentes de RNA total foram transferidas para membranas de nylon (GeneScreen Plus; NEN Life Science products, Inc., Boston, Mass., USA) através de um aparato específico para os ensaios de Slot Blot, como descrito por Sambrook *et al.* (SAMBROOK *et al.*, 1989). As amostras de RNA foram fixadas nas membranas por luz ultravioleta e então hibridizadas com sondas preparadas a partir de fragmentos dos genes de interesse. As sondas foram previamente marcadas com a substância psoralen-biotin, utilizando-se o kit Brightstar (Ambion, Inc., Austin, Texas, USA). Hibridizações e lavagens de membranas foram realizadas sob condições de alta estringência. Os sinais obtidos nas autoradiografias foram quantificados por densitometria através do sistema de imagens digital IS1000 (Alpha Innotech Corp., San Leandro, Calif., USA).

7.8. Western Blot e Géis de Eletroforese bidimensionais (2D)

Células bacterianas concentradas foram lavadas duas vezes com tampão TBS (Tris-HCl 10 mM, NaCl 0,9%, pH 7,4), e ressuspensas em 500μL do mesmo tampão. O lisado celular foi obtido por ruptura mecânica em Bead Beater, utilizando-se pérolas de vidro e quatro ciclos de 30 segundos, com posterior centrifugação à 14.000 rpm/ 25minutos/ 4°C. A concentração de proteínas das amostras foi determinada por ensaios com ácido bicinchoninic (BCA; Sigma, St. Louis, Mo.). Os lisados de proteínas (10 μg) foram separados por géis de eletroforese contendo

SDS em poliacrilamida, transferidos para membranas Immunobilon P (Millipore, Bedford, Mass., USA) e submetidos à análises de Western Blot com técnicas previamente padronizadas (AUSUBEL *et al.*, 1987). As membranas foram incubadas com anticorpos anti-*dna*K e anti-*gro*EL de *S. pyogenes*, ou específicos para os genes codificadores das proteínas EI e HPr de *S. salivarius*, e diluídos em TBS nas respectivas concentrações 1:1000, 1:500, 1:200 e 1:200. Os anticorpos específicos para DnaK e GroEL fazem parte da coleção do laboratório do Prof. Robert A. Burne (University of Florida, Flórida, USA), enquanto os anticorpos contra EI e HPr foram gentilmente cedidos pelo Prof. C. Vadenbocoeur (Universite Laval, Quebec, Canada). A reatividade foi detectada por incubação das membranas em solução contendo a enzima perixidase conjugada com IgG anti-coelho seguida pela detecção com reagente 4-cloro-3-naftol.

O mesmo preparado protéico foi utilizado para a realização dos géis de eletroforese bidimensionais (2-D) pelo laboratório Kendrick (Madison, Wis.), seguindo-se o protocolo descrito por O'Farrell (O'FARRELL, 1975), e os géis corados com prata. Análises de densitometria foram usadas na comparação da intensidade de spots.

8. Anexos

8.1. Confirmação do recebimento do artigo referente ao Capítulo 1

Para: rgoncalves@fop.unicamp.br, mmatosn@yahoo.com.br
Assunto: Manuscript submitted - MECO-2004-0150
De: jill.walters@pnl.gov
Data: Tue, 13 Jul 2004 15:03:46 - 0400 (EDT)

Dear Dr. Goncalves,

We have received your manuscript, "ORAL BACTERIA SUBMITTED TO NVIRONMENTAL STRESS". It will be assigned to a regional ditor, for consideration. Please note: If the manuscript umber ends with "R1", it is being considered as a revised manuscript.

Thank you for your interest in Microbial Ecology.

Sincerely,

Jill Walters (Mrs.)
Managing Editor

8.2. Aceite do artigo referente ao Capítulo 2

Data: Thu, 8 Jul 2004 11:21:09 -0400
De: DENK@uthscsa.edu
Para: rburne@dental.ufl.edu
Assunto: Decision on manuscript JB00602-04 Version 2

Dr. Robert A. Burne
University of Florida
Department of Oral Biology
College of Dentistry, Room #: D5-18
College of Dentistry, Room #: D5-18
Gainesville, FL 32610
United States

Re: The Adaptive Acid Tolerance Response of Streptococcus sobrinus (JB00602-04 Version 2)

Dear Dr. Burne:

Your manuscript has been accepted, and I am forwarding it to the ASM Journals Department for publication. For your reference, ASM Journals' address is given below. Before it can be scheduled for publication, your manuscript must be checked by the ASM production editor to make sure that all elements meet the technical requirements for publication. Jack Kenney, the production editor for the Journal of Bacteriology (JB), will contact you if anything needs to be revised before copyediting and production can begin.
An official letter of acceptance and a copyright transfer agreement will be sent to the person listed as the corresponding author on the manuscript title page once your article has been scheduled for an issue.

Thank you for submitting your paper to JB.

Sincerely,
William G. Haldenwang
Editor, Journal of Bacteriology (JB)
Journals Department
American Society for Microbiology
1752 N St., NW, Washington, DC 20036
jkenney@asmusa.org
1-202-942-9243
1-202-942-9355