Archives of Oral Biology 81 (2017) 74–80

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Streptococcus mutans membrane lipid composition: Virulence factors and structural parameters

M.A. Bojanich[a,*], R.O. Calderón[b]

[a] Department of Biological Chemistry, Oral Biology Department, School of Dentistry, National University of Córdoba, Argentina
[b] Department of Cell Biology, Histology Embryology, School of Medical Sciences, National University of Córdoba, Argentina

ARTICLE INFO

Keywords:
Oral microorganisms
ATPase activity
Fatty acids

ABSTRACT

Objective: The aim of the present work was to analyze whether the location of the dental biofilm was associated with shifts in the membrane fatty acid profile, and whether such shifts could affect certain virulence factors of strains of *Streptococcus mutans*.

Design: An experimental study was conducted to assess the behavior of S. mutans strains isolated from dental biofilm collected from sound and carious smooth and occlusal surfaces of the oral cavity of children. The lipid composition of the bacterial membrane, structural membrane parameters, acid survival, and ATPase activity were tested at pH 7 and 5.

Results: At pH 5, an increase in both the unsaturated and the long chain fatty acids as well as in proton ATPase hydrolytic activity was observed in strains isolated from carious smooth surface biofilm but not in carious occlusal surface strains. The observed changes correlated with the studied structural parameters, and were found to be associated with membrane organization. The changes correlated with a decreased in ΔC (sn-1 and sn-2 acyl chain inequivalence), indicative of increased acyl chain interaction.

Conclusions: The obtained results suggest that the acidic environment (pH 5) of smooth surface caries affects membrane organization, inducing a shift in membrane lipid profile, which would likely induce better *protein/lipid* hydrophobic *matching*, resulting in increased ATPase activity and higher acid survival.

1. Introduction

Streptococcus mutans (S. mutans) is considered one of the principal agents in the development of dental caries in children (Shimada et al., 2015; Ge, Caufield, Fisch & Li, 2008). This microorganism has important virulence factors that enable it to dominate the dental biofilm and induce the development of caries (Struzycka, 2014). It is known that when environmental conditions change, such as a drop in pH, *S. mutans* changes its membrane fatty acid profile, thus inducing an alteration in membrane permeability (Fozo & Quivey, 2004). Shifts in the membrane fatty acid profile of *S. mutans* as a survival mechanism in acidic environments, are evidenced by increases in the amount of monounsaturated fatty acids (Fozo & Quivey, 2004).

The acid generation capacity (acidogenicity) necessarily implies the presence of a mechanism that enables these microorganisms to tolerate an acidic environment (aciduricity). It is known that this tolerance depends, at least in part, on the ATPases located in the plasma membrane. These enzymes are responsible for cytoplasmic proton extrusion (Guo, McLean, Lux, He & Shi, 2015). There are no studies in the literature comparing the behavior of *S. mutans* isolated from biofilm that forms on two different types of tooth surfaces in the same mouth. The hypothesis of the present study was that the response of native *S. mutans* strains to an acidic environment (intermediate pH value of 5) is associated with type of tooth surface where the biofilm develops. Hence, the present study aimed to analyze whether the location of the biofilm, i.e. carious or sound, smooth (S) or occlusal (O) tooth surfaces, is associated with shifts in the membrane fatty acid profile, and whether such shifts could affect certain virulence factors (acid survival and ATPase activity) of native *S. mutans* strains.

2. Materials and methods

2.1. Patients

The study protocol was approved by the Ethics Committee for activities involving human subjects, of the School of Medical Sciences of the National University of Cordoba, Argentina. All participating patients' parents or caretakers gave their written informed consent. All

* Corresponding author at: Facultad de Odontología, Cátedra "A" de Química Biológica, Universidad Nacional de Córdoba, Haya de la Torre SN, Pabellón Argentina, Ciudad Universitaria, 5016 Córdoba, Argentina.
E-mail address: mariaalejandraboja@gmail.com (M.A. Bojanich).

http://dx.doi.org/10.1016/j.archoralbio.2017.04.023
Received 25 August 2016; Received in revised form 23 March 2017; Accepted 17 April 2017

study samples were obtained from boys and girls living in Córdoba City, average age 6.2 ± 1.4 years, and receiving oral care at the Department of Pediatric Dentistry, School of Dentistry, National University of Cordoba, Argentina. Hence, the strains were considered indigenous strains. All children underwent dental examination, performed by a fully qualified, calibrated dental health professional and Faculty member of the School of Dentistry, UNC. The children had either pure primary dentition or early mixed dentition, with and without caries. Children receiving antibiotics, topical application of fluoride and/or using antiseptic mouth rinses within three months prior to samples collection were excluded from the study. First mandibular molars diagnosed with active carious lesions and their sound contralateral molar (with no sign or symptom of caries) were studied, following the criteria established by Pitts (2001). Bitewing radiographs were taken and used as a complementary method to diagnose caries, in keeping with Kidd, Mejare, and Nyvad (2003).

2.2. Strains and growth conditions

S. mutans ATCC 25175 from the American Type Culture Collection (ATCC, Manassas, VA) was used as a standard control strain. Occlusal and smooth surface biofilm samples were obtained from carious and sound sites of mandibular molars of the studied patients. In total, forty-eight samples of native strains were obtained (n = 48). Twenty-four strains were isolated from smooth surface biofilm (SS), twelve of which corresponded to carious smooth tooth surfaces (CSTS), and twelve corresponded to sound smooth tooth surfaces (SSTS). The twenty-four remaining strains were isolated from occlusal surfaces (OS): twelve from carious occlusal tooth surfaces (COTS) and twelve from sound occlusal tooth surfaces (SOTS). The collected material was placed immediately in sterile physiological solution and incubated in mitis salivarius agar with bacitracin in an anaerobic atmosphere (DIFCO®, Becton Dickinson, France) at 37 °C for 48 h. Species identification was based on colony morphological characteristics compatible with *S. mutans,* and confirmed by conventional biochemical tests (Diatabs®, Doughnut Diagnoses, Denmark) following criteria established by Facklam (2002) and Whiley and Beighton (1998). *S. mutans* were grown in brain heart infusion broth (DIFCO), and the initial pH was adjusted to about 7 with 81% lactic acid solution. Half of the bacterial suspensions were kept at pH 7, whereas the remaining suspensions were added with 55.6 mM of glucose and allowed to grow until the suspension reached a pH of 5 (Harper & Loesche, 1984).

2.3. Extraction of the membrane fraction

Membrane fraction was extracted following the method described by Magalhães, Paulino, Thedei Jr., and Ciancaglini (2005). Briefly, 1 g (wet weight) of cells from each of the tooth surfaces was washed with 15 ml of sterile distilled water, centrifuged at 9000 x *g* at 20 °C for 10 min. The pellets were weighed, resuspended, and homogenized in 25 mM MES buffer, pH 6.2, containing 97 mM of NaSCN. The absorbance (at 700 nm) of the solution was monitored until attaining a value of about 0.4. The obtained solution was added with 0.4 mg/ml of Lysozyme (Sigma), incubated at 37 °C for 3 h, and centrifuged at 9000 x *g* at 4 °C for 20 min. The pellets were homogenized in 50 mM MES buffer, pH 6.2, containing 10 mM $MgSO_4$ and 0.8 M NaCl (osmotic buffer), and sonicated (tip sonicator, Vibracell 600 V, 50% amplitude) at 4 °C for 20 min. Homogenates were incubated with 25 U DNAse/ml homogenate and 0.25 ml protease inhibitor cocktail/g cell at room temperature for 45 min and ultracentrifuged at 100000 x *g* at 4 °C for 60 min (Beckman L5–50 B ultracentrifuge). The pellets (membrane fraction) were resuspended in 25 mM MES buffer, pH 6.2, containing 97 mM NaSCN, and then incubated with lysozyme 1:1 (mg protein/mg lysozyme) at room temperature for 2 h. The homogenates were ultracentrifuged under the same conditions, and the pellets were resuspended in 40 ml 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgSO4 (stock buffer), and were stored at −20 °C until use.

2.4. Determination of proteins and fatty acids

Membrane lipids were extracted following the method described by Folch, Lees, and Sloane Stanley (1957). The fatty acid methyl esters were prepared from membrane total lipids with toluene (500 µl) and sodium methoxide (1 ml, 0.5 M) at 4 °C overnight and extracted with hexane (Cantellops, Reid, Eitenmiller & Long, 1999). The methyl esters were isolated using a gas chromatographer (Perkin Elmer, Waltham, USA), and identified using commercial standards. Reagents and organic solvents were provided by Sigma Chemical Co., St. Louis, USA, and FA standards by Nu-Check Prep Inc., Elysian, USA. Total proteins were determined by the Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951).

2.5. Determination of structural membrane parameters

Following determination of membrane fatty acid composition of the isolated strains, the following structural parameters were calculated according to Mason, Huang, and Biltonen (1981): 1) average fatty acid chain length (CL), 2) the proportional (weighted) contribution of each fatty acid to weighted average chain length (WACL), 3) the effective inequivalence (ΔC) between the saturated and unsaturated acyl chains (sn-1 and sn-2 respectively), and 4) hydrophobic core thickness (HC) of the membrane (sum of weighted average chain lengths of both saturated and unsaturated fatty acyl chains). In line with Mason's report, we assumed that: a) the acyl chains in the sn-1 and sn-2 positions are saturated and unsaturated, respectively; b) fatty acids are mixed randomly in phospholipids, and c) the chain length (CL) of saturated and unsaturated chains, expressed as C−C bonds, is equal to (n1–1) and (n2–1 to 1.5–X) respectively. Parameters n1 and n2 represent the number of carbon atoms in the sn-1 and sn-2 chains respectively; 1.5 represents the shortening due to bending of C_2, and X is the shortening of each double bond. Each fatty acid chain length was used to determine the proportional (weighted) contribution of that fatty acid to the weighted average chain length (WACL) of the sn-1 (saturated) and sn-2 (unsaturated) chains. The difference between the WACL of the saturated and unsaturated chains is equal to ΔC, which represents the inequivalence between the two chains. The thickness of the bilayer hydrophobic core (HC), equal to the sum of both saturated and unsaturated WACL, was calculated according to Wang, Lin, Li, and Huang (1995).

2.6. pH determination and acid survival

Each sample was grown in brain-heart broth (DIFCO) under anaerobic atmosphere conditions at 37 °C for 24 h, centrifuged, and a bacterial density of 10 mg of dry weight of cells was obtained. The samples were then washed twice in 10 ml of a solution containing 50 mM of KCl and 1 mM of MgCl2, and resuspended in the aforementioned solution to obtain a bacterial density of 2 mg of dry weight of cells. Suspension pH was adjusted to 7.2 ± 0.01 (baseline pH) using KOH. Following, each bacterial suspension was added with 55.6 mM of glucose, and pH was determined at room temperature at time 0 (baseline pH) and at 30 min (final pH) (pHmeter SANXIN Code: PHS-3D-03. Shanghai. San-Xin Instrumentation, Inc. China).

Acid survival was assessed by determining bacterial viability at the same time points as pH determinations (time 0 and at 30 min). For this purpose, 0.1 ml of each bacterial suspension were grown on brain-heart agar plates (DIFCO). The plates were then incubated under anaerobic atmosphere conditions at 37 °C for 24 h. The number of colony forming units per milliliter of bacterial suspension (UFC/ml) was then determined in each plate. All microbial suspensions contained the same number of viable colonies at time 0 (Belli & Marquis, 1991).