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Toluene permeabilization differentially affects F- and P-type ATPase activities present in the plasma membrane of *Streptococcus mutans*

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Streptococcus mutans membrane-bound P- and F-type ATPases are responsible for H⁺ extrusion from the cytoplasm thus keeping intracellular pH appropriate for cell metabolism. Toluene-permeabilized bacterial cells have long been used to study total membrane-bound ATPase activity, and to compare the properties of ATPase *in situ* with those in membrane-rich fractions. The aim of the present research was to determine if toluene permeabilization can significantly modify the activity of membrane-bound ATPase of both F-type and P-type. ATPase activity was assayed discontinuously by measuring phosphate release from ATP as substrate. Treatment of *S. mutans* membrane fractions with toluene reduced total ATPase activity by approximately 80% and did not allow differentiation between F- and P-type ATPase activities by use of the standard inhibitors vanadate (3 μM) and oligomycin (4 μg/mL). Transmission electron microscopy shows that, after *S. mutans* cells permeabilization with toluene, bacterial cell wall and plasma membrane are severely injured, causing cytoplasmic leakage. As a consequence, loss of cell viability and disruption of H⁺ extrusion were observed. These data suggest that treatment of *S. mutans* with toluene is an efficient method for cell disruption, but care should be taken in the interpretation of ATPase activity when toluene-permeabilized cells are used, because results may not reflect the real P- and F-type ATPase activities present in intact cell membranes. The mild conditions used for the preparation of membrane fractions may be more suitable to study specific ATPase activity in the presence of biological agents, since this method preserves ATPase selectivity for standard inhibitors.

Key words: Cell permeabilization; F-type ATPase; P-type ATPase; Toluene permeabilization; *Streptococcus mutans*

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Introduction

Dental caries have long been recognized as an infectious disease related to the presence of dental plaque (1). Dental diseases pose a distinct challenge when it comes to determining their microbial etiology. Disease occurs at sites with a pre-existing natural and diverse microflora,

while even more complex but distinct consortia of microorganisms are implicated in the pathology (2). The oral bacteria that are most closely associated with caries development are the mutans streptococci, mainly *Streptococcus mutans* and *S. sobrinus*, noted for their ability to produce acids from the fermentation of dietary carbohydrates, which are directly associated with tooth mineral

dissolution (3). *S. mutans* adheres to the tooth surface and forms dental plaque, a classic biofilm. The pH of the oral cavity is predominantly acidic; within the dental plaque, *S. mutans* induces an acid-tolerance response that favors its survival under low pH conditions (4).

S. mutans survives in an extremely diverse, high cell density biofilm on the tooth surface. This bacterium is strongly associated with caries formation. Following adhesion and accumulation of cells on surfaces to form multilayered cell clusters, the bacterial biofilm demonstrates a radically phenotypic difference from the planktonic state (5). Therefore, this acidogenic potential is recognized as an important virulence factor of these cariogenic species (1). Additionally, these microorganisms often must adapt to their acidic environment and can do so by modulating the activity of the plasma membrane (6,7). Acid tolerance by *S. mutans* has been studied in some detail, and it has been established that this microorganism possesses an inherent acid resistance that distinguishes it from other microorganisms not commonly associated with dental caries (8-10). This aciduricity has been largely attributed to the fact that *S. mutans* has both an F-type ATPase, which is expressed at higher levels in mutans streptococci plasma membrane and in many oral bacteria (11,12), and a P-type ATPase, which was identified and characterized for the first time by Magalhães et al. (13,14). Both membrane enzymes are responsible for cytoplasmic proton extrusion and regulation of the internal pH. Consequently, acid adaptation may play an important role in the cariogenicity of mutans streptococci since prolonged plaque acidification may enhance tooth damage by promoting more severe demineralization (15).

Carbohydrate metabolism by oral streptococci via the glycolytic pathway is of considerable importance to the ecology of dental plaque biofilm, since the resulting acid formation lowers the plaque pH (16). The degree and rate of acidification increase with the carbohydrate concentration in food, particularly that of refined sugars, so that the plaque pH can reach values below 5.0 and remain at low levels for some time (16). Under these conditions, the low pH can lead to enamel demineralization, as well as selection of strains that are able to adapt and survive in this acidic environment. Hence, an acid-tolerant microflora will emerge, which in turn will lead to further demineralization and the development of caries (16).

Furthermore, the cytoplasmic membrane, which serves as the cell barrier, not only permits excretion of waste products, but also allows selective uptake of desired substrates (17). Hence, another important membrane function of mutans streptococci that is strictly related to this organism's cariogenicity is the nutrient acquisition pro-

cess, which involves the transport of carbohydrates from the external environment through the cell barrier into the cytoplasm, where metabolism occurs (18).

Much attention has been given to the investigation of the roles and regulation of these virulence attributes, so as to better understand dental caries formation and establish control action. Also, membrane targets are likely to be more important than cytoplasmic targets simply because they are directly exposed to biological agents added from the outside (19). On the other hand, most studies assessing membrane enzyme activities use isolated membrane fractions or toluene-permeabilized cells. Some correlations with membrane enzyme activity in intact cells have been contradictory possibly due to damage caused to the bacterial plasma membrane by the permeabilization process. Moreover, according to Nguyen et al. (19), it is always difficult to relate results obtained with fractions from lysed cells with those obtained with intact cells.

The main objective of the present research was to determine if toluene permeabilization can affect F- and P-type ATPase activities in *S. mutans* cells and membrane fractions isolated from these cells.

Material and Methods

Material

All solutions were prepared using Millipore Direct-Q ultra pure apyrogenic water. Tris[hydroxymethyl]amino-methane (Tris), bis[2-hydroxyethyl]imino-Tris[hydroxymethyl]methane (Bis-Tris), imidazole, trichloroacetic acid (TCA), 2-N-morpholine ethenesulfonic acid (MES), 2-amino-2-methyl-propan-1-ol, triethanolamine, sodium orthovanadate, oligomycin, protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin), deoxyribonuclease I (DNase I), adenosine 5'-triphosphate Tris salt (ATP), lysozyme, and bovine serum albumin (BSA) were purchased from Sigma (USA). Tryptic soy agar (TSA) was acquired from Difco (USA). Analytical grade reagents were used without further purification.

Strain and growth conditions

The ATCC 25175 strain of *S. mutans* was purchased from Fundação André Tosello (Brazil). Bacterial cells were stocked at -20°C in 40% glycerol (v/v) or in a candle jar for frequent utilization upon weekly subculturing on TSA. Large amounts of cells (about 1 g wet weight) were obtained from 1-liter cultures of complete medium incubated in a candle jar under magnetic stirring, as described by Magalhães et al. (13), and grown until the pH reached 4.2. Growth curves were constructed by inoculating ~10⁷ cells/mL of complete

medium (4 mL). Samples were collected for monitoring pH and turbidity, as well as total membrane ATPase activity determination.

Extraction of ATPase-rich membrane fraction

H⁺-ATPase-rich membrane fractions were obtained as described by Magalhães et al. (13). Approximately 1.0 g (wet weight) of cells was washed twice with 15 mL water and centrifuged at 9000 *g* for 10 min at 20°C. The pellet was weighed (wet weight), resuspended, and homogenized in 25 mM MES buffer, pH 6.2, containing 97 mM NaSCN. The absorbance of the suspension at 700 nm was monitored until a value of about 0.4 was achieved, and 0.4 mg/mL lysozyme was then added. The mixture was incubated for 3 h at 37°C with gentle agitation, and centrifuged at 9000 *g* for 20 min at 4°C. The resulting pellet was resuspended and homogenized in 50 mM MES buffer, pH 6.2, containing 10 mM MgSO₄ and 0.8 M NaCl (osmotic buffer), and sonicated (tip sonicator, Vibracell 600V, 50% amplitude) for 20 min at 4°C (50 mL buffer/g cell). Cell lysis was monitored by light microscopy. The homogenate was incubated with DNase (25 U/mL homogenate) and 0.25 mL protease inhibitor cocktail/g cell with gentle agitation for 45 min at room temperature. The mixture was ultracentrifuged at 100,000 *g* for 1 h at 4°C. The pellet containing the membrane fraction was resuspended in 40 mL 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgSO₄ (denoted stock buffer) and ultracentrifuged again under the same conditions. The pellet containing the membrane fraction was again suspended in 20 mL stock buffer and stored at -80°C. Membrane fractions obtained from 25 mL cell culture were also subjected to toluene treatment.

Toluene permeabilization of *S. mutans* cells or ATPase-rich membrane fractions

Permeabilized *S. mutans* cells were prepared as described by Belli et al. (11), with modifications. Two hundred and fifty-milliliter cultures (about 0.25 g wet weight cells) were harvested in the early stationary phase of growth by centrifugation at 4°C. Each culture sample was washed once and resuspended in 25 mL of a 75 mmol/L Tris-HCl buffer, pH 7.0, containing 10 mmol/L MgSO₄. This buffer was used rather than that of Belli et al. (11), because phosphate buffer interferes with our activity assays.

Toluene (2.5 mL) was added to each cell suspension prior to vigorous vortex mixing for 30 s and incubation for 5 min at 37°C. Each cell suspension was then subjected to two freezing cycles in a dry ice-ethanol bath and thawing at 37°C. Samples of permeabilized cells were collected and washed five times in buffer by centrifugation. They were resuspended in the same Tris buffer containing magne-

sium in dense suspensions containing 1 mg dry weight/mL, divided into aliquots of 1 mL in polypropylene tubes, and used immediately for all assays. The total amount of protein in permeabilized cell aliquots was determined according to Hartree (20).

The membrane fraction from *S. mutans* was treated with toluene using the same conditions.

Microscopy

Both intact (control) and toluene-permeabilized cells were examined by electron microscopy. The pellet obtained after centrifugation at 14,000 *g* was resuspended in 1% (w/v) osmium tetroxide prepared in 0.1 M sodium phosphate buffer. After 1 h, the suspension was washed in 0.1 M sodium phosphate buffer, pH 7.3, centrifuged, and the pellet was dehydrated with acetone/water mixtures of increasing acetone concentration, and the final dehydration step was carried out with 3 washes of 100% acetone for 10 min. Finally, the pellet was infiltrated in an 1:1 araldite resin at 37°C for 48 h and included in pure araldite for 72 h at 60°C. A thin section (0.5 μm) was cut with a microtome and stained with 4% (w/v) uranyl acetate, pH 12, for 10 min, followed by 0.3% (w/v) lead citrate, pH 12, for 10 min. The sample was then examined using a Philips (Model 208, Germany) transmission electron microscope.

Cell viability

The number of viable cells in both intact and permeabilized cell aliquots was determined by the standard pour plate method. Sequential dilutions of cell aliquots in sterile 0.9% NaCl were mixed in brain heart infusion agar, poured onto Petri dishes, and incubated in candle jars at 37°C for 48 h. The number of colonies of *S. mutans* was then determined with a light stereomicroscope.

Glycolytic pH-drop assay

S. mutans cells were freshly harvested in the early stationary phase of growth, washed once by centrifugation, and resuspended in the washing buffer (Tris buffer with magnesium) in dense suspensions containing 1 mg dry weight/mL, and were divided into aliquots of 4 mg (dry weight) in polypropylene tubes. For the measurement of acid production, 4-mg aliquots of both intact (control) and toluene-permeabilized cell pellets were resuspended in 50 mM KCl and 1 mM MgCl₂. Suspensions were titrated with KOH to a pH of about 7.2. Then, glucose was added to an initial concentration of 55.6 mM. The resulting fall in pH was monitored with a thin-bulb glass pH electrode (model 8103, Orion, USA). Alternatively, pH data were converted to nmol H⁺ (i.e., pH = -log [H⁺]), and glycolytic activities of both intact and toluene-permeabilized cells were reported

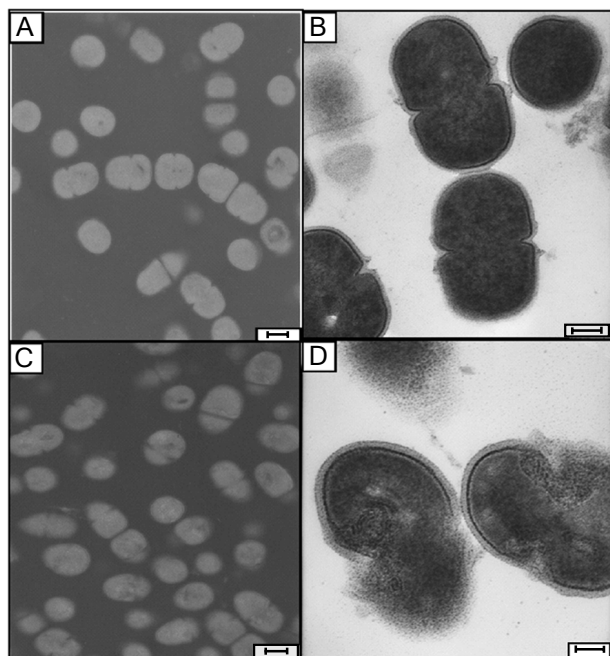


Figure 1. Electron micrographs showing the effect of toluene on *Streptococcus mutans*. A and B, Not-treated. C and D, Treated with toluene. Magnification bar: 300 nm in A and C, 100 nm in B and D. Notice severe injury of both cell wall and plasma membrane in most of the cells.

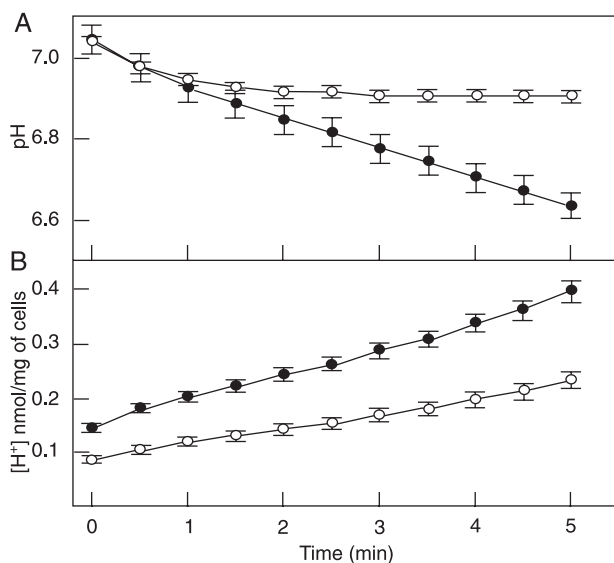


Figure 2. Glycolytic pH profile (A) and proton release reported as nmol H⁺/mg (dry weight) of cells (B) by intact *Streptococcus mutans* ATCC 25175 (filled circles) and permeabilized cells (open circles) incubated with a limiting supply of glucose (10 mM).

as either pH decay or nmol H⁺ produced per mg dry weight of cells.

ATPase assays

Total ATPase activity was assayed discontinuously at 37°C by measuring of phosphate release, as described by Magalhães et al. (13). The reaction was initiated by addition of the enzyme (in membrane fractions or permeabilized cells), stopped with 0.5 mL cold 30% TCA at appropriate time intervals, and centrifuged at 4,000 *g* immediately prior to phosphate determination according to the procedure described by Heinonen and Lahti (21). Standard conditions were 50 mM Bis-Tris buffer, pH 6.0, containing 5 mM ATP, 10 mM MgCl₂. P-type ATPase activity was determined in the presence of 4 μg/mL oligomycin and F-type ATPase activity was determined in the presence of 3 μM vanadate. The activity determination was carried out in triplicate, and the initial velocity was constant for at least 30 min, provided that less than 5% of the substrate was hydrolyzed. Controls without added enzyme were included in each experiment to control the non-enzymatic hydrolysis of the substrate. One enzyme unit (U) is the amount of enzyme, which hydrolyzes 1.0 nmol of substrate per minute at 37°C. ATPase-specific activity is reported as U/mg total protein. Data are reported as means ± SD of triplicate measurements from three different enzyme preparations.

Protein analysis

Protein concentration was measured as described by Hartree (20) in the presence of 2% (w/w) SDS, using BSA as standard.

Results

In order to evaluate the extent of bacterial cell damage caused by toluene treatment, samples of toluenized and intact cells were submitted to electron microscopy and details of cellular structure were compared (Figure 1). Toluene permeabilization resulted in partial exposure of the plasma membrane as well as severe injury of both cell wall and plasma membrane in most of the cells, causing leakage of intracellular material into the immediate environment. After toluene treatment, a reduced number of cells remained intact and only 8% of the cells formed viable colony forming units compared with cells not submitted to toluene permeabilization.

Toluene-permeabilized cells only weakly acidified the external medium after a glucose pulse, that is less than 0.1 pH unit in 5 min in the glycolytic pH-drop assay, whereas intact cells continuously secreted H⁺, reduced the pH at a rate of 0.3 pH units 5 min after glucose addition (Figure 2).

An ATPase-rich membrane fraction of *S. mutans*, which had not been treated with toluene and used as control, presented P-type and F-type ATPase activities, as indicated by the specific inhibitors vanadate and oligomycin. P- and F-type activities corresponded to 60 and 40% of the total ATPase activity, respectively. Together, the specific enzyme activities of P- and F-type ATPase activities totaled 120.3 U/mg protein, which is consistent with the total ATPase activity determined (119.4 ± 1.7 U/mg protein) in this assay (Table 1).

However, there was a reduction of about 80% in total ATPase activity in the toluene-treated membrane fraction compared with the control (22.5 ± 0.2 U/mg protein). This suggests that toluene was deleterious to enzyme activity. In addition, toluene treatment did not allow differentiation between F- and P-type ATPase activities, which were recorded as 23.9 ± 0.3 and 22.6 ± 0.4 U/mg protein, respectively, suggesting that toluene interfered with the inhibiting mechanisms of both oligomycin and vanadate upon enzymes.

A similar effect was observed when toluene-permeabilized *S. mutans* cells were employed in the ATPase assay. Very similar values of enzymatic specific activity were recorded for F-type and P-type ATPases (equal to 229.7 ± 3.4 and 216.7 ± 1.8 U/mg protein, respectively), demonstrating that specific inhibition of both types of enzyme by oligomycin and vanadate was affected (Table 1). On the other hand, a slightly higher value was recorded for total ATPase activity in permeabilized cells compared with the sum of both F- and P-type activities. It was not possible to evaluate whether toluene permeabilization reduced the total ATPase activity in relation to intact cells since it was impossible to monitor ATP hydrolysis and inorganic phosphate release from membrane-bound ATPase without removing the bacterial cell wall.

Discussion

Toluene-permeabilized microbial cells have long been used to study the activity of membrane-bound enzymes or enzymatic complexes such as the H⁺-ATPases, since this procedure removes portions of the cell wall, exposing plasma membrane enzymes to the action of biological agents and reagents added to the reaction mixture. Permeabilized cells are also used to compare the properties of ATPase *in situ* with those of ATPase in membrane-rich fractions.

In the present study, it has been demonstrated that

Table 1. Specific activity of P- and F-ATPase present in toluene-permeabilized *Streptococcus mutans* cells and membrane fractions.

Sample	Toluene treatment	ATPase activity (U/mg)		
		Non-specific ATPase	F-type	P-type
Membrane fraction	No	119.4 ± 1.7	49.1 ± 0.3	71.2 ± 0.9
Membrane fraction	Yes	22.5 ± 0.2	23.9 ± 0.3	22.6 ± 0.4
<i>S. mutans</i> cells	Yes	251.8 ± 2.8	229.7 ± 3.4	216.7 ± 1.8

F-type ATPase and P-type ATPase activities were determined in the absence of inhibitors or in the presence of specific inhibitors of ATPase activity: oligomycin ($4 \mu\text{g/mL}$) and vanadate ($3 \mu\text{M}$), respectively. Assays of each membrane fraction and *S. mutans* cells were carried out on the same sample, thus permitting specific activity to be reported. Data are reported as means \pm SD of specific activity for triplicate analysis of three preparations.

toluene treatment of an ATPase-rich membrane fraction of *S. mutans* caused severe reduction (about 80%) in total ATPase activity, suggesting that toluene is deleterious to enzyme function. Furthermore, toluene treatment did not allow differentiation between F- and P-type ATPase activities. The percent of inhibition seen with vanadate (40%) and the 60% inhibition obtained with oligomycin on control cell membranes were not detected with toluene-treated membranes, suggesting that toluene interferes with the inhibiting mechanisms of both oligomycin and vanadate upon enzymes (22,23). This effect suggests that this organic solvent may affect membrane architecture, inducing alterations in the enzyme microenvironment, which reflects on its properties, as already stated for other enzymes such as glycosyltransferases and the phosphoenolpyruvate phosphotransferase system from *S. mutans* (24,25). Moreover, toluene treatment may affect protein structure, altering enzyme-substrate-inhibitor interactions.

A similar effect has been observed for toluene-permeabilized cells, demonstrating that specific inhibition of both types of enzyme by oligomycin and vanadate is depleted, possibly by the presence of residual toluene in cell preparations. An analogous attempt to determine the specific activity of membrane-bound ATPase in toluene-permeabilized hyphae of *Penicillium simplicissimum* has also failed (26). In that paper, the authors observed that vanadate inhibition of permeabilized hyphae was lower and poorly reproducible and no distinct optimum pH for vanadate-sensitive ATPase activity was observed after toluene treatment.

Unfortunately, experiments with permeabilized cells could not confirm whether toluene permeabilization reduces the total ATPase activity in relation to intact cells, as observed in experiments with ATPase-rich membrane fractions, since it was not possible to record membrane-bound

ATP activity in intact cells. Despite this, experiments using toluene-treated membrane fractions have offered enough evidence to believe so.

Furthermore, the glycolytic pH-drop assay has demonstrated that toluene-permeabilized cells only poorly acidified the external medium after a glucose pulse, compared with intact cells. This suggests that toluene should interfere with bacterial ATPase-dependent H⁺ extrusion. However, microscopic evidence of plasma membrane rupture and a drastic reduction in cell viability indicate that reduction in transmembrane H⁺ extrusion is more likely to be caused by cell death than to be direct ATPase inhibition by toluene. It is known that the initial stages of organic solvent damage upon bacterial cells consist of binding and penetration into the plasma membrane lipid bilayer. As a consequence, membrane fluidity is affected and bacteria thus launch appropriate responses to diminish the disruptive effects. Membrane fluidity is then re-adjusted, primarily by altering the composition of the lipid bilayer through compensatory mechanisms that resemble some of those observed in response to physical and chemical stresses imposed by the environment (27). It has also been reported that mitochondrial uncoupling via ATP depletion might be responsible for cell toxicity to toluene and xylene in eukaryotic cells, confirming the role of these aromatic volatile organic compounds in the modification of biological membrane structures and reduction of cell viability (28).

Analysis of permeabilized *S. mutans* cells by electron microscopy revealed that toluene treatment not only removes portions of the cell wall, but also ruptures the plasma membrane in most cells, thus causing cytoplasm outflow. This effect suggests that the total ATPase activity detected in permeabilized cells may have resulted from the coupled action of membrane-bound ATPases and cyto-

plasmic ATPases from the intracellular material that leaked into the immediate environment. This would justify the slightly higher values recorded for total ATPase activity in permeabilized cells compared with the sum of both F- and P-type-specific activities. Extra ATPase activity could be due to cytoplasmic ATPases, which would not be subjected to specific inhibition by oligomycin or vanadate.

It should be noted that many studies concerning the membrane-bound ATPase of *S. mutans* using toluene-permeabilized cells have been performed (11,18,29,30). This means that such enzymatic determinations represent a sum of both F- and P-type activities, and perhaps cytoplasmic ATPases, with exception of the studies made with purified F-type ATPase (8). Also, these studies cannot detect the presence of distinctive P-type and F-type activities since toluenization makes the ATPases insensitive to vanadate and oligomycin inhibitors. Our results suggest that, despite being efficient for removal of the cellular wall, toluene-permeabilization of cells is not recommended if the aim is to quantify specific activities of membrane-bound P-type and/or F-type ATPase, whereas the mild conditions used for the preparation of membrane fractions, achieved with enzymatic digestion of the bacterial cell wall, may be more suitable for the study of specific ATPase activity in the presence of biological agents, since these membrane fractions preserve ATPase selectivity for standard inhibitors.

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