Reprinted from *Journal of Dental Research*, June, 1987

Membrane-associated and Solubilized ATPases of *Streptococcus mutans* and *Streptococcus sanguis*

S. V. W. SUTTON and R. E. MARQUIS

Departments of Microbiology and Dental Research, The University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

The proton-translocating, membrane ATPases of oral streptococci have been implicated in cytoplasmic pH regulation, acidurance, and cariogenicity. Membranes were isolated from Streptococcus mutans *GS-5 and* Streptococcus sanguis *NCTC 10904 following salt-induced lysis of cells treated with lysozyme and mutanolysin. The ATPase activities of these membranes were 1.8 and 1.1 units per mg membrane protein, respectively. F_1 ATPases were washed free from the membranes and purified by fast protein liquid chromatography (FPLC). Hydrolytic activities of the F_1 ATPases were maximal at pH values between 6.0 and 6.6, whereas the membrane-bound enzymes had pH maxima of 7.5 (*S. sanguis*) and 6.0 (*S. mutans*). The F_1 ATPases of the streptococci were similar to the well-characterized enzyme of* Escherichia coli*; they consisted of five different polypeptides and had apparent, aggregate molecular weights of from 335 to 350 Kd. The membrane-bound ATPases were characterized biochemically and found to be similar to those of proton-translocating ATPases of* E. coli *and* Streptococcus faecalis. *K_m values for the membranes with respect to ATP were found to be 0.9 and 1.0 mmol/L for* S. mutans *and* S. sanguis, *respectively. Both enzymes had specificities for purine triphosphates and were active with a variety of divalent cations, although optimal activity occurred with ATP and Mg. The membrane-associated enzymes were sensitive to the inhibitors dicyclohexylcarbodiimide (DCCD) and azide, but insensitive to ouabain and vanadate. Overall, it appears that the membrane-associated ATPases of* S. mutans *and* S. sanguis *are similar to the extensively studied proton-translocating ATPases of* E. coli *and* S. faecalis, *and that differences in pH sensitivities of the enzymes from the oral bacteria are greater for the membrane-bound, proton-translocating forms than for the soluble, F_1 forms.*

J Dent Res 66(6):1095–1098, June, 1987

Introduction.

Virulence factors of *Streptococcus mutans* have been recently reviewed by Curtiss (1985), who describes three stages for pathogenicity. The first stage is sucrose-independent attachment of the bacteria to the pellicle on teeth. The second is a firmer attachment which is sucrose-dependent and mediated by the glucosyltransferases of the bacterium. The third stage is the production of acids from glycolytic substrates. Acidogenesis can be distinguished from acidurance, which also plays a major role in pathogenicity, although this role is not fully understood. Many acidogenic plaque bacteria appear not to contribute significantly to caries formation, because they cannot glycolyze in the acid environment of a caries lesion at pH values below about 5.0 (Bunick and Kashket, 1981; Iwami and Yamada, 1980; Thibodeau and Marquis, 1983). Although even a transient pH drop in plaque can result in some demineralization of the tooth surface, this damage may be repaired through remineralization following acid neutralization by the buffers of saliva. The cycle of a drop in pH value followed by a rise, commonly to values greater than 7.0, was first described by Stephan (1940) for whole plaque. Little irreversible damage occurs to the tooth unless the plaque population can function at the low pH values necessary for extensive demineralization. Therefore, acidurance can be considered an important element in the pathogenicity of a cariogenic bacterium such as *S. mutans*.

Cariogenic *S. mutans* organisms are generally more aciduric than the less-cariogenic *S. sanguis*. The difference appears to depend primarily on membrane physiology rather than on inherent molecular properties of enzymes or other cell components (Bender *et al.*, 1985). For example, glycolytic enzymes of the aciduric GS-5 strain of *S. mutans* were not appreciably more acid-resistant than were similar enzymes from the less aciduric, NCTC 10904 strain of *S. sanguis* (Thibodeau and Marquis, 1983). The acidurance of *S. mutans* may be interpreted mainly in terms of its ability to maintain a relatively alkaline cytoplasmic pH by the export of protons. The principal engine involved in cytoplasmic pH regulation in the streptococci appears to be the proton-translocating ATPase (H^+-ATPase) (Kobayashi, 1985).

Earlier studies from our laboratory have shown the importance of the H^+-ATPase in maintaining the barrier functions of the protoplast membrane to proton flow in the oral, lactic-acid bacteria (Bender *et al.*, 1986). In this paper, molecular and biochemical characteristics of the streptococcal enzymes are presented.

Materials and methods.

Bacteria and growth conditions. — *S. mutans* GS-5, *S. sanguis* NCTC 10904, *S. faecalis* ATCC 9790, and *L. casei* ATCC 4646 were maintained in the laboratory by weekly subculture on tryptone-Marmite-glucose agar (Marquis *et al.*, 1973).

Membrane isolation. — Membrane fractions with high ATPase activity were isolated as described by Bender *et al.* (1986) from cells grown in Brain-Heart-Infusion (BHI) broth with 2% glucose and 20 mmol/L DL-threonine, or in tryptone-Marmite-glucose broth. The cells were converted to spheroplasts by use of lysozyme or lysozyme plus mutanolysin and were then lysed. The membrane fractions were isolated by means of differential centrifugation.

F_1 ATPase isolation. — F_1 enzyme was isolated from the membrane fractions by the procedure of Senior *et al.* (1979), modified for use with fast protein liquid chromatography (FPLC). The ATPase-active washes were concentrated over an ultrafiltration membrane (YM100 Filter, Amicon Corp., Danvers, MA) to approximately 10 mL, and then re-suspended in Column Buffer (20 mmol/L Tris, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol [DTT], pH 6.9). This suspension was then concentrated to approximately 10 mL and loaded onto a strong, anion-exchange column (Mono Q Column, Pharmacia Biotechnology, Piscataway, NJ). The ATPase activity eluted as a single peak at a NaCl concentration of about 500 mmol/L when the column was run with a 0 - 1 mol/L NaCl gradient. The active fractions were then pooled and separated by size on a TSK-250 gel permeation column (Biorad Laboratories, Richmond, CA). The pooled ATPase fractions from this separation

Received for publication August 6, 1986
Accepted for publication December 12, 1986
This work was supported by USPHS Research Grants RO1-DE06127 and P50-DE07003 from the National Institute of Dental Research.

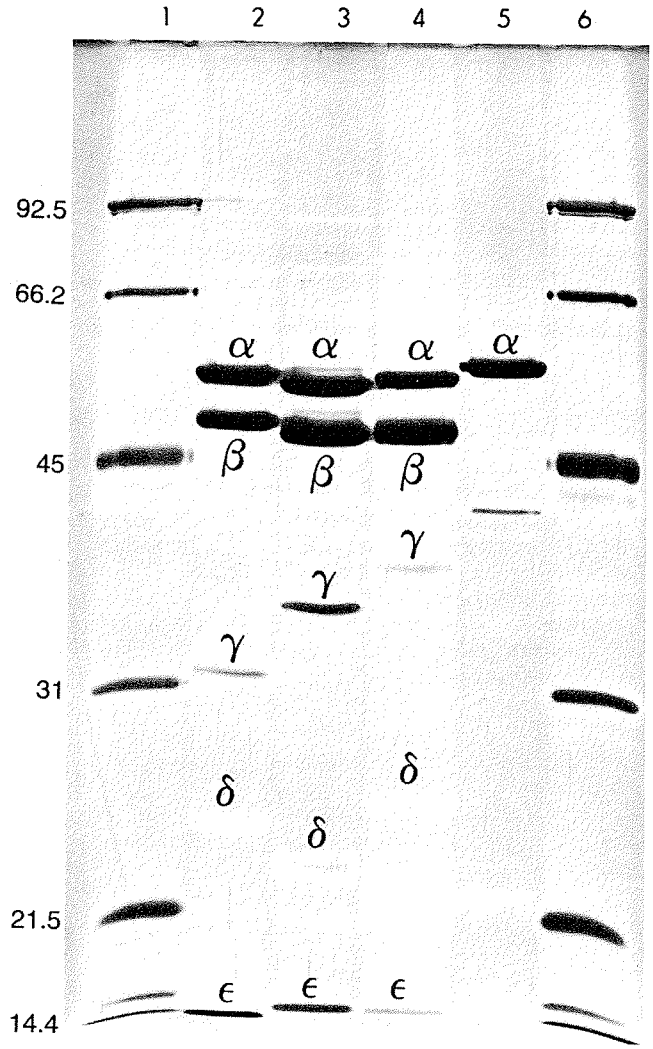

Fig. 1 — SDS-Polyacrylamide Gel Electrophoresis of F_1 ATPases. F_1 ATPases of *E. coli* (Lane 2), *S. mutans* GS-5 (Lane 3), *S. sanguis* ATCC 10904 (Lane 4), and *L. casei* ATCC 4646 (Lane 5). Lanes 1 and 6 contain BioRad low-molecular-weight markers; the molecular weights of the markers are: 92.5 Kd, 66.2 Kd, 45 Kd, 31 Kd, 21.5 Kd, and 14.4 Kd. See Table 1 for an analysis of the molecular weights of the major bands seen in the gel. Non-labeled bands were considered to be proteolytic-breakdown products commonly observed in preparations of isolated F_1 ATPases.

were used for F_1 enzyme preparations. (*E. coli* F_1 ATPase was a generous gift of Dr. Alan Senior.)

ATPase assays. — All assays were performed with a Reaction Buffer containing 50 mmol/L Tris Maleate and 10 mmol/L $MgSO_4$ at the optimal pH for the particular ATPase assayed, unless otherwise noted. The reaction was initiated by addition of ATP to 5 mmol/L to a suspension of membranes pre-warmed to 38°C. F_1 ATPase assays were initiated by the addition of the enzyme. The unit of ATPase activity was defined in terms of μmoles phosphorus released per minute per mg membrane protein (or total protein for the F_1 assays). Phosphate was assayed by a modification of the Fiske-SubbaRow method with reagents obtained from the American Monitor Corp. (Indianapolis, IN) for membranes and by the procedure of Taussky and Schorr (1953) for the F_1 assays. The colorimetric assay was carried out with the use of a spectrophotometer (Model 55, Perkin-Elmer Corp., Norwalk, CT) at 650 nm.

Enzyme kinetics. — K_m values for ATP and Mg were determined by a least-squares fit of data on a Lineweaver-Burk plot of inverse reaction velocity against inverse ATP concentration. The least-squares analysis gives the values c = intercept and m = slope for the straight line equation $y = mx + c$. In the Lineweaver-Burk plot, c can be defined as $1/V_{max}$, and the intercept is $-1/K_m$.

Ion requirements. — Individual salts were added to a suspension of membrane and 50 mmol/L Tris Maleate buffer and incubated for 10 minutes at 38°C. The reaction was initiated by addition of Tris-ATP to 5 mmol/L. Tris Maleate was not contaminated by significant amounts of Mg or Ca, as determined by atomic absorption spectrophotometry.

Drug inhibition assays. — All enzyme preparations were pre-incubated in the presence of the individual drug for 10 min at 38°C before the reaction was initiated by addition of ATP.

Protein determinations. — Membrane protein was assayed by the method of Lowry *et al.* (1951). Soluble, F_1 protein determinations were performed with commercial reagents (Biorad Laboratories, Richmond, CA) utilizing Coomassie staining of the proteins. The assays were calibrated with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) used as the protein standard.

SDS-PAGE. — Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The gels were stained with silver as described by Morrissey (1981).

Chemicals. — DCCD was obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO) and Boehringer Mannheim Biochemicals (Indianapolis, IN).

TABLE 1
PROCARYOTIC F_1 ATPase SUBUNIT STRUCTURE*

Subunit	Organism				
	E. coli	*S. faecalis*	*S. mutans*	*S. sanguis*	*L. casei*
α	58	60	58	59	62
β	54	55	52	51	—
γ	31	37	41	40	—
δ	18	20	27	26	—
ϵ	14	12	18	18	—

*All values are given in terms of Kd. The stoichiometry of the subunit assembly for *E. coli* is $\alpha_3\beta_3\gamma\delta\epsilon$. The entire F_1 ATPase from *E. coli* is 360 Kd (by deduced amino acid sequence), 385 Kd in *S. faecalis*, 350 and 335 Kd by FPLC analysis for *S. mutans* and *S. sanguis*, respectively, and 360 Kd for *L. casei*. *L. casei* F_1 ATPase apparently consists of only one subunit, present in six copies. See text for references.

Results.

Isolation of F_1 ATPase. — The pH-activity profiles described previously for the membrane-bound ATPases of streptococci and *Lactobacillus casei* suggested that the enzymes might have different structures. The F_1 ATPase of *L. casei* ATCC 7469 had been reported to be composed of six copies of a single subunit of molecular weight 42 Kd (Biketov *et al.*, 1982), whereas F_1 ATPases of organisms such as *S. faecalis* and *E. coli* were composed of five different subunits, some of which were present in multiple copies in the native enzyme. As shown in Fig. 1, SDS-PAGE analysis of the enzymes from *S. mutans* and *S. sanguis* showed that the streptococcal enzymes were more closely related to the enzyme of *E. coli* (and *S. faecalis*) than to that of *L. casei*, here the ATCC 4646 strain. The F_1 enzyme of *L. casei* ATCC 4646 was significantly larger than that of *L. casei* ATCC 7469 but still consisted of multiple copies of a single polypeptide. Table 1 gives molecular weights

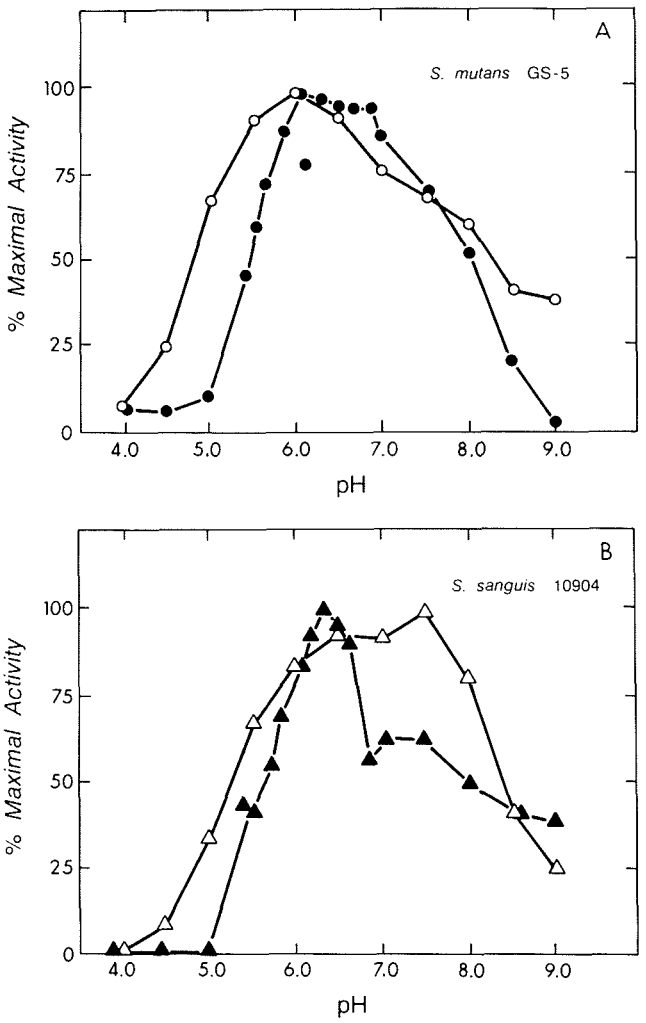

Fig. 2 — pH Profiles of membrane-bound and F_1 ATPases. The points show average values for experiments with each F_1 (closed symbols) or membrane-bound ATPase (open symbols), in 50 mmol/L Tris Maleate with 10 mmol/L $MgSO_4$. The reactions were initiated by addition of ATP to 5 mmol/L (membrane assays) or 10 mmol/L (F_1 assays). Samples were taken at various times to assay phosphate release. 100% activity was defined as the maximum activity seen from the averaged values. These values were 57.8 and 65.3 units per mg protein for the F_1 ATPases of *S. mutans* and *S. sanguis*, respectively, and 1.8 and 1.1 units per mg membrane protein for the membrane-bound enzymes.

for F_1 ATPase subunits determined as described or as reported in the literature.

pH profiles of F_1 ATPases. — The soluble F_1 ATPase for *S. mutans* GS-5 showed a maximal activity between pH values of approximately 6.0 to 6.8, as shown in Fig. 2a. The activity profile for the membrane-bound enzyme is included for comparison. In contrast, the F_1 enzyme of *S. sanguis* showed maximal activity at a pH value of 6.4 (Fig. 2b). For both organisms, the F_1 enzyme was more acid-sensitive than the membrane-associated enzyme. The membrane-associated enzyme of *S. mutans* showed optimal hydrolytic activity at a pH value of about 6.0, compared with a pH value of about 7.5 for the *S. sanguis* enzyme.

Substrate specificity and kinetics. — The membrane-associated enzymes had specificity for purine triphosphates as substrates (Table 2). Nitrophenylphosphate, a substrate for acid- or alkaline-phosphatase, did not serve effectively as a substrate, nor did the glycolytic intermediates tested, except for barely detectable release of phosphate from fructose-1,6 bisphosphate. The relative capacities of various salts to replace $MgCl_2$ in the ATP hydrolytic reaction are presented in Table 3. Comparable results obtained with the well-characterized, membrane-associated enzyme of *S. faecalis* 9790 (isolated in a manner similar to that used for the membranes of *S. mutans* and *S. sanguis* and assayed in our buffer system) are presented in Tables 2 and 3. All three enzymes could catalyze hydrolysis of a number of purine triphosphates but were most active with ATP. Also, they were activated optimally by Mg^{++} and to a lesser degree by certain other divalent cations.

The K_m values for ATP were found to be 0.9 and 1.0 mmol/L for *S. mutans* and *S. sanguis*, respectively, and for Mg^{++}, they were 1.47 and 0.64 mmol/L, respectively.

Inhibitors. — The inhibitory effects of azide, DCCD, vanadate, and ouabain are indicated by the data in Table 4. DCCD is specific for proton-translocating ATPases, and the membrane-associated ATPase of each organism was inhibited by

TABLE 2
SUBSTRATE SPECIFICITIES OF STREPTOCOCCAL MEMBRANE ATPases

Substrate	Relative Activity (%)		
	S. mutans	*S. sanguis*	*S. faecalis*
ATP	100.0	100.0	100.0
GTP	49.3	74.5	84.3
ITP	57.5	71.6	107.6
UTP	13.4	13.5	14.9
CTP	12.0	13.7	6.6
TTP	5.9	9.7	7.1
ADP	7.6	9.5	11.3
NPP	1.4	1.1	7.5
Glu-6-P	0.0	0.0	0.0
PEP	0.0	0.0	0.3
Fru-bis-P	1.5	0.5	3.6

Assays were done twice and the results averaged. The specific activity of ATP under assay conditions was defined as 100%. This activity was 0.74 and 0.18 units per mg membrane protein for *S. mutans* and *S. sanguis*, respectively, and 0.26 units per mg membrane protein for *S. faecalis*. Abbreviations used were: NPP, nitrophenylphosphate; Glu-6-P, glucose-6-phosphate; PEP, phosphoenolpyruvate; and Fru-bis-P, fructose-1,6 bisphosphate.

TABLE 3
CATION SPECIFICITY OF THE STREPTOCOCCAL MEMBRANE ATPases

Salt	Relative Activity (%)		
	S. mutans	*S. sanguis*	*S. faecalis*
$MgCl_2$	100.0	100.0	100.0
$CaCl_2$	27.2	28.3	10.9
$ZnSO_4$	28.9	2.6	2.3
$SrCl_2$	1.4	1.8	0.0
$NiCl_2$	32.6	10.2	19.1
$MnCl_2$	77.0	12.4	62.2
$CoCl_2$	53.4	17.4	60.8
$BaCl_2$	6.9	8.4	0.8
$CuCl_2$	7.3	3.9	30.8
NaCl	4.1	6.4	5.1
Buffer	4.9	9.8	0.4

These results are average values for two assays done in a reaction buffer of 50 mmol/L Tris-Maleate, 5 mmol/L ATP, and 10 mmol/L cation. The reaction mix was pre-incubated at 38°C with 0.125 mg membrane protein (total volume 0.5 mL) and started with the addition of Tris-ATP (Sigma). Specific activity for the reaction utilizing $MgCl_2$ was defined as 100%. This activity was 0.57 units per mg membrane protein for *S. mutans*, 1.13 units per mg membrane protein for *S. sanguis*, and 0.20 units per mg membrane protein for *S. faecalis*.

TABLE 4
INHIBITION OF STREPTOCOCCAL MEMBRANE ATPases

	% Inhibition			
	S. mutans		*S. sanguis*	
Inhibitor	Holoenzyme	F_1	Holoenzyme	F_1
DCCD (0.1 mmol/L)	45	0	61	0
Azide (5 mmol/L)	68	94	54	100
Vanadate (50 - 200 nmol/L)	0	ND	0	ND
Ouabain (50 - 200 nmol/L)	0	ND	0	ND

These results are average values from duplicate experiments. ND stands for "not determined".

this compound. The soluble, uncoupled F_1 ATPases were not inhibited by DCCD. Azide was a potent inhibitor of both the membrane-associated and the soluble ATPases. Neither vanadate, a specific inhibitor of the potassium-translocating ATPase, nor ouabain, an inhibitor of the sodium-potassium activated ATPases, inhibited the streptococcal ATPases.

Discussion.

In this study, the basic biochemistry of the DCCD-sensitive, membrane-bound ATPases of *S. mutans*, *S. sanguis*, and *S. faecalis* was examined. These enzymes appear to be important for acidurance of whole cells and for cytoplasmic pH regulation (Kobayashi, 1985; Bender *et al.*, 1986).

The oxybiontic bacterium *E. coli* generates a proton gradient through respiration which is sufficiently large to drive the synthesis of ATP through the H^+-ATPase, and a major function of the enzyme is in ATP generation. The streptococci lack a functional respiratory chain and depend on substrate-level phosphorylation for ATP synthesis. The primary function of the proton-translocating ATPase in these organisms appears to be in acid-base physiology, and the bacteria have been found to maintain a transmembrane pH gradient as great as 1 unit when they are in an acidic environment (Kashket and Kashket, 1985). Treatment of intact cells with the ATPase inhibitor DCCD markedly increases proton permeability of the membrane (Bender *et al.*, 1986) and dissipates the pH gradient.

The membrane-associated ATPases of *S. mutans* and *S. sanguis* appear to be biochemically similar to the proton-translocating ATPases of *S. faecalis* and *E. coli*. This similarity was demonstrated in terms of substrate preference, cation preference, and inhibitor sensitivities. Physically, the isolated F_1 ATPases of *S. mutans* and *S. sanguis* were similar to those of *E. coli* and *S. faecalis*, with an apparent composition of five dissimilar subunits and a similar apparent aggregate molecular weight. The characteristics of the enzymes of *E. coli* and *S. faecalis* have been reviewed by Futai and Kanazawa (1983) and Abrams (1985). The F_1 ATPase of *L. casei* ATCC 4646 was found to have a different structure, similar to that reported for *L. casei* ATCC 7469 by Biketov *et al.* (1982).

These findings open new possibilities for the study of the pathogenicity of *S. mutans* and other plaque bacteria in terms of the physiology of acidurance, which appears to depend upon the dynamic relationship between the influx of protons across the membrane (or their cytoplasmic production) and the subsequent expulsion of these protons by enzymes of the cell membrane. The biochemical study of the membrane ATPases of these bacteria may well lead to new methods for the moderation of the cariogenicity of these organisms by use of agents that affect proton current across the membrane.

Acknowledgments.

The authors wish to thank Drs. John Wise and Alan Senior for their many helpful comments and contributions, and Kurt Schilling for providing instruction on the use of the HPLC equipment. We thank Gary Bender for his expert technical assistance.

REFERENCES

ABRAMS, A. (1985): The Proton-Translocating Membrane ATPase (F_1F_0) in *Streptococcus faecalis (faecium)*. In: **The Enzymes of Biological Membranes**, Vol. 4, A.N. Martonosic, Ed., New York, NY: Plenum Press, pp. 177–193.

BENDER, G.R.; THIBODEAU, E.; and MARQUIS, R.E. (1985): Reduction of Acidurance of Streptococcal Growth and Glycolysis by Fluoride and Gramicidin, *J Dent Res* 64:90–95.

BENDER, G.R.; SUTTON, S.V.W.; and MARQUIS, R.E. (1986): Proton Permeabilities, Acidurance, and Membrane ATPases of Oral Streptococci, *Infect Immun* 53:331–338.

BIKETOV, S.F.; KASHO, V.N.; KOZLOV, I.A.; MILEYKOVSKAYA, Y.I.; OSTROVSKY, D.N.; SKULACHEV, V.P.; TIKHONOVA, G.V.; and TSUPRUN, V.L. (1982): F_1-like ATPase from Anaerobic Bacterium *Lactobacillus casei* Contains Six Similar Subunits, *Eur J Biochem* 129:241–250.

BUNICK, J.F. and KASHKET, S. (1981): Enolases from Fluoride-resistant and Fluoride-sensitive Streptococci, *Infect Immun* 34:856–863.

CURTISS, R. (1985): Genetic Analysis of *Streptococcus mutans* Virulence, *Curr Top Microbiol Immunol* 118:253–277.

FUTAI, M. and KANAZAWA, H. (1983): Structure and Function of Proton-translocating Adenosine Triphosphatase (F_0F_1): Biochemical and Molecular Biological Approaches, *Microbiol Rev* 47:285–312.

IWAMI, Y. and YAMADA, T. (1980): Rate-limiting Steps of the Glycolytic Pathway in the Oral Bacteria *Streptococcus mutans* and *Streptococcus sanguis* and the Influence of Acidic pH on the Glucose Metabolism, *Arch Oral Biol* 25:163–169.

KASHKET, S. and KASHKET, E.R. (1985): Dissipation of the Proton Motive Force in Oral Streptococci by Fluoride, *Infect Immun* 48:19–22.

KOBAYASHI, H. (1985): A Proton-translocating ATPase Regulates pH of the Bacterial Cytoplasm, *J Biol Chem* 260:72–76.

LAEMMLI, U.K. (1970): Cleavage of Structural Proteins During Assembly of the Head of Bacteriophage T_4, *Nature* 227:680–685.

LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L.; and RANDALL, R.J. (1951): Protein Measurement with the Folin Phenol Reagent, *J Biol Chem* 193:265–275.

MARQUIS, R.E.; PORTERFIELD, N.; and MATSUMURA, P. (1973): Acid-base Titration of Streptococci and the Physical States of Intracellular Ions, *J Bact* 114:491–498.

MORRISSEY, J.H. (1981): Silver Stain for Proteins in Polyacrylamide Gels: A Modified Procedure with Enhanced Uniform Sensitivity, *Anal Biochem* 117:307–310.

SENIOR, A.E.; DOWNIE, J.A.; COX, G.B.; GIBSON, F.; LANGMAN, L.; and FAYLE, D.R.H. (1979): The *uncA* Gene Codes for the Alpha-subunit of the Adenosine Triphosphatase of *Escherichia coli*, *Biochem J* 180:103–109.

STEPHAN, R.M. (1940): Changes in Hydrogen-ion Concentration on Tooth Surfaces and in Carious Lesions, *J Am Dent Assoc* 27:718–723.

TAUSSKY, H.H. and SCHORR, E.J. (1953): A Microcolorimetric Method for the Determination of Inorganic Phosphorus, *J Biol Chem* 202:675–685.

THIBODEAU, E.A. and MARQUIS, R.E. (1983): Acid Sensitivity of Glycolysis in Normal and Proton-permeable Cells of *Streptococcus mutans* GS-5, *J Dent Res* 62:1174–1178.