Genetic Studies of Sugar Transport in *Streptococcus mutans*

by

Dennis Gerard Cvitkovitch

A Thesis
Submitted to the Faculty of Graduate Studies
In partial fulfillment of the requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Oral Biology
Faculty of Dentistry
University of Manitoba
Winnipeg, Manitoba

(c) July, 1995, D.G. Cvitkovitch

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-13056-8

Canada

Name DENNIS GERARD CVITKOVITCH

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Microbiology
SUBJECT TERM

0410
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
- Architecture 0729
- Art History 0377
- Cinema 0900
- Dance 0378
- Fine Arts 0357
- Information Science 0723
- Journalism 0391
- Library Science 0399
- Mass Communications 0708
- Music 0413
- Speech Communication 0459
- Theater 0465

EDUCATION
- General 0515
- Administration 0514
- Adult and Continuing 0516
- Agricultural 0517
- Art 0273
- Bilingual and Multicultural 0282
- Business 0688
- Community College 0275
- Curriculum and Instruction 0727
- Early Childhood 0518
- Elementary 0524
- Finance 0277
- Guidance and Counseling 0519
- Health 0680
- Higher 0745
- History of 0520
- Home Economics 0278
- Industrial 0521
- Language and Literature 0279
- Mathematics 0280
- Music 0522
- Philosophy of 0998
- Physical 0523
- Psychology 0525
- Reading 0535
- Religious 0527
- Sciences 0714
- Secondary 0533
- Social Sciences 0534
- Sociology of 0340
- Special 0529
- Teacher Training 0530
- Technology 0710
- Tests and Measurements 0288
- Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
- Language
  - General 0679
  - Ancient 0289
  - Linguistics 0290
  - Modern 0291
- Literature
  - General 0401
  - Classical 0294
  - Comparative 0295
  - Medieval 0297
  - Modern 0298
  - African 0316
  - American 0591
  - Asian 0305
  - Canadian (English) 0352
  - Canadian (French) 0355
  - English 0593
  - Germanic 0311
  - Latin American 0312
  - Middle Eastern 0315
  - Romance 0313
  - Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
- Philosophy 0422
- Religion
  - General 0318
  - Biblical Studies 0321
  - Clergy 0319
  - History of 0320
  - Philosophy of 0322
- Theology 0469

SOCIAL SCIENCES
- American Studies 0323
- Anthropology
  - Archaeology 0324
  - Cultural 0326
  - Physical 0327
- Business Administration
  - General 0310
  - Accounting 0272
  - Banking 0770
  - Management 0454
  - Marketing 0338
- Canadian Studies 0385
- Economics
  - General 0501
  - Agricultural 0503
  - Commerce-Business 0505
  - Finance 0508
  - History 0509
  - Labor 0510
  - Theory 0511
- Folklore 0358
- Geography 0366
- Gerontology 0351
- History
  - General 0578
  - Ancient 0579
  - Medieval 0581
  - Modern 0582
  - Black 0328
  - African 0331
  - Asia, Australia and Oceania 0332
  - Canadian 0334
  - European 0335
  - Latin American 0336
  - Middle Eastern 0333
  - United States 0337
- History of Science 0585
- Law 0398
- Political Science
  - General 0615
  - International Law and Relations 0616
  - Public Administration 0617
- Recreation 0814
- Social Work 0452
- Sociology
  - General 0626
  - Criminology and Penology 0627
  - Demography 0938
  - Ethnic and Racial Studies 0631
  - Individual and Family Studies 0628
  - Industrial and Labor Relations 0629
  - Public and Social Welfare 0630
  - Social Structure and Development 0700
  - Theory and Methods 0344
- Transportation 0709
- Urban and Regional Planning 0999
- Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
- Agriculture
  - General 0473
  - Agronomy 0285
  - Animal Culture and Nutrition 0475
  - Animal Pathology 0476
  - Food Science and Technology 0359
  - Forestry and Wildlife 0478
  - Plant Culture 0479
  - Plant Pathology 0480
  - Plant Physiology 0817
  - Range Management 0777
  - Wood Technology 0746
- Biology
  - General 0306
  - Anatomy 0287
  - Biostatistics 0308
  - Botany 0309
  - Cell 0379
  - Ecology 0329
  - Entomology 0353
  - Genetics 0369
  - Limnology 0793
  - Microbiology 0410
  - Molecular 0307
  - Neuroscience 0317
  - Oceanography 0416
  - Physiology 0433
  - Radiation 0821
  - Veterinary Science 0778
  - Zoology 0472
- Biophysics
  - General 0786
  - Medical 0760

EARTH SCIENCES
- Biogeochemistry 0425
- Geochemistry 0996
- Geodesy 0370
- Geology 0372
- Geophysics 0373
- Hydrology 0388
- Mineralogy 0411
- Paleobotany 0345
- Paleoecology 0426
- Paleontology 0418
- Paleozoology 0985
- Palynology 0427
- Physical Geography 0368
- Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
- Environmental Sciences 0768
- Health Sciences
  - General 0566
  - Audiology 0300
  - Chemotherapy 0992
  - Dentistry 0567
  - Education 0350
  - Hospital Management 0769
  - Human Development 0758
  - Immunology 0982
  - Medicine and Surgery 0564
  - Mental Health 0347
  - Nursing 0569
  - Nutrition 0570
  - Obstetrics and Gynecology 0380
  - Occupational Health and Therapy 0354
  - Ophthalmology 0381
  - Pathology 0571
  - Pharmacology 0419
  - Pharmacy 0572
  - Physical Therapy 0382
  - Public Health 0573
  - Radiology 0574
  - Recreation 0575
  - Speech Pathology 0460
  - Toxicology 0383
- Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences
- Chemistry
  - General 0485
  - Agricultural 0749
  - Analytical 0486
  - Biochemistry 0487
  - Inorganic 0488
  - Nuclear 0738
  - Organic 0490
  - Pharmaceutical 0491
  - Physical 0494
  - Polymer 0495
  - Radiation 0754
- Mathematics 0405
- Physics
  - General 0605
  - Acoustics 0986
  - Astronomy and Astrophysics 0606
  - Atmospheric Science 0608
  - Atomic 0748
  - Electronics and Electricity 0607
  - Elementary Particles and High Energy 0798
  - Fluid and Plasma 0759
  - Molecular 0609
  - Nuclear 0610
  - Optics 0752
  - Radiation 0756
  - Solid State 0611
- Statistics 0463

Applied Sciences
- Applied Mechanics 0346
- Computer Science 0984
- Engineering
  - General 0537
  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
  - Electronics and Electrical 0544
  - Heat and Thermodynamics 0348
  - Hydraulic 0545
  - Industrial 0546
  - Marine 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
  - Sanitary and Municipal 0554
  - System Science 0790
- Geotechnology 0428
- Operations Research 0796
- Plastics Technology 0795
- Textile Technology 0994

PSYCHOLOGY
- General 0621
- Behavioral 0384
- Clinical 0622
- Developmental 0620
- Experimental 0623
- Industrial 0624
- Personality 0625
- Physiological 0989
- Psychobiology 0349
- Psychometrics 0632
- Social 0451

GENETIC STUDIES OF SUGAR TRANSPORT IN STREPTOCOCCUS MUTANS

BY

DENNIS GERARD CVITKOVITCH

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

© 1995

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and LIBRARY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or other-wise reproduced without the author's written permission.

To my Family.

Susan, Katherine, and Graham.

Their company is always the brightest time of my day

Table of Contents

Acknowledgements

The author wishes to express his utmost gratitude and appreciation to Dr. Ian Hamilton, who, with his invaluable experience, patience, support and encouragement allowed me to fulfill my pursuits. Many thanks to Dr. George Bowden, who provided me with guidance through his invaluable insight and wisdom of the 'big picture' of oral ecology. I also owe special thanks to Mr. Dave Boyd who made his excellent technical background available to me throughout the course of this work and taught me the 'hands-on' skills of molecular biology. Thanks also to Dr. Song Lee for providing me with invaluable advice in the design of countless experiments. I am also indebted to Dr. Christian Vadeboncoeur and Dr. Michel Frenette for antibodies, probes and advice, which were paramount to the success of this project.

I would also like to acknowledge the support of Ms. Tracy Thevenot, who demonstrated her ability to master crossed immunoelectrophoresis while raising twins. Thanks to Dr. Nicole Buckley who built a strong biochemical basis for the preceding work.

I also thank Beverly Grimshire for many years of support and for helping me locate those long forgotten items, and to Elke Grief and Nora Nolette for much appreciated assistance.

Abstract

Streptococcus mutans is the principal etiological agent of dental caries, the demineralization of tooth enamel by the acid end-products of carbohydrate metabolism. This organism transports a variety of carbohydrates including glucose via the phosphoenolpyruvate: sugar phosphotransferase system (PTS), a group translocation process whereby the sugar is simultaneously transported across the cell membrane and phosphorylated at the expense of phosphoenolpyruvate (PEP). Non-PTS sugar transport systems in this organism include the well-characterized multiple sugar metabolism (MSM) system, which transports raffinose, melibiose, and isomaltosaccharides and a yet uncharacterized glucose transport system, suggested to exist from indirect biochemical evidence.

The aim of this thesis was to investigate the non-PTS glucose transport processes and to examine PTS-mediated regulation of non-PTS sugar transport. This was accomplished by first cloning the *ptsI* and *ptsH* genes coding for the general PTS proteins Enzyme I (EI) and HPr, respectively. The nucleotide and deduced amino acid sequences of these genes were determined and compared to those of other bacteria, where they showed significant homology. The expression of the functional enzymes in *E. coli* hosts was demonstrated in vitro by [^{32}P]-PEP phosphorylation that indicated transfer of phosphate from the *S. mutans* EI to the *E. coli* HPr.

Complementation of *pts* mutations in *E. coli* hosts harboring plasmids containing the *S. mutans ptsH* or *ptsI* gene was also demonstrated by restoration of glucose fermentation.

The cloned *ptsI* gene was then utilized to construct an integration vector pDC-5 to transform the parent strain *S. mutans* BM71 to generate an isogenic *ptsI* mutant DC10. DC10 was unable to ferment PTS substrates because it expressed a non-functional EI as demonstrated by it inabilility to be phosphorylated by [^{32}P]-PEP and subsequently transfer the phosphate to HPr. DC10 was however able to transport glucose via the secondary system which had a K_s of 125 μM and a Vmax of 0.87 nmol mg (dry weight) of cells^{-1} min^{-1}. Glucose transported via this system was shown to be phosphorylated by ATP and not by PEP. Sugar competition experiments with DC10 indicated that this system had high specificity for glucose, since glucose transport was not significantly inhibited by a 100-fold molar excess of several competing sugars, including the glucose analogs 2-deoxyglucose (2-DG) and α-methylglucoside (α-MG). These results indicate that *S. mutans* has a glucose transport system that can function independently of the PTS.

DC10 was also shown to have a reduced capacity for growth, acid production and transport with the MSM substrate raffinose, having rates 50%, 7% and 10%, that of the parent BM71, respectively. This suggested PTS-mediated control of MSM transport, which was subsequently confirmed by the ability of the PTS substrates glucose, 2-DG, and α-MG to

inhibit transport of [^{3}H]-raffinose. A 59 kDa protein was phosphorylated via [^{32}P]-PEP in cell extracts of raffinose grown BM71, but was absent in DC10, suggesting a possible regulatory role for this protein.

Chapter 1

Introduction

(A) The Role of *Streptococcus mutans* as an Oral Pathogen

1. The oral environment. The oral cavity provides microorganisms with a variety of habitats including hard and soft tissues that can harbor complex microbial communities comprised of a variety of genera of bacteria (14). Some of these bacteria are found exclusively in the mouth and this mixed flora usually exist without ill effects to the host (14,110). Most oral bacteria reside in plaque, a biofilm composed of an aggregate of bacteria and extracellular matrix comprised primarily of polysaccharide (62). Plaque can contain many genera including *Actinobacillus, Actinomyces, Bacteroides, Bifodobacterium, Corynebacterium, Eikenella, Eubacterium, Fusobacterium, Hemophilus, Lactobacillus, Leptotrichia, Neisseria, Peptococcus, Peptostreptococcus, Prevotella, Propionibacterium, Rothia, Selenomonas, Streptococcus, Treponema, and Veillonella* (68).

The composition of the microbial communities found at the various locations in the mouth is affected by a number of environmental factors, including, the site, the available nutrient supply, the oxygen concentration, numerous microbial interactions and the flow of saliva (12,105,196). The composition of the microbial community also changes as the plaque ages or matures with a succession of microorganisms commencing in a complex climax community (14). The

environmental conditions in plaque can differ greatly at various times and locations resulting in different habitats each supporting their own microbial communities.

The diversity and relative proportion of each species of bacteria found at a particular site is a direct reflection of the local environment experienced by the plaque community (12,14). The variability of some of these environmental parameters and their effects on the oral bacteria have been extensively studied (see reviews: 14,64,105). The oral flora has evolved in response to the local environment and thus has resulted in the development of physiological traits necessary to contend with the many fluctuations within this microcosm. For example, it has been demonstrated that oral bacteria can rely on the components of saliva as a sole source of nutrients between meals, when the free sugar concentration is very low (~50 μM) (13,31), and yet, at times immediately following the ingestion of food by the host they are able to cope with increases in the concentration of sugar by as much as 10,000-fold (22). Many oral bacteria are capable of rapid metabolism of these carbohydrates, and in some instances, the production of acid-end products by these organisms leads to an acidification of the surrounding matrix. The pH of plaque has also been shown to vary from above 8.0 on smooth surfaces to below 4.0 in caries lesions as a consequence of the metabolism of these dietary sugars (42,83,180,181).

Another factor influencing the composition of the plaque community is the availability of oxygen, which can vary from as high as 16% on the surface of the tongue to as low as 0.3% or less in the buccal fold (between the cheek and gum) (93,104). 'Climax' plaque contains less oxygen than 'commensal' plaque and the deepest areas of plaque, such as in pits and fissures of teeth, contain virtually no detectable oxygen. The oxygen is utilized as a terminal electron acceptor by facultative anaerobes living in the outer layers, allowing the deep areas of plaque to be inhabited by strict anaerobes (177).

The buffering and clearing powers of saliva also influence the bacterial populations in plaque. Saliva not only removes acids from the tooth surface but also contains anti-microbial components, such as, lysozyme, lactoferrin, and peroxidase-thiocyanate which attack susceptible organisms (109). Saliva also contains immunoglobin A (IgA) which is postulated to prevent bacteria from adhering to oral surfaces. The bacteria that reside in 'normal' plaque are generally resistant to these antimicrobial factors in saliva. Therefore, their populations are protected from colonization and competition by bacteria from the environment which are suceptible to these antimicrobials. The importance of saliva in maintaining oral health is clearly seen in patients with xerostomia, a condition where the salivary glands are non-functional. Xerostomia often results from radiotherapy for

treatment of head and neck cancer (17). If they are not treated these patients can experience a characteristically high caries rate. Treatment involves rinsing their mouths frequently, restricting their intake of dietary carbohydrates, and fluoride treatment to maintain health.

2. Dental caries and streptococci. The streptococci are often predominant members of plaque communities, and considerable attention has been given to their role as pathogens in the initiation and progression of dental caries. Caries is a disease of the tooth characterized by dissolution of the enamel by organic acids resulting from carbohydrate metabolism by bacteria on the tooth surface (105). This association of the streptococci with this disease stems from the ability of several species of these bacteria to degrade dietary carbohydrates to produce acid end-products that are a characteristic of caries-causing 'acidogenic plaque'(12,68,105).

Caries has a multifactorial etiology requiring: (i) a susceptible site, (ii) a diet high in carbohydrate, and (iii) caries causing bacteria (94). The bacteria that reside in dental plaque are usually harmless to the host and, in many ways, are actually beneficial, since a stable plaque community discourages oral colonization by other potentially pathogenic bacteria (110,111). Under the appropriate environmental conditions, the composition of the plaque community changes to produce 'cariogenic plaque'. An example

of this would be an increased intake of refined sugar and a decrease in oral hygiene by the host. Under such conditions, acidogenic plaque bacteria generate acid from the metabolism of dietary sugars, which in turn, lowers the local pH in the biofilm. Thus, as a result of succession, the bacteria that cannot adapt to acid tolerance are gradually displaced by those that are acid tolerant or aciduric (12,196). Continued carbohydrate metabolism by this 'pathogenic' plaque community can eventually lead to the initiation and progression of dental caries. This occurs when the rate of demineralization of the tooth enamel by the bacterial acids exceeds the rate of remineralization of enamel by saliva for a prolonged period (12,195).

The oral streptococci are among the dominant members of the oral flora and several species can be found in acidogenic plaque. Currently, the oral streptococci include *Streptococcus oralis, S. salivarius, S. mitis, S. sanguis sensu stricto, S. gordonii, S. parasanguis, S. crista, S. intermedius, S. anginosus, S. intermedius,* and *S. constellatus* and the 'mutans' streptococci which are comprised of *S. mutans, S. sobrinus, S. cricetus, S. rattus, S. ferus, S. downeii and S. macacae* (43,105). Of these species, *S. mutans* and *S. sobrinus* species are generally regarded as the principal agents of dental caries in humans (105,111,185,195), although some 'non-mutans' streptococci

have also been implicated as potentially cariogenic (166,195,199).

Species of other genera have also been associated with dental caries, including *Lactobacillus casei* (17,87,106), *Actinomyces odontolyticus* (15) and *Actinomyces viscosus* (now *Actinomyces naeslundii* genospecies II) (116). These organisms may play a significant role in the development of carious lesions, but the extent of their involvement is uncertain at this time. It is conceivable, however, that if these organisms can successfully compete with the acidogenic streptococci they may share similar 'pathogenic' characteristics and may, therefore, be capable of contributing to caries (12). The presence of an acidogenic plaque community is not, however, the only requirement for dental caries to develop. This is illustrated in a study of school children in Sudan who had high levels of 'mutans' streptococci, but low levels of caries (20). The reason for this is unknown, but it is suspected that a diet low in refined sugar is partially responsible for the health of these individuals, re-enforcing the concept that caries is a multifactorial disease.

Of all the bacteria associated with dental caries, the 'mutans' streptococci have been implicated as the principal causative agents of the disease. Earlier studies which used colony morphology to identify *S. mutans* as opposed to serological identification failed to distinguish between

S. mutans and *S. sobrinus*. Recent work has however demonstrated that *S. mutans* is isolated from carious lesions with a higher frequency then *S. sobrinus* resulting in *S. mutans* being the subject of more research (12,64,195). The strong affiliation of *S. mutans* with caries arises from its association with the disease in humans, its cariogenic potential in experimental animals, and its physiological properties several of which are considered to be virulence factors (64,195).

3. Virulence factors of *S. mutans*. Since a strong case has been built for the association of *S. mutans* with dental caries, its physiology has been studied extensively in order to understand the mechanisms it utilizes to colonize and dominate plaque communities during the initiation and progression of the disease (64,105). Many physiological properties have been implicated as virulence factors and a few of these properties have recently been characterized at the genetic level (152). The use of molecular biology has allowed construction of defined mutants defective in the supposed virulence factor of interest. The identification of each individual property as a virulence factor has often been strengthened by experiments comparing the cariogenic potential of these mutants to their parental strains. The mutants often demonstrated a decreased cariogenicity in animal models. Principal among the virulence factors are: (i) the ability to generate and tolerate (acidurance) acid

end-products, (ii) extracellular polysaccharide production; and (iii) intracellular polysaccharide storage and metabolism. Each of these factors has been extensively studied (64,105,152) and will, therefore, be discussed here with emphasis on the recent findings, particularly with respect to the application of molecular biology.

(i) Acidurance. A primary factor that contributes to the ability of *S. mutans* to survive and dominate carious sites is its acidogenic and aciduric properties. These permit the organism not only to rapidly generate acid end-products from the metabolism of carbohydrates, but allow it to grow and tolerate acidic environments (12,64,80.105). *S. mutans* was originally believed to be homofermentative and was presumed to generate lactate as a sole end-product of metabolism. It was subsequently demonstrated that this organism was actually homofementive and heterofermentive producing lactate with excess glucose, but also acetate, formate, and ethanol under conditions of limiting glucose (21). Recent studies have also demonstrated that lactate is the principal organic acid found in carious dentin, comprising 88% of the total measured acid (83).

The acidogenic and aciduric properties of *S. mutans* are illustrated in recent in vitro studies designed to test the ability of *S. mutans* and other plaque bacteria to grow and metabolize carbohydrates at pH values below 7.0 (79,80). When grown in batch culture, several *S. mutans* strains, a

strain of *L. casei*, and also a strain of *Streptococcus (Enterococcus) faecalis*, were able to grow and metabolize carbohydrate at pH 5.0, while strains of *S. sanguis*, *S. mitis*, *S. salivarius* and *A. viscosus* were incapable of doing so. Of these aciduric bacteria, *S. mutans* strains were the most acid tolerant, capable of carbohydrate metabolism at pH 4.0, with an optimal pH of 5.0. These and subsequent experiments illustrate the aciduric properties of *S. mutans* and provide insight into its role as a dominant organism in the low pH environments of carious lesions.

Bacteria, including *S. mutans*, have evolved homeostatic systems that allow them to persist under variable levels of external pH. Under acidic conditions these systems maintain the internal pH of the organism in a range suitable for cytoplasmic enzymes to function. This protection from acid is accomplished by several means, including: (i) the extrusion of protons, (ii) a decrease in the permeability of the cell to protons (9), or (iii) even by alteration of the external pH by the production of deaminases and decarboxylases (4,55). Defensive mechanisms involving the activation of multi-gene systems, or stimulons, to protect bacteria against acid have been well characterized in several species of bacteria including *Escherichia coli* (115,172,176,202), *Salmonella typhimurium* (47,49,50), *Vibrio cholerae* (128) *Thiobacillus ferrooxidans* (3), and *Rhizobium meliloti* (61).

The application of molecular biology to the analysis of pH adaptive responses has led to the discovery of several genes that are under the control of pH-induced signals. The best characterized response to an acid pH has been observed in *Sal. typhimurium* (47,48,49,50,51). This organism employs three distinct physiological responses to protect itself from external acid. Two are pH-dependent with the first termed the log-phase 'acidification tolerance response' (ATR) which is comprised of pre-acid shock and acid shock, the former is induced when exponential-phase cells are exposed to mild acid (pH 5.8), and involves synthesis of ATR-specific homeostatic systems to augment the constitutive pH homeostatic mechanisms. The latter log-phase ATR process (acid shock) is activated when cells experience rapid changes in pH levels from neutral to as low as pH 4.0. Although the pre-acid shock and the acid shock systems are activated independently, both are required for the survival of cells of *Sal. typhimurium* at pH 3.3 (48,49,50). The second pH-dependent system is termed the stationary phase ATR as the proteins specific to this response are expressed under acidic conditions in the stationary phase of growth (101). A third acid response is not induced by low pH but is activated as part of a general stress response modulated by a growth-phase-dependent sigma factor *rpoS* (σ^s), which initiates transcription of several genes when the bacteria enter stationary phase (101).

Together in *Sal. typhimurium* these three mechanisms afford protection from pH levels as low as 2.0, a condition encountered by these organisms when ingested by phagolysosomes of macrophages (48). Currently, there is evidence suggesting that *S. mutans* also exhibits specific adaptive responses to a lowering of its surrounding pH (6,13,66,69). The ATR of *Sal.typhimurium* could serve as a model for the acid adaptive response in *S. mutans,* allowing the use of established methods to study this phenomenon. An experiment performed using continuous culture has clearly demonstrated that a gradual reduction in external pH allows *S. mutans* to adapt to acidic environments (66). The pH of *S. mutans* cultures was maintained at pH 7.0 in a chemostat by the automatic addition of KOH. When the the pH of the culture was rapidly decreased to pH 4.8 by the addition of HCl, the culture was unable to persist and was 'washed out' of the chemostat within 4 days. Conversely, when the pH controller was shut off and the pH of the culture allowed to fall to 4.8 due to the production of acids from glucose metabolism, this 'biologically generated' pH drop resulted in a reduction of cell numbers by a factor of 20, but the cell density remained at a constant level for the 6 day duration of the experiment at low pH. Adaptation to acidic pH was also demonstrated by the glycolytic rate of these cells which was greatly diminished following the initial drop in pH but increased over time to rates near those of neutral pH-grown

cells. In a separate study pre-exposure to mild acids of pH 5.0 was also shown to increase the organisms ability to survive low pH (2.5)(6). These experiments strongly suggest that *S. mutans* is capable of adaptation to acid when pre-exposed to mildly acidic conditions.

Subsequent experiments to investigate the mechanisms of adaptation to acid used by *S. mutans* concentrated on alterations in selected phenotypic properties of the cells on exposure to low environmental pH. The best characterized of these responses was the increased expression of a membrane-bound H^+/ATPase (7) which increased 4-fold in cells grown at pH 5.5 compared to cells grown at pH 7.0 (69). This increase in H^+/ATPase activity enhanced the cell's ability to extrude protons from the cytoplasm in order to maintain an intracellular pH range where cytoplasmic enzymes will function. This stabilization of the internal pH allowed glycolysis to continue in the cells exposed to external pH values as low as 4.0 (7). Other important protective mechanism observed when *S. mutans* was grown at low pH included decreased pH optima for sugar transport and glycolysis and an increase in proton impermeability (69). The ability of *S. mutans* to grow at low pH is likely the result of the mechanisms mentioned above and a number of uncharacterized physiological changes.

The role of the adaptive response to acid in enhancing the ability of *S. mutans* to compete against other aciduric

bacteria has been clearly illustrated in a continuous culture experiment utilizing a mixture of *L. casei* and *S. mutans*. (13). When the pH of the culture was rapidly reduced to 4.8 by the addition of HCl, the *S. mutans* population was dramatically reduced and the *L. casei* dominated the culture. When the cultures were individually pre-adapted to pH 5.5 and then mixed in a single chemostat at pH 5.5, the *S. mutans* population dominated. This suggests that a gradual reduction in environmental pH is a signal for the organism to initiate expression of genes coding for specific enzymes which protect the cell against acids giving it a selective advantage over other bacteria in the plaque environment.

Little is known of the genetics of the acid tolerance response in *S. mutans* and at present there is only one published report of the isolation of a gene believed to be involved in acid tolerance (205). In this study, Yamashita et al. (1994) used transposon mutagenesis to isolate a mutant that was able to grow at pH 7.0, but was unable to grow at pH 5.5. Isolation and nucleotide sequence analysis of the gene indicated it was a homolog of diacylglycerol kinase of *E. coli*, an enzyme involved in phosphatidic acid formation (201). It is suspected that diacylglycerol kinase may also play a role in a general stress response by *S. mutans*, since the pH-sensitive mutant also exhibited increased sensitivity to high temperature and high osmolarity relative to the parent strain (205). Caution must, however, be exercised

when assigning roles to inactivated genes based on the resultant phenotype since genes coding for constitutive 'housekeeping functions' may play a general role in the hardiness of an organism, but may not be involved in a specific response to environmental stimuli. Our understanding of the ATR-like response in *S. mutans* will undoubtedly be enhanced by an in-depth study of the physiological and genetic mechanisms involved.

(ii) Extracellular polysaccharides. The study of sucrose utilization by oral streptococci, and *S. mutans* in particular, has received a great deal of attention since the unequivocal link between this disaccharide and caries has been well established (63, 119). Sucrose is readily metabolized and converted to acid end-products by *S. mutans*. The modes of sucrose transport and metabolism have been studied extensively and will be discussed later. A small percentage (< 10%) of this dietary sucrose is, however, acted upon by extracellular glucosyl- (GTF,EC 2.4.1.10) and fructosyl- (FTF,EC 2.4.1.5) transferases which form extracellular glucans and fructans, respectively (64). Glucosyltransferases transfer the glucose moiety of sucrose onto a heterogeneous group of extracellular glucans and release free fructose. Fructosyltransferases transfer the fructose moiety of sucrose onto inulin-like fructans and release free glucose (64). These polymers act as an exogenous source of energy, modify the biofilm matrix

increasing its permeability to solutes (198) and were originally believed to be essential in the attachment of the bacteria to the tooth surface (105). Currently, however, it is believed that their role in attachment is minimal (105).

The complete nucleotide sequences of at least 9 GTF genes from oral streptococci are known and several more are presently being characterized (152). *S. mutans* has 3 distinct GTF genes (*gtfB*, *gtfC* and *gtfD*) (152) with several polymorphic variations being observed among strains, sometimes affecting the structure of the synthesized product (27,204). It appears that they are all approximately 1,500 amino acids in length and share several common regions, including a signal peptide of about 30 amino acid residues that shares homology with other secreted proteins (151). This region is followed by a highly variable stretch of about 200 amino acids which, in turn, is followed by a 1000 bp region that contains interspersed variable and conserved regions (151). The molecular structure of some of these enzymes has also been determined and site-directed-mutagenesis studies are currently underway to determine the regions of the enzymes involved in the binding of sucrose and its conversion to the polymeric glucans (92,120). As shown in animal models, insertional inactivation of these genes to generate GTF-deficient mutants has demonstrated that strains lacking a functional enzyme often have a decreased cariogenic potential relative to the parent strain (121,204).

Many studies have also focused on the role of the fructosyltransferases in the cariogenicity of *S. mutans* (64,121,171), since most isolates produce at least one FTF enzyme (64). Several of these studies have employed the molecular approaches used to study the glucosyltransferases and most are focused on the role of the enzyme in generating fructan for adhesion and energy storage (96,167,173,203). The gene encoding the FTF from *S. mutans* GS5 has been cloned and sequenced, and has been designated *ftf* (167,173). This gene has been inactivated in a cariogenic parent strain and the *ftf* mutant was found to be less cariogenic then the parental strain (171). The resultant loss of cariogenicity was originally believed to result from the inability of the mutant to generate extracellular fructan for use as an exogenous energy source (171). However, a separate study, utilized a mutant defective in *fruA*, the gene coding for the extracellular fructanase which degrades extracellular fructan to fructose for subsequent metabolism. In vivo experiments using a rat model demonstrated that this *fruA* mutant retained its cariogenic potential, when compared to the parent strain (203). These results suggested that the adhesive and aggregative properties of fructan likely provided a greater contribution to the cariogenic potential of *S. mutans* than the ability to degrade the polymer for energy.

(iii) Intracellular polysaccharide storage and metabolism. The ability of *S. mutans* to produce and utilize intracellular

storage polysaccharides (IPS) has been associated with the cariogenicity of the organism for many years (60). Interest in this phenomenon began when it was discovered that *S. mutans* strains isolated from carious lesions contained large amounts of iodine-staining polysaccharide (60). This storage compound has been identified as glycogen and is comprised of glucose molecules joined in an $\alpha(1-4)$ backbone with $\alpha(1-6)$ branching (28). The association of IPS production with dental caries stems from the observation that *S. mutans* can utilize IPS under conditions of low nutrient availability and consequently generate acid end-products for prolonged periods in the absence of dietary carbohydrate (65). This continued production of acids by *S. mutans*, in combination with its aciduric nature, contributes to its dominance in cariogenic plaque (105).

The cellular processes involved in IPS synthesis in *S. mutans* have been studied in detail and it has been established that the (ADP)-glucose synthase (pyrophosphorylase) (EC 2.7.7b) synthesizes ADP-glucose from ATP and glucose and ADP-glucose:glycogen glucosyltransferase (EC 2.4.1a) catalyzes the addition of glucose to a glycogen primer (8). Under conditions of low or depleted external carbohydrate, glycogen phosphorylase and a de-branching enzyme can liberate glucose-1-P from glycogen (179), and is thus readily converted to glucose-6-P in the glycolytic pathway (86).

The genes encoding the glycogen synthetic and degradative enzymes have not yet been cloned or characterized, however recent work by Spatafora-Harris et al. (178) has identified a gene (*glgR*) believed to be involved in the regulation of glycogen synthesis. A stable isogenic mutant harboring a defective copy of the gene was unable to produce IPS and, when tested for its ability to generate caries in a rat model, was found to be non-cariogenic. These results along with previous studies (186,208) lend strong support to the role of IPS as a virulence factor in *S. mutans*.

(B) Transport

In most environments where bacteria live, including the oral cavity, the substrates that they require are often available at very low concentrations. Consequently, bacteria have evolved sets of proteins which comprise a diverse array of transport systems that allow them to accumulate nutrients to intracellular levels that will support their metabolic processes. The importance and diversity of these systems becomes immediately obvious when one considers that *E. coli* alone has over 100 genetically-distinct transport systems (122). The types of transport mechanisms used by bacteria include: simple diffusion, facilitated diffusion, and active transport via proton motive force, binding-protein-dependent transport and group translocation (39). Each will be briefly

described and the characterization of these systems in oral streptococci, and especially *S. mutans*, will be discussed.

1. Simple diffusion. The membrane that surrounds the bacterial cell is impermeable to most solutes. Some small molecules, such as, O_2, CO_2, NH_3 and H_2O, however, are able to diffuse across the membrane. This diffusion requires a concentration gradient across the cell membrane, with the solute moving from the side of the membrane with the higher concentration to the side with the lower concentration until equilibrium is reached. The rate of movement across the membrane is determined by the membrane's permeability to a particular solute, the surface area of the membrane, and the difference in concentration across the membrane (122). This type of transport is called simple or passive diffusion as it has no requirement for energy.

2. Facilitated diffusion. In order for bacteria to transport nutrients that are normally impermeable to the membrane, they must have a carrier protein associated with the membrane that acts as a gate to allow the uptake of specific molecules. The simplest transport process of this type is termed facilitated diffusion. A carrier has similar properties to an enzyme in that it has specificity for a certain molecule and acts somewhat as a 'catalyst' allowing the rate of transport to reach much higher rates than that of simple diffusion. This process requires no energy and results in the accumulation of substrates only to levels

equal to their exterior concentration (122). This type of transport is relatively rare in bacteria and the best characterized example of this type of process is the glycerol facilitator of *E. coli* (165). Growth of this organsim on glycerol is characterized by a decrease in growth rate as the external substrate concentration is depleted. This occurs since the internal concentration cannot exceed the exterior concentration, and as the growing culture depletes it from the medium, less glycerol is available for the intracellular metabolic enzymes (165).

3. Active Transport. The processes of simple and facilitated diffusion are very limited in their capacity to support growth of bacteria in the conditions encountered in most environments. In order for a solute to be accumulated in the cell at a higher concentration than that of the external medium, the bacterium must generate a concentration gradient, rather than exploit one. The elegant processes that have evolved in bacteria to accomplish this task require energy, usually derived from the high-energy-phosphate bonds of ATP, PEP, or from the electrochemical gradient generated by proton motive force (149). In some cases, these transport systems are able to accumulate solutes at up to 10^4 times their exterior concentration as illustrated with K^+ uptake in *E. coli* (78).

(i) Proton motive force (PMF). Since a detailed description of PMF is beyond the scope of this thesis the

process will be described briefly (see reviews: 77,91). The theory of PMF, developed by Mitchell in 1961, hypothesized that that energy derived from biological oxidations could be stored across a membrane in the form of a proton electrochemical potential difference (117). This hypothesis was not readily accepted by many fellow biochemists since this type of biological energy transduction involving an equilibrium between chemical and electrical gradients was a radically new idea. This chemiosmotic theory has, however, established itself as the central mode of energy transfer from electron transport to ATP synthesis in mitochondria, chloroplasts and bacteria. The validity of Mitchell's theory was gradually accepted, following confirmation by a multitude of studies and he eventually received the Nobel prize in Chemistry in 1978 for his hypothesis. In bacteria, PMF has not only been implicated in active transport, but also for maintaining the cell's turgor, maintaining intracellular pH, flagellar locomotion, reduction of NAD to $NADH_2$ by reversing electron flow through the respiratory chain, and generating ATP via F_1F_0 ATPases (39,122).

Briefly, the chemiosmotic theory states that biological oxidations result in the expulsion of protons from the cell to generate a chemical gradient of hydrogen ions, which can be detected as a pH difference between the interior and the exterior of the cell (ΔpH), and as an electric potential difference across the membrane ($\Delta\mu_{H+}$), similar to the current

generated in a battery. The general equation that describes this electrochemical gradient is:

$$\Delta\mu_{H+} = \Delta\psi - Z\,\Delta pH$$

where $\Delta\psi$ is the membrane potential and Z is the factor for converting pH units to millivolts.

In many bacteria, including *S. mutans*, this gradient is generated by the efflux of protons with the end-product lactate, known as end-product efflux, as well as by their expulsion by a membrane-bound proton-translocating ATPase, which extrudes protons at the expense of ATP and helps maintain a stable intracellular pH in acidic environments (76,78,183). Proton extrusion to generate PMF is termed primary active transport and supports secondary active transport systems to concentrate solutes internally (39,78).

There are three types of secondary active transport: symport, antiport and uniport. Symport is described as the transport of two substrates in the same direction by a single carrier. Antiport is the simultaneous transport of two solutes in opposite directions through a common carrier. Active uniport is a flow of ions driven directly by an ion gradient. Several examples of these systems have been characterized in bacteria (78,132), and in streptococci (30,52,132,183,190).

To date, only one system that utilizes PMF to drive transport has been recognized in *S. mutans* and it is involved in the transport of amino acids (30). This system was shown to transport leucine with the intracellular accumulation of this amino acid correlated to the magnitude of the PMF. The system also demonstrated affinity for isoleucine and valine in uptake competition experiments, suggesting that it is specific for branched-chain amino acids. Originally, the transport of glucose in *S. mutans* was also believed to be driven by PMF (73), however, uptake studies with 6-deoxyglucose (29) and measurements of the components of the transmembrane electrochemical proton gradients in cells of *S. mutans* and *S. sobrinus* grown in continuous culture under a variety of conditions (67) have questioned this model. This aspect of glucose transport by *S. mutans* is discussed in detail in Chapter 5.

(ii) Shock-sensitive and binding-protein dependent systems. When Gram-negative bacteria are osmotically shocked by transferring them from a solution of 20% sucrose to a solution of 0.5 mM $MgCl_2$, the proteins present in the periplasmic space will leak out into the surrounding media (122) As a result, these osmotically-shocked bacteria lose the ability to transport a number of solutes, hence the name 'osmotic-shock-sensitive transport systems'. Some of the transport proteins lost from the periplasmic space are termed binding proteins by virtue of their high affinity for

specific nutrients (53). The first of these binding proteins was discovered by A. Pardee in 1966 and was shown to be involved in the transport of sulfate by *Sal. typhimurium* (53,82). Since that time, a multitude of binding protein-dependent transport systems involved in the transport of amino acids, nucleic acids, peptides, carbohydrates and vitamins have been characterized in Gram-negative bacteria (53).

The osmotic-shock-sensitive transport systems are active transport systems comprised of a family of transporters that are much more complex in nature than the PMF-dependent secondary active transport systems so far described. These transporters consist of four distinct protein domains, two integral, hydrophobic regions that are involved in translocation and two membrane-associated, hydrophilic regions that bind ATP. These domains may consist of a single polypeptide or may exist as four distinct polypeptides. One feature of these transporters is a highly conserved 200 amino acid sequence that binds ATP, termed the ATP-binding cassette (ABC) (53,82). It has been proposed by Higgins et al. that this family of transport systems should be renamed the 'ABC transporters'(82). Although controversy remains as to the energy source utilized by these systems, it is accepted that they are PMF independent and most, if not all, derive the energy needed to accumulate solutes from the hydrolysis of ATP (148).

(iii) The multiple sugar metabolism system of *S. mutans*. Until recently ABC-type active transport systems were not known to exist in Gram-positive bacteria. The discovery of the multiple sugar metabolism system (MSM) in *S. mutans* (153) and the oligopeptide transporter system (Ami) of *S. pneumoniae* (2) has confirmed the existence of similar systems in Gram-positive bacteria. The MSM has been shown to transport a variety of sugars, including melibiose, raffinose and isomaltosaccharides (153). This system was originally identified by genetic means and is comprised of eight genes including four which appear to be similar to the ABC transporter domains: *msmK*, which shares homology to the ATP-binding protein of ABC-type systems; *msmF* and *msmG*, which code for membrane-bound proteins similar to those of the maltose permease of *E. coli*, and *msmE*, which has homology to the maltose-binding periplasmic-protein of *E. coli* (46,155). The products of the other genes are cytoplasmic enzymes involved in the metabolism of MSM substrates: *dexB* codes for dextran glucosidase, an enzyme that cleaves single glucose units from isomaltosaccharides (19,154); *aga* expresses α-galactosidase, an enzyme acting on both melibiose and raffinose to generate free galactose glucose and sucrose, respectively (182), while *gtfA* encodes sucrose phosphorylase that cleaves intracellular sucrose (generated from the α-galactosidase-mediated breakdown of raffinose) to glucose-1-P and fructose (1). A possible arrangement of the MSM

proteins, their transport and metabolic functions is illustrated in Fig. 1.

The expression of the *msm* operon appears to be under the control of a positive regulator encoded by *msmR*, which was originally believed to be induced by either melibiose or raffinose (153). Recent work by Tao et al., utilizing gel retardation assays to determine the substrates responsible for the initiation of transcription of *msmR*, indicates that the true inducer(s) of the system is likely to be glucose-1-P or fructose, the MSM-mediated breakdown products of raffinose (188). Regulation of transport via the MSM is one of the main topics of this thesis and is discussed in detail in Chapter 6.

One interesting feature of the MSM system is the structure of the sugar-binding protein encoded by *msmE*. Since *S. mutans*, being a Gram-positive bacterium has no periplasmic space, the structure of the protein was believed to differ from its counterpart in Gram-negative bacteria. Metabolic labelling by [^{14}C]-palmitic acid revealed that this protein contained a lipid moiety believed to anchor it to the membrane (180). Since the discovery of *msmE*, other similar substrate-binding ABC-like lipoproteins have been discovered in other Gram-positive bacteria, including a protein from *Corynebacterium diptheriae* postulated to bind iron (170), an oligonucleotide transport protein from *Lactococcus lactis*

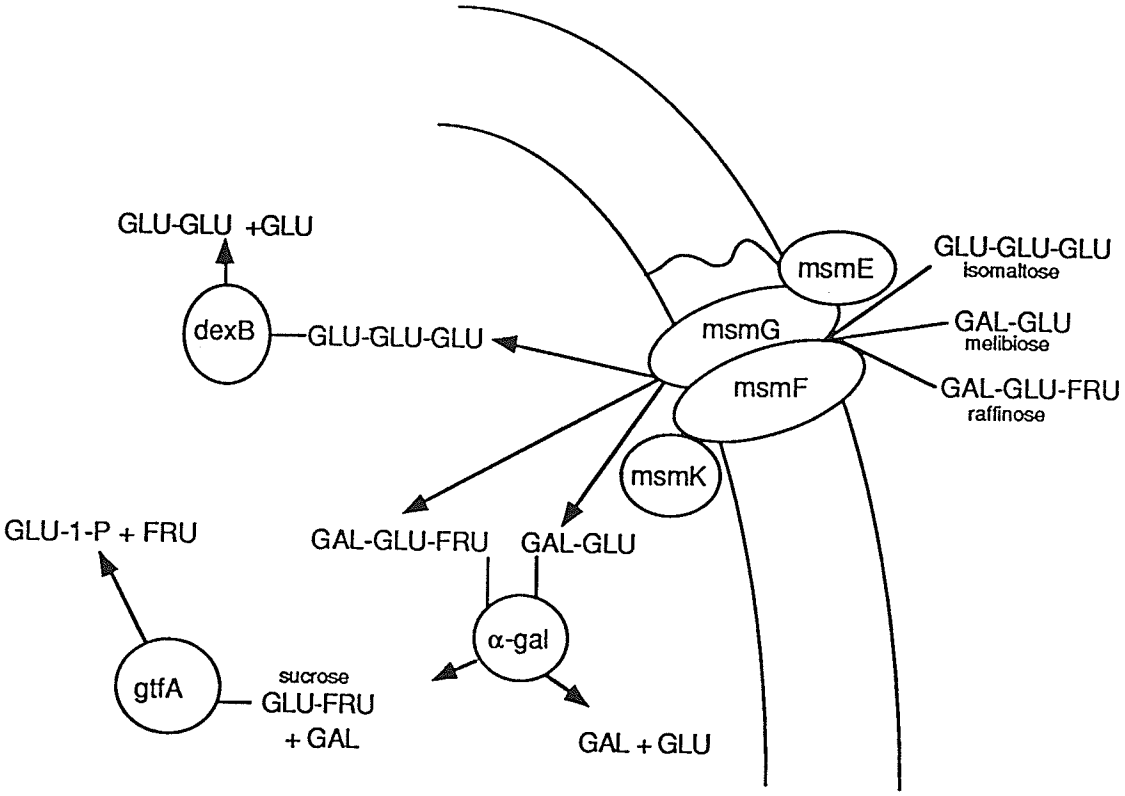


Fig. 1. Diagramatic representation of the multiple sugar metabolism (MSM) transport system of *S. mutans*. Abbreviations: GLU, glucose; GAL, galactose; FRU, fructose; α-gal, α-galactosidase; gtfA, glucosyltransferase A (sucrose phosphorylase); dexB, dextran glucosidase.

(191) and a phosphate-binding protein from *Mycobacterium tuberculosis* (23).

The discovery of the MSM system in *S. mutans* will undoubtedly lead researchers to examine other transport systems to see if they share properties with ABC transporter systems that utilize substrate-binding lipoproteins. To date, only one other operon with significant homology to those of ABC systems has been partially characterized in oral streptococci, in *S. gordonii*. An operon found in this organism is comprised of 6 open reading frames (ORFs) expressed from a single promoter with three of the ORFs sharing homology to ABC-type transporter systems (97). The *scaA* gene of this operon is believed to code for a surface lipoprotein that may function in binding small molecules that can be transported by the ABC-type system. This protein has been shown to have adhesive properties and appears to be involved in co-aggregation with *A. naeslundii*. The function of the system coded for by this operon is not known but its primary function may be to transport peptides (97).

4. Group translocation

(i) The PEP:PTS. The primary sugar transport system utilized by most bacteria, including *S. mutans*, is the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). The PTS is a form of transport termed group translocation, since it involves the transfer of a high

energy phosphate group from PEP via soluble and membrane-bound proteins to an incoming sugar. Transport by these systems is energy dependent since they utilize PEP, but, by definition, it is not an active transport system since a true solute concentration gradient is not established (122). The net effect of PTS transport is a higher concentration of phosphorylated substrate inside the cell relative to the concentration of free unphosphorylated substrate outside the cell. Transport by the PTS is more energy efficient than active transport, since the substrate is phosphorylated as it enters the cell and can, therefore, enter the metabolic pathway without the additional energy expenditure of a phosphorylation step (122). The PTS has received a great deal of attention by oral microbiologists since it is the primary sugar transport system of oral streptococci and the first step in sugar metabolism leading to the production of acid end-products implicated in the progression of dental caries (64).

Ghosh and Roseman (98) reported the discovery of the PTS in *E. coli* more than thirty years ago. Since that time, the PTS has been established not only as the primary sugar transport system of most bacteria, but also as a complex protein kinase signal transduction system that plays a key role in the regulation of a wide variety of metabolic processes as well as the expression of several genes. The research on the bacterial PTS is so extensive that a recent review article

(134) cited 551 published articles with over 400 articles published in the last 10 years. Since there are several current general reviews of the PTS (114,133,134,145), but no comprehensive review of the system in oral streptococci, the system will be described briefly, with emphasis on recent findings involving the oral streptococci.

As in most bacteria, the PTS in oral streptococci consists of two cytoplasmic, general proteins, Enzyme 1 (EI) and HPr, that are involved in the phosphorylation of all PTS carbohydrates. As seen in Fig. 2, the phosphate group from PEP is first transferred to EI and then to HPr followed by phosphoryl transfer to a sugar-specific Enzyme II (EII) and finally to the incoming sugar. EI is common to all bacteria containing a functional PTS and usually consists of two identical monomers that self-assemble to form a functional dimeric enzyme (134). The monomers have been shown to range in size from 58-85 kDa and contain a histidyl residue which accepts the phosphoryl group from PEP and a region of conserved amino acids believed to bind PEP (114), a process in enteric bacteria known to be dependent on Mg^{2+} (75).

The second general, soluble component of the PTS is HPr, a small heat-stable molecule which can range in size from 6.7-15 kDa (114). In all cases where the phosphorylation site has been characterized, the phosphorylation via EI occurs at a histidyl residue (114). In Gram-positive bacteria, HPr can also be phosphorylated at a seryl residue by a kinase

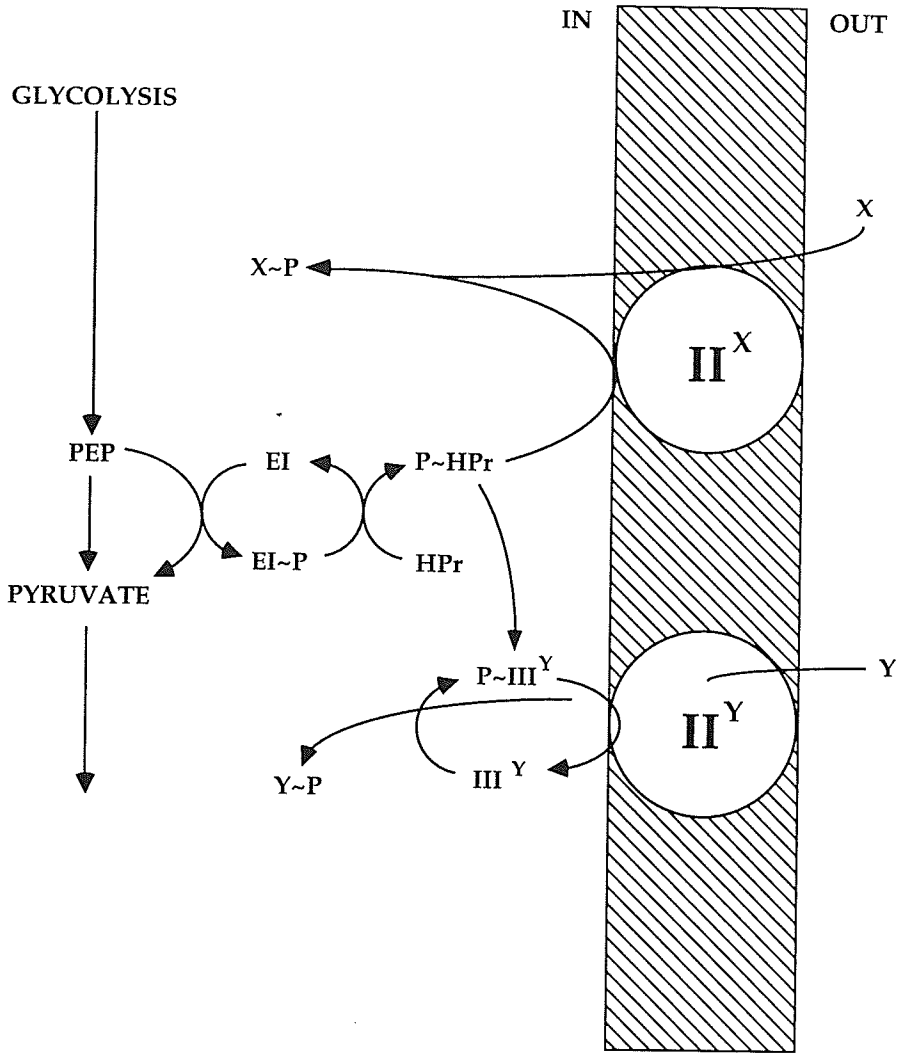


Fig. 2. The phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) of *S. mutans* consists of two general cytoplasmic proteins EI and HPr, as well as, membrane-associated sugar-specific EIIs that function as both a permease and kinase. EIIs can exist as a single entity II^X or may require a sugar-specific cytoplasmic-associated EIII or IIA (III^Y-II^Y).

[(ser)HPr kinase], a process believed to be involved in the regulation of the transport of non-PTS substrates (144). HPr has been found in bacteria that do not contain a functional PTS, such as, *L. brevis* and *L. buchneri*, where its role is believed to be entirely regulatory (142).

The permeases (EIIs) of the bacterial PTS may consist of one to four distinct polypeptide chains and may be fused to domains which usually exist as the soluble general components EI and/or HPr (134,160). Recently, sequence comparisons of many of these permeases from a diverse group of bacteria have suggested an evolutionary relatedness (164). The first striking similarity is that most have a similar size of about 635 amino acid residues and nearly all of these permeases exist in one of two common forms: as a separate EII or as an EII-III pair. Irrespective of the number of polypeptide chains comprising these permeases, there are always three distinct functional domains: a III-like region containing the first phosphorylation site (always a histidyl residue), a second hydrophilic peptide or domain containing the second phosphorylation site (either a histidyl or a cystidyl residue), and a third region comprised largely of hydrophobic residues spanning the membrane to bind and translocate the sugar substrate (102,134,160). Saier and Reizer (161) have suggested that, due to the integrity of these domains in EIIs and EII-III pairs, these regions should be classified as domains IIA, IIB, IIC, respectively. There are also unique

regions associated with some systems as observed with the IID domain of the *E. coli* mannose permease. Throughout this thesis the terms EIII, III and IIA are used interchangably to conform with the terminology used in the individual citations. In each case these terms refer to a distinct polypeptide existing as a soluble enzyme, except when described differently. The enzyme IIs are refered to as EII^{sugar} with the superscript designating the sugar that the system is capable of transporting. Fig. 3 illustrates some examples of the different arrangements of the sugar-specific EIIs seen in *E. coli*, and these are also representative of the arrangements found in other bacteria. The mannitol permease of *E. coli* was the first PTS permease to be characterized and sequenced (100). It consists of 637 amino acids and is observed to have three recognizable and distinct domains: a hydrophobic transmembrane region (IIC) and two hydrophilic domains, each containing a histidyl phosphorylation site (197). The region near the C-terminal domain is designated IIA, a III^{glc}-like domain that is fused to the membrane-bound EII^{glc}. The second, internal region is designated as IIB and contains the second phosphorylation site (160,197,200).

The *E. coli* glucose permease is classically described as an EII-III pair with the EII portion being membrane-bound and the III (IIA) portion existing as a distinct, soluble component (Fig. 3). The two components, when observed

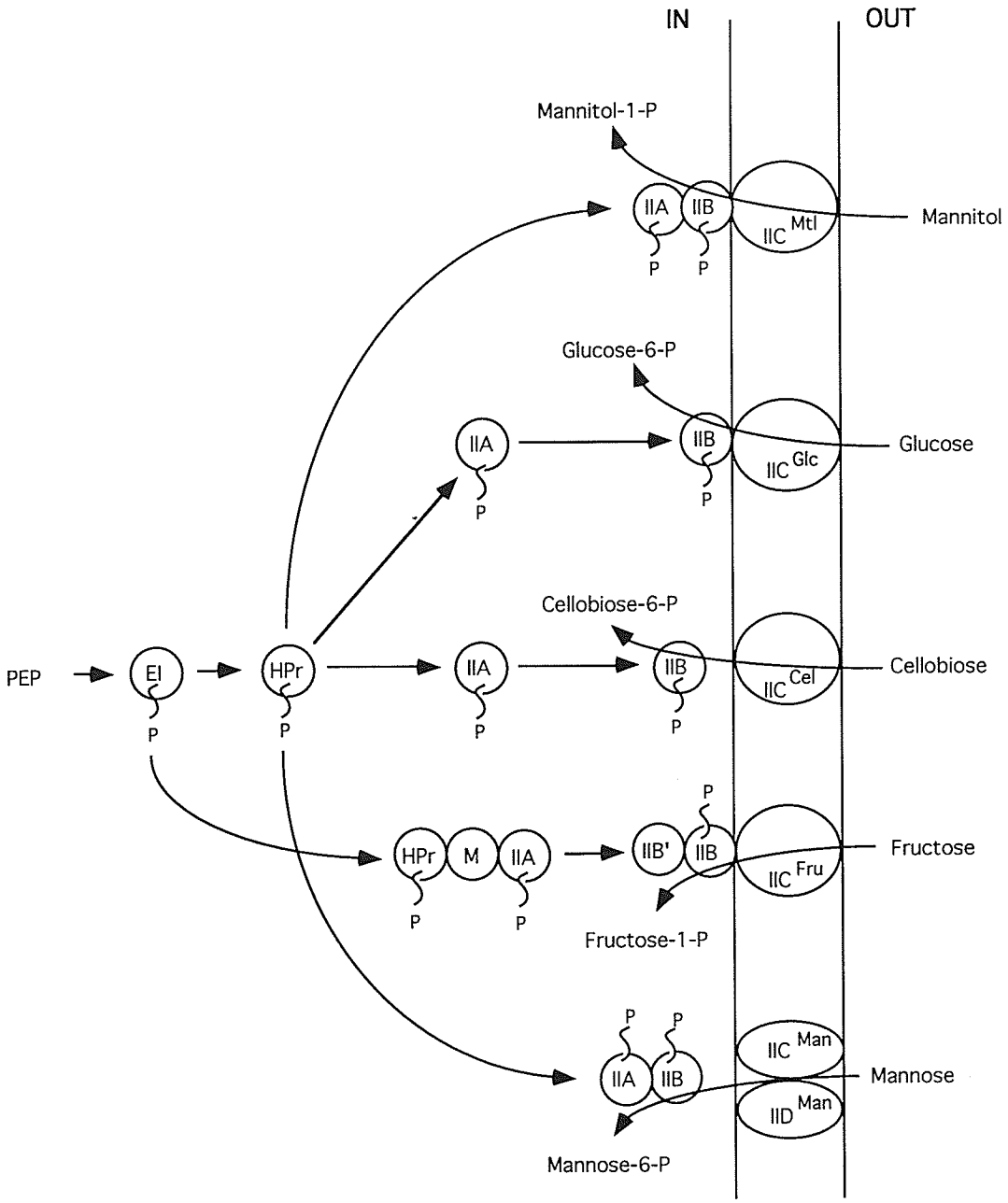


Fig. 3. Schematic representation of phosphoryl transfer via five representative PTS permeases from *E. coli* along with the energy coupling proteins EI and HPr.

together, contain similar functional domains analogous to the single component of the mannitol permease with only the C and B domains fused together, i.e. the membrane-bound IIC region and the second phosphorylation site-containing IIB domain. The third domain containing the first phosphorylation site exists as distinct, soluble enzyme III (IIA) (44,160).

The cellobiose permease of *E.coli* represents a third type of system encompassing three separate components (Fig. 3) with a combined size of 639 residues designated as IIC (membrane-bound), IIB (soluble) and IIA (soluble) in this proposed scheme. Together these components serve the same function as the traditional EII-III pair (127,160).

The *E. coli* fructose permease is similar to the PTS permeases specific for glucose and mannitol, but is somewhat more complex. The membrane-bound component of the system contains two homologous B-type domains near the N-terminus of the protein, called IIB' and IIB by the scheme outlined in Fig. 3 with the latter unit presumed to be phosphorylated at a cystidyl residue. These regions are believed to have originated by an internal gene duplication event which occurred during the evolution of this permease. The IIA domain is located as a separate, soluble enzyme III, containing not only the phosphorylation site near the N-terminus, but two other domains as well: a central region

believed to have a regulatory role (M region) and a third C-terminal region containing an HPr-like domain (58,160).

The last type of PTS permease found in *E. coli* is specific for mannose and is quite different from the other permeases sharing little sequence homology with them. It does have similarities in that it contains a transmembrane component (IIC) and a cytoplasmic component (IIAB) with two phosphorylation sites (45). The uniqueness of this system is exhibited by the presence of a second transmembrane protein that has a fairly high degree of hydrophilic residues and bears little resemblance to other PTS permeases. This component is designated as IID in the Saier-Reizer scheme (160,161), and although its functional role has yet to be elucidated, it is suspected of being involved with the injection of lambda phage DNA during infection (102).

The homologies observed between these distinct PTS permeases of *E. coli* suggest that, during their evolution, the various hydrophobic and hydrophilic domains have become fused to each other in different orders and combinations with the possibility that each domain can act as a region of a large multi-functional protein, or as a distinct protein as part of a multi-enzyme system. The homogeneity of the system is emphasized by the fact that regardless of the association or independence of the domains, they must all be present and act together to facilitate the transport and phosphorylation of their respective sugar substrates (133,134). In

considering the relatedness of the permeases of the *E. coli* PTS, it is easy to visualize that the mechanisms of intragenic fusion, splicing, shuffling and possibly deletion have occurred during the evolution of these various transporters. It appears that the similarity in the domains may be universal among bacteria which employ PTS transport and for this reason Saier and Reizer (161) suggest that the nomenclature they have developed (IIA, B, C, etc.) be adopted to reflect the function of the various PTS enzymes and genes that are currently being characterized.

(ii) The PTS components of *S. mutans*. The PTS is utilized by *S. mutans* for intracellular transport of a variety of sugars including glucose, mannose, sucrose, sorbitol, fructose, mannitol, maltose, N-acetylglucosamine, and trehalose (84,88,192). Many of these transport systems have only been characterized at the biochemical level. Biochemical evidence suggests that the PTS systems for glucose, mannose, and fructose require a soluble III (IIA) factor (10). It is likely that the sorbitol permease requires a soluble III component as well, since this requirement has been demonstrated in the oral streptococcus, *S. sanguis* (184), an organism with a PTS similar to that of *S. mutans* as discussed in Chapter 3. The glucose and mannose systems have also been extensively characterized at the biochemical level and appear to play a key role in the

regulation of sugar transport in the oral streptococci (56) and this will be discussed in greater detail later.

One of the most extensively studied transport systems in *S. mutans* is the sucrose PTS since it constitutes the primary route of entry for this sugar (89,174,175). The system has been characterized at the biochemical level and the organization and nucleotide sequence of the genes has also been determined (81,89,168,174). Uptake begins with the transport and phosphorylation of sucrose via the sucrose-specific membrane-bound permease, EII^{Scr}, followed by hydrolysis by sucrose-6-phosphate hydrolase, generating glucose-6-phosphate and fructose (24,156). To enter the glycolytic pathway, fructose is phosphorylated to fructose-6-phosphate by fructokinase (169). The genes encoding EII^{Scr} (*scrA*) and the sucrose-6-phosphate hydrolase (*scrB*) have been cloned from *S. mutans* (81,107) and from *S. sobrinus* (25). The gene encoding the fructokinase is also located in this region immediately downstream from *scrA* (169). The permease found in both species is EIII independent with all three II domains (IIA, IIB, and IIC) located on a single membrane-associated polypeptide (26,168) in a similar arrangement to the II^{mtl} complex of the *E. coli* mannitol PTS (Fig. 3).

The metabolism of lactose in *S. mutans* via the lactose PTS and the tagatose pathway is well characterized (71,72,90), as is the arrangement and sequence (85,150) of the genes for the components comprising the system. Transported lactose is

phosphorylated as it enters the cell through the EII^{lac} and is cleaved by phospho-β-galactosidase to produce galactose-6-phosphate and glucose. The resultant galactose-6-phosphate is then converted first to tagatose-6-phosphate by an isomerase, and to tagatose-1,6-bisphosphate by a kinase. The metabolism proceeds by an aldolase which converts tagatose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (71).

The *lac* operon in *S. mutans* is comprised of seven genes: *lacRABCDFE*, with *lacF* coding for the III^{lac} component (IIA) and *lacE* coding for II^{lac} (IIBC)(85) with the organization of these domains similar to the glucose PTS of *E. coli* (Fig. 3). In *S. mutans*, the genes coding for the tagatose-6-phosphate pathway are co-transcribed with the PTS genes and include: *lacA* and *lacB* (galactose-6-phosphate isomerase), *lacC* (tagatose-6-phosphate kinase), *lacD* (tagatose-1,6-bisphosphate aldolase), *lacE*, (Enzyme II^{lac}), and *lacG* (phospho-β-galactosidase) (85,150). The transcription of these genes appears to be regulated by the lactose repressor, the product of *lacR* (150).

At present, the only other sugar-specific component of the *S. mutans* PTS characterized at the genetic level is the factor III (IIA) of the mannitol system (*mtlF*)(84). This gene is located downstream from *mtlD*, the gene for mannitol-1-phosphate dehydrogenase. Mannitol, when transported by the PTS, is converted to mannitol-1-phosphate and then to

fructose-6-phosphate by the dehydrogenase (18,112). The presence of a distinct III (IIA) factor gene indicates that the arrangement of the permease domains in the mannitol system is similar to the lactose system described above and the *E. coli* glucose permease (Fig. 2).

5. PTS-mediated regulation. It has been well established that the PTS plays a central role in the regulation of carbohydrate transport and metabolism (134). In most bacteria, the glucose-PTS system exerts control over the expression of the genes coding for the general proteins, the substrate-specific operons, and genes coding for both sugar-specific PTS and non-PTS target systems (134). The molecular mechanisms involved in the regulation of both PTS and non-PTS systems are often similar and for this reason the two types of target systems will be discussed together.

The recent development of an extensive literature on the regulation of the PTS and the role of the PTS in regulation of other transport systems is an indication of its diversity and complexity among bacteria, however, it is apparent that the regulatory mechanisms utilized by Gram-positive bacteria are quite different than those of Gram-negative enteric bacteria. For this reason, the regulatory mechanisms in enteric organisms will be briefly described and more emphasis placed on the Gram-positive bacteria, with special attention given to oral streptococci and *S. mutans*.

(i) Regulation of the general PTS proteins. In all bacteria that have been genetically characterized, the genes coding for the general proteins of the PTS (*ptsH, ptsI*) are located in a single operon (134). In *E. coli* the operon also contains the *crr* gene, which codes for the soluble factor III (IIA) of the glucose PTS (32). The regulation of the operon is complex, however, the levels of the transcripts (32) and the resultant amounts of the proteins only vary insignificantly by about three-fold, reaching their maximum levels in the presence of PTS substrates under anaerobic conditions (113). In this regard, *S. mutans* is the best characterized of the Gram-positive bacteria with the levels of EI and HPr shown to vary by as much as 4-fold with the components being repressed under conditions of high extracellular glucose concentrations (70). Recent work has demonstrated that the regulation of transcription of the *pts* operon of *S. salivarius* involves an antitermination mechanism (54). This mechanism regulates the expression of the *ptsI* and *ptsH* genes and is reflected by an intracellular ratio of HPr:EI of 100:1. This process is believed to be controlled by the ATP-dependent phosphorylation of HPr by (Ser)HPr kinase, a mechanism believed to be central to PTS-mediated regulation of non-PTS sugar transport systems in Gram-positive bacteria (54).

(ii) PTS regulatory functions in enteric bacteria. The study of PTS regulation in Gram-negative bacteria has

virtually exploded since Monod began to characterize the 'glucose effect' in the 1940's (118). In these early experiments, the growth rates of *E. coli*, *B. subtilis*, and *Sal. typhimurium* were measured in chemically-defined media with a mixture of two sugars. In many cases, Monod observed that when one of the two sugars was glucose the bacteria would deplete the glucose and then assume a lag period before growing on the second sugar, generating a typical biphasic or 'diauxic' growth curve.

Although many researchers were able to reproduce this glucose effect with glucose plus a number of secondary sugars, the physiological basis of this phenomenon had only begun to be understood in the 1960's. Pastan and Perlman (129) demonstrated with *E. coli* that many of the catabolic enzymes repressed by glucose were under the control of cyclic AMP in a process termed catabolite repression. Catabolite repression was later shown to require a functional *crr* gene encoding IIA^{glc} of the PTS (162). The involvement of the PTS in the regulation of sugar uptake was also observed when mutants defective in the *ptsI* (EI) or *ptsH* (HPr) genes were unable to transport a variety of sugars, including both PTS and non-PTS substrates (59,130,163). These experiments provided the first insight that the PTS controlled sugar transport and metabolism through a variety of sugar-specific systems.

Since the early work on PTS-mediated regulation, it has been suggested that IIA^{glc} is a key regulator of sugar transport and metabolism in Gram-negative bacteria (157). Experimental evidence suggests that IIA^{glc} allosterically controls the activity of target permeases and adenylate cyclase (157). The modulation of transport is accomplished by a protein-protein interaction of the free unphosphorylated form of IIA^{glc}, normally at a high concentration when PTS substrates are available, with the target permeases or catabolic enzymes of the regulated systems (40,157). This interaction appears to inhibit transport or metabolic function of the target protein, resulting in 'inducer exclusion'. Inducer exclusion is when inducers of the target systems are prevented from entering the cell, or being formed within the cytoplasm (123,157). Conversely, the phosphorylated form of IIA^{glc}, at a high concentration in the absence of PTS substrates, appears to activate adenylate cyclase to generate cyclic AMP which, upon interaction with the catabolite-gene-activator protein (CAP), subsequently, activates transcription of target systems (131,159). When glucose is present, the operons coding for the secondary transport systems remain repressed, hence the term 'catabolite repression'. Current research in PTS-mediated regulation is concentrated on the intricate protein-protein interactions that control these processes (158).

Recent experiments with many species of bacteria has also suggested that the PTS has a much broader effect on metabolism than simply regulating the transport of sugars into the cell. It is believed that PTS auxiliary proteins, such as the fructose repressor of *E. coli* (FruR), not only control the transcription of PTS components, but may also regulate the expression of other sets of genes encoding several other metabolic pathways including: glycolysis, the Krebs cycle, electron transport, the glyoxylate shunt and possibly the Entner-Duodoroff pathway (158). Genetic studies have also suggested that other processes, including the production of carbon and energy storage compounds, such as, poly-β-hydroxybutyrate may be controlled by the PTS (136). The transcription of σ^{54}-dependent genes involved in nitrogen metabolism is also believed to be controlled by the PTS, establishing a possible link between carbon and nitrogen metabolism (5,143).

(iii) PTS-mediated regulation in Gram-positive bacteria. Many species of Gram-positive bacteria exhibit catabolite repression and diauxic growth similar to that observed with enteric bacteria. Although the PTS components of Gram-negative and Gram-positive bacteria are closely related, several of the mechanisms utilized to achieve this regulatory process by the two types of bacteria are quite distinct (145). Unlike the enteric bacteria, many Gram-positive bacteria do not make cyclic AMP. Also, in those that do

produce cyclic AMP, including *S. salivarius* (95) a regulatory role for this molecule has not been substantially demonstrated (134,145). The unique regulatory mechanisms utilized by the Gram-positive bacteria include ATP-dependent phosphorylation of HPr, which appears to serve a transport control function similar to that of IIA^{glc} in enteric bacteria.

The regulation of sugar uptake is essential to saccharolytic bacteria, since an intracellular accumulaton of sugar phosphate is toxic to the cell (134). While enteric bacteria prevent this from occurring by the mechanism of inducer exclusion, Gram-positive bacteria appear to deal with this problem differently. Mechanisms for decreasing intracellular sugar phosphate concentrations unique to Gram-positive bacteria include: (i) the efflux of free sugar following dephosphorylation in the cell, (ii) exchange of intracellular sugar phosphate with external phosphate by an antiporter, or (iii) exchange of the intracellular sugar-phosphate with extracellular sugar via PTS-mediated transphosphorylation reaction (145).

Catabolite repression in *S. mutans* was first observed in its preferential utilization of glucose over lactose (72). This study demonstrated that the glucose PTS was used preferentially over the lactose PTS with a classical diauxic growth curve, generated when the culture was grown with a mixture of these two sugars. As previously discussed, *S.*

mutans uses the tagatose-6-phosphate pathway to metabolize lactose (71). The exact mechanism of regulation is not known, but the genes involved in lactose transport and metabolism appear to be under the control of a transcriptional repressor (*lacR*)(150). It is likely that under the appropriate conditions of low glucose and high lactose concentrations the operon becomes derepressed.

Regulatory mechanisms appear to differ between strains of *S. mutans* since EII's that are constitutive in one strain may be inducible in another, as observed with strains GS-5 and NCTC 10449, which yield typical diauxic growth curves with mixtures of glucose and lactose, while strain ATCC 27352 uses both sugars simultaneously (194). It has also been demonstrated that a strain 6715 of *S. mutans* utilized lactose preferentially before glucose with the EIIs for glucose, mannose, and fructose being repressed during growth on lactose (194).

In *S. mutans*, glucose promotes inducer exclusion by repressing the synthesis of the enzymes required for both mannitol and sorbitol metabolism (18,41). This work demonstrated that transport of hexoses inhibited the uptake of sugar alcohols suggesting that the various EIIs must compete for P~(HPr) in a hierarchical fashion as observed with enteric bacteria (157). In *S. sanguis*, the addition of glucose to cultures growing on sorbitol resulted in the rapid

expulsion of sorbitol from the cells indicating a mechanism of inducer expulsion was functioning in this organism (74).

Several of the studies of PTS in oral streptococci have implicated the mannose PTS as the primary glucose transport system and principal regulator of PTS transport. The focus on the mannose PTS began when it was discovered that mutations affecting the *S. mutans* system cause a number of pleiotropic effects, including losses of diauxic growth on glucose and lactose (56,103,192), catabolite repression (124), and inducer exclusion (41). Recent studies utilizing *S. salivarius* have given some insight into the regulatory role of the mannose PTS in this organsim (57). In this bacterium, as well as in *S. mutans*, the mannose PTS is the primary glucose transport system that has affinity for mannose, glucose and 2-deoxyglucose (192). It was discovered that the mannose PTS of *S. salivarius* has two forms of EIII, designated III_H^{man} and III_l^{man}, which have molecular weights of 38.9 kDa and 35.2 kDa, respectively (10). III_l^{man} has been implicated as a key regulatory protein in this organsim having been associated with: (i) induction of an EII^{fru} and activation of a cryptic EII^{glc} (11), (ii) alterations in the protein profiles of the cellular envelope (16), and (iii) alteration of expression of proteins as revealed by two dimensional gel electrophoresis profiles of membrane (16) and cytoplasmic (99) components. These data strongly implicate III_l^{man} as a regulator of several multigene systems.

The regulation of the sucrose PTS in *S. mutans* appears to be quite complex, since sucrose has at least four routes of entry into the cell. These mechanisms include: (i) transport via a high-affinity sucrose PTS (174,175), (ii) transport via the trehalose PTS, which was originally believed to be a low affinity sucrose PTS (135), (iii) transport via the multiple sugar metabolism operon (MSM) (187), and (iv) transport of glucose or fructose generated from the extracellular action of fructosyl- or glucosyltransferases, respectively (151). The regulation of the sucrose PTS is not well understood, however, it appears that at high sucrose concentrations, the expression of the sucrose PTS operon is likely repressed by high intracellular levels of sucrose-6-P (108). One interesting aspect of PTS-mediated regulation of sucrose utilization is observed with the control of fructosyltransferase activity (96). The expression of the gene encoding this extracellular enzyme (*ftf*) was repressed in the presence of PTS substrates, with the apparent involvement of a factor encoded outside the region of this gene (96).

The center of the PTS regulatory network in Gram-positive bacteria has been suggested to involve ATP-mediated phosphorylation of HPr on the serine-46 residue (145,146). This process has been central to the study of PTS-mediated regulation in Gram-positive bacteria since 1983 when Reizer et al. (141) discovered a metabolite-activated protein kinase

in *Streptococcus pyogenes*, believed to function in the process of inducer expulsion. This process expels non-metabolizable intracellular sugar phosphates accumulated by the PTS upon addition of a metabolizable carbohydrate (138,139,140). Further investigation revealed that this protein kinase was involved in the phosphorylation of HPr at seryl residue 46 (37) with the phosphoryl group unable to be transfered to any subsequent PTS proteins or sugars, supporting its role in regulation (144).

The ATP-dependent phosphorylation of (Ser)-HPr has been observed in a number of other Gram-positive bacteria including *S. salivarius* and *S. mutans* (193), but it has not yet been detected in Gram-negative bacteria (134,144). The reaction has also been identified in *L. brevis*, which has an HPr, but lacks EI and EIIs (142). The phosphorylation of the *L. brevis* HPr at a seryl residue has been demonstrated to regulate the transport of glucose (206) and lactose (207), both transported via proton symport mechanisms. Transport via these permeases appears to be allosterically regulated by the intracellular concentration of P-(Ser)-HPr, which in turn is affected by the intracellular concentrations of glycolytic intermediates (137). The P-(Ser)-HPr is believed to interact directly with the glucose and lactose symporters resulting, not only in the characteristic inducer efflux, but also inducer exclusion in a manner similar to the binding of IIA^{glc} to target permeases in *E. coli*.

As mentioned previously, the level of phosphorylation of HPr at the seryl residue is controlled by the action of the ATP-dependent (Ser)HPr kinase and by an inorganic phosphate (Pi)-dependent P-(Ser)-HPr phosphatase (35,141,142). The (Ser)HPr kinase has been partially purified from *E. faecalis* (35), *S. pyogenes* (141), *L. brevis* (147), and *B. subtilis* (144) and, in some cases, has been shown to be stimulated by metabolites, such as, fructose-1,6-bisphosphate (FBP), gluconate-6-P, and 2-P-glycerate (35,141,144,147). The P-(Ser)-HPr phosphatase has been partially purified from *E. faecalis* and its activity has been found to be dependent on a phosphate concentration of approximately 50 mM (36).

Recent work by Thevenot et al. (189) has demonstrated that the (Ser)HPr kinases of *S. salivarius* and *S. mutans* are only slightly activated by FBP. A 2-fold increase in activity was observed by the addition of FBP at an ATP concentration of 0.05 mM, but this intermediate showed moderate inhibitory effects when added to reactions containing 1.0 mM ATP. Fructose-6-phosphate, glyceraldehyde-3-phosphate, 2-phosphoglycerate, 2,3-diphosphoglycerate, and pyruvate also had little effect on the enzyme activity under the conditions tested. Quantitative assays of all four forms of HPr [free HPr, P~(His)HPr, P-(ser)HPr, and P~(His)-P-(Ser)-HPr] were also performed on cells grown at varying glucose concentrations in continuous culture. The total HPr pool increased with an increase in glucose concentration with

significant increases of P-(Ser)-HPr and P~(His)-P-(Ser)-HPr concentrations. The total ATP-dependent phosphorylation was found to increase 12-fold at glucose concentrations of 100 mM and 200 mM when compared to the concentration at 10 mM glucose. The activity of the (Ser)HPr kinase was found to be unchanged in cells grown at 10, 50, and 100 mM glucose, but was shown to increase 3-fold at 200 mM glucose, a condition where the PTS is known to be repressed (70). The activity of (Ser)HPr kinase did not correlate with the intracellular concentration of FBP, suggesting the system may be activated in a different manner than in other Gram-positive bacteria examined to date. Furthermore, previous studies with other Gram-positive bacteria did not account for the possibility of the doubly phosphorylated form of HPr being capable of transferring its phosphoryl group from P~His to EIIs, as demonstrated with the oral streptococci. Further study of this system will undoubtedly shed light on the processes used to regulate transport in oral streptococci and other Gram-positive bacteria.

A second PTS-mediated regulatory mechanism utilized by Gram-positive bacteria involves direct phosphorylation of target molecules via P~(His)-HPr (144). In 1985, the discovery of a 55 kDa protein that was phosphorylated by HPr at the expense of [^{32}P]-PEP in *E. faecalis* initiated an investigation into this process (33). After purification of this protein, it was identified as glycerol kinase and was

found to have a nine-fold increase in activity when phosphorylated (38). The characterization of this positive-control mechanism describes a novel form of metabolic regulation utilized by Gram-positive bacteria (34). This process is believed to function by glycerol kinase competing for P~(His)-HPr or P~(His)-P-(Ser)-HPr with IIA domains of PTS sugar transport systems. When the PTS substrates are unavailable, the concentration of these two forms of HPr will increase to a level where glycerol kinase will become phosphorylated, thereby, increasing its activity. This process is discussed in greater detail in Chapter 6, where a similar process involving PTS-mediated phosphorylation in *S. mutans* is demonstrated to be associated with MSM transport activity.

Our knowledge of how *S. mutans* and other streptococci transport and metabolize sugars to eventually convert them to acid end-products has greatly expanded in the last decade. The application of molecular genetics to the study of these processes in oral streptococci, and bacteria in general, has shed light upon the structure and regulation of many of these systems. Studies which began on a single transport process have broadened our understanding of microbial physiology immensely and are now directing people to investigate the innumerable chemical, physical and electrical interactions occurring within the cell. The division between molecular biologist, microbial physiologist and microbial ecologist is

now becoming indistinguishable and, as the combined evidence is accumulated, the interaction of bacteria with their environment and molecular events associated with them are now beginning to be understood. Future research will inevitably lead to a greater understanding of how bacteria interact with, and respond to, their environment allowing us to design means to effectively control pathogens and to better exploit microorganisms for a variety of useful purposes.

References

1. Aduse-Opoku, J., L. Tao, J. J. Ferretti, and R. R. B. Russell. 1991. Biochemical and genetic analysis of *Streptococcus mutans* α-galactosidase. J. Gen. Microbiol. **137**: 757-764.
2. Alloing, G., P. de Philip, and J.-P. Claverys. 1994. Three highly homologous lipoproteins participate in oligopeptide transport by the Ami system of the Gram-positive *Streptococcus pneumonia*. J. Mol. Biol. **241**: 44-58.
3. Amaro, A. M., D. Chamorro, M. Seeger, R. Arredondo, I. Peirano, and C. A. Jerez. 1991. Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. J. Bacteriol. **173**: 910-915.
4. Auger, E. A., K. E. Redding, T. Plumb, L. C. Childs, S. Y. Meng, and G. N. Bennett. 1989. Construction of lac fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli*. Mol. Microbiol. **3**: 609-620.
5. Begley, G. S., and G. R. Jacobson. 1995. Overexpression, phosphorylation, and growth effects of

ORF162, a *Klebsiella pneumoniae* protein that is encoded by a gene linked to *rpoN*, the gene encoding σ^{54}. FEMS Microbiol. Lett. in Press

6. Belli, W. A., and R. E. Marquis. 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in culture. Appl. Environ. Microbiol. **57**: 1134-1138.

7. Bender, G. R., S. V. W. Sutton, and R. E. Marquis. 1986. Acid tolerance, proton permeabilities and membrane ATPases of oral streptococci. Infect. Immun. **53**: 331-338.

8. Birkhed, D. and J. M. Tanzer. 1979. Glycogen synthesis pathway in *Streptococcus mutans* strain NCTC 10449 and its glycogen-synthesis defective mutant. Arch. Oral. Biol. **24**: 67-73.

9. Booth, I. R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49** :359-378.

10. Bourassa, S., L. Gauthier, R. Giguere, and C. Vadeboncoeur 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. Oral Microbiol. Immunol. **5**: 288-297.

11. Bourassa, S., and C. Vadeboncoeur. 1992. Expression of an inducible enzyme II fructose and activation of a cryptic enzyme II glucose in glucose-grown cells of spontaneous mutants of *Streptococcus salivarius* lacking the low-molecular-mass form of III^{man}, a component of the phosphoenolpyruvate:mannose phosphotransferase system. J. Gen. Micro. **138**: 769-777.

12. Bowden, G. H. 1990. Which bacteria are cariogenic in Humans? p. 266-286. *In* N. W. Johnson (ed.), Dental caries vol 1, Markers of high and low risk groups and individuals. Academic Press, London.

13. Bowden, G. H., and Hamilton, I. R. 1989. Competition between *Streptococcus mutans* and *Lactobacillus casei* in mixed continuous culture. Oral Microbiol. Immunol. **4**: 57-64.

14. Bowden, G. H., I. R. Hamilton, and D. H. Ellwood. 1979. Microbial ecology of the oral cavity, p. 1-75, *In* I. Kleinberg, S. A. Ellison, and I. D. Mandel (ed.), Saliva and dental caries Sp. Supp. Microbiology Abstracts, Information Retrieval Inc., New York.

15. Boyar, R. M., and G. H. Bowden. 1985. The microflora associated with the progression of incipient caries lesions in teeth of children living in a water flouridated area. Caries Res. **19**: 298-306.

16. Brochu, D., L. Trahan, M. Jacques, M.C. Lavoie, M. Frenette, and C. Vadeboncoeur. Alterations in the cellular envelope of spontaneous III_L^{man}-defective mutants of *Streptococcus salivarius*. J. Gen. Microbiol. **139**: 1291-1300.

17. Brown, L. R., S. Dreizen, and S. Handler. 1976. Effects of selected caries preventative regimens on microbial changes following irradiation-induced xerostomia in cancer patients, p. 275-290. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed), Microbial aspects of dental caries, I, Sp. Suppl. Microbiology Abstracts, Information Retrieval Inc., New York.

18. Brown, A. T., and C. L. Wittenberger. 1973. Mannitol and sorbitol catabolism in *Streptococcus mutans*. Arch. Oral Biol. **18**: 127-131.

19. Burne, R. A., R. A., B. Rubinfeld, W. H. Bowen, and R. E. Yasbin. 1986. Tight genetic linkage of a glucosyltransferase and dextranase of *Streptococcus mutans* GS-5. J. Dent. Res. **65**: 1392-1401.

20. Carlsson, P., I. A. Gandour, B. Olsson, B. Rickardsson, and K. Abbas. 1987. High prevalence of mutans streptococci in a population with extremely low prevalence of dental caries. Oral Microbiol. Immunol. **2**: 121-124.

21. Carlsson, J., and C. J. Griffith. 1974. Fermentation products and bacterial yields in glucose-limited and

nitrogen-limited cultures of streptococci. Archs. Oral Biol. **19**: 1105-1109.

22. Carlsson J, and I. R. Hamilton 1994. Metabolic activity of oral bacteria p. 71-88. *In* Thylstrup, A., and O. Fejerskov (ed.), Textbook of clinical cariology, Munksgaard, Copenhagen.

23. Chang, Z., A. Choudhary, R. Lathigra, and F. A. Quiocho. 1994. The immunodominant 38-kDa lipoprotein antigen of *Mycobacterium tuberculosis* is a phosphate-binding protein. J. Biol. Chem. **269**: 1956-1958.

24. Chassy, B. M., and E. V. Porter. 1982. Sucrose-6-phosphate hydrolase from *Streptococcus mutans* 6715. Infect. Immun. **90**: 556-559.

25. Chen, Y. M., and D. L. LeBlanc. 1992. Genetic analysis of *scrA* and *scrB* from *Streptococcus sobrinus* 6715. Infect. Immun. **60**:3739-3749.

26. Chen, Y. M., L. N. Lee, and D. L. LeBlanc. 1993. Sequence analysis of *scrA* and *scrB* from *Streptococcus sobrinus* 6715. Infect. Immun. **60**: 3739-3749.

27. Chia, J. S., T. Y. Hsu, L. J. Teng, J. Y Chen, L. J. Hahn, and C. S. Yang. 1991. Glucosyltransferase gene polymorphism among *Streptococcus mutans* strains. Infect. Immun. **59**: 1656-1660.

28. Critchley, P. J., C. Wood, and S. Leach. 1976. The polymerisation of dietary sugars by dental plaque. Caries Res. **1**: 112-129.

29. Dashper, S. G., and E. C. Reynolds. 1990. Characterization of transmembrane movement of glucose and glucose analogues in *Streptococcus mutans* Ingbritt. J. Bacteriol. **172**: 556-563.

30. Dashper, S. G., and E. C. Reynolds. 1993. Branched-chain amino acid transport in *Streptococcus mutans* Ingbritt. Oral Microbiol. Immunol. **8**: 167-71.

31. De Jong, M. H., and J. S. van der Hoeven. 1987. The growth of oral bacteria on saliva. J. Dent. Res. **66**: 498-505.

32. De Reuse H., and A. Danchin. 1988. The *ptsH, ptsI* and *crr* genes of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system: a complex operon with several modes of transcription. J. Bacteriol. **170**: 3827-3837.

33. Deutscher, J. 1985. Phosphoenolpyruvate-dependent phosphorylation of a 55 kDa protein of *Streptococcus faecalis* catalyzed by the phosphotransferase system. FEMS Microbiol. Lett. **29**: 237-243.

34. Deutscher, J., B. Bauer, and H. Sauerwald. 1993. Regulation of glycerol metabolism in *Enterococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation of glycerol kinase catalyzed by enzyme I and HPr of the phosphotransferase system. J. Bacteriol. **175**: 3730-3733.

35. Deutscher, J, and R. Engelmann. 1984. Purification and characterization of an ATP-dependent protein kinase from *Streptococcus faecalis*. FEMS Microbiol. Lett. **23**: 157-162.

36. Deutscher, J., U. Kessler, and W. Hengstenberg. 1985. Streptococcal phosphoenolpyruvate:sugar phosphotransferase system: purification and characterization of a phosphoprotein phosphatase which hydrolyzes the phosphoryl bond in seryl-phosphorylated histidine-containing protein. J. Bacteriol. **163**: 1203-1209.

37. Deutscher, J., and M. H. Saier Jr. 1983. ATP-dependent protein kinase catalyzed phosphorylation of a seryl residue in HPr, a phosphate carrier protein of the phosphotransferase system in *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **80**: 6790-6794.

38. Deutscher, J., and H. Sauerwald. 1986. Stimulation of dihydroxyacetone/glycerol kinase activity in *Streptococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation catalyzed by EI and HPr of the

phosphotransferase system. J. Bacteriol. **166**: 829-836.

39. Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**: 385-418.

40. Dills, S. S., M. R. Schmidt, and M. H. Saier Jr. 1982. Regulation of lactose transport by the phosphoenolpyruvate-sugar phosphotransferase system in membrane vesicles of *Escherichia coli*. J. Cell. Biochem. **18**: 239-244.

41. Dills, S. S., and S. Seno. 1983. Regulation of hexitol catabolism in *Streptococcus mutans*. J. Bacteriol. **166**: 861-866.

42. Dirksen, T. R., M. F. Little, B. G. Bibby, and S. L. Crump. 1962. The pH of carious cavities I. Effect of glucose and phosphate buffer on cavity pH. Arch. Oral Biol. **7**: 49-57.

43. Douglas, C. W. I., J. Heath, K. K. Hampton, and F. E. Preston. 1993. Identity of viridans streptococci isolated from cases of infective endocarditis. J. Med. Microbiol. **39**: 179-182.

44. Erni, B., and B. Zanolari. 1986. Glucose-permease of the bacterial phosphotransferase system. J. Biol. Chem. **261**: 16398-16403.

45. Erni, B., B. Zanolari, P. Graff, and H. P. Kocher. 1989. Mannose Permease of *Escherichia coli*. Domain structure and function of the phosphorylating subunit. J. Biol. Chem. **264**: 18733-18741.

46. Ferretti, J. J., T. T. Huang, and R. R. B. Russell. 1988. Sequence analysis of the glucosyltransferase A gene (*gtfA*) from *Streptococcus mutans* Ingbritt. Infect. Immun. **56**: 1585-1588.

47. Foster, J. W. 1991. *Salmonella* acid shock proteins are required for the acid tolerance response. J. Bacteriol. **173**: 6896-6902.

48. Foster, J. W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **175**: 1981-1987.

49. Foster, J. W., and H. K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172**: 771-778.

50. Foster, J. W., and H. K. Hall. 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173**: 5129-5135.

51. Foster, J. W., and H. K. Hall. 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174**: 4317-4323.

52. Foucaud, C., and B. Poolman. 1992. Lactose transport system of *Streptococcus thermophilus*. Functional reconstitution of the protein and characterization of the kinetic mechanism of transport. J. Biol. Chem. **267**: 22087-22094.

53. Furlong, C. E. 1987. Osmotic-shock-sensitive transport systems, 768-796. *In* F. C. Neidhardt (ed.), *Esherichia coli* and *Salmonella typhimurium* cellular and molecular biology. Am. Soc. Microbiol. Washington, D. C.

54. Gagnon, G., L. Gauthier, C. Vadeboncoeur, and M. Frenette. 1995. Regulation of transcription of the *pts* operon of *Streptococcus salivarius* ATCC 25975 involves an antitermination mechanism. Mol. Microbiol. In Press.

55. Gale, E. F., and H. M. R. Epps. 1942. The effect of pH of the medium during growth on the enzyme activities of bacteria (*Escherichia coli* and *Micrococcus lysodiekticus*) and the biological significance of the changes produced. Biochem. J. **36**: 600-619.

56. Gauthier, L., S. Bourassa, D. Brochu, and C. Vadeboncoeur. 1990. Control of sugar utilization in oral streptococci. Properties of phenotypically distinct 2-deoxyglucose-resistant mutants of *Streptococcus salivarius*. **5**: 352-359.

57. Gauthier, L., S. Thomas, G. Gagnon, M. Frenette, L. Trahan, and C. Vadeboncoeur. 1994. Positive selection for resistance to 2-deoxyglucose gives rise, in *Streptococcus salivarius*, to seven classes of pleiotropic mutants, including *ptsH*, and *ptsI* missense mutants. Mol. Microbiol. **13**: 1101-1109.

58. Geerse, R. H., F. Izzo, and P. W. Postma. 1989. The PEP:fructose phosphotransferase system in *Salmonella typhimurium*: FPr combines enzyme III^{fru} and psuedo-HPr activities. Mol. Gen. Genetics. **216**: 517-525.

59. Gershanovitch, V. N., G. I. Bourd, N. V. Jorovitz-Kaya, A. G. Shavronskaya, V. V. Klyutchova, and V. P. Shabolenko. 1967. β-galacosidase induction in cells of *Escherichia coli* not utilizing glucose. Biochim. Biophys. Acta **134**: 188-190.

60. Gibbons, R. J., and S. S. Socransky. 1962. Intracellular polysaccharide storage by organisms in dental plaques. Arch. Oral Biol. **7**:73-80.

61. Goss, T. J., G. W. O'Hara, M. J. Dilworth, and A. R. Glenn. 1990. Cloning, characterization, and complementation of lesions causing acid sensitivity in *Tn*5-induced mutants of *Rhizobium meliloti* WSM419. J. Bacteriol. **172**: 5173-5179.

62. Guggenheim, B. 1970. Extracellular polysaccharides and microbial plaque. Int. Dent. J. **20**: 657-678.

63. Gustafsson, B. E., C. E. Quensel, L. S. Lanke, H. Grahmen, B. E. Bonow, and B. Krasse. 1954. The Vipeholm dental study. The effect of different levels of carbohydrate intake on caries activity in 436 individuals observed for 5 years. Acta Odontol. Scand. **11**: 232-364.

64. Hamada, S., and H. D. Slade. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331-284.

65. Hamilton, I. R. 1976. Intracellular polysaccharide synthesis by cariogenic microorganisms, p. 683-701. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings microbial aspects of dental caries. Sp. suppl. Microbiol. Abstr., Vol 3. Information Retrieval Inc., Washington, D. C.

66. Hamilton, I. R. 1986. Growth, metabolism and acid production of *Streptococcus mutans,* p. 145-155. *In* S. Hamada S. M. Michalek, H. Kiyono, L. Menaker and J. R. McGhee. (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishers, Amsterdam.

67. Hamilton, I. R. 1990. Maintenance of proton motive force by *Streptococcus mutans* and *Streptococcus sobrinus* during growth in continuous culture. Oral Microbiol. Immunol. **5**: 280-287.

68. Hamilton, I.R., and G. H. Bowden. 1992. Oral Microbiology, p 269-282. *In* J. Lederberg (ed.) Encyclopedia of Microbiology. Academic Press Inc., San Diego. USA.

69. Hamilton, I. R., and N.D. Buckley. 1991. Adaptation of *Streptococcus mutans* to acid tolerance. Oral Microbiol. Immun. **6** : 65-71.

70. Hamilton, I. R., L. Gauthier, B. Desjardins, and C. Vadeboncoeur. 1989. Concentration-dependent repression of the soluble and membrane components of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus mutans* by glucose. J. Bacteriol. **171**: 2942-2948.

71. Hamilton, I.R., and H. Lebtag. 1979. Lactose metabolism by *Streptococcus mutans*: Evidence for induction of the tagatose 6-phosphate pathway. J. Bacteriol. **140**: 1102-1104.

72. Hamilton, I., R., and G. C. Y. Lo. 1978. Co-induction of ß-galactosidase and the lactose P-enolpyruvate phosphotransferase system in *Streptococcus mutans* and *Streptococcus salivarius*. J. Bacteriol. **136**: 900-908.

73. Hamilton, I. R., and E. J. St. Martin. 1982. Evidence for the involvement of protonmotive force in the transport of glucose by a mutant of *Streptococcus mutans* Strain DR0001 defective in the glucose phosphoenolpyruvate phosphotransferase system. Infect. Immun. **36**: 567-575.

74. Hamilton, I. R., and G. Svensater. 1991. Sorbitol inhibition of glucose metabolism by *Streptococcus sanguis* 160. Oral Microbiol. Immunol. **6**: 151-159.14.

75. Han, M. K., S. Roseman, and L. Brand. 1990. Sugar transport by the bacterial phosphotransferase system. Characterization of the sulfhydryl groups and site-specific labelling of Enzyme I. J. Biol. Chem. **265**: 1985-1995.

76. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**: 172-230.

77. Harold, F. M. 1974. Chemiosmotic interpretation of active transport in bacteria. Ann. N. Y. Acad. Sci. **227**: 297-302.

78. Harold, F.M., and Y. Kakinuma. 1985. Primary and secondary transport of cations in bacteria. Annals. New York Acad. Sci. **456**: 375-383.

79. Harper, D. S., and W. J. Loesche. 1983. Effect of sucrose and glucose catabolism by the various genogroups of *Streptococcus mutans*. J. Dent. Res. **62**: 526-531.

80. Harper, D. S., and W. J. Loesche. 1984. Growth and acid tolerance of human dental plaque bacteria. Arch. Oral Biol. **29**: 843-848.

81. Hayakawa, M., H. Aoki, and H. K. Kuramitsu. 1986. Isolation and characterization of the sucrose-6-

phosphate hydrolase gene from *Streptococcus mutans*. Infect. Immun. **53**: 582-586.

82. Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gill, and M. P. Gallagher. 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomemb. **22**: 571-592.

83. Hojo, S., M. Komatsu, R. Okuda, N. Takahashi,and T. Yamada. 1994. Acid profiles and pH of carious dentin in active and arrested lesions. J. Dent. Res. **73**: 1853-1857.

84. Honeyman, A. L., and R. Curtiss III. 1992. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* mannitol-phosphate dehydrogenase gene and the mannitol-specific Factor III gene of the phosphoenolpyruvate phosphotransferase system. Infect. Immun. **60**: 3369-3375.

85. Honeyman, A. L., and R. Curtiss III. 1993. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* lactose-phosphate Enzyme II (*lacE*) gene of the PTS and the phospho-ß-galactosidase (*lacG*) gene. J. Gen. Microbiol. **139**: 2685-2694.

86. Huis in't Veld, J. H. J., and O. Backer-Dirks. 1978. Intracellular polysaccharide metabolism in *Streptococcus mutans*. Caries Res. **12**: 243-249.

87. Ikeda, T., H. J. Sandham, and E. L. Bradley. 1973. Changes in *Streptococcus mutans* and lactobacilli in plaque in relation to the initiation of dental caries in negro children. Arch. Oral Biol. **18**: 555-566.

88. Jacobson, G. R., J. Lodge, and F. Poy. 1989. Carbohydrate uptake in the oral pathogen *Streptococcus mutans*: mechanism and regulation by protein phosphorylation. Biochimie **71**: 997-1004.

89. Jacobson, G. R., C. S. Mimura, P. J. Scott. and P. W. Thompson. 1984. Identification and properties of distinct sucrose and glucose phosphotransferase enzyme

II in *Streptococcus mutans* 6715g. Infect. Immun. **46**: 854-856.

90. Jagusztyn-Krynicka, E. K., J. B. Hansen, V. L. Crow, T. D. Thomas, A. L. Honeyman, and R. Curtiss III. 1992. *Streptococcus mutans* serotype c tagatose 6-phosphate pathway gene cluster. J. Bacteriol. **174**: 6152-6158.

91. Kashket, E. 1985. The protonmotive force in bacteria: a critical assessment of methods. Ann. Rev. Microbiol. **39**: 219-242.

92. Kato, C., and H. K. Kuramitsu. 1990. Carboxyl-terminal deletion analysis of the *Streptococcus mutans* glucosyltransferase-I enzyme. FEMS Microbiol. Lett. **72**:299-302.

93. Kenney, E. B., and M. M. Ash. 1969. Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. J. Periodontol. **40**: 630-632.

94. Keyes, P. H., and H. V. Jordan. 1963. Factors influencing the initiation, transmission and inhibition of dental caries. p 261-283. *In* R. S. Harris (ed.), Mechanisms of hard tissue destruction. Academic Press, New York.

95. Khandelwal, R. J., and I. R. Hamilton. 1971. Purification and properties of adenyl cyclase From *Streptococcus salivarius*. J. Biol. Chem. **246**: 3297-3304.

96. Kiska, D. L., and F. L. Macrina. 1994. Genetic regulation of fructosyltransferase in *Streptococcus mutans*. Infect. Immun. **62**: 1241-1251.

97. Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar. 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. Infect. Immun. **62**: 4469-80.

98. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to a histidine in a protein as an intermediate in

a novel phospho-transferase system. Proc. Natl. Acad. Sci. U.S.A. **52**: 1067-1074.

99. Lapointe, R., M. Frenette, and C. Vadeboncoeur. 1993. Altered expression of several genes in III_L^{man}-defective mutants of *Streptococcus salivarius* demonstrated by two-dimensional gel electrophoresis of cytoplasmic proteins. Res. Microbiol. **144**: 305-316.

100. Lee, C. A., and M. H. Saier. 1983. Mannitol-specific enzyme II of the bacterial phosphotransferase system-III the nucleotide sequence of the permease gene. J. Biol. Chem. **258**: 10761-10767.

101. Lee, I. S., J. Slonczewski, and J. W. Foster. 1994. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. **174**: 1537-1543.

102. Lengeler, J.W. 1990. Molecular analysis of the enzyme- II complexes of the bacterial phosphotransferase system (PTS) as carbohydrate transport systems. Biochim. Biophys. Acta. **1018**: 55-159.

103. Liberman, E. S., and A. S. Bleiweis. 1984. Role of the phosphoenolpyruvate-dependent glucose phosphotransferase system of *S. mutans* GS5 in the regulation of lactose uptake. Infect. Immun. **43**: 536-542.

104. Loesche W. J. 1976. Chemotherapy of dental plaque infections. Oral Sci. Rev. **9**: 65-107.

105. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50**: 353-380.

106. Loesche, W. J., and L. H. Straffon. 1979. Longitudinal investigation of the role of *Streptococcus mutans* in human fissure decay. Infect. Immun. **26**: 498-507.

107. Lunsford, R. D., and F. L. Macrina. 1986. Molecular cloning and characterization of *scrB*, the structural gene for the *Streptococcus mutans* phosphoenolpyruvate-

dependent sucrose phosphotransferase system sucrose-6-phosphate hydrolase. J. Bacteriol. **166**: 426-434.

108. Macrina, F. L., K. R. Jones, C. A. Alpert, B. M. Chassy, and S. M. Michalek. 1991. Repeated DNA sequence involved in mutations affecting transport of sucrose into *Streptococcus mutans* V403 via the phosphoenolpyruvate phosphotransferase system. Infect. Immun. **59**: 1535-1543.

109. Mandel, I. D. 1974. Relation of saliva and plaque to caries. J. Dent. Res. **53**: 246-266.

110. Marsh, P. 1989. Host defences and microbial homeostasis: role of microbial interactions. J. Dent Res. **68**: 1567-1575.

111. Marsh, P., and M. Martin. 1984. The resident oral flora p. 27-54. *In* Marsh, P., and M. Martin (ed.) Oral Microbiology. Chapman and Hall, New York

112. Maryanski, J. H., and C. L. Wittenberger. 1975. Mannitol transport in *Streptococcus mutans*. J. Bacteriol. **124**: 1475-1481.

113. Mattoo, R. L., and E. B. Waygood. 1983. Determination of the levels of HPr and Enzyme I of the phosphoenolpyruvate-sugar phosphotransferase system in *Escherichia coli* and *Salmonella typhimurium*. Can. J. Biochem. **61**: 29-37.

114. Meadow, N. D, D. K Fox, and S. Roseman. 1990. The Bacterial phosphoenolpyruvate:glycose phosphotransferase system. Ann. Rev. Biochem. **59**: 497-542.

115. Meng, S. Y., and G. N. Bennett. 1992. Regulation of the *Escherichia coli cad* operon: location of a site required for acid induction. J. Bacteriol. **174**: 2670-2678.

116. Milnes, A. R. 1987. A longitudinal investigation of the microflora associated with developing lesions of nursing caries. Ph.D. thesis, University of Manitoba, Winnipeg, Canada.

117. Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature **191**: 25-37.

118. Monod, J. 1942. Recherches sur la croissance des cultures bacteriennes. Hermann et Cie, Paris.

119. Moore, W. J., and E. Corbett. 1973. The distribution of dental caries in ancient British populations. Caries Res. **7**: 139-159.

120. Mooser, G., S. A. Hefta, R. J. Paxton, J. E. Shively, and T. D. Lee. 1991. Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* α-glucosyltransferases. J. Biol. Chem. **266**: 8916-8922.

121. Munro, C., S. M. Michalek, and F. L. Macrina. 1991. Cariogenicity of *Streptococcus mutans* V403 glucosyltransferase and fructosyltransferase mutants constructed by allelic exchange. Infect. Immun. **59**: 2316-2323.

122. Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the bacterial cell. Sinauer Associates, Inc., Sunderland, Massachusetts.

123. Nelson, S. O., J. K. Wright, and P. W. Postma. 1983. The mechanism of inducer exclusion. Direct interaction between purified III^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system and the lactose carrier from *Escherichia coli*. EMBO J. **2**: 715-720.

124. Neron, S., and C. Vadeboncoeur. 1987. Two functionally different glucose phosphotransferase systems in *Streptococcus mutans* and *Streptococcus sobrinus* Oral Microbiol. Immun. **2**: 171-177.

125. Pardee, A. B. 1966. A binding site for sulfate and its relation to sulfate transport into *Salmonella typhimurium*. J. Biol. Chem. **241**: 3962-3969.

126. Pardee, A. B. 1966. Purification and properties of a sulfate-binding protein from *Salmonella typhimurium*. J. Biol. Chem. **241**: 5886-5892.

127. Parker, L. L., and B. G. Hall. 1990. Characterization and nucleotide sequence of the cryptic *cel* operon of *Escherichia coli*. Genetics. **124**: 455-471.

128. Parsot, C., and J. J. Mekalanos. (1991). Expression of the *Vibrio cholerae* gene encoding aldehyde dehydrogenase is under control of *ToxR*, the cholera toxin transcriptional activator. J. Bacteriol. **173**: 2842-2851.

129. Pastan, I., and R. L. Perlman. 1969. Cyclic adenosine monophosphate in bacteria. Science **169**: 339-344.

130. Pastan, I., and R. L. Perlman. 1969. Repression of β-galactosidase synthesis by glucose in phosphotransferase mutants of *Escherichia coli*. J. Biol. Chem. **244**: 5836-5842.

131. Peterkofsky, A., and C. Gazdur. 1975. Interaction of enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system with adenylate cyclase of *Escherichia coli*. Proc. Nat'l. Acad. Sci. USA **72**: 2920-2924.

132. Poolman, B. 1993. Energy transduction in lactic acid bacteria. FEMS Microbiol. Rev. **12**: 125-147.

133. Postma, P. W., and J. W. Lengeler. 1985. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49**: 232-269.

134. Postma, P. W., J. W. Lengeler, and G. G. Jacobson. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **57**: 543-594.

135. Poy, F., and G. R. Jacobson. 1990. Evidence that a low-affinity sucrose phosphotransferase activity in *Streptococcus mutans* GS-5 is a high-affinity trehalose uptake system. Infect. Immun. **58**: 1479-1480.

136. Pries, A., H. Priefert, N. Kruger, and A. Steinbuchel. 1991. Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(β-hydroxybutyric acid)-leaky phenotype which exhibit homology to *ptsH* and *ptsI* of *Escherichia coli*. J. Bacteriol. **173**: 5843-5853.

137. Reizer, J., J. Deutscher, and M. H. Saier Jr. 1989. Metabolite-sensitive, ATP-dependent, protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system in Gram-positive bacteria. Biochimie **71**: 989-996.

138. Reizer, J., J. Deutscher, S. Sutrina, J. Thompson, and M. Saier, Jr. 1985. Sugar accumulation in Gram-positive bacteria: exclusion and expulsion mechanisms. Trends. Biochem. Sci. **10**: 32-35.

139. Reizer, J., J. Deutscher, S. Sutrina, J. Thompson, and M. Saier, Jr. 1985. Sugar accumulation in Gram-positive bacteria: exclusion and expulsion mechanisms. Trends. Biochem. Sci. **10**: 32-35.

140. Reizer, J., M. J. Novotny, C. Panos, and M. J. Saier Jr. 1983. Mechanism of inducer-expulsion in *Streptococcus pyogenes:* a two-step process activated by ATP. J. Bacteriol. **156**: 354-361.

141. Reizer, J., M. J. Novotny, W. Hengstenberg, C. Panos, and M. J. Saier Jr. 1984. Properties of ATP-dependent protein kinase from *Streptococcus pyogenes* that phosphorylates a seryl residue in HPr, a phosphocarrier protein of the phosphotransferase system. J. Bacteriol. **160**: 333-340.

142. Reizer, J., A. Peterkofsky, and A. H. Romano. 1988. Evidence for the presence of heat-stable protein (HPr) and ATP-dependent HPr kinase in heterofermentive lactobacilli lacking a phosphoenolpyruvate: glycose phosphotransferase activity. Proc. Nat'l. Acad. Sci. U.S.A. **85**: 2041-2045.

143. Reizer, J., A. Reizer, M. H. Saier, and G. R. Jacobson. 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci. **1**: 722-726.

144. Reizer, J., A. H. Romano, and J. Deutscher. 1993. The role of phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, in the regulation of carbon metabolism in Gram-positive bacteria. J. Cell. Biochem. **51**: 19-24.

145. Reizer, J., M. H. Saier Jr., J. Deutscher, F. Grenier, J. Thompson, and W. Hengstenberg. 1989. The phosphoenolpyruvate phosphotransferase system in Gram-positive bacteria: properties, mechanism and regulation. CRC Crit. Rev. Microbiol. **15**: 297-338.

146. Reizer, J., S. L. Sutrina, M. H. Saier, G. C. Stewart, A. Peterkofsky, and P. Reddy. 1989. Mechanistic and physiological consequences of HPr(ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: studies with site-specific mutants of HPr. EMBO J. **8**: 2111-2120.

147. Romano, A. H., G. Brino, A. Peterkofsky, and J. Reizer. 1987. Regulation of β-galactoside transport and accumulation in heterofermentative lactic acid bacteria. J. Bacteriol. **169**: 5589-5596.

148. Rosen, B. P. 1987. ATP-coupled solute transport systems, p. 760-767. *In* F. C. Neidhardt (ed.), *Esherichia coli* and *Salmonella typhimurium* cellular and molecular biology. Am. Soc. Microbiol. Washington, D. C.

149. Rosen, B.P., and E.R. Kashket, 1978. Energetics of Active Transport, p. 559-620. *In* B.P. Rosen (ed.), Bacterial transport. Marcel Dekker Inc., New York.

150. Rosey, E. L., and G. C. Stewart. 1992. Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-

phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans*. J. Bacteriol. **174**: 6159-6170.

151. Russell, R. R. B. 1990. Molecular genetics of glucan metabolism in oral streptococci. Arch. Oral Biol. **35**: 53D-58S.

152. Russell, R. R. B. 1994. The application of molecular genetics to the microbiology of dental caries. Caries Res. **28**: 69-82.

153. Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao, and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. *J. Biol. Chem.* **267**: 4631-4637.

154. Russell, R. R. B., and J. J. Ferretti. 1990. Nucleotide sequence of the dextran glucosidase (*dexB*) gene of *Streptococcus mutans*. J. Gen. Microbiol. **136**: 803-810.

155. Russell, R. R. B., H. Mukasa, A. Shimamura, and J. J. Ferretti. 1988. *Streptococcus mutans gtfA* gene specifies sucrose phosphorylase. Infect. Immun. **56**: 2763-2765.

156. St. Martin, E. J., and C. L. Wittenberger. 1979. Regulation and function of sucrose 6-phosphate hydrolase in *Streptococcus mutans*. Infect. Immun. **26**: 487-491.

157. Saier, M. H. 1989. Protein phosphorylation and allosteric control of inducer exclusion by the bacterial phosphoenolpyruvate; sugar phosphotransferase system. Microbiol. Rev. **53**: 109-120.

158. Saier, M. H. Jr. 1994. The bacterial phosphotransferase system: new frontiers 30 years later. Mol. Microbiol. **13**: 755-764.

159. Saier , M. H., and B. U. Feucht. 1975. Coordinate regulation of adenylate cyclase and carbohydrate

permeases by the phosphoenolpyruvate:sugar phosphotransferase system in *Salmonella typhimurium*. J. Biol. Chem. **250**: 7078-7080.

160. Saier, M. H., and J. Reizer. 1990. Domain shuffling during evolution of the proteins of the bacterial phosphotransferase system. Res. Microbiol. **141**: 1033-1038.

161. Saier, M. H. Jr., and J. Reizer. 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. **174**: 1433-1438.

162. Saier, M. H. Jr., and S. Roseman. 1976. Sugar transport. IX. The *crr* mutation: its effect on repression of enzyme synthesis. *J. Biol. Chem.* **25**: 6598-6605.

163. Saier, M. H. Jr., R. D. Simoni, and S. Roseman. 1970. The physiological behavior of Enzyme I and heat-stable protein mutants of a bacterial phosphotransferase system. J. Biol. Chem. **245**: 5870-5873.

164. Saier, M. H., M. Yamada, B. Erni, K. Suda, J. Lengeler, R. Ebner, P. Argos, B. Rak, K. Schenetz, C. A. Lee, G. C. Stewart, F. Breidt, E. B. Waygood, K. Peri, and R. F. Doolittle. 1988. Sugar permeases of the bacterial phosphoenolpyruvate-dependent phosphotransferase system: sequence comparisons. FASEB J. **2**: 199-208.

165. Sanno, Y., T. H. Wilson, and E. C. C. Lin. 1968. Control of permeation to glycerol in cells of *Escherichia coli*. Biochem. Biophys. Res. Commun. **32**: 344-349.

166. Sansome, C., J. van Houte, K. Joshipura, R. Kent, and H. C. Margolis. 1993. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J. Dent. Res. **72**: 508-516.

167. Sato, S., and H. K. Kuramitsu. 1986. Isolation and characterization of a fructosyltransferase from *Streptococcus mutans* GS-5. Infect. Immun. **52**: 166-170.

168. Sato, Y., F. Poy, G. R. Jacobson, and H. K. Kuramitsu. 1989. Characterization and sequence analysis of the *scrA* gene encoding enzyme II^{Scr} of the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system. J. Bacteriol. **171**: 263-271.

169. Sato, Y., Y. Yamamoto, H. Kizaki, and H. K. Kuramitsu. 1993. Isolation, characterization and sequence analysis of the *scrK* gene encoding fructokinase of *Streptococcus mutans*. J. Gen. Microbiol. **139**: 921-927.

170. Schmitt, M. P., and R. K. Holmes. 1994. Cloning, sequence, and footprint analysis of two promoter/operators from *Corynebacterium diptheriae* toxin repressor (*DtxR*) and iron. J. Bacteriol. **176**: 1141-1149.

171. Schroeder, V. A., S. M. Michalek, and F. L. Macrina. 1989. Biochemical characterization and evaluation of virulence of a fructosyltransferase-deficient mutant of *Streptococcus mutans* V403. Infect. Immun. **57**: 3560-3569.

172. Shi, X., B. C. Waasdorp, and G. N. Bennett. 1993. Modulation of acid-induced amino acid decarboxylase gene expression by *hns* in *Escherichia coli*. J. Bacteriol. **175**: 1182-1186.

173. Shiroza, T., and H. K. Kuramitsu. 1988. Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. J. Bacteriol. **170**: 810-816.

174. Slee, A. M., and J. M. Tanzer. 1979. Phosphoenolpyruvate-dependent sucrose

phosphotransferase activity in *Streptococcus mutans* NCTC 10449. Infect. Immun. **24**: 821-828.

175. Slee, A. M., and J. M. Tanzer. 1979. Phosphoenolpyruvate-dependent sucrose phosphotransferase activity in five serotypes of *Streptococcus mutans*. Infect. Immun. **26**: 783-786.

176. Slonczewski, J., T. N. Gonzalez, F. M. Bartholomew, and N. J. Holt. 1987. Mu d-directed *lacZ* fusions regulated by low pH in *Escherichia coli*. J. Bacteriol. **169**: 3001-3006.

177. Socransky, S. S., A. D. Manganiello, D. Propas, V. Oram, and J. van Houte. 1977. Bacteriological studies of developing supragingival dental plaque. J. Periodont. Res. **12**: 90-106.

178. Spatafora-Harris, G., S. M. Michalek, and R. Curtiss III. 1992. Cloning of a locus involved in *Streptococcus mutans* intracellular polysaccharide accumulation and virulence testing of an intracellular polysaccharide-deficient mutant. Infect. Immun. **60**: 3175-3185.

179. Spearman, T. N., R. L. Khandelwal, and I. R. Hamilton. 1973. Some regulatory properties of glycogen phosphorylase from *Streptococcus salivarius*. Arch. Biochim. Biophys. **154**: 306-313.

180. Stephan, R. M. 1940. Changes in hydrogen ion concentrations on tooth surfaces and on carious lesions. J. Am. Dent. Assoc. **27**: 718-723.

181. Stephan, R. M. 1944. Intra-oral hydrogen-ion concentrations associated with dental caries activity. J. Dent. Res. **23**: 257-266.

182. Sutcliffe, I. C., L. Tao, J. J. Ferretti, and R. R. B. Russell. 1993. *MsmE*, a lipoprotein involved in sugar transport in *Streptococcus mutans*. J. Bacteriol. **175**: 1853-1855.

183. Sutton, S. T. W., and R. E. Marquis. 1987. Membrane-associated and solubilized ATPases of *Streptococcus*

mutans and *Streptococcus sanguis*. J. Dent. Res. **66**: 1095-1098.

184. Svensater, G., and I. R. Hamilton. 1991. Sorbitol transport by *Streptococcus sanguis* 160. Oral Microbiol. Immunol. **6**: 160-168.

185. Tanzer, J. M. 1989. On changing the cariogenic chemistry of coronal plaque. J. Dent. Res. **68**: 1576-1587.

186. Tanzer, J. M., M. L. Freedman, F. N. Woodiel, R. L. Eifert, and L. A. Rinehimer. 1976. Association of *Streptococcus mutans* virulence with synthesis of intracellular polysaccharide, p. 597-616. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings Microbial Aspects of Dental Caries (sp. suppl. Microbiol. Abstr.), Vol 3. Information Retrieval Inc., Washington, D. C.

187. Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti. 1993. Transport of sugars, including sucrose, by the *msm* transport system of *Streptococcus mutans*. J. Dent. Res. **267**: 4631-4637.

188. Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti. 1995. Regulation of the multiple sugar metabolism operon in *S. mutans*. *In* J. J. Ferretti (ed.), Genetics of streptococci, lactococci, and enterococci. Int'l Assoc. Biol. Stand. (In press).

189. Thevenot, T., D. Brochu, C. Vadeboncoeur, and I. R. Hamilton. 1995. Regulation of ATP-dependent P-(Ser)-HPr formation in *Streptococcus mutans* and *Streptococcus salivarius*. J. Bacteriol. **177**: 2751-2759.

190. Trombe, M. C. 1993. Characterization of a calcium porter of *Streptococcus pneumoniae* involved in calcium regulation of growth and competence. J. Gen. Microbiol. **139**: 433-439.

191. Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characteristics of the oligopeptide

transport system of *Lactococcus lactis*. J. Bacteriol. **175**: 7523-7532.

192. Vadeboncoeur, C. 1984. Structure and properties of the phosphoenolpyruvate: glucose phosphotransferase system of oral streptococci. Can. J. Microbiol. **30**: 495-502.

193. Vadeboncoeur, C., D. Brochu, and J. Reizer. 1991. Quantitative determination of the intracellular concentration of the various forms of HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system in growing cells of oral streptococci. Anal. Bioch. **196**: 24-30.

194. Vadeboncoeur, C., and L. Trahan. 1983. Comparative study of the *Streptococcus mutans* laboratory strains and fresh isolates from carious and caries-free tooth surfaces and from subjects with hereditary fructose intolerance. Infect. Immun. **40**: 81-90.

195. van Houte, J. 1994. Role of micro-organsims in caries etiology. J. Dent. Res. **73**: 672-681.

196. van Houte J., J. Lopman, and R. Kent. 1994. The predominant cultivable flora of sound and carious human root surfaces. J. Dent. Res. **73**: 1727-1734.

197. Vanweeghel, R. P, G. H. Meyer, W. Keck, and G. T. Robillard. 1991. Phosphoenolpyruvate-dependent mannitol phosphotransferase system of *Escherichia coli* -overexpression, purification, and characterization of the enzymatically active C-terminal domain of enzyme-II^{mtl} equivalent to enzyme-III^{mtl}. Biochem. **30**: 1774-1779.

198. van Houte, J., J. Russo, and K. S. Prostak. 1989. Increased pH-lowering ability of *Streptococcus mutans* cell masses associated with extracellular glucan glucan-rich material and the mechanisms involved. J. Dent. Res. **68**: 451-459.

199. van Houte, J., C. Sansone, K. Joshipura, and R. Kent. 1991. Mutans streptococci and non-mutans streptococci

acidogenic at low pH, and in vitro acidogenic potential of dental plaque in two different areas of the human dentition. J. Dent. Res. **70**: 1503-1507.

200. Vanweeghel, R. P., Y. Y. Vanderhoek, H. H. Pas, M. Elferink, W. Keck, and G. T. Robillard. 1991. Details of mannitol transport in *Escherichia coli* elucidated by site-specific mutagenesis and complementation of phosphorylation site mutants of the phosphoenolpyruvate-dependent mannitol-specific phosphotransferase system. Biochem. **30**: 1768-1773.

201. Walsh, J. P., C. R. Loomis, and R. M. Bell. 1990. *sn*-1,2-Diacylglycerol kinase of *Escherichia coli*. J. Biol. Chem. **261**: 11021-11027.

202. Watson, N., D. S. Dunyak, E. L. Rosey, J. L. Slonczewski, and E. R. Olson. 1992. Identification of elements involved in transcriptional regulation of the *Esherichia coli cad* operon by external pH. J. Bacteriol. **174**: 530-540.

203. Wexler, D. L., J. E. C. Penders, W. H. Bowen, and R. A. Burne. 1992. Characteristics and cariogenicity of a fructanase-defective *Streptococcus mutans* strain. Infect. Immun. **60**: 3673-3681.

204. Yamashita, Y., W. H. Bowen, and H. K. Kuramitsu. 1992. Molecular analysis of a *Streptococcus mutans* strain exhibiting polymorphism in the tandem *gtfB* and *gtfC* genes. Infect. Immun. **60**: 1618-1624.

205. Yamashita, Y., T. Takehara, and H. K. Kuramitsu. 1993. Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. J. Bacteriol. **175**: 6220-6228.

206. Ye, J.J., J. W. Neal, J. Reizer, X. Cui, and M. H. Saier Jr. 1994. Regulation of the glucose:H^+ symporter by metabolite-activated ATP-dependent phosphorylation of HPr in *Lactobacillus brevis*. J. Bacteriol. **176**: 3484-3492.

207. Ye, J.J., J. Reizer, X. Cui, and M. H. Saier Jr. 1994. ATP-dependent phosphorylation of serine-46 in the phosphocarrier protein HPr regulates the lactose/H^+ symport in *Lactobacillus brevis*. Proc. Nat'l. Acad. Sci. USA. **91**: 3102-3106. 190.

208. Zero, D. T., J. van Houte, and J. Russo. 1986. Enamel demineralization by acid produced from endogenous substrate in oral streptococci. Arch. Oral Biol. **31**: 229-234.

Chapter 2

Background to the Research

Several previous studies in our laboratory have focused on the regulation of the glucose PTS in *S. mutans*. Many of these experiments have employed the technique of continuous culture in order to control the growth rate, limiting growth component and the pH of a culture to simulate conditions typical of those encountered in the mouth (7). The application of this technique to the study of sugar transport in *S. mutans* has demonstrated that the glucose PTS is repressed under conditions of low pH (3), high growth rates, and with excess glucose (4). Since the overall rate of glycolysis by cells growing under these conditions was not inhibited to the same degree as the PTS, it was hypothesized that glucose transport occurred by a non-PTS process. Previous studies in this laboratory have suggested that non-PTS glucose transport was dependent on proton-motive force (5,6), however, subsequent experiments have questioned this model (2). Recent work by Buckley and Hamilton utilizing membrane vesicles has demonstrated the presence of a carrier capable of transporting glucose into the cell (1). This work led to the suggestion that the non-PTS system was dependent on a phosphate bond for energy to drive transport.

The overall goal of the research was to utilize molecular genetic techniques to generate a PTS defective mutant in order to examine this non-PTS glucose transport system in

more detail. The project had three specific aims to eventually reach this goal:

1. To clone and sequence the genes coding for Enzyme 1 (*ptsI*) and HPr (*ptsH*) from *S. mutans*.
2. To generate a mutant of *S. mutans* defective in Enzyme 1 of the PTS by allelic exchange using the cloned *ptsI* gene.
3. To utilize the PTS-defective mutant to examine non-PTS glucose transport.

The chapters that follow in this thesis are arranged chronologically and were originally written as full length papers that have, with the exception of Chapter 4, been published or submitted to refereed journals.

Chapter 3 begins with a paper that describes the cloning, nucleotide sequence and expression of the *S. mutans ptsH* and *ptsI* genes in *E. coli*. These genes, coding for the soluble enzymes EI (*ptsI*) and HPr (*ptsH*) of the PTS, were the first target of our research, since we believed they would be useful in constructing integration vectors to generate PTS defective mutants. Inactivation of either of the general proteins would block phosphoryl transfer to the sugar-specific EIIs rendering the PTS non-functional. This paper describes the successful cloning and nucleotide sequencing of the genes and the comparison to known *ptsH/I* sequences from other bacteria. The proteins were also expressed in *E. coli* hosts, where they were able to complement *E. coli* PTS$^-$

mutants, as demonstrated by restoration of the ability of the transformants to metabolize glucose. The results of this work were published in Infection and Immunity (62: 1156-1165, 1994).

The second Specific Aim of the project was to generate a PTS-defective mutant by allelic exchange. The generation of such a mutant would allow us to study non-PTS transport systems in this organism without competition by the PTS and would also serve as a tool for investigating the regulation of sugar transport systems. The initial phase of this project, described in Chapter 4, was started by the construction of an integration vector harboring a fragment of the cloned *ptsI* gene of *S. salivarius*. The gene from the latter organism was used with the kind cooperation of Dr. Michel Frenette, Laval University, Quebec, since we had not, at that time, cloned the genes from *S. mutans*. The results of Southern hybridization experiments had indicated that the genes from the two species were highly homologous and when *S. mutans* was transformed with this vector, we expected the phenotype of the resultant transformant to be PTS^-.

Transformations of *S. mutans* with the *S. salivarius-ptsI*-containing vector failed to generate a PTS-defective mutant, however, a mutant incapable of utilizing mannose was isolated. Subsequent examination of this transformant revealed that the plasmid had inserted into the multiple sugar metabolism operon (*msm*). We were, however, unable to

generate this phenotype in subsequent transformations with integrations into the same region of the chromosome, leading us to believe that the mutation causing the man$^-$ phenotype occurred independently of the plasmid insertion.

At this point in time, the cloning of the *ptsH* and *ptsI* genes from *S. mutans* was completed and I was able to construct integration vectors harboring fragments of these genes. One such vector, pDC-5, containing an internal fragment of the *ptsI* gene that had been disrupted by an erythromycin-resistance cassette, was successful in generating a PTS defective mutant. Chapter 5 describes the isolation of this mutant by inactivation of the *ptsI* gene by allelic exchange with this vector. The resultant *ptsI* mutant, DC10, was unable to ferment all known PTS substrates with the exception of glucose, which was believed to be transported by the non-PTS system. [^{32}P]-PEP phosphorylation experiments as well as quantification of the various intracellular forms of HPr demonstrated a non-functional PTS in this strain.

The generation of the PTS defective mutant allowed us to proceed with Specific Aim 3 in order to examine non-PTS glucose transport in *S. mutans*. Chapter 5 describes this transport process in regard to kinetics, substrate specificity and potential phosphoryl donors. The system appears to fit the model of glucose transport previously suggested by work with *S. mutans* membrane vesicles (1). The

transport system was shown to have a high specificity for glucose, but exhibited a lower affinity for glucose than the PTS. Transport via this system also relied on ATP and not PEP to generate phosphorylated glucose. This work has recently appeared in the Journal of Bacteriology (177: 2251-2258, 1995)

This mutant not only proved useful in the analysis of non-PTS glucose transport, but was also utilized in the characterization of PTS-mediated MSM transport (Chapter 6). Although not an original goal of the project, the observation that growth of the PTS defective mutant on the MSM-substrate raffinose was slower then the wild-type strain prompted us to examine this phenomenon. This difference in growth rates suggested that there was a possible interaction between the MSM transport system and the PTS so we set out to examine possible modes of regulation between the two systems.

The first experiments to test for PTS-mediated control of the MSM demonstrated diauxic growth of *S. mutans* when grown in a mixture of melibiose and glucose, with glucose used preferentially. An examination of MSM-mediated growth, transport, and acid production with MSM substrate raffinose revealed that these processes were diminished in the mutant relative to the parent. The transport of ^{3}H-raffinose was also inhibited by glucose and the non-metabolizable glucose analogs, 2-deoxyglucose and α-methylglucoside. [^{32}P]-PEP phosphorylation experiments also revealed that a 59 kDa

phosphoprotein appeared to be specific to MSM-substrate grown cells. These results suggested that glucose repressed the *msm* operon as well as inhibited uptake via the MSM system. This work has been submitted for publication to the Journal of Bacteriology.

Authors contribution to research. The beginning of the thesis project involving the cloning of the *S. mutans ptsH* and *ptsI* genes, I worked closely with Mr. Dave Boyd to gain experience with the techniques of gene cloning and manipulation. During the course of this work, described in Chapter 5, I contributed to the overall design of the experiments, the genomic library construction, in both plasmid and phage hosts, the cloning of the operon in the EI$^-$ *E. coli* host CHE11, the subcloning of fragments and the evaluation of expression of the genes in the host strains. I also performed the [^{32}P]-PEP autoradiography. The deletion subcloning and nucleotide sequencing were essentially performed by David Boyd. In all the subsequent chapters the experiments were primarily carried out by myself with the exception of the rocket immunoelectrophoresis used to quantitate the various forms of HPr, as described in Chapter 5, which was performed by Mrs. Tracy Thevenot.

References

1. Buckley, N. D., and I. R. Hamilton. 1994. Vesicles prepared from *Streptococcus mutans* demonstrate the

presence of a second glucose transport system. Microbiology **140**: 2639-2648.

2. Dashper, S. G., and E. C. Reynolds. 1990. Characterization of transmembrane movement of glucose and glucose analogues in *Streptococcus mutans* Ingbritt. J. Bacteriol. **172**: 556-563.
3. Ellwood, D. C., and I. R. Hamilton. 1982. Properties of *Streptococcus mutans* Ingbritt growing in limiting sucrose in a chemostat: Repression of the phosphoenolpyruvate phosphotransferase transport system. Infect. Immun. **36**: 576-581.
4. Ellwood, D. C., P. J. Phipps, and I. R. Hamilton. 1979. Effect of growth rate and glucose concentration on the activity of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans* Ingbritt grown in continuous culture. Infect. Immun. **23**: 224-231.
5. Hamilton, I. R. 1990. Maintenance of proton motive force by *Streptococcus mutans* and *Streptococcus sobrinus* during growth in continuous culture. Oral Microbiol. Immunol. **5**: 280-287.
6. Hamilton, I. R., and E. J. St. Martin. 1982. Evidence for the involvement of protonmotive force in the transport of glucose by a mutant of *Streptococcus mutans* Strain DR0001 defective in the glucose phosphoenolpyruvate phosphotransferase system. Infect. Immun. **36**: 567-575.
7. Tempest, D.W. 1970. The place of continuous culture in microbiological research. Adv. Microbiol. Phys. **4** : 223-249.

Chapter 3

Sequence and Expression of the Genes for HPr (*ptsH*) and Enzyme I (*ptsI*) of the Phosphoenolpyruvate-dependent Phosphotransferase Transport System from *Streptococcus mutans*

David A. Boyd, Dennis G. Cvitkovitch and Ian R. Hamilton*

Department of Oral Biology

University of Manitoba

Winnipeg, Manitoba

R3E 0W3

Published in Infection and Immunity 62: 1156-1165 April, 1994

Abstract

We report the sequencing of a 2242 bp region of the *Streptococcus mutans* NG5 genome containing the genes for *ptsH* and *ptsI*, which encode HPr and Enzyme I (EI), respectively, of the phosphoenolpyruvate-dependent phosphotransferase transport system (PTS). The sequence was obtained from two cloned overlapping genomic fragments, one which expresses HPr and a truncated EI, while the other expresses a full length EI in *E. coli* as determined by Western immunoblotting. The *ptsI* gene appeared to be expressed from a region located in the *ptsH* gene. The *S. mutans* NG5 *pts* operon does not appear to be linked to other PTS proteins as has been found in other bacteria. A positive fermentation pattern on Maconkey-glucose plates by an *E. coli ptsI* mutant harboring the *S. mutans* NG5 *ptsI* gene on a plasmid indicated that the *S. mutans* NG5 EI can complement a defect in the *E. coli* gene. This was confirmed by protein phosphorylation experiments utilizing [^{32}P]-PEP indicating phosphotransfer from the *S. mutans* NG5 EI to the *E. coli* HPr. Two forms of the cloned EI, both truncated to varying degrees in the C-terminal region, were inefficiently phosphorylated and unable to complement fully the *ptsI* defect in the *E. coli* mutant. The deduced amino acid sequence of HPr shows a high degree of homology, particularly around the active site, to the same protein from other Gram-positive bacteria, notably *Streptococcus salivarius* and to a lesser extent with those of Gram-negative

bacteria. The deduced amino acid sequence of *S. mutans* NG5 EI also shares several regions of homology with other sequenced EIs, notably with the region around the active site, a region that contains the only conserved cystidyl residue amongst the various proteins and may be involved in substrate binding.

Introduction

Dental caries is caused by the dissolution of tooth enamel by acid end-products generated during carbohydrate metabolism by acidogenic bacteria in the biofilms on the tooth surface (dental plaque). A principle etiological agent of this disease is *Streptococcus mutans* (5,30), an organism capable of high levels of glycolytic activity in the presence of mono- and disaccharides. *S. mutans* transports such sugars via the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) (38,46). The PTS is a group translocation process which utilizes PEP as an energy source and substrate, and catalyzes the sequential phosphorylation of the general, non-sugar specific proteins, enzyme I (EI) and HPr required for the uptake of all PTS sugars. In many cases, phospho-HPr (HPr-P), generated from phospho-EI, transfers the phosphoryl group directly to carbohydrate-specific, membrane-bound enzyme II (EII), which in turn phosphorylates the incoming sugar. In other cases, HPr-P transfers the phosphoryl group to a third soluble protein called enzyme III (EIII) prior to interaction with EII (4,15,49,50).

Earlier continuous culture studies with *S. mutans* Ingbritt, using decryptified cells to measure phosphotransferase activity, indicated that the growth environment regulates PEP-dependent PTS sugar transport (21). Subsequently, this regulation was shown to be associated with repression of the membrane, sugar-specific EII component. For example, EII

activity for glucose (EII^{glc}) and mannose (EII^{man}), measured in isolated membrane preparations in the presence of excess EI and HPr, was repressed by as much as 62-fold under conditions of low pH, high growth rates and with excess glucose compared to optimal PTS conditions (i.e., 5 mM glucose, D=0.1 h^{-1} and pH 7.0)(52). The cellular concentration of HPr varied two-fold under the same conditions. Similar repression of the EII activities for glucose, mannose and 2-deoxyglucose of the PTS was recently reported for a fresh strain of *S. mutans* growing in continuous culture (22), however, the cellular concentrations of EI, but not HPr, also varied significantly with the growth conditions.

Concentration-dependent glucose repression (33-fold) of EII^{glc} in membrane preparations of *S. mutans* Ingbritt occurs during growth in continuous culture at glucose levels above 3 mM (pH 7.0 and D=0.1 h^{-1}) even during conditions of glucose limitation (22). EI and HPr cellular levels varied 4-fold under the same conditions. More recently, the effect of growth pH on EII activity for glucose, mannose and 2-deoxyglucose was examined with cells of *S. mutans* Ingbritt grown in continuous culture at D=0.1 h^{-1} with 10 mM glucose (glucose limited)(53). In this study, progressively lower EII activity was observed as the growth pH was reduced from pH 8.0 with cells grown at pH 5.0 completely devoid of EII activity. Similarly, cells of the organism grown at high

growth rates (e.g. 0.5 and 1.0 h^{-1}) also exhibited little EII^{glc} and EII^{man} activity. In the same study, it was observed that $EIII^{man}$ synthesis was repressed at conditions above and below pH 7.0 and was particularly sensitive to growth at high growth rates.

These studies indicate that the regulation of the PTS in *S. mutans* is a complex matter undoubtedly involving a variety of mechanisms. As a consequence, we have undertaken to study the regulation of the glucose-PTS in *S. mutans* and, as a first step, have begun to isolate and characterize the genes for the various components of the PTS. We report here the cloning and sequencing of the genes for HPr (*ptsH*) and Enzyme I (*ptsI*) from *S. mutans* NG-5 and their expression in *E.coli*.

Materials and Methods

Bacterial strains, plasmids, bacteriophage, and media. *S. mutans* strain NG5 was maintained on blood plates (Oxoid blood base agar #2, 5% sheeps blood, 0.0005% hemin) and grown for DNA isolation in TYE-glucose broth (1% tryptone, 0.5% yeast extract, 20 mM glucose and 17 mM K_2PO_4). For routine subcloning, *E. coli* XL1-blue or SURE (Stratagene) were used as the hosts for plasmids constructed using the vectors pBluescript II (Stratagene) or pUC9 (55). *E. coli* strains were maintained on LB plates (35) supplemented when

needed with the appropriate drugs and/or chemicals at the following concentrations: 100 µg/ml ampicillin, 13 µg/ml tetracycline, 80 µg/ml isopropyl-ß-D-thiogalactopyranoside (IPTG), 33 µg/ml 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal).

S. mutans NG5 genomic libraries were constructed in λgt11 (25) using *E. coli* Y1090r$^-$ (25) as a host and in λEMBL3 (13) using *E. coli* KW251 (Promega) as a host. The *ptsI* mutant, *E. coli* CHE11 (18), was used as a host for a recombinant phage and plasmid harboring cloned *S. mutans* NG5 *ptsH* gene regions. Two plasmids, pEIP8 containing a 1.1 kb *Eco*RI fragment from the C-terminal region of the *S. salivarius ptsI* gene and pEIP14-II containing a 0.45 kb *Hind*III/*Xba*I fragment from the N-terminal region of the gene, both cloned into pUC18 (14), were a generous gift from Michel Frenette, Laval University, Quebec.

DNA isolation. For the isolation of DNA, *S. mutans* was inoculated into 500 ml of TYE broth and incubated anaerobically at 37°C. The cells were centrifuged, resuspended in 5 ml of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and the genomic DNA isolated as follows. The cells were heated at 60°C for 20 min, the suspension cooled to 37°C and lysozyme (20 mg/ml) and mutanolysin (200 units/ml) added and the mixture incubated for 1 h. Proteinase K (100 µg/ml) was then added, the mixture incubated for a further 1 h and

then the cells lysed by the addition of sodium dodecyl sulfate (SDS) to 4% with gentle inversion of the mixture. The NaCl concentration was adjusted to 0.7 M and then 1/10 vol of 0.7 M NaCl-10% cetyltrimethylammoniumbromide (CTAB) was added, the mixture incubated at 65°C for 20 min and then extracted with an equal volume of chloroform. The upper aqueous phase was collected and extracted once with phenol:chloroform:isoamylalcohol (25:24:1 vol/vol) and once with chloroform:isoamylalcohol (24:1 vol/vol). The DNA was precipitated from the aqueous phase with 2 vol ethanol, washed once with 70% ethanol, and resuspended in TE buffer at 1 mg/ml.

Rapid screening of plasmids was done from 1.5 ml *E. coli* cultures by using alkaline lysis (3). Plasmid DNA to be sequenced or for use in creating unidirectional deletions was isolated from 3-10 ml *E. coli* cultures using the Magic Minipreps Kit (Promega). Phage DNA was isolated from liquid lysates by the method of Kaslow (26).

Recombinant DNA methodology. Agarose gel electrophoresis carried out in 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0, and plaque and Southern hybridizations, were as described in Maniatis et al. (33). All hybridizations were carried out in aqueous solutions. Transfer of DNA was to Hybond N+ (Amersham) for detection by radiolabelled probes and to Photogene Nylon membranes (Gibco/BRL) for detection by

biotin-labelled probes. Radiolabelled DNA probes were prepared by using the Nick Translation or Random Primers Labelling Kits, and biotin-labelled DNA probes were prepared using the Bio-Nick Labelling Kit (Gibco/BRL). Recombinant plasmids used for sequencing were produced by subcloning specific restriction fragments after purification from agarose gels using the GENECLEAN Kit (Bio 101), restriction digestion deletion/religation to produce specfic subclones, or by unidirectional exonuclease III digestion (23) using the Erase-a-Base system (Promega).

E.coli was transformed by electroporation using a Gene Pulser apparatus (Bio-Rad) according to the method of Dower et al. (11). Sequencing was carried out on double stranded DNA by the dideoxy termination method (44) using [α^{35}S]-dATP and the Sequenase Version 2.0 Kit (U.S. Biochemicals). The sequence was confirmed by sequencing overlapping clones and determining the sequence on both strands. For sequencing the N-terminal region of the *ptsH* gene, we used a 16-mer sequencing primer we had synthesized (University of Manitoba DNA Laboratory) based on a previously-sequenced region of the *ptsH* gene. The sequence of the primer was 5'-TGTAATATCTGAGGCA-3'.

Preparation of a cytoplasmic fraction from *S. mutans*. The preparation of a *S. mutans* cytoplasmic fraction

containing HPr and EI for use as a positive control in Western immunoblotting has previously been described (54).

Protein electrophoresis and Western immunoblotting. Crude protein preparations from *E. coli* harboring plasmids were prepared by emulsifying a 1-2 cm streak from an LB plate containing the appropriate antibiotics in 500 µl of 50 mM Na_2HPO_4 (pH 7.4), 150 mM NaCl, 10 mM EDTA, adding 250 µl of 187.5 mM Tris (pH 6.8), 6% SDS, 15% ß-mercaptoethanol, 0.006% bromophenol blue, 30% glycerol, and boiling for 2 min. Proteins were separated by SDS-PAGE according to Laemmli (29), and transferred to Immobilon-P membranes (Millipore) according to Towbin et al. (48). After incubation of membranes with the appropriate primary antibody (1:750 dilution in Tris/saline buffer), EI and HPr-specific bands were detected by incubation with goat anti-rabbit IgG alkaline phosphate conjugate followed by detection with Nitroblue Tetrazolium Chloride (NBT) and 5-Bromo-4-chloro-3-indolylphosphate p-Toluidine (BCIP).

In vivo complementation of the *ptsI* mutant *E. coli* CHE11. To determine if the cloned *S. mutans* NG5 *ptsI* gene or truncated forms of it could complement the *ptsI* mutation in *E. coli* CHE11, pDB102, pDB105, and pDB201 were transformed into into CHE11 and streaked onto Maconkey plates containing 1% glucose. Positive controls were *E. coli* XL1-Blue with or without pBluescript and the negative control was *E. coli*

CHE11 with or without pBluescript. The color of the streaks was compared to determine if the glucose was fermented.

In vitro phosphorylation of PTS proteins. Membrane-free cytoplasmic extracts were made from *E. coli* CHE11 harboring the appropriate plasmids as follows: cells were grown overnight at 37°C in 2 L of LB medium containing 50 µg/ml ampicillin, the cells were harvested by centrifugation, washed once in 20 ml of Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and the final cell pellet suspended in the same buffer at a 1:1 ratio (wt:vol). Cell lysis was accomplished by two passages through a French pressure cell at 10,000 psi and the whole cells removed by a centrifugation at 10,000 g for 15 min. Membranes were removed by centrifugation at 200,000 g for 16 h, followed by centrifugation of the cytoplasmic fraction at 200,000 g for 4 h. The supernatant was then dialysed at 4°C against 4 L of PEMPP buffer (10 mM KPO_4, pH 7.5, 1 mM EDTA, 14 mM 2-mercaptoethanol [2-ME], 0.1 µM Pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride) with one change. Protein concentrations of the cytoplasmic fractions were determined by the method of Lowry (31).

Phosphorylation of PTS proteins with [^{32}P]-PEP and subsequent autoradiography were done according to the method of Bourassa et al. (4) with slight modification. [^{32}P]-PEP was synthesized by the method of Mattoo and Waygood (34) using purified carboxykinase from *E. coli*, kindly provided by

Dr. H. Goldie, University of Saskatchewan, Saskatoon, Canada. Since relative amounts of EI and the truncated EI proteins varied in the *E. coli* extracts, the ratios of EI to total protein were determined by an ELISA using anti-EI antibody with an extract from *E. coli* harboring pBluescript as a background control. The EI-to-protein ratios were used to determine the amounts of total protein added to the phosphorylation reactions and were as follows: 12 µg for pDB102, 108 µg for pDB105 and 216 µg for pDB201. Positive and negative controls contained 20 µg of protein from membrane-free extract of *S. mutans* NG5 and 216 µg of *E. coli* CHE11 harboring pBluescript II, respectively. Samples were incubated at room temperature in a 25 µl reaction mixture containing 0.1 mM [^{32}P]-PEP (4.0×10^3 dpm, $nmol^{-1}$), 5 mM $MgCl_2$, 12.5 mM NaF and 10 mM Hepes (pH 7.5). The reactions were stopped after 5 min by the addition of 10 µl of 188 mM Tris-HCl (pH 8.0), which contained 6% (w/v) SDS, 30% (v/v) glycerol, 6% (v/v) 2-ME, and 0.005% bromophenol blue. Samples were boiled for 1 min and loaded onto a 12.5% 1.5 mm thick SDS-polyacrylamide gel which was electrophoresed for 1 h at 100 V in a Biorad Mini Protean II apparatus (Biorad Laboratories Richmond CA). The dried gel was placed on X-ray film with an intensifying screen at -70°C for 22 h.

Chemicals and enzymes. Restriction enzymes, T4 DNA ligase, goat anti-rabbit IgG alkaline phosphatase conjugate, DNA and protein molecular weight standards were from

Gibco/BRL and used as directed by the supplier. Radiolabelled nucleotides [α^{32}P]-dATP, [α^{32}P]-dCTP, and [α^{35}S]-dATP were obtained from Dupont New England Nuclear. All other chemicals were obtained from Sigma Chemical Company, Fisher Scientific Limited, or Difco. Rabbit anti-EI (*S. salivarius*) and anti-HPr (*S. mutans*) polyclonal antibody were a generous gift from Christian Vadeboncoeur, Laval University, Ste-Foy, Quebec, Canada.

Computer-aided analysis. The similarity of the *S. mutans* NG5 EI and HPr proteins to similar proteins in other organisms was assessed by using the ALIGN program (6). Multiple sequence alignment of the various HPr and EI proteins was carried out using the CLUSTALV program (24).

Nucleotide sequence accession number. The sequence shown in Fig. 3 has been submitted to the GenBank database under accession number L15191.

Results

Cloning of the *ptsI* gene. Gagnon et al. (14) have cloned and sequenced the *ptsI* gene from a genomic DNA library of *S. salivarius*. Two plasmids from that study, pEIP8 and pEIP14-II, clones carrying portions of the C-terminal and N-terminal regions, respectively, of the *ptsI* gene of *S.*

salivarius were used in this study. Both pEIP8 and pEIP14-II were used as probes to screen various digests of *S. mutans* NG5 DNA (data not shown) in order to construct a crude restriction map of the *ptsI* region (Fig. 1[A]). Since both plasmids hybridized to a 4.8 kb *Eco*RI fragment, we constructed a genomic DNA library in λgt11 using a complete *Eco*RI digest of *S. mutans* NG5 DNA as a first step in cloning of the *ptsI* gene. Screening of this library with pEIP8 produced 3-5 positive plaques for every 1500 plaques screened. We purified the DNA from four of these plaques, digested them with *Eco*RI, and found that all four contained a 4.8 kb *Eco*RI fragment as an insert to which pEIP8 hybridized (data not shown). Since the initial probing of *S. mutans* NG5 genomic DNA with pEIP8 indicated that the *ptsI* gene is localized on a 3.6 kb *Eco*RI/*Sst*I fragment, we subcloned this fragment from one of the positive λgt11 clones into pBluescript II SK and named this clone pDB101 (Fig. 1[B]).

Location, orientation, and expression of the *ptsI* gene. To determine if the complete *ptsI* gene is expressed from pDB101, we performed a Western immunoblot of a crude protein preparation from *E. coli* harboring pDB101 using anti-EI Ab raised against *S. salivarius* EI. Vadeboncoeur et al. (51) had previously shown that these Ab cross-react with *S. mutans* EI. A 68 kDa band was detected both in the lane containing a sample from pDB101 and in the positive control lane containing a sample of a supernatant fraction from

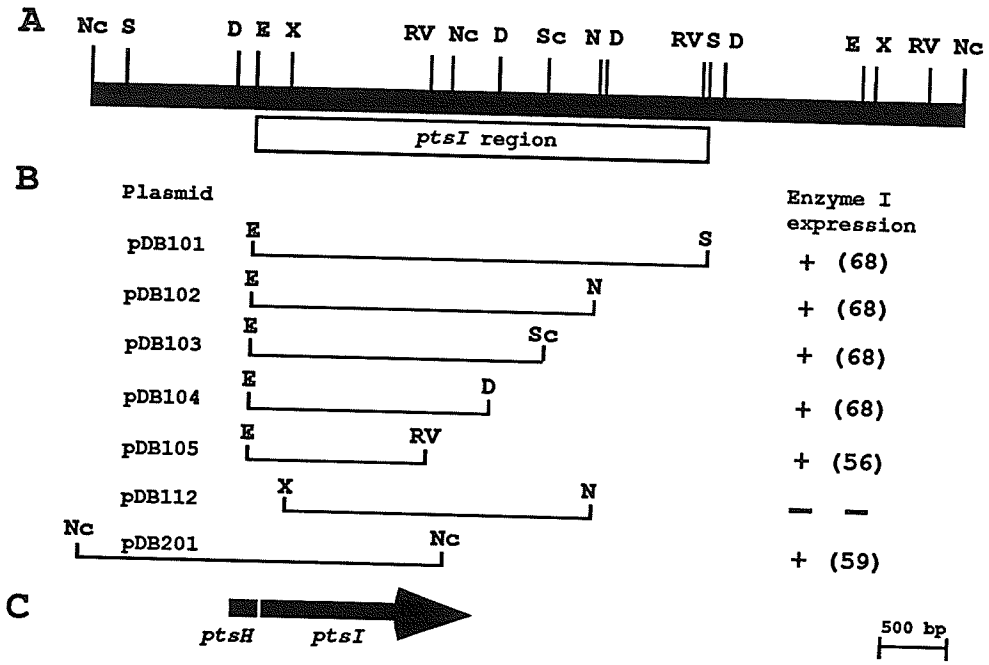


Fig. 1 [A]. Restriction map of the *Streptococcus mutans* NG5 genomic *ptsI* region as determined by Southern blot hybridization of genomic digests with pEIP8 and pEIP14-II, plasmids carrying portions of the *ptsI* gene from *S. salivarius* (10). [B]. Restriction fragments subcloned into plasmids and used for sequencing and expression studies. Expression data is from Western immunoblot analysis of plasmids as shown in Fig. 2. Number in brackets is size in kDa of expressed EI. [C]. Precise location and orientation of the ptsH and *ptsI* genes as determined from sequence analysis (Fig. 3). Restriction sites are; D, *Dra*I; E, *Eco*RI; Nc, *Nco*I; N, *Nsp*V; RV, *Eco*RV; S, *Sst*I; Sc, *Sca*I; X, *Xba*I.

S. mutans (see Fig. 2, lanes 1 and 2) indicating a complete full length *ptsI* gene was contained on the cloned 3.6 kb *Eco*RI/*Sst*I fragment. To determine more precisely the location and orientation of the *ptsI* gene on pDB101 a series of deletion subclones were constructed (Fig. 1[B]). Crude protein preparations from *E. coli* harboring the subclones were subjected to Western immunoblotting using anti-EI Ab and the size, if present, of the EI bands analysed (Fig. 2). All the plasmids, with the exception of pDB112, expressed the *ptsI* gene. In the case of pDB105, however, the EI band was truncated, showing a size of 56 kDa (Fig. 2, lane 6). These results indicated that the *ptsI* gene is oriented on pDB101 with its 5' end located in the vicinity of the *Xba*I site and the 3' end of the gene between the first *Eco*RV site and first*Dra*I site. The promoter-acting sequences are located between the *Eco*RI and *Xba*I sites (Fig. 1[A]).

The *ptsH* gene encoding HPr is located immediately upstream of the *ptsI* gene in *S. salivarius* (14) as it is in other Gram-positive (8,17,27) and in Gram-negative bacteria (7,8,39,43). Western immunoblotting of proteins from *E. coli* harboring pDB101 using anti-HPr antibodies failed to detect any HPr protein (data not shown).

Sequence of the *ptsI* gene. Once the relative location of the *ptsI* gene on pDB101 was ascertained from expression studies, the sequence of this region was determined. The

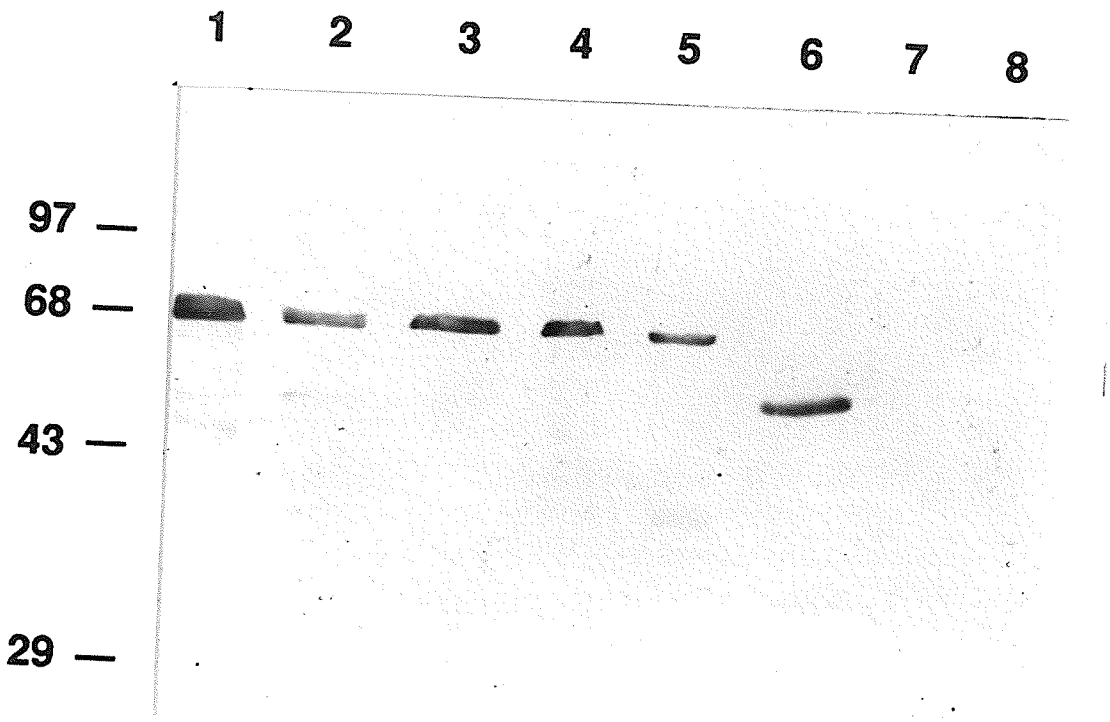


Fig. 2. Expression of *Streptococcus mutans* NG5 *ptsI* gene in *E. coli* as determined by Western immunoblot analysis. Anti-EI Ab was reacted with crude protein preparations from *E. coli* harboring plasmids carrying various portions of the *ptsI* region from *S. mutans* NG5 (see Fig. 1). Lane 1, NG5 supernatant; lane 2, pDB101; lane 3, pDB102; lane 4, pDB103; lane 5, pDB104; lane 6, pDB105; lane 7, pDB112; lane 8, pBluescript. Size standards (kDa) are indicated on the left.

sequence including 140 bp upstream of the *Eco*RI site (see below) is presented in Fig. 3. Analysis of the sequence revealed the presence of two open reading frames (ORFs) between the *Eco*RI site and the first downstream DraI site. The longer one of 1734 nucleotides begins with an AUG codon at position 372 and codes for a putative protein of 577 amino acids with a molecular weight of 63282 daltons. This ORF could be identified as *ptsI* as the amino acid sequence of its putative translation product exhibited 89.4% identity to the EI from *S. salivarius* (14), 62.4% identity with the EI of *Staph. carnosus* (27), 61% identity with the EI of *B. subtilis* (16,40), 47.4% identity with the EI of *E. coli* (7,43), 39.5% identity with the EI domain of the MTP of *R. capsulatus* (56), and 37.8% identity with the EI-like *phbI* gene product protein I of *A. eutrophus* (39). Located 33 bp downstream of the *ptsI* gene stop codon is the beginning of a region of dyad symmetry, positions 2138 to 2160, which could play a role in transcriptional termination (Fig. 3). However, due to the relative instability of the putative hairpin with a ΔG of -8.5 kcal/mol (47), the possibility of significant read through in this region cannot be ruled out.

Cloning, expression, and sequence of the *ptsH* gene. Sequence analysis upstream of the *ptsI* gene revealed a shorter ORF in the same orientation and separated from it by 4 bp that continues to the *Eco*RI site defining the insert/vector junction in pDB101. Translation of this ORF

Fig. 3. Nucleotide sequence of the *Streptococcus mutans* NG5 *ptsH* and *ptsI* genes and their deduced amino acid sequences. Putative -35 and -10 boxes and ribosome binding sites (RBS) are overlined. A putative transcriptional terminator-like region is indicated by inverted arrows. The HPr active site histidyl residue (no. 15) and the EI active site residue (no. 191) are indicated by asterisks.

revealed a putative protein of 74 amino acids that had >80% identity with the HPr of *S. salivarius* (Michel Frenette, personal communication), and >35% identity with the HPr of *E. coli* (8). As the full length of the *S. salivarius* HPr is 87 amino acids, it appeared that the pDB101 insert was probably missing the first 13 N-terminal residues of the *ptsH* gene. This would explain why we could not detect any expression of HPr in Western immunoblots of pDB101 using anti-HPr Ab. In order to clone the complete *ptsH* gene, we constructed a library in λEMBL3 using 15-20 kb fragments from a partial Sau3A digest of *S. mutans* NG5 genomic DNA. The library was screened with pDB101, positive plaques were isolated and their DNA purified. The phage DNA yields were low in the *E. coli* KW251 background and the phage genome appeared to be subject to deletions and/or rearrangements. Overexpression of the cloned *S. salivarius ptsH* gene was found to be toxic to *E. coli* hosts with an intact PTS (Michel Frenette, personal communication). Consequently, using a *ptsI* mutant, *E. coli* CHE11 (18) as a host, phage DNA yields increased and restriction analysis revealed that the one phage genome contained a stable 13-15 kb insert. Southern hybridization with pDB101 as a probe revealed that the phage insert included the 3.5 kb NcoI fragment that included part of the *ptsI* gene and continued upstream of the *ptsH* gene for approx 1.9 kb (Fig. 1). To overcome the potential toxic effect of *ptsH* gene overexpression, we subcloned this fragment into the unique NcoI site of the streptococcal intergration vector

pVA891 (32), which has a lower copy number than pUC-type vectors, and used *E. coli* CHE-11 as the host. The plasmid, pDB201, was stably maintained.

Expression of the *ptsH* gene from pDB201 was examined by Western immunoblotting a crude protein preparation from *E. coli* CHE11 harboring pDB201 using anti-HPr Ab raised against *S mutans* HPr. Two bands approximately 14 kDa in size were detected in both the lane containing a sample from pDB201 and in the positive control lane containing a sample of a supernatant fraction from *S. mutans* (Fig. 4, lanes 1 and 2). Though various streptococcal species contain two forms of HPr differing only in the presence or absence of the N-terminal methionine (41,52), the additional band observed probably represents a degradation product as we would not expect *E. coli* to process the streptococcal HPr. The higher molecular weight band seen in the pDB201 extract and pBluescript extract (Fig. 4, lanes 2 and 3) must be a host protein reacting with anti-*E. coli* Abs contained in the anti-HPr Ab preparation. To complete the sequence of the *ptsH* gene through and upstream of the *Eco*RI site, we used a custom-made primer synthesized with a previously-sequenced region of the *ptsH* gene and pDB201 as a template. The complete *ptsH* ORF is 264 nucleotides begining with an AUG codon at position 104 (Fig. 3) and codes for a putative protein of 87 amino acids with a molecular weight of 8926. The *S. mutans* HPr amino acid sequence exhibits 97% identity to the *HPr* from

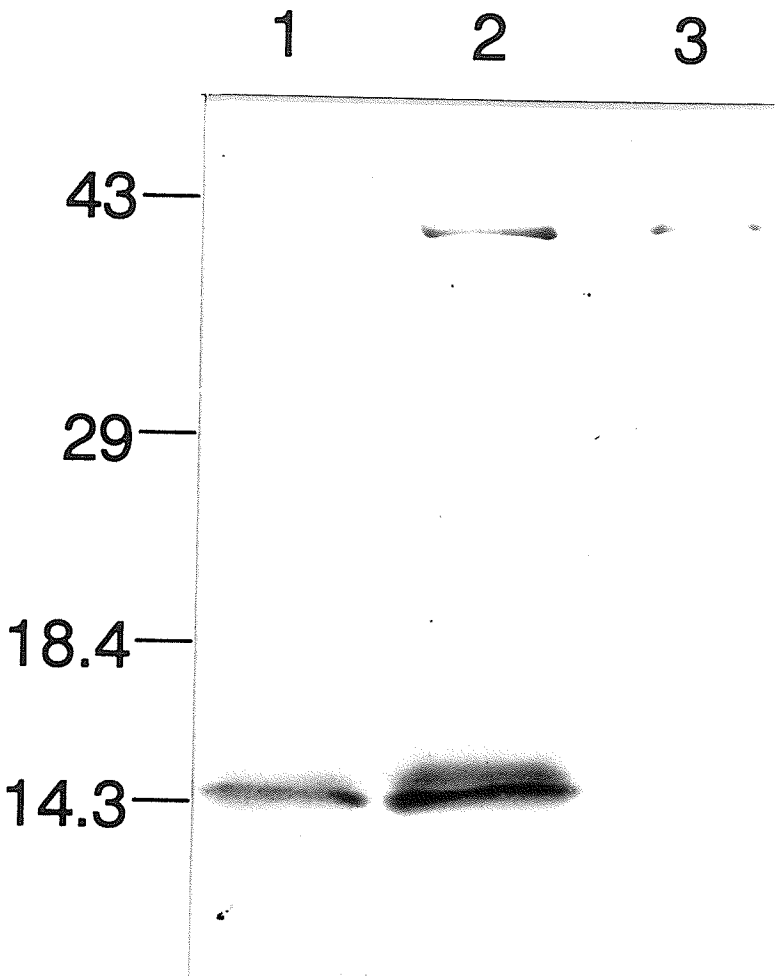


Fig. 4. Expression of *Streptococcus mutans* NG5 *ptsH* gene in *E. coli* as determined by Western immunoblot analysis. Anti-HPr Ab was reacted with crude protein preparation from *E. coli* harboring pDB201 (see Fig 4A). Lane 1, NG5 supernatant; lane 2, pDB201; lane 3, pVA891.

S. salivarius (Michel Frenette, personal communication), 78% identity with the HPr of *Enteroccocus faecalis* (9), 65% identity with the HPrs of *Staph. aureus* (2) and *Staph. carnosus* (12), 62% identity with the HPr of *B. subtilis* (17), 38% identity with the FPr domain of the MTP of *R. capsulatus* (56), 37% with the HPr of *E. coli* (8) and 34% with the HPr-like *phbH* gene product protein H from *Alcaligenes eutrophus* (39).

Base composition. The base composition of the overall sequence is 60.2% A+T with *ptsH* being 60.6% A+T and *ptsI* being 58.4% A+T. This is consistent with the data showing that *S. mutans* A+T content is between 60 and 64% (20). As might be expected from the base composition, the wobble position of the codons are occupied by an A or T in 82.8% of the *ptsH* codons and 72.8% of the *ptsI* codons (data not shown). The above observations are similar to those found for the *ptsH* and *ptsI* genes of *S. salivarius* (14).

In vivo complementation. *E. coli* CHE11 was transformed with pDB102, which expresses a full-length EI, and with pDB105 and pDB201, which express C-terminally truncated 56 kDa and 59 kDa EI proteins, respectively. Transformants were streaked on Maconkey plates containing 1% glucose and compared to CHE11, with or without pBluescript, and to *E. coli* XL1-Blue, with or without pBluescript (data not shown). The streaks of *E. coli* CHE11, with or without pBluescript,

were pale pink in color and there was no coloration of the surrounding medium. On the other hand, streaks of *E. coli* XL1-Blue, with or without pBluescript, and of *E. coli* CHE11 harboring pDB102 were dark red and there was dark red coloration of the medium surrounding the streaks after overnight incubation at 37°C. The streaks of the transformants harboring either pDB105 or pDB201 were a very light red in color and there was no coloration of the surrounding medium. Thus, it appeared that the full length mutans *ptsI* gene complemented the *ptsI* mutation in *E. coli* CHE11, whereas two truncated forms missing 20% or 26% of the wild-type protein C-terminus showed little, if any, complementation.

Phosphorylation of PTS proteins in *E. coli* CHE11 harboring cloned S. mutans NG5 *ptsI* genes. To examine directly if the cloned *S. mutans* NG5 EI protein could function in the phosphotransfer reactions of the PTS in *E. coli* CHE11, protein phosphorylation experiments were carried out using exogenously supplied PEP and membrane-free cell extracts of *E. coli* CHE11 harboring various recombinant plasmids (Fig. 5). Using membrane-free cell extracts from *S. mutans* NG5 as a positive control, three proteins became phosphorylated; EI, HPr, and a 34 kDa protein which was most likely an Enzyme III (Fig. 5, lane 1). No phosphorylated proteins were observed in the negative control, *E. coli* CHE11 harboring pBluescript (Fig. 5, lane 5). An extract, prepared

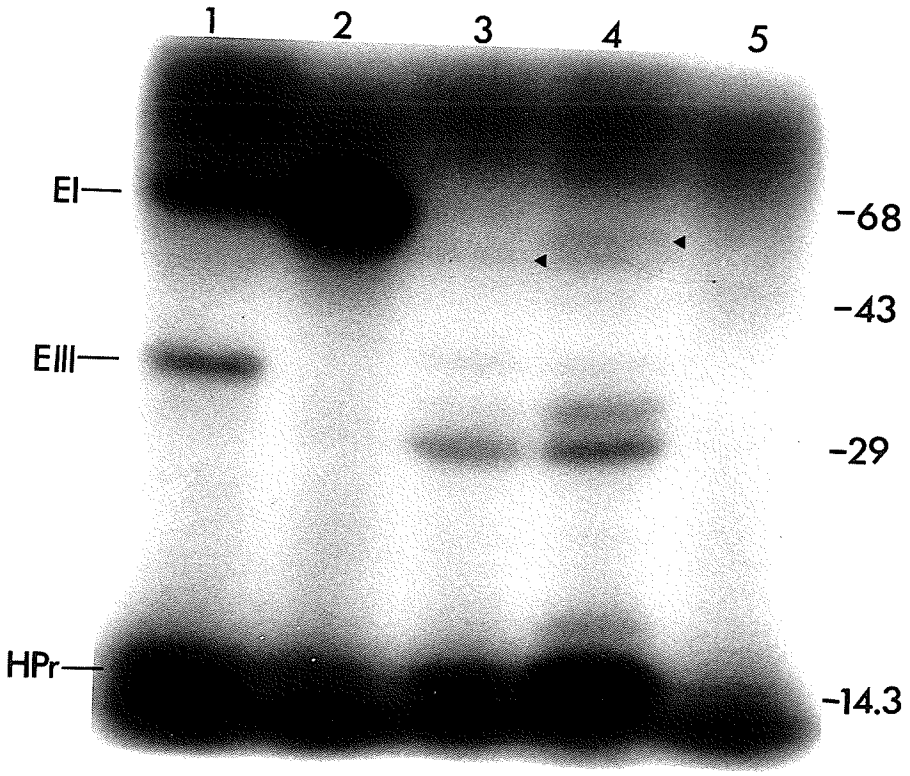


Fig. 5. Polyacrylamide gel electrophoresis of phosphorylated PTS-related proteins in reactions using [^{32}P]-PEP and extracts prepared from *E. coli* CHE11 harboring various forms of the cloned *S. mutans* NG5 *ptsI* gene. Extracts in each reaction were from; lane 1, *S. mutans;* lane 2, *E. coli* CHE11 harboring pDB102; lane 3, *E. coli* CHE11 harboring pDB105; lane 4, *E. coli* CHE11 harboring pDB201; lane 5, *E. coli* CHE11 harboring pBluescript. The identities of the major phosphorylated proteins are indicated on the left and size standards (kDa) are indicated on the right. The arrowheads indicate the truncated *S. mutans* EIs of 56 kDa (lane 3) and 59 kDa (lane 4) as expressed from pDB105 and pDB201, respectively

from *E. coli* CHE11 harboring the full length *S. mutans* EI expressed from pDB102, exhibited EI phosphorylation, as did the *E. coli* HPr (Fig. 5, lane 2). When extracts prepared from *E. coli* CHE11 harboring pDB105 or pDB201 were used, very faint bands corresponding in size to the truncated EIs appeared (Fig. 5, lanes 3 and 4, arrowheads) indicating the mutant EIs may be capable of very limited phosphorylation under conditons where the EI levels, as determined by ELISA, were the same in each extract. Less tenuous evidence that the truncated EIs may retain some limited function is the presence in these reactions of phospho-HPr. Intermediate-sized phosphoproteins were seen in the reactions with extracts from pDB105 and pDB201 are likely EIII proteins and/or proteolytic degradation products of EI (Fig. 5, lanes 3 and 4). No intermediate-sized phosphoproteins were seen in the reaction using the extract from pDB102 probably due to the lesser total protein, and thus lower levels of *E. coli* PTS components, in the reactions. The appearance of phospho-HPr in the reactions would indicate that the *S. mutans* EI can function in the phosphotransfer function of the *E. coli* PTS and that the truncated EI's on pDB105 and pDB201 retain limited phosphotransfer capabilities.

Discussion

The principal aim of this study was to gain insight into the arrangement and sequence of the region of the *S. mutans*

NG5 chromosome that harbours the genes coding for the general proteins, EI and HPr, of the PEP: sugar phosphotransferase system. This information is intended to initiate further research to enrich our understanding of the mechanisms controlling the expression of these genes under various environmental conditions (53).

The cloning of the *ptsI* gene proceeded via selection of *ptsI*-hybridizing clones from a λgt11 genomic library and the eventual subcloning of a 3.6 kb *Eco*RI/*Sst*I fragment (pDB101) that expressed EI, but did not express HPr. Sequence analysis of this fragment revealed the presence of two ORFS, a truncated *ptsH* followed 4 bp downstream by *ptsI*. Another construct with the complete *ptsH* gene on a 3.5 kb NcoI fragment (pDB201) was isolated and used as a template to complete the sequence of *ptsH*. The order of these two genes is typical of the *pts* operons of both Gram-positive and Gram-negative organisms (7,8,12,14,17,27,39,43,56). Analysis of the sequence upstream of *ptsH* revealed a putative RBS AAGGAGA at positions 89-95 and a putative promoter region with a -10 box TATAAT at positions 32-37 and a -35 box TTGAAA at positions 9-14 (Fig. 3). This structure is very similar to a Gram-positive consensus promoter (19). Analysis of the 3'-end of *ptsH* identified a putative RBS AGAAGGA at positions 352-358 (Fig. 3).

Sequence analysis revealed that the inserts from the plasmids used in this study were cloned in the opposite orientation to the *lacZ* gene of the vector, yet all of them except one (pDB112) expressed the *ptsI* gene in *E. coli* (Figs. 1 and 2). The fact that the subclone in which all the *ptsH* sequences had been deleted showed no EI expression would indicate that the *ptsH* gene contains promoter-acting sequences allowing expression of the *ptsI* gene. Similar results were found with *S. salivarius* in which a putative RBS, as well as putative -10 and -35 boxes, were identified in the *ptsH* coding sequence (14). For *Staphylococcus carnosus*, a plasmid containing the 3'-end of *ptsH* and the complete *ptsI* gene did not express EI in *E. coli* when the insert was cloned in the antisense orientation to the *lacZ* gene of the vector (27). With *Bacillus subtilis* and *E. coli*, no active promoter for *ptsI* could be detected within the *ptsH* gene (7,17,43).

The intracellular concentration of HPr and EI in oral streptococci can vary according to growth conditions and the level of HPr is higher than that of EI (22,45,53,54). It has been suggested (27) that *ptsI* gene expression may be attenuated by possession of its own weak RBS and by stem loop structures near the beginning of its coding sequence, situations found for the *ptsI* genes of *Staph. carnosus* (27), *B. subtilis* (17), and *E. coli* (7,43). Although we have identified a putative RBS, we could not identify any stem

loop structures near the 5'-end of the *S. mutans* NG5 *ptsI* gene. While the *ptsI* genes of *S. mutans* NG5 and *S. salivarius* are probably co-transcribed with *ptsH* under certain conditions, as has been shown in the *pts* operons of *B. subtilis* and *E. coli* (7,43), existence of their own promoters could provide an explanation for the differing expression of *ptsH* and *ptsI* in the oral streptococci in vivo. It should be noted, however, that due to the high A+T content of streptococcal DNA, it is highly likely that random sequences of DNA could resemble *E. coli* promoter sequences (36). It has been observed that *S. pneumoniae* sequences that do not normally function as promoters can do so when cloned in *E. coli* (10). Thus, it is possible that the expression of the cloned *S. mutans* NG5 *ptsI* gene could have been initiated fortuitously from an A+T-rich region in the *ptsH* sequence. Transcript analysis of this region of the genome will be needed to clarify these questions and whether the stem-loop structure identified following the *ptsI* gene functions in transcriptional termination.

We have sequenced 103 bp upstream of *ptsH* and 137 bp downstream of *ptsI* and have not detected any other ORFS. In other bacteria, the *pts* operons have been found to be associated with genes coding for other PTS enzymes. In *B. subtilis*, the *ptsG* gene coding for EII^{glc}-$EIII^{glc}$ is located 100 bp upstream of the *ptsH* gene (17), while in *E. coli* the *ptsI* gene is followed by the *crr* gene coding for $EIII^{glc}$

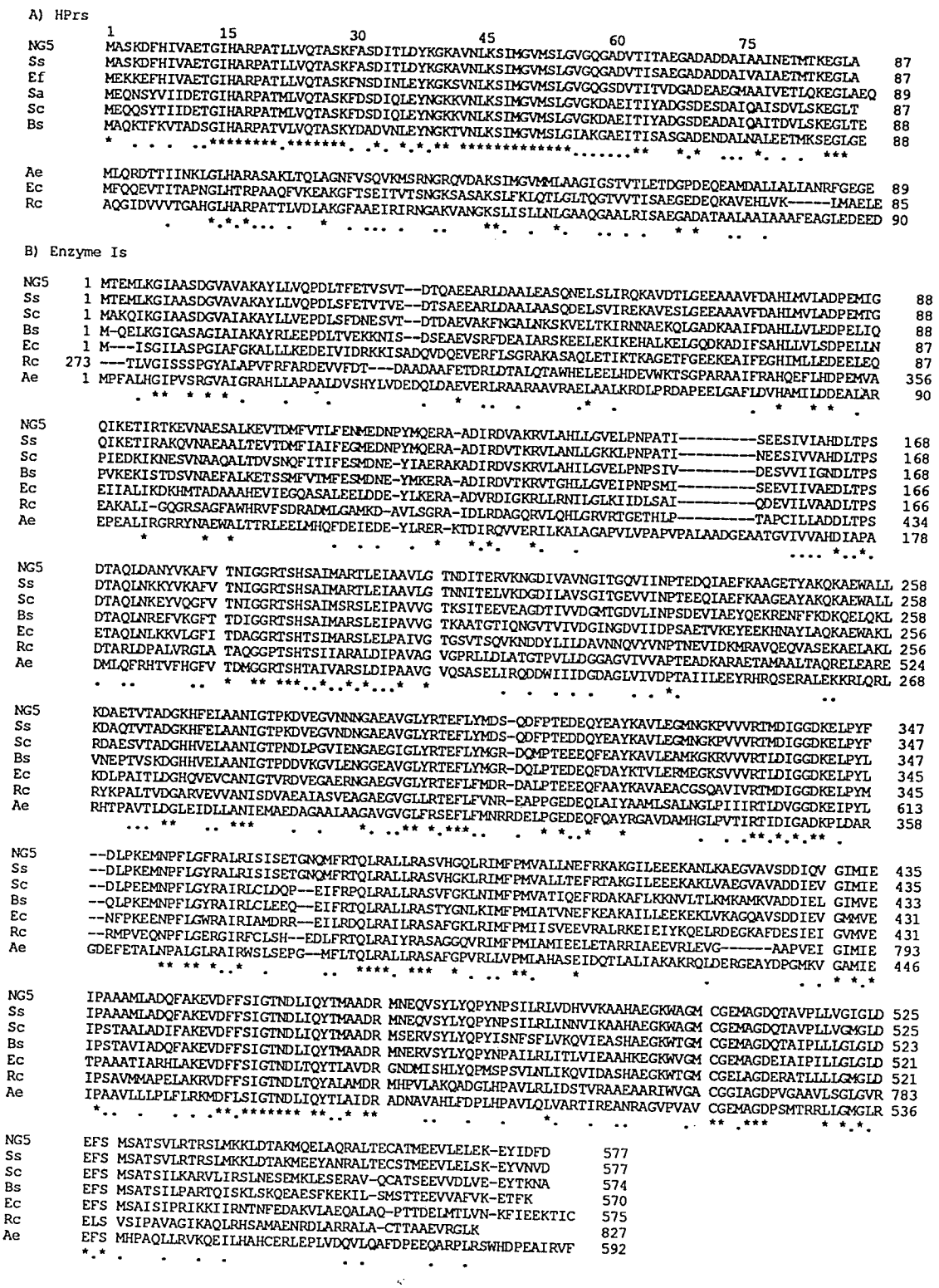
(7,43). In the fructose-PTS operon of *Rhodobacter capsulatus*, *fruB* codes for a multiphosphoryl transfer protein (MTP) in which the C-terminal EI domain is preceded by domains for FPr and $EIII^{fru}$ (56). Downstream of the *fruB* gene is *fruk* coding for fructose 1-phosphate kinase followed by *fruA*, coding for the EII^{fru}. The genes associated with the *ptsH* and *ptsI* genes in *Staph. carnosus* and *S. salivarius* have yet to be identified, although large amounts of these sequences have been analysed in *S. salivarius* (14,27). Thus, it appears that the *pts* operons from Gram-positive cocci may have distinct arrangements differing from other known *pts* operons, although further sequence analysis will be needed to clarify this.

To determine if the *S. mutans* NG5 EI could complement a mutation in the *ptsI* gene of *E. coli* CHE11 in vivo, we transformed it with pDB101. PTS function in the transformed CHE11 appeared to be restored by the appearance of dark red colonies and red coloration of the surrounding medium when grown on Maconkey-glucose plates. This finding is in agreement with the observation that the EIs of the Gram-positive bacteria, *S. carnosus* and *B. subtilis*, are able to complement an EI defect in *E. coli* (27,16). We were also interested to determine if two truncated forms of EI would complement the *ptsI* mutation in *E. coli* CHE11. Western-blot analysis indicated that pDB105 and pDB201 expressed C-terminally truncated forms of EI 56 kDa and 59 kDa in size,

respectively. Colonies of *E. coli* CHE11 transformed with either of these two plasmids when grown on Maconkey-glucose medium appeared as very light red, but with no red coloration of the surrounding medium. Protein phosphorylation experiments were conducted using extracts from *E. coli* CHE11 harboring the appropriate plasmids to determine whether these truncated EI's had a limited capacity for phosphotransfer (Fig. 5). As expected, the wild-type EI, as expressed from pDB102, was efficiently phosphorylated and was able to transfer the phosphate to the *E. coli* HPr (Fig. 5, lane 2). However, when extracts containing either of the truncated EI's were used, it appeared that a small amount of each EI form may have been phosphorylated (Fig. 5, lanes 3 and 4, arrowheads) and the phosphate passed to the *E. coli* HPr. This small amount of PTS phosphotransfer could explain the weak Maconkey-glucose reactions with *E. coli* CHE11 harboring either pDB105 or pDB201. Based on sequence analysis of the *S. mutans* NG5 *ptsI* gene, the EI expressed from pDB105 would be missing 149 C-terminal residues of the wild type EI, while the EI expressed from pDB201 would be missing 112 C-terminal residues of the gene. Thus, the 112 C-terminal residues of EI appears to be essential for optimal phosphoryl transfer function in the phosphotransferase system. While the exact nature of the role of this sequence to EI function in *S. mutans* is unknown, this segment contains the conserved cystidyl residue at position 506, which could be important to phosphotransfer coupling to HPr.

Fig. 6(A) shows an alignment of the amino acid sequences of eleven HPr proteins. The HPrs from the Gram-positive bacteria exhibit high homology overall, especially around the PEP-dependent phosphorylation site, histidine-15, and the site of ATP-dependent phosphorylation in Gram-positive bacteria, serine-46 (2). The HPrs of the Gram-negative bacteria are less homologous to the Gram-positive HPr, although still exhibiting significant homology around histidine-15, with glycine-13, arginine-17, and alanine-26 conserved in all the HPrs. Arginine-17 has recently been shown to be essential for normal HPr activity in *E. coli* (1) and *Staph. carnosus* (28). The sequence around serine-46 of the Gram-negative HPrs, which does not function as a phosphorylation site in these organisms, is less conserved, although lysine-45 and leucine-53 are present in all the HPrs. Interestingly, the *A. eutrophus phbH* product, protein H, has a serine-46 region more similar to that in the Gram-positive HPrs and one wonders whether the serine-46 residue can be phosphorylated by an ATP-dependent kinase. The functions of the *phbH* and *phbI* products from *A. eutrophus* are unknown although they appear to be related to poly(β-hydroxybutyric acid) metabolism (39). A more complete analysis of the relatedness of sequenced HPrs has recently been published (40).

An alignment of seven EI proteins revealed several regions of homology spread over most areas of the protein, but


A) HPrs
B) Enzyme Is

showing that the 150 residues N-terminal are generally less conserved than the rest of the protein (Fig. 6B). The end of this region in *A. eutrophus* is of interest as it has a putative proline-alanine rich linker (residues 152-165) occurring in what is a gap region in all the other EIs. This region may serve as a flexible hinge connecting two rigid domains (40). Of the homologous regions of note is the region from residues 181-197 (*S. mutans* NG5 residues) containing the histidyl residue (#191), which is the site of PEP-dependent phosphorylation. Also of note is the conserved region, residues 431-469, in which there are 18 of 39 residues (46%) strictly conserved and 29 of 39 (74%) either identical or similar. Gagnon et al. (14) have postulated that this may be a domain important in PEP-binding. Residues 504-529 is also of interest because of the location of the only conserved cystidyl residue (#506) amongst the EI proteins. This residue may provide an essential catalytic thiol group to the

---

Fig. 6. Alignments of the amino acid sequences of *S. mutans* NG5 HPr and EI with other related proteins. Under the groups of sequences, an asterisks (*) indicates identical residues and a dot (·) indicates similar residues in the above group(s). Residue numbers are indicated; gaps are not counted in the numbering scheme. Abbreviations are NG5 for *Streptococcus mutans* NG5, Ss for *Streptococcus. salivarius* , Ef for *Enterococcus facaelis*, Sc for *Staphylococcus carnosus*, Sa for *Staphylococcus aureus*, Bs for *Bacillus subtilis*, Ec for *Escherichia coli*, Rc for *Rhodobacter capsulatus*, Ae for *Alcaligenes eutrophus*. [A]. Alignment of HPrs. Top group is the sequences from Gram-positive organisms and bottom group is the sequences from Gram-negative organisms. Rc is the FPr domain from its MTP, and Ae is protein H, the product of *phbH*. [B]. Alignment of EIs. Conserved regions dicussed in the text are boxed. Rc is the EI domain of its MTP, Ae is protein I, the *phbI* product.

active site (56). Recent reports have shown that the EI proteins share these regions of homology, and at least one other (residues 354-363), with the pyruvate:orthophosphate dikinase (PDK) from maize and *Bacteroides symbiosus*, as well as the phosphoenolpyruvate synthase (PPS) from *E. coli* (37,40,56). Similar to EI, the PDK and PPS enzymes also bind PEP and catalyze the transfer of a phophoryl group via a phosphohistidine intermediate. Extensive analysis of their primary structures has indicated all of the above proteins belong to an Enzyme I family (40).

Clearly, our results show that the EI and HPr of *S. mutans* NG5, products of the *ptsI* and *ptsH* genes, are expressed in *E. coli* and that the EI can complement a *ptsI* defect in *E. coli*. The *ptsI* gene can be expressed from a promoter-like sequence in the *ptsH* gene and, if this sequence is a true streptococcal promoter, this transcriptional organization would be unique amongst *pts* operons, having been described so far only in oral streptococci. The *S. mutans* NG5 *ptsH* and *ptsI* genes do not appear to be linked with with either EIIs or EIIIs, which is in contrast to the same genes in *B. subtilis* and *E. coli*.

Acknowledgment

This research was supported by Grant MT-11673 from the Medical Research Council of Canada.

References

1. Anderson, J. W., Pullen, K., Georges, F., Klevit, R. E., and E. B. Waygood. 1993. The involvement of the arginine-17 residue in the active site of the histidine-containing protein, HPr, of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli*. J. Biol. Chem. **268**: 12325-12333.
2. Beyreuther, K., H. Raufuss, O. Schrecker, and W. Hengstenberg. 1977. The phosphoenolpyruvate-dependent phosphotransferase system of *Staphylococcus aureus*. I. Amino acid sequence of the phosphocarrier HPr. Eur. J. Biochem. **75**: 275-286.
3. Birnboim, H. C. 1979. A rapid alkaline extraction method for isolation of plasmid DNA. Method. Enzymol. **100**: 243-255.
4. Bourassa, S., L. Gauthier, R. Giguere and C. Vadeboncoeur. 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. Oral Microbiol. Immunol. **5**: 288-297.
5. Bowden, G.H.W., 1991. Which Bacteria are Cariogenic in Humans?, p. 266-286 *In* N.W. Johnson (ed.) Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Cambridge University Press, Cambridge,England.
6. Dayhoff, M. O., Barker, W. C., and L. T. Hunt. 1983. Establishing homologies in protein sequences. Methods Enzymol. **91**: 524-545.
7. De Reuse, H., and A. Danchin. 1988. The *ptsH*, *ptsI* and *crr* genes of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system: a complex operon with several modes of transcription. J. Bacteriol. **170**: 3827-3837.

8. De Reuse, H., A . Roy, and A. Danchin. 1985. Analysis of the *ptsH-ptsI* region of the *Escherichia coli* K-12: nucleotide sequence of the *ptsH* gene. Gene 35: 199-207.

9. Deutscher, J., B. Pevec, K. Beyreuther, H. H. Kiltz, and W. Hengstenberg. 1986. Streptococcal phosphoenolpyruvate-sugar phosphotransferase system: amino acid sequence and site of ATP-dependent phosphorylation of HPr. Biochemistry **25**: 6543-6551.

10. Dillard, J. P., and J. Yother. 1991. Analysis of *Streptococcus pneumoniae* sequences cloned into *Escherichia coli*: effect of promoter strength and transcription terminators. J. Bact. **173**: 5105-5109.

11. Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127-6145.

12. Eisermann, R., A. Fisher, A. Kessler, A. Neubauer, and W. Hengstenberg. 1991. Staphylococcal phosphoenolpyruvate-dependent phosphotransferase system: purification and protein sequencing of the *Staphylococcus carnosus* histidine-containing protein, and cloning and DNA sequencing of the *ptsH* gene. Eur. J. Biochem. **197**: 9-14.

13. Frischauf, A-M, H. Lehrach, A. Polstka, and N. M. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**: 827-842.

14. Gagnon, G., C. Vadeboncoeur, R. C. Levesque and M. Frenette. 1992. Cloning, sequencing and expression in *Escherichia coli* of the *ptsI* gene encoding enzyme I of the phosphoenolpyruvate phosphotransferase system transport system from *Streptococcus salivarius*. Gene **121**: 71-78.

15. Gauthier, L., D. Mayrand and C. Vadeboncoeur. 1984. Isolation of a novel protein involved in the transport of fructose by an inducible phosphoenolpyruvate fructose phosphotransferase system in *Streptococcus mutans*. J. Bacteriol. **160**: 755-763.

16. Gonzy-Treboul, G., and M. Steinmetz. 1987. Phosphoenolpyruvate:sugar phosphotransferase system of *Bacillus subtilis:* Cloning of the region containing the *ptsH* and *ptsI* genes and evidence for a *crr*-like gene. 1987. J. Bact. **169**: 2287-2290.

17. Gonzy-Treboul, G., M. Zagorec, M-C. Rain-Guion and M. Steinmetz. 1989. Phosphoenolpyruvate:sugar phosphotransferase system in *Bacillus subtilis*: nucleotide sequence of *ptsX*, *ptsH* and the 5'-end of *ptsI* and evidence for the *ptsHI* operon. Mol. Microbiol. **3**: 103-112.

18. Gottesman M., M. Hicks, and M. Gellert. 1973. Genetics and function of DNA ligase in *Escherichia coli*. J. Mol. Biol. **77**: 531-547.

19. Graves, M.C., and J.C. Rabinowitz. 1986. In vivo and in vitro transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for "extended" promoter elements. J. Biol. Chem. **261**: 11409-11415.

20. Hamada, S. and H. D. Slade. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331-384.

21. Hamilton, I. R. 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94-133 *In* J. Reizer and A. Peterkofsky (ed.) Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.

22. Hamilton, I. R., L. Gauthier, B. Desjardins and C. Vadeboncoeur. 1989. Concentration-dependent repression of the soluble and membrane components of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus mutans* by glucose. J. Bacteriol. **171**: 2942-2948.

23. Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351-359.

24. Higgins, D. G., Bleasby, A. J., and R. Fuchs. 1991. CLUSTALV: improved software for multiple sequence alignment. Comp. Appl. Biosc. **8**: 189-191..

25. Huynh, T. V., R. A. Young, and R. A. Davis. 1984. Constructing and screening cDNA libraries in λgt10 and λgt11, p. 49-78. *In* D. M. Glover (ed.), DNA Cloning Techniques: A pratical approach, IRL Press, Oxford.

26. Kaslow, D.C. 1986. A rapid biochemical method for purifying lambda DNA from phage lysates. Nucleic Acids Res. **14**: 6767.

27. Kohlbrecher, D., R. Eisermann, and W. Hengstenberg. 1992. Staphylococcal phosphoenolpyruvate-dependent phosphotransferase system: molecular cloning and nucleotide sequence of the *Staphylococcus carnosus ptsI* gene and expression and complementation studies of the gene product. J. Bacteriol. **174**: 2208-2214.

28. Kruse, R., Hengstenburg, W., Beneicke, W., and H. R. Kalbitzer. 1993. Involvement of various amino-terminal and carboxy-terminal residues in the active site of the histidine-containing protein HPr of the phosphoenolpyruvate-dependent phosphotransferase system of *Staphylococcus carnosus* - site-directed mutagenesis with the *ptsH* gene, biochemical characterization and NMR studies of the mutant proteins. Protein Eng. **6**: 417-423.

29. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**: 680-685.

30. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50**: 353-380.

31. Lowry, O. H., Rosebrough, A. L., Farr, A. L., and Randall, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

32. Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones. 1983. Novel shuttle

plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25**: 145-150.

33. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
34. Mattoo, R. L., and Waygood E. B. 1983 An Enzymatic method for [^{32}P]phosphoenolpyruvate synthesis. Anal. Biochem. **128**: 245-249.
35. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
36. Mulligan, M. E., and W. R. McClure. 1985. Analysis of the occurence of promoter sites in DNA. Nucleic Acids Res. **14**: 109-126.
37. Pocalyko, D. J., L. J. Carroll, B. M. Martin, P. C. Babbitt, and D. Dunaway-Mariano. 1990. Analysis of sequence homologies in plant and bacterial pyruvate phosphate dikinase, enzyme I of the bacterial phosphoenolpyruvate: sugar phosphotransferase system and other PEP-utilizing enzymes. Identification of potential catalytic and regulatory motifs. Biochemistry **29**: 10757-10765.
38. Postma, P. W. and J. W. Lengeler. 1985. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49**: 232-269.
39. Preis, A., H. Priefert, N. Kruger and A. Steinbuchel. 1991. Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(ß-hydroxybutyric acid)-leaky phenotype which exhibit homology to *ptsH* and *ptsI* of *Escherichia coli*. J. Bacteriol. **173**: 5843-5853.
40. Reizer, J., Hoischen, C., Reizer, A., Pham, T. N., and M. H. Saier Jr. 1993. Sequence analyses and evolutionary relationships among the energy-coupling proteins Enzyme I and HPr of the bacterial phophoenolpyruvate:sugar phosphotransferase system. Protein Sci. **2**: 506-521.

41. Robitaille, D., L. Gauthier, and C. Vadeboncoeur. 1991. The presence of two forms of the phosphocarrier protein HPr of the phosphoenolpyruvate:sugar phosphotransferase system in streptococci. Biochimie **73**: 573-581.

42. Roeske, C. A., R. M. Kutny, R. A. Budde, and R. Chollet. 1988. Sequence of the phosphothreonyl regulatory site peptide from inactive maize leaf pyruvate:orthophosphate dikinase. J. Biol. Chem. **263**: 6683-6687.

43. Saffen, D. W., K. A. Presper, T. L. Doering and S. Roseman. 1987. Sugar transport by the bacterial phosphotransferase system: molecular cloning and structural analysis of the *Escherichia coli ptsH*, *ptsI*, and *crr* genes J. Biol. Chem. **262**: 16241-16253.

44. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**: 5463-5467.

45. Thibault, L., and C. Vadeboncoeur. 1985. Phosphoenolpyruvate-sugar phosphotransferase transport system of *Streptococcus mutans*: purification of HPr and Enzyme I and determination of their intracellular concentrations by rocket immunoelectrophoresis. Infect. Immun. **51**: 817-825.

46. Thompson, J. 1987. Sugar transport in the lactic acid bacteria, p.13-38. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.

47. Tinoco, I., Uhlenbeck, O. C., Crothers, D. M., and J. Gralla. 1973. Improved estimation of secondary structure in ribonucleic acids. Nature **246**: 40-41.

48. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophorectic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**: 4350-4354.

49. Vadeboncoeur, C. and L. Gauthier 1987. The phosphoenolpyruvate: sugar phosphotransferase system in

Streptococcus salivarius. Identification of a III^{man} protein. Can. J. Microbiol. **33**: 118-122.

50. Vadeboncoeur, C. and M. Proulx. 1984. Lactose transport in *Streptococcus mutans* Isolation and characterization of factor III^{lac} a specific component of the phosphoenolpyruvate-lactose phosphotransferase system. Infect. Immun. **46**: 213-219.

51. Vadeboncouer, C., M. Proulx, and L. Trahan. 1983. Purification of proteins similar to HPr and Enzyme I from the oral bacterium *Streptococcus salivarius*. Biochemical and immunological properties. Can. J. Microbiol. **29**: 1694-1705.

52. Vadeboncoeur, C., Y. Konishi, F. Dumas, L. Gauthier, and M. Frenette. 1991. HPr polymorphism in oral streptococci is caused by partial removal of the N-terminal methionine. Biochimie **73**: 1427-1430.

53. Vadeboncoeur, C., S. St. Martin, D. Brochu and I. R. Hamilton. 1991. Effect of growth rate and pH on intracellular levels and activities of the components of the phosphoenolpyruvate: sugar phosphotransferase system in *Streptococcus mutans* Ingbritt Infect. Immun. **59**: 900-906.

54. Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson and I. R. Hamilton. 1987. Effect of growth conditions on levels of components of the phosphoenolpyruvate: sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus sobrinus* grown in continuous culture. J. Bacteriol. **169**: 5686-5691.

55. Vieria, J. and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**: 259-268.

56. Wu, L-F., J. M. Tomich, and M. M. Saier Jr. 1990. Structure and evolution of a multidomain multiphosphoryl transfer protein. Nucleotide sequence of the *fruB* (HI) gene in *Rhodobacter capsulatus* and comparison with

homologous genes from other organisms. J. Mol. Biol. **213**: 687-703.

Chapter 4

Isolation of a mutant of *Streptococcus mutans* unable to utilize mannose.

Dennis Cvitkovitch, David Boyd and Ian R. Hamilton

Department of Oral Biology
University of Manitoba
Winnipeg, Manitoba
R3E 0W3

Abstract

Streptococcus mutans is capable of fermenting several sugar substrates, including mannose, to acid end-products that can dissolve tooth enamel and result in dental caries. The aim of this study was to gain insight into the mechanism of mannose utilization by examining the properties of a mutant of *S. mutans* BM71, defective in mannose utilization. Mannose negative (man^-) mutants were generated by transformation of naturally competent *S. mutans* BM71 with an integration vector pVAE1 harboring a 630 bp fragment of the EI gene (*ptsI*) of the phophoenolpyruvate sugar phosphotransferase system from the closely related strain, *Streptococcus salivarius*. The transformation was originally intended to select EI^- mutants of *S. mutans*, however, all viable transformants were found to carry an intact EI gene as revealed by Southern blot analysis and restriction mapping, and also expressed an intact EI protein as revealed by Western blot analysis. The integration vector had, in fact, recombined at a specific secondary location in the genome to generate several clones defective in mannose utilization. Nucleotide sequence analysis of the integration region revealed that the plasmid had inserted into a region of the multiple sugar metabolism operon (*msm*). An examination of one of the mutants (B7) revealed slower growth rates on glucose and mannose relative to the parental strain and a decrease in phosphorylated III^{man} as revealed by $[^{32}P]$-PEP phosphorylation of cell extract

proteins followed by autoradiography. The mutant B7 was, however, unstable and was able to revert to a phenotype that could ferment mannose, while retaining the inserted plasmid. These results, along with the inability to generate the man$^-$ phenotype with subsequent transformations with a vector designed to integrate into the same gene interrupted in B7 suggested that the mannose-negative phenotype was likely the result of a secondary mutation affecting mannose transport or metabolism.

Introduction

Streptococcus mutans is the principal etiological agent of dental caries (9,18). This disease is caused by the demineralization of tooth enamel by the acid end-products generated from carbohydrate metabolism by this organism. The phosphoenolpyruvate sugar phosphotransferase system (PEP:PTS) is the primary sugar transport system used by this bacterium. In most bacteria including streptococci, the PTS is comprised of the general, soluble cytoplasmic proteins enzyme I (EI) and HPr, which transfer a phosphoryl group from PEP to one of a variety of sugar-specific membrane-bound Enzymes II (EIIs). The phosphorylation of the EII may be directly from HPr or may proceed via an intermediate soluble component known as Enzyme III. The sugar is then translocated and phosphorylated by the sugar-specific EII and is subsequently metabolized. The PTS is also central to the regulation of sugar uptake and catabolism and these aspects of the system have been extensively studied (20).

The original goal of this project was to generate a mutant of *S. mutans* defective in Enzyme I (EI). Since EI and HPr are general proteins required for the transport and phosphorylation of all sugars transported via the PTS, a mutant defective in EI would be unable to utilize all sugars that are transported exclusively via the PTS. Such a mutant strain would be an invaluable asset in our laboratory for

study of non-PTS sugar transport systems in *S. mutans* since there is a considerable amount of evidence for the transport of glucose by the organism (10). This non-PTS system is believed to function under the conditions of high growth rates, high sugar concentration, and low pH, with the concurrent repression of the PTS (10).

The multiple sugar metabolism operon (*msm*) codes for another non-PTS sugar transport system used by *S. mutans* to transport and metabolize a variety of sugar substrates including raffinose, melibiose and isomaltosaccharides (21). This transport system is comprised of an operon containing eight characterized genes, some of which share homology to osmotic-shock-sensitive transport systems of enteric bacteria (21). The generation of a PTS-defective mutant was undertaken with intent to study these non-PTS transport systems.

This report describes the methodology used in an attempt to generate a PTS defective mutant by inactivation of the *ptsI* gene, and the subsequent isolation of a mutant incapable of mannose utilization. The characterization of the mutant and the relevance of the plasmid insertion to the resultant phenotype is also discussed.

Materials and methods

Bacterial strains, media, and growth conditions. *E. coli* DH5α [Ø80d lac ZΔ M15, end A1, rec A1, hsdR17 (r_{k-},

m_{k+}), sup E44, thi-1, λ-, gyrA, relA1, F-, Δ(lacZYA- arg F), U169] was used as a host strain for isolation and maintenance of plasmids in this study and was maintained in LB broth and on plates (15) containing ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), kanamycin (50 μg/ml) or erythromycin (500 μg/ml). *E. coli* used for large scale plasmid isolation were grown in TB medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.23% KH_2PO_4 and 1.25% K_2HPO_4 with phosphates autoclaved separately). Various selective media were utilized in an attempt to isolate PTS-defective streptococcal transformants including; semi-defined media plates (ADM)(1) containing 1% melibiose and 10 μg/ml erythromycin; ADM plates containing 1% glucose and 10 μg/ml erythromycin (pH 5.5) and TYE plates containing 1% glucose and 10 μg/ml erythromycin.

To prepare frozen competent *E. coli* cells for transformation by electroporation, 1 L cultures of were grown in LB media at 37°C with vigorous shaking. When the culture reached mid-log phase (0.5-1.0 OD_{600}), they were cooled on ice and centrifuged at 4000 x g for 15 min at 4°C. The cells were resuspended in 1 L of ice cold water, centrifuged as above, resuspended in 0.5 L of water, centrifuged and resuspended in 20 ml of 10% (v/v) glycerol, again centrifuged and finally resuspended in 2-3 ml of 10% glycerol. The cells were dispensed in 100 μl volumes in microcentrifuge tubes, rapidly frozen in a -70°C ethanol bath and stored at -70°C.

Several streptococcal strains were utilized as parent strains in the construction of mutants including: the laboratory strains *S. mutans* Ingbritt, *S. mutans* ATCC 10449, *S. salivarius* ATCC 25975, and *S. sanguis* 10556, as well as *S. mutans* fresh isolates BM71, DC1, and 1546. Streptococci were grown and maintained in tryptone yeast extract (TYE) glucose broth or plates (1.0% tryptone, 0.5% yeast extract, 0.35% K_2HPO_4 and 0.2% glucose).

DNA Manipulations. Digestions with restriction endonucleases (BRL Inc., Bethesda MD) were carried out as recommended by the supplier for 2-4 h at 37°C in 30-50 µl volumes containing 2-10 µg of DNA. Ligation reactions using T4 DNA ligase (BRL) were performed as described by the supplier in 10 µl volumes containing 100-500 ng of plasmid DNA and 200-1000 ng insert DNA. Incubations were at 4°C overnight folowed by precipitation with 2 volumes of ethanol, incubation at -20°C for 20 min and then centrifugation at 15,000 x g for 10 min in a microcentrifuge. The precipitated DNA was then washed once in 70% ethanol, centrifuged for 2 min at 15,000 x g and resuspended in 5 µl of distilled, deionized water (ddH_2O), and 1-5 µl used to transform *E. coli* hosts by electroporation.

Calf intestinal alkaline phosphatase (International Biotechnologies, Inc,: Promega Biotec) treatment was performed as recommended by the manufacturer. DNA fragments

were isolated from agarose gels (0.8-1.2%) in Tris-acetate-EDTA (TAE) buffer using the GENECLEAN II® system (Bio 101, La Jolla CA) as recommended by the manufacturer. Sequencing was carried out on double stranded DNA by the dideoxy termination method (22) using [α^{35}S]dATP and the Sequenase Version 2.0 Kit (U.S. Biochemicals).

Preparation of plasmid DNA. Mini preparations of plasmid DNA were prepared by an alkaline lysis method (23). Large scale CsCl-EtBr preparations of plasmids were performed as described by Sambrook et al. (23), where 400-2000 µg of purified DNA was typically recovered from 1 L overnight cultures grown in TB media. Plasmid DNA to be sequenced was isolated from 3-10 ml *E. coli* cultures using the Magic Minipreps Kit (Promega). Phage DNA was isolated from liquid lysates by the method of Kaslow (13).

Small scale preparation of *S. mutans* genomic DNA. Small scale preparations of *S. mutans* genomic DNA were prepared from 5 ml overnight cultures grown anaerobically at 37°C in TYE-glucose media. The cells were washed twice in TE buffer (50 mM Tris 1 mM EDTA, pH 8.0), resuspended in 250 µl TE buffer and heated at 65°C for 20 min The suspensions were cooled on ice and then incubated at 37°C for 1 hr with the addition of 50 units of mutanolysin and 5 mg of lysozyme. Proteinase K (25 µg) (Sigma) was added and the incubation continued for an additional 1 h followed by the addition of 150 µl of 10% SDS with incubation at 50°C for 10 min. The

reactions were then centrifuged at 15,000 x g for 3 min to remove cell debris. The mixtures were then extracted twice with an equal volume of phenol:chloroform (1:1), once with chloroform and the DNA precipitated with 2 volumes of ice cold ethanol, incubated at -20°C for 20 min and centrifuged at 15,000 x g for 12 min. The DNA pellets were then washed once with ice cold 70% ethanol, dried for 10 min in a vacuum desicator and resuspended in 75 µl of TE buffer.

Southern blotting and DNA hybridizations. Restriction fragments were electrophoresed on 0.8-1.5% agarose gels and transferred to a charged nylon membrane (Hybond N+, Amersham UK) using 0.4 N NaOH as described in the 'DNA/RNA alkali blotting procedure' detailed by the manufacturer. Radiolabeled probes were prepared from plasmid or restriction fragment DNA and labeled with [^{32}P]-CTP by nick translation using the BRL 'nick translation kit' (BRL) as described by the supplier. [^{32}P]-CTP radiolabeled probes were prepared from pVA891 and pDL276 plasmid DNA purified by CsCl density gradient centrifugation, from restriction fragments EIP8 and EIP14-II containing regions of the *ptsI* gene from *S. salivarius* (Fig. 1) and EIS2 and EI-E1.1 which are restriction fragments of the *S. mutans* EI genomic region, (Fig. 2) isolated from agarose gels as described above. Labelled probes were separated from unincorporated nucleotides by passage through a Sephadex G-50 column (23). Blots were prehybridized for 1 h in 6x SSC, 5x Denhardt's

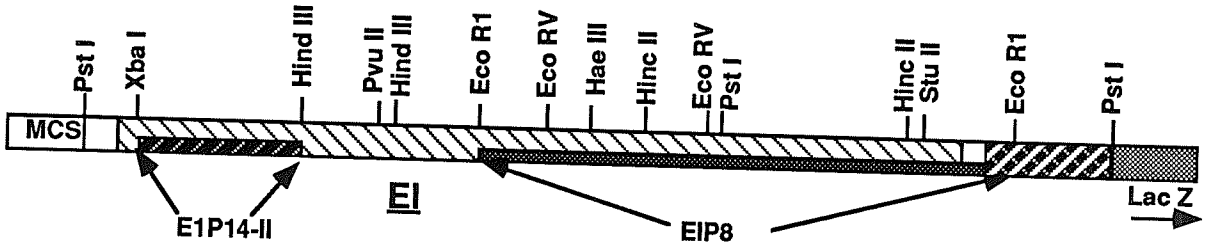


Fig. 1. Cloned regions of the EI gene from *Streptococcus salivarius* (generously provided by Dr. C. Vadeboncoeur, Laval University PQ) used as probes in Southern hybridization reactions as described in the text.

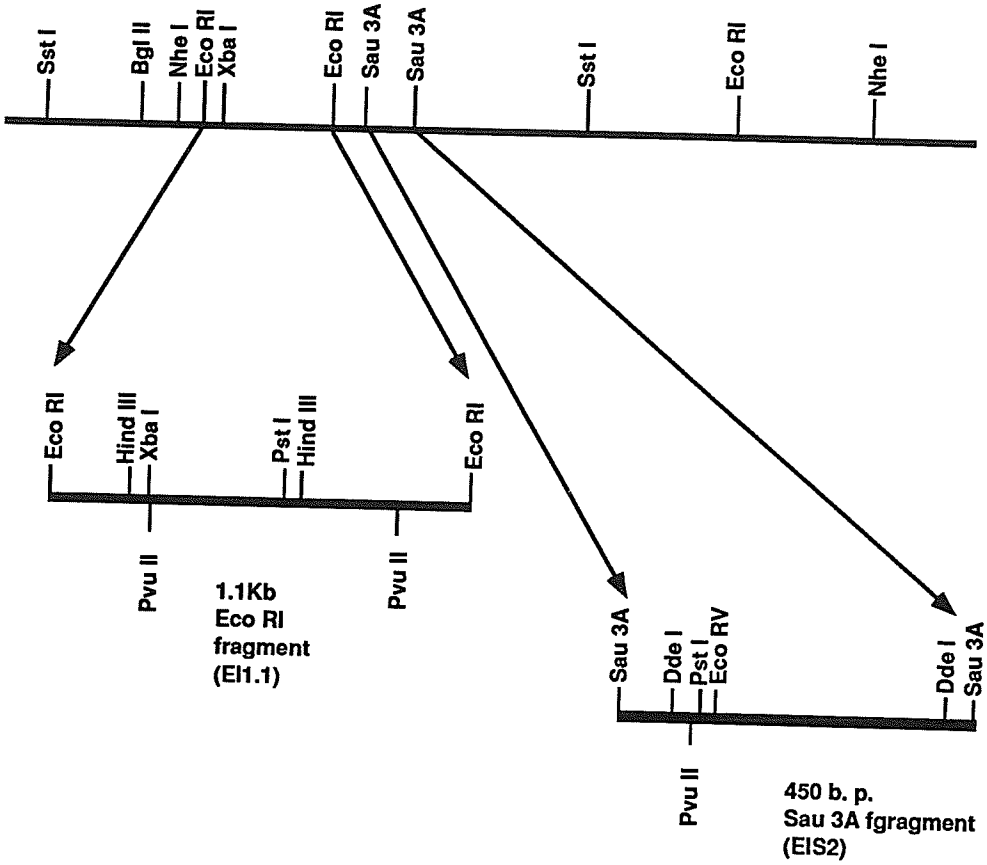


Fig. 2. Cloned regions of the *Streptococcus mutans* EI gene used as probes in Southern hybridization reactions and for the construction of integration vectors as described in the text.

solution, 0.5% SDS and 100 µg/ml salmon sperm DNA as described by Mantiantis et al. (23). Probes were boiled for 5 minutes, cooled rapidly on ice, added to the prehybridized blots and incubated at the desired temperature (50-65°C) overnight. The blots were then washed first in 2x SSC, 0.1% SDS at room temperature, next in 0.2x SSC, 0.1% SDS at room temperature and finally in 0.1x SSC, 1.0% SDS at the restrictive temperature. They were then dried and placed on x-ray film for 18 h at either -70°C. Each lane contained 1 µg total DNA.

Fermentation tests. *S. mutans* transformants and parent strain BM71 were tested for their ability to ferment a number of sugar substrates. Overnight cultures of each strain were used as inocula for sugar fermentation tests that were carried out on sugar indicator plates containing 1.6% agar, 1% tryptone, 0.5% yeast extract, 0.3% K_2HPO_4, 0.0001% bromocresol purple and 1% of the desired sugar. The plates were incubated anaerobically at 37°C for 48 h after which they were scored for fermentation. A bright yellow color indicated positive ferentation, a purple color was scored as negative while a light yellow color was termed weak fermentation.

Bacterial transformations. *E. coli* DH5α was transformed by electroporation using the gene pulser (Biorad

laboratories, Richmond CA) by a modification of the method of Dower (8). A pulse controller was constructed according to the circuit described by Dower (8) to allow the use of 0.1 cm and 0.2 cm cuvettes which generate higher field strengths than the standard 0.4 cm cuvettes which result in much higher transformation efficiencies.

Prior to use the frozen competent cells were thawed on ice and 20 µl of the suspension and 1-5 µl of the ligation reactions were added to the gene pulser cuvette, allowed to stand on ice for 1 min and then pulsed at 25 µF capacitance, 1.25 kV with the pulse controller set at 400 Ω, these conditions generated a field strength of 12.5 kV/cm and a pulse length of approximately 9 ms. One ml of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to resuspend the cells that were subsequently transferred to 17 X 100 mm polypropylene tubes for incubation with shaking at 225 rpm for 1 h at 37°C at which time 50 µl of 1:1, 1:10, and 1:100 dilutions were plated on the appropriate selective media.

S. mutans strains were transformed by a modification of the method of Caufield et al. (6). *S. mutans* cells were made competent by inoculation of 5 ml of Todd Hewitt broth (Difco) containing 10% heat inactivated horse serum (THBS) (Sigma) with a single colony followed by overnight anaerobic incubation at 37°C. These cells were then diluted 1:40 in

THBS and allowed to achieve competence after 3 h growth at 37°C where they reached an OD_{600} of 0.15-0.25.

Saturating concentrations of plasmid DNA (10 µg in 0.70 ml) were added to 0.33 ml of this mixture and incubation was continued for another 90 min. The cells were then washed twice in sterile distilled water to remove residual glucose, were plated onto one of the various selective media previously described, and the plates incubated anaerobically and growth monitored daily for up to 10 days.

A variety of techniques were utilized in an attempt to transform streptococci by electroporation. These techniques were modifications of protocols suggested by Biorad's technical support staff, as well as by Debbie Macintyre from the University of Minnesota. Generally, cells from an overnight 100 ml culture or from a chemostat, were harvested, washed three times in ice cold water and resuspended at a concentration of 5 X 10^{10} cell/ml in water. The gene pulser cuvettes were chilled on ice and 20-200 µl of cells and 1 µg of plasmid DNA were added to this mixture and was allowed to stand on ice for 1 min. The cells were then pulsed at times ranging from 2-15 milliseconds at field strengths between 5 and 25 kV/cm. One ml of TYE media containing either 1% glucose or melibiose, or 1 ml of SOC media, was then added to the cuvettes to resuspend the cells which were then transferred to a test tube and incubated at 37°C for 3 h or overnight to allow recovery. The cells were then either

washed twice in water or plated directly on to the various selective plates as described previously for the natural competence experiments.

SDS-PAGE and Western blotting. Membrane-free cell extracts were prepared using an alumina-grinding technique (25). SDS-PAGE was performed in 10% polyacrylamide gels run at 200 V for 40 min by the method of Laemmli (15) using the Biorad Mini protein II apparatus (Biorad laboratories, Richmond CA). Western blotting to PVDF membranes (Immobilon-P, Millipore, Bedford MA) was performed using the Biorad transblot apparatus as recommended by the manufacturer for 1 h at 100 V. Immunodetection was performed using primary antibody (anti-EI to *S. salivarius* kindly supplied by C. Vadeboncoeur, Laval University, Quebec), alkaline phosphatase conjugated secondary antibody (goat anti-Rabbit) and color development reagents provided in the Pico Blue® immunodetection kit (Stratagene Inc., La Jolla, CA).

[^{32}P]-PEP phosphorylation of PTS proteins. Phosphorylation of PTS proteins with [^{32}P]-PEP and subsequent autoradiography was according to the method of Bourassa et al. (3) with a slight modification. [^{32}P]-PEP was synthesized by the method of Mattoo and Waygood (19) using purified carboxykinase from *E. coli*, kindly provided by Dr. H. Goldie, University of Saskatchewan, Saskatoon. Reactions contained 35 µg of total protein comprised of membrane or cytoplasmic fractions alone or in combination. Samples were

incubated at room temperature in a 25 µl reaction mixture containing 0.1 mM [^{32}P]-PEP (180 Ci, mole^{-1}), 5 mM $MgCl_2$, 12.5 mM NaF and 10 mM HEPES (pH 7.5). The reactions were stopped after 5 min by the addition of 10 µl of 188 mM Tris-HCl (pH 8.0), containing 6% (w/v) SDS, 30% (v/v) glycerol, 6% (v/v) 2-mercaptoethanol, and 0.005% bromophenol blue. Samples were loaded onto a 4-15% SDS-polyacrylamide continuous gradient gel and electrophoresed for 1 h at 200 V in a Biorad Mini Protean II apparatus (Biorad). The dried gel was placed on X-ray film with an intensifying screen at -70°C for 22 h.

Sugar competition. The specificity of mannose transport was tested by sugar competition experiments in which competing sugars were added to mannose transport reactions at a 100-fold excess. Cells were grown to mid log phase in TYE media with 0.2% mannose, harvested by centrifugation and washed twice in 50 mM Na/K phosphate buffer (pH 7.0) (PB). Assays contained 0.5 mg (dry wt.) cells, 1 mM [^{14}C]-mannose (9 Ci, mole^{-1}) and 100 mM of competing carbohydrate in PB and were carried out in a total volume of 0.5 ml. After 1 min of incubation at 37°C, the reaction mixtures were filtered through 0.45 µm Millipore filters using a vacuum manifold, washed 3 times with 1 ml PB prewarmed to 37°C and counted for activity in a liquid scintillation counter. Samples were taken in triplicate and the mean values were used to determine the relative activities.

Results

Construction of plasmids. Plasmid pVAEI (see Fig. 3) was constructed by digestion of pVA891 by a double restriction digest with EcoRI and PvuII as suggested by Sambrook et al. (23). The 630 bp insert fragment containing an internal region of the EI gene from *S. salivarius* was generated by an *Eco*RI-*Hae*III double digest of EIP8, an internal segment of the *S. salivarius* EI gene (Fig. 3). Following excision and purification from a 1.2% agarose gel, the fragment was ligated to pVA891 at 4°C overnight. *E. coli* DH5α was transformed by electroporation and 5 erythromycin-resistant colonies were picked, grown in 10 ml LB broths containing 500 μg/ml erythromycin and screened for plasmids. To confirm that the resultant plasmids contained the desired insert they were digested with *Eco*RI, *Pvu*II and *Xba*I, transferred to nylon membranes by Southern blotting and the blots probed by hybridization with P^{32}-labeled EIP8 at 65°C overnight (final wash 0.1x SSC, 1% SDS, 45 min). After 20 h of exposure, the X-ray film was developed and the 1.4 kb fragment released from pVAEI was confirmed to carry the correct insert (not shown).

Transformation of *E. coli*. Several parameters for the transformation of *E. coli* by electroporation were tested including field strengths ranging from 10 kV/cm to 17.5 kV/cm (voltage settings of 1.0 kV to 1.75 kV) and pulse lengths of

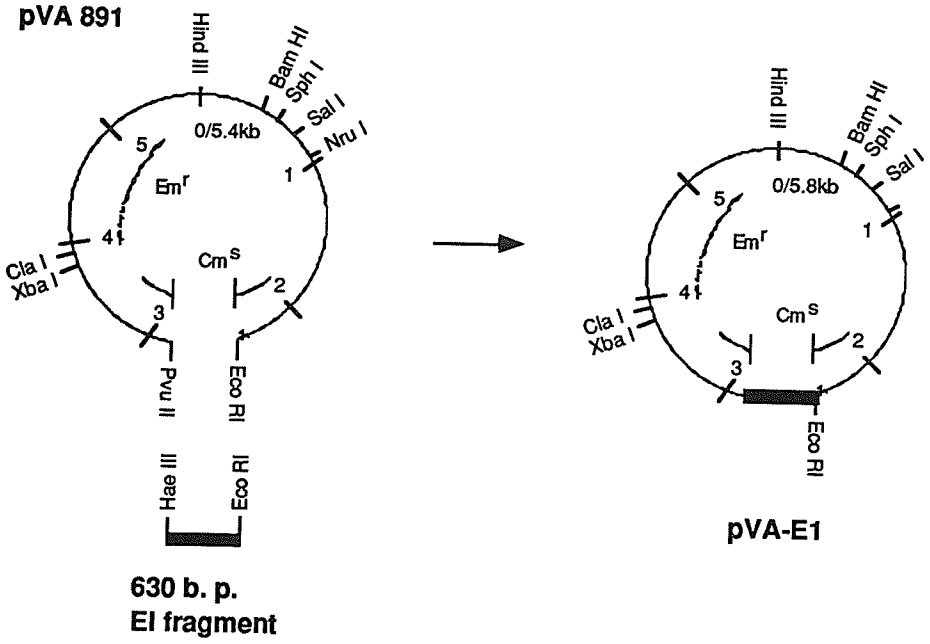


Fig. 3. The construction of pVAE1 is illustrated showing ligation of the 650 bp region of the EI gene of *Streptococcus salivarius* to the *Eco*RI-*Pvu*II site of the integration vector pVA891. Cm^s = Chloramphenicol sensitivity; Em^r = Erythromycin resistance.

between 2 ms and 16.6 milliseconds (pulse controller settings 200 Ω-1000 Ω). The highest transformation efficiency was obtained using 23 ng of pUC19 DNA with 20 µl of cells, 25 µF capacitance, 1.25 kV and pulse controller setting of 400 Ω using the 0.1 cm cuvette to give a transformation efficiency of 6.0×10^8 transformants/µg DNA. Lower or higher voltages resulted in lower efficiency of transformation with a secondary problem of arcing occurring at voltages over 1.5 kV resulting in unreliable current delivery. These parameters are similar to those determined optimal by Dower et al. (8), but are adapted to use with the 0.1 cm cuvettes allowing small volumes of cells (20 µl) to be used.

Transformation of *S. mutans* strains. Several attempts were made to transform *S. mutans* Ingbritt by electroporation, but all were unsuccessful. Typically, 0-30 colonies emerged on the selective plates following 2-10 days of incubation. Unfortunately, none of the resultant organisms were *S. mutans* as revealed by Gram stains with the contaminants predominantly Gram-positive diplococci or Gram-negative rods. The transformation of protoplasts by electroporation was also unsuccessful as no resistant colonies were isolated under the conditions tested.

The unsuccessful results obtained with the electroporation method led to the use of a natural competence technique for the transformation of streptococci reported by Caufield and

co-workers (6). These researchers had observed a wide range of transformation efficiencies with various *S. mutans* strains, so we used several strains of *S. mutans* in our subsequent experiments, including the laboratory strains *S. mutans* Ingbritt, *S. mutans* ATCC 10449 and *S. sanguis* ATCC 10556 and fresh *S. mutans* isolates BM71, DC-1, and 1546. The transformation reactions were plated out immediately after the 90 minute incubation and were then grown anaerobically at 37°C for 3 days. Transformation efficiencies were quite low with only five BM71 transformants and three 10449 transformants with pDL276. All resultant colonies were picked and grown in 5 ml cultures for further characterization. Southern hybridization was performed on small-scale DNA preparations using radiolabeled pDL276 as a probe and all the transformants were confirmed to carry the plasmid.

Since successful transformation was observed in strains 10449 and BM71, these strains were utilized for a subsequent transformation, again using the natural competence technique with pVAE1. As a result of this experiment, fourteen BM71 transformants were isolated on TYE-glucose plates containing 8 µg/ml erythromycin. DNA was isolated from these transformants, digested with *EcoRI*, electrophoresed, blotted and probed with pVA891. The results of the experiment depicted in Fig. 4 indicated that pVAEI had successfully integrated into the chromosome of 3 of the 4 BM71

transformants, but no integration was observed with the 10449 or 1546 strains. The expected integration event is illustrated in Fig. 5. The BM71 transformant B7 was subjected to sugar plate tests to determine if they had lost the ability to ferment known PTS sugars and the results indicated that the strain B7 had lost its ablity to ferment mannose, so we decided to investigate this phenomenon in greater detail.

Immunodetection of EI, HPr and EIII^{man}. Membrane-free cell extracts of three BM71 transformants, containing pVAEI inserts, and the wild-type BM71 strain were prepared and screened with EI antibodies raised against EI, HPr and EIII^{man}. From the resultant blots, illustrated in Fig. 6 (panels I), it is obvious that the EI protein is intact in all the strains tested running with an apparent molecular weight of approximately 68 kDa, the same as the parent strain, indicating that an intact copy of the EI gene is present in these isolates. No apparent differences between the mutant and parent were observed with HPr (panel II) or EIII^{man} (panel III).

[^{32}P]-PEP phosphorylation of cell extracts. To further examine the function of EI with respect to its ability to catalyze phosphotransfer from PEP to HPr and subsequently to the specific soluble components and permeases, we examined the SDS-PAGE profiles of the phosphoproteins that could be observed by autoradiography following phosphorylation of cell

extracts with [^{32}P]-PEP . The phosphoprotein profiles of wild-type BM71 and the transformant B7 (Fig. 7) indicate the presence of intact EI and HPr in all of the lanes, including those of the mutant and wild-type strains. The wild type strain exhibited three other bands at 47, 38 and 35 kDa. It appeared that the mutant B7 had a lower level of phosphorylation of the 38 kDa band, while the 35 and 47 kDa bands were not visible. The 38 kDa band is likely EIII^{man}, while the identity of the other bands is as yet unknown.

Identification of the insertion point of pVAEI. Since the vector had integrated into the chromosome in a region distinct from the EI gene as indicated by Southern blot analysis, we undertook to determine exactly where this insertion had taken place. To do this, we first constructed a B7 genomic library in λGEM 11 (Promega) by ligating partially digested 15-20 kb sucrose-density-gradient-sized *Sau*3A DNA fagments of B7 genomic DNA to the pre-cut and dephosphorylated phage arms which were subsequently packaged in vitro as described by the manufacturer. We then selected positive clones with pVA891 as a probe. From clone λB7-4 (Fig 8), a 2.0 kb *EcoRI-BamHI* fragment was subcloned into pBSII for sequence analysis, since it contained the integration point for the vector. Sequence analysis of this region revealed that the pVAEI vector had integrated into into the *msmG* gene, a region of the multiple sugar metabolism (*msm*) operon just upstream of the *gtfA* gene. This gene codes

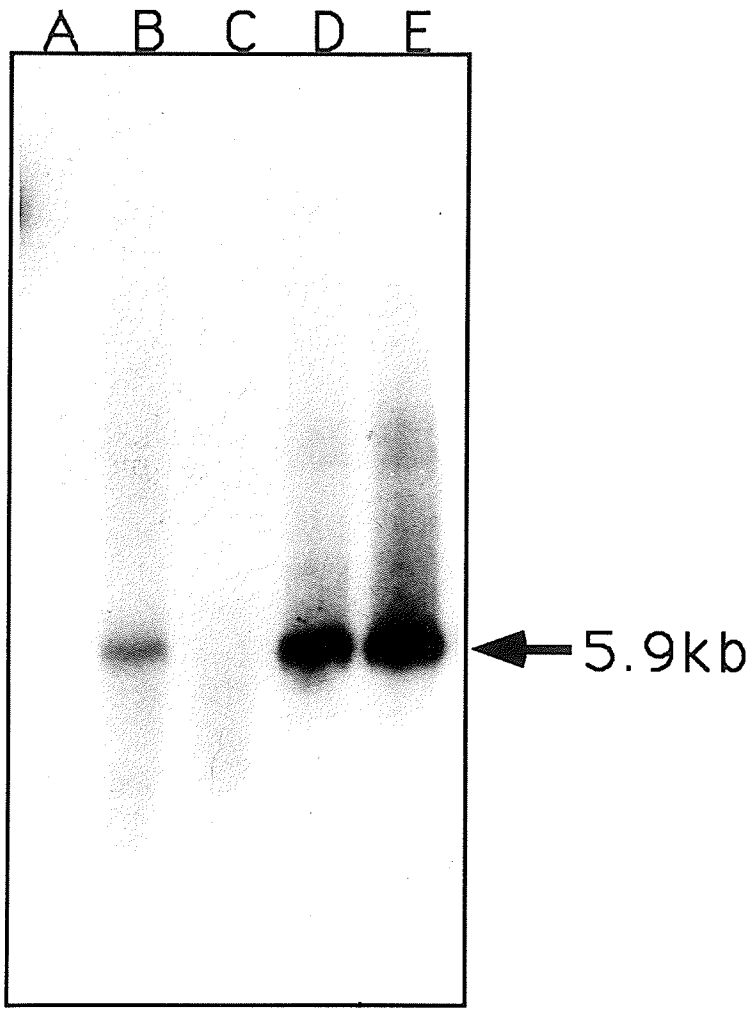


Fig. 4. Southern blot of *Eco*RI digested genomic DNA of *S. mutans* BM71 (lane A) and erythromycin-resistant transformants B1 (lane B), B3 (lane C) B4, (lane D) and B7 (lane E) probed with pVA891.

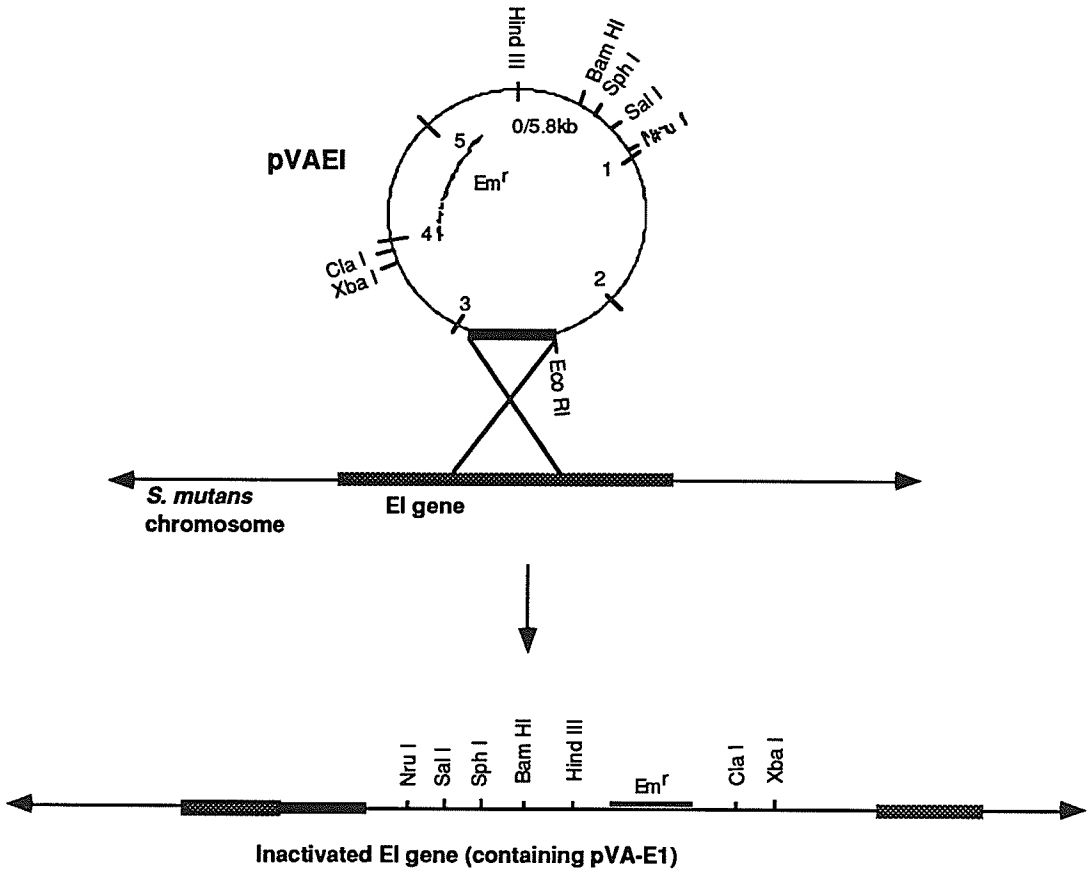


Fig. 5. Expected site of integration of vector pVAEI into the *S. mutans* chromosome. Vector pVAEI was constructed by inserting a 640 bp *Eco*RI-*Hae*III fragment of the *ptsI* gene from *S. salivarius* into the *Eco*RI-*Pvu*II site of pVA891. Transformants are selected by the conference of erythromycin resistance by the erythromycin resistance gene (Em^r).

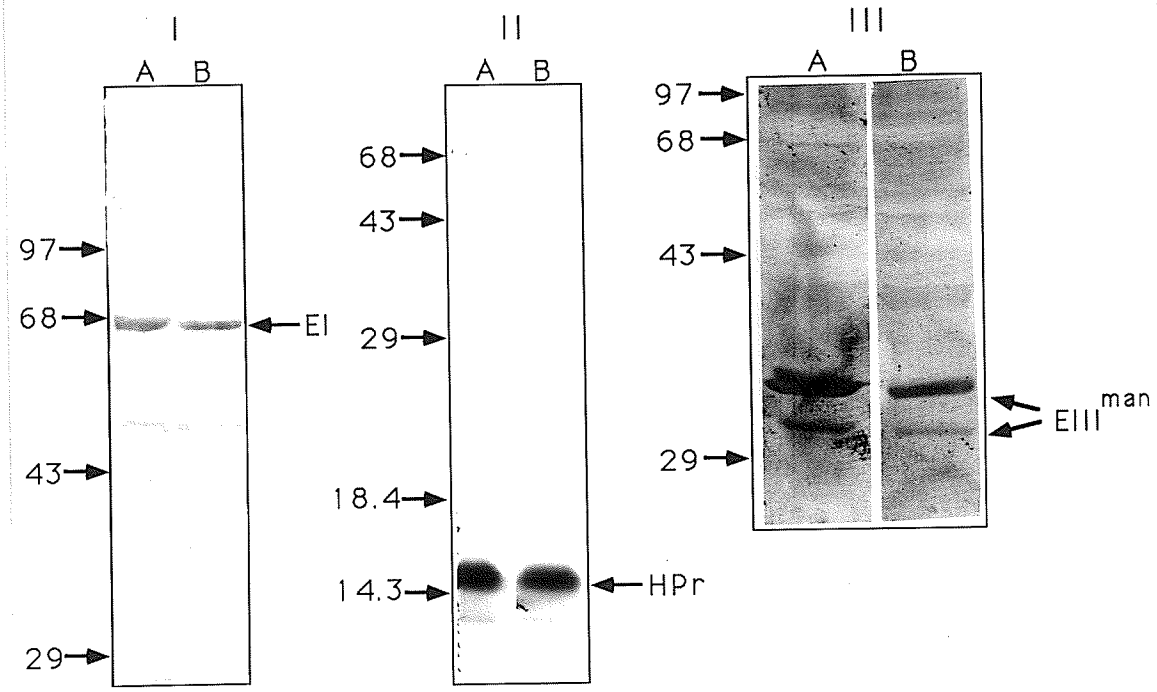


Fig. 6. Western blots of soluble extracts of parent strain *S. mutans* BM71 (A lanes) and mutant B7 (B lanes). SDS-PAGE was performed in 10% (I and III) and 15% (II) polyacrylamide gels which were run at 200 V for 40 min. and were blotted to to PVDF membranes for 1 h at 100 V. Immunodetection was performed using primary antibody against purified *S. salivarius* EI (I), HPr(II) and $EIII^{man}$, (III) as described in Materials and Methods.

for a membrane-bound protein which likely forms part of the *msm* permease (21).

Phenotypic properties of strain B7. In order to access the loss of mannose utilization in mutant B7, the growth rates of the mutant and wild-type was recorded with glucose and mannose (Fig. 9). The mutant had a similar growth rate to the parent on glucose with an observed doubling time of 0.5 generations, h-1. The mutant B7 initially failed to grow on TYE-mannose plates, but after 48 hours of incubation several small colonies would appear. One of these apparent revertants was isolated and was designated 7R. Revertant 7R was observed to grow slightly slower on mannose exhibiting a doubling time of 2.5 h, compared to the parent strain which doubled every 2.0 h. Strain 7R had retained its copy of pVAEI, as revevaled by Southern blot analysis, and it also remained resistant to erythromycin.

To test whether the plasmid integration was responsible for the mannose-negative phenotype, the vector pVAGTFA was constructed to transform BM71 to interrupt the region of the chromosome where pVAEI had integrated. This vector was constructed by ligation of a 2.4 kb *EcoRI* fragment from pDP6, a plasmid harboring the *GTFA* gene from *S. mutans* (generously provided by R. Burne University of Rochester, N. Y.), to *Eco*RI digested and dephosphorylated pVA891. This fragment contains part of the *msmG* gene, which codes for an integral membrane protein, and the *gtfA* gene of the *msm*, encoding a

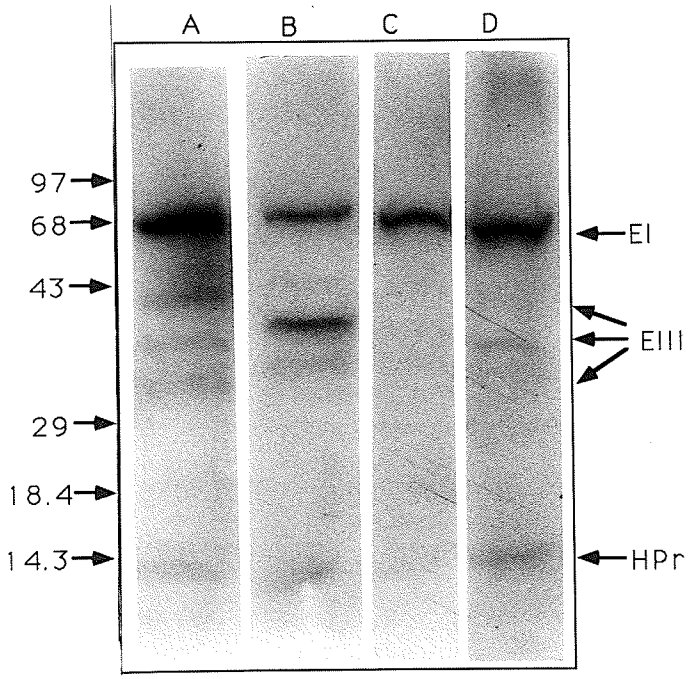


Fig. 7. [^{32}P]-PEP phosphorylated cell extract proteins of *S. mutans* BM71 and mutant B7 were electrophoresed on a 4-15% SDS-polyacrylamide continuous gradient gel for 1h at 200 V. The dried gel was placed on x-ray film with an intensifying screen for 6 days at - 70°C. A, BM71 cytoplasmic fraction; B, BM71 cytoplasmic + membrane fraction; C, B7 cytoplasmic fraction; D, B7 cytoplasmic + membrane fraction.

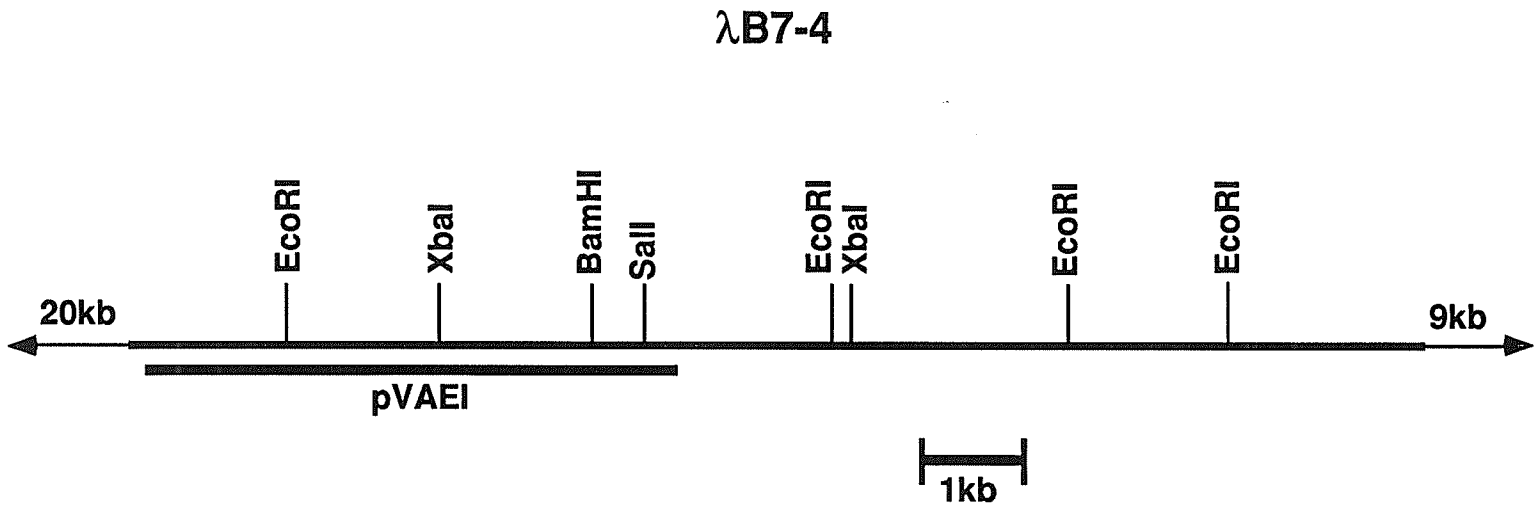


Fig. 8. Clone λB7-4 was selected from a λGEM11 (Promega Inc.) genomic library constructed with *Streptococcus mutans* B7 genomic DNA using the vector pVA891 as a probe. It contains the entire pVAEI integration vector as well as 8.5kb of flanking DNA.

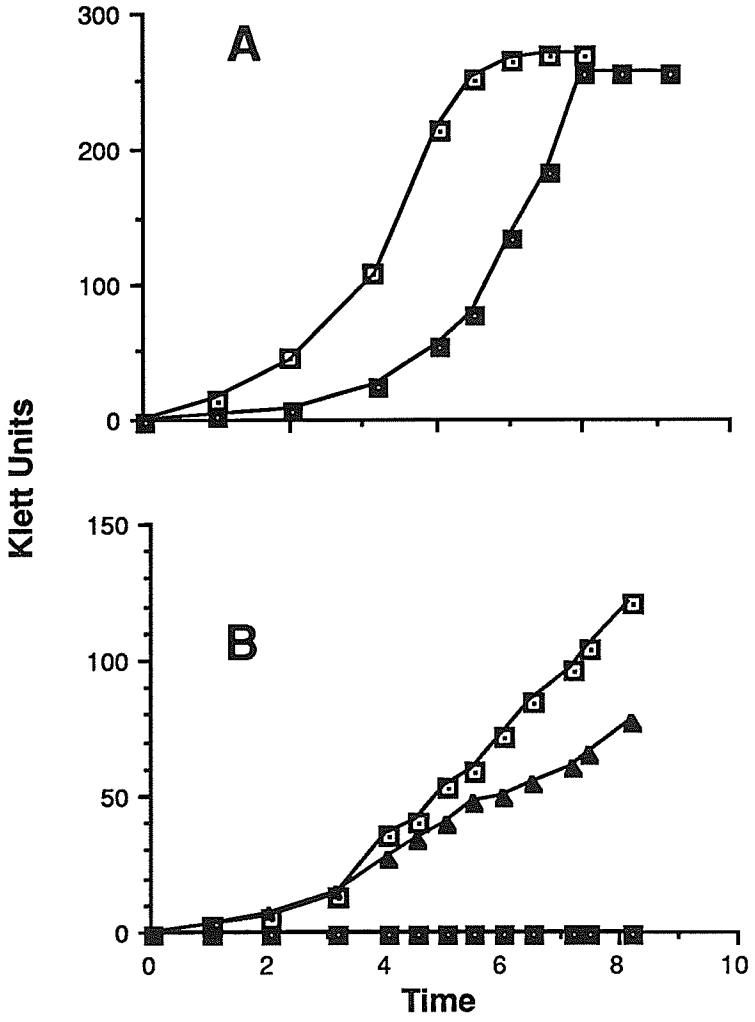


Fig. 9. Growth curves of *S. mutans* wild-type BM71 -□-; mutant B7 -■-; and revertant 7R -▲- grown on glucose **(A)** and mannose **(B)**.

sucrose phosphorylase of *S. mutans* (4). After transformation of the parent strain with pVAGTFA, erythromycin-resistant colonies were isolated and the insertion of the vector determined by Southern blot analysis of the DNA from these transformants with pVA891 as a probe. Although the integration of this vector was in the same gene as pVAEI, the mannose-negative phenotype could not be generated. Subsequent transformations with pVAEI also failed to produce the desired phenotype suggesting that the mutation causing the loss of mannose fermentation was likely the result of a secondary mutation, possibly independent from the insertion.

In order to gain insight into the nature of mannose transport by the revertant 7R, we compared the uptake of [^{14}C]-mannose by the parent strain, BM71, and the revertant, 7R, in the presence of a 100-fold molar excess of competing carbohydrates, glucose, fructose, α-methylglucoside, and 2-deoxyglucose. The results, illustrated in Fig. 10(A), demonstrate that strain BM71 transports mannose via the mannose PTS which is specific for mannose, glucose and 2-deoxyglucose, since these substrates are able to strongly inhibit the uptake of radiolabelled mannose (3). With the revertant 7R (Fig. 10B), it appears that mannose transport is occurring via a system with specificity for mannose, fructose and 2-deoxyglucose, since these substrates are able to significantly inhibit mannose transport.

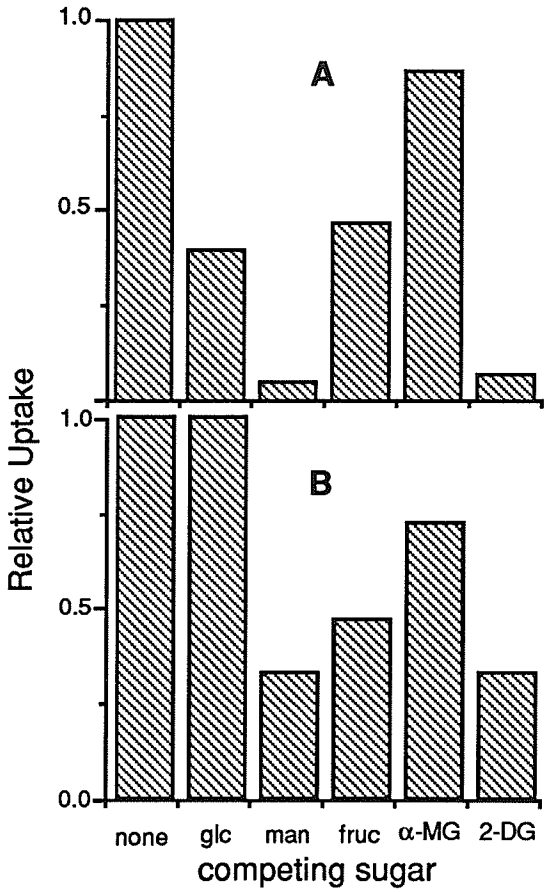


Fig. 10. Graph depicting the effect of a 100-fold concentration of excess carbohydrate on mannose uptake in mannose grown parent strain BM71 (A) and mannose utilizing revertant 7R (B). Abbreviations: glc = glucose. man = mannose, fruc = fructose, α-MG = α-methlyglucoside, 2-DG = 2-deoxyglucose.

Discussion

All attempts to isolate EI mutants of *S. mutans* with the *S. salivarius*-EI-containing integration vector were unsuccessful. Several obstacles were, however, overcome during this project that allow us to quickly proceed with new attempts to generate these mutants using the insertional inactivation technique including: the use of high efficiency electrotransformation of *E. coli* host strains to facilitate the proliferation of new plasmid vectors and the use of the natural competence technique for the transformation of oral streptococci. The mini-DNA preparation technique for rapid screening of genomic streptococcal DNA should also prove to be a valuable technique, requiring less time and smaller culture volumes then some previously published techniques (5,11).

Several problems were encountered in the attempts to transform streptococci by electroporation. Although this technique has been successfully implemented by other investigators (17), several others have abandoned it in favor of the natural competence technique of transformation (3,14). One important observation is that transformation appears to be quite strain specific (6). The exclusive use of *S. mutans* Ingbritt in several initial transformation attempts undoubtably impeded our investigations.

The isolation of BM71 erythromycin-resistant transformants with plasmid pVAEI was initially quite encouraging. The demonstration that pVAEI had successfully and stably integrated into the chromosome of BM71 by the Southern hybridization with pVA891 was also a desirable result. The integration event appeared to be stable since all transformants retained resistance to erythromycin when subcultured three times on plates devoid of the antibiotic. The plasmid pVAEI appeared to have integrated into a single *EcoRI* fragment of approximately 5.9 kb in size in 12 of the transformants indicating that the plasmid probably integrated into a wild-type *EcoRI* fragment of approximately 200 bp. This data did not match the size of either the *EcoRI* fragments carrying the EI gene in *S. mutans* Ingbritt [1.1 and 3.5 kb (data not shown)]. Information obtained by probing genomic restriction digests of both the wild type (BM71) and one of the transformants (B7) allowed us to construct a genomic map of the EI region of BM71 and it was determined that the EI gene was located on a single 5.1 kb *EcoRI* fragment. This, unfortunately, did not encourage us to believe that the insertion occurred at the desired location, where one would expect a resultant *Eco RI* fragment to be approximately 10.5 kb (5.4 kb pVAEI + 5.1 kb EI) instead of the observed 5.9 kb. The fact that EIS2 and EI1.1 (Fig. 2), both carrying portions of the S. *mutans* EI gene, hybridized to the same size restriction fragments in both wild-type and

transformant digests also indicated that the EI gene was still intact in the transformants (not shown).

The sugar fermentation tests were performed on the transformants to test if any phenotypic alteration had occurred as a result of the integration of pVAEI. The desired phenotype of a true EI mutant was expected to be pleiotropically unable to ferment most, if not all, PTS sugars. The results of the fermentation tests indicated that the transformants retained the ability to ferment all of the PTS sugars, with the exception of mannose. The negative or weak fermentation results observed on the mannose plates with strain B7 warranted further examination.

The data obtained from the Western blots conclusively demonstrated that pVAEI had not integrated into the EI gene, since the EI protein appeared as an intact fragment running with the same apparent molecular weight (68 kDa) as the enzyme in the wild type-strain (Fig. 6(I)). If the gene had been successfully interrupted, we would expect either a smaller immunoreactive fragment(s) if integration had occurred in the structural domain, or no fragment if a regulatory or promoter region had been disrupted.

The fact that integration did occur, but at an undesired location, was in itself an interesting observation. We would suspect that integration into the *msm* operon would occur at a lower frequency than integration into the EI gene, since

little homology between the integration plasmid harboring the EI fragnment and the *msm* region was expected. In order to determine the degree of homology between the 650 bp EI fragment harbored on pVAEI to the *msm* region of the chromosome, the two sequences were aligned using the ALIGN program (7). This analysis revealed that there was a region with a 182 nucleotide overlap with 56.6% homology. This low degree of homology would likely result in no recombination or a very low efficiency of recombination at this site.

Since the Southern blot data suggested that pVAEI has significant homology to the EI gene, we would expect it to be the primary target for integration, and indeed, integration likely occurred, but these transformants may have been unable to grow well under the chosen selective conditions. Melibiose was the sugar of choice in our selective media since it is transported via the MSM transport system (24). The independence of this system from components of the PTS has not yet been investigated, so we are only assuming that EI is not required for the uptake and metabolism of melibiose. The fact that the BM71 transformants isolated in this study were selected on glucose plates and grew as well as wild-type strains should have given us reason to immediately question their merit.

The possibility that the EI gene is essential to *S. mutans* also exists. Other investigators were able to isolate several *S. mutans* mutants that were unable to ferment

carbohydrates using streptozotocin, yet, quite strangely, with the exception of one leaky and unstable mutant, none appeared to de deficient in either of the two general PTS components, HPr or EI (12). If EI proves to be essential for the growth of *S. mutans,* this would be quite a unique feature since EI mutants have been readily isolated from several species that are known to contain the PTS (20).

This paper describes our initial attempts to isolate pleiotrophic PTS mutants. Although the results were unsuccessful in this regard several useful techniques and information were acquired including the observation that some strains transform better then others. The characterization of the mannose-negative phenotype of transformant B7 is likely the result of a mutation secondary to the plasmid insertion. This hypothesis is supported by the fact that the strain readily reverted to ferment mannose while retaining the inserted plasmid. We were also unable to reproduce the man$^-$ phenotype by re-transformation with either pVAGTF-A or pVAEI. The fact that the phenotype could not be generated in subsequent experiments with these vectors also supports this hypothesis. The mutation causing the phenotype may have occurred in a gene affecting phosphorylation of one or both of the forms of EIII^{man} as suggested by the [^{32}P]-PEP phosphorylation experiments. EIII^{man} likely has a central role in the regulation of several physiological processes in this organism (3).

The reversion of the phenotype may have occurred at the site of the mutation, but since strain 7R had a slower rate of growth then the wild-type, it is more likely that the mutation causing reversion occurred elsewhere. The results of sugar competition experiments with the revertant strain 7R and the parent BM71 suggest that mannose is being transported via an EII that has acquired a mutation altering its specificity, or via a secondary or cryptic system that can only be activated in the absence of the primary mannose PTS. This system appears to be specific for mannose and fructose and may possibly be the fructose PTS. A similar result has been observed for the utilization of glucose which can be transported via a cryptic system that is only activated in the abscence of mannose PTS function (16).

Acknowledgements

I thank Drs. C. Vadeboncoeur, and M. Frenette from the University of Laval, Quebec for the *S. salivarius* EI clones, the anti-EI antibody, and useful advice, and Dr. R. Burne from The University of Rochester, N. Y. for helpful discussions, the GTF-A clone and the *S. rattus* strain

References

1. Bowden, G.H., J. M. Hardie and E. D. Fillery. 1976. Antigens from *Actinomyces* species and their value in identification. J. Dent. Res. **55** (Special Issue A): A192-A204.
2. Bender, G. R., G. R., S. V. W. Sutton, and R. E. Marquis. 1986. Acid tolerance, proton permeabilities and membrane ATPases of oral streptococci. Infect. Immun. **53**: 331-338.
3. Bourassa, S., L. Gauthier, R. Giguere, and C. Vadeboncoeur 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. Oral Microbiol. Immunol. **5**: 288-297.
4. Burne, R. A., B. Rubinfeld, W. H. Bowen, and R. E. Yasbin. 1986. Tight genetic linkage of a glucosyltransferase and dextranase of *Streptococcus mutans* GS-5. J. Dent. Res. **65**: 1392-1401.
5. Caufield, P. W., and T. M. Walker. 1989. Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. J. Clin. Microbiol. **27**:274-278.
6. Caufield, P. W., G. R. Shah, and S. K. Hollingshead. 1990. Use of transposon *Tn*916 to inactivate and isolate a mutacin-associated gene from *Streptococcus mutans*. Infect. Immun. **58**: 4126-4135.
7. Dayhoff, M. O., W. C. Barker, and L. T. Hunt. 1983. Establishing homologies in protein sequences. Methods Enzymol. **91**: 524-545.
8. Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *Escerichia coli* by high voltage electroporation. Nucleic Acids Res. **16**: 6127-6145.
9. Hamada, S., and H. D. Slade. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331-284.

10. Hamilton, I. R., and E. J. St. Martin. 1982. Evidence for involvement of protonmotive force in the transport of glucose by a mutant of *Streptococcus mutans* strain DR0001 defective in glucose-phosphoenolpyruvate phosphotransferase activity. Infect. Immun. **36:** 567-575.
11. Hudson M. C., and R. Curtiss III. 1990. Regulation of expression of *Streptococcus mutans* genes important to virulence. Infect. Immun. **58:** 464-470.
12. Jacobson, G. R., F. Poy, and J. W. Lengeler. 1990. Inhibition of *Streptococcus mutans* by the antibiotic streptozotocin: mechanisms of uptake and the selection of carbohydrate-negative mutants. Infect. Immun. **58:** 543-549.
13. Kaslow, D.C. 1986. A rapid biochemical method for purifying lambda DNA from phage lysates. Nucleic Acids Res. **14:** 6767.
14. Koga, T., N. Okahashi, I. Takahashi, T. Kanamoto, H. Asakawa, and M. Iwaki. 1990. Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype c. Infect. Immun. **58:** 289-296.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.
16. Lapointe, R., M. Frenette, and C. Vadeboncoeur. 1993. Altered expression of several genes in $III^{man}L$-defective mutants of *Streptococcus salivarius* demonstrated by two-dimensional gel electrophoresis of cytoplasmic proteins. Res. Microbiol. **144:** 305-316.
17. Lee, S. F., A. Progulske-Fox, G. W. Erdos, D. A. Piacentini, G. Y. Ayakawa, P. J. Crowley, and A. S. Bleiweis. 1989. Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/II). Infect. Immun. **57:** 3306-3313.

18. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. Micobiol. Rev. **50**: 353-380.

19. Mattoo, R. L., and E. B. Waygood. 1983. An enzymatic method for [^{32}P]phosphoenolpyruvate synthesis. Anal. Biochem. **128**: 245-249.

20. Postma, P. W., and J. W. Lengeler. 1985. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49**: 232-269.

21. Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao, and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. *J. Biol. Chem.* **267**: 4631-4637.

22. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat'l. Acad. Sci. U.S.A. **74**: 5463-5467.

23. Sambrook, J. Mantiatis, T., and E. F. Fritsch. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

24. Tao, L., R. R. B. Russell, and J. J. Ferretti. 1990. Insertional inactivation of genes concerned with melibiose and isomaltosaccharides in *Streptococcus mutans*. Abstr., 3rd Int'l Conference on Streptococcal Genetics, abstr. B40.

25. Vadeboncoeur, C. L., L. Thibault, S. Neron, H. Halvorson, and I. R. Hamilton. 1987. Effect of growth conditions on levels of the phosphoenolpyruvate: sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus salivarius*. J. Bacteriol. **169**: 5686-5691.

Chapter 5

Glucose Transport by a Mutant of *Streptococcus mutans* Unable to Accumulate Sugars via the Phosphoenolpyruvate Phosphotransferase System

Dennis G. Cvitkovitch, David A. Boyd, Tracy Thevenot and Ian R. Hamilton*

Department of Oral Biology
Faculty of Dentistry,
University of Manitoba,
Winnipeg, Canada R3E 0W2

Published in The Journal of Bacteriology 177: 2251-2258, 1995

Abstract

Streptococcus mutans transports glucose via the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS). Earlier studies indicated that an alternate glucose transport system functions in this organism under conditions of high growth rates, low pH or excess glucose. To identify this system, *S. mutans* BM71 was transformed with integration vector pDC-5 to generate a mutant, DC10, defective in the general PTS protein, enzyme I (EI). This mutant expressed a defective EI that had been truncated by approximately 150 amino acids at the carboxyl terminus as revealed by Western blot (immunoblot) analysis with anti-EI antibody and Southern hybridizations with a fragment of the wild-type EI gene as a probe. Phosphotransfer assays utilizing [^{32}P]-PEP indicated that DC10 was incapable of phosphorylating HPr and EIIA^{man}, indicating a nonfunctional PTS. This was confirmed by the fact that DC10 was able to ferment glucose but not a variety of other PTS substrates and phosphorylated glucose with ATP and not PEP. Kinetic assays indicated that the non-PTS system exhibited an apparent K_s of 125 μM for glucose and a Vmax of 0.87 nmoles mg (dry weight) of cells^{-1}, min^{-1}. Sugar competition experiments with DC10 indicated that the non-PTS transport system had high specificity for glucose since glucose uptake was not significantly inhibited by a 100-fold molar excess of several competing sugar substrates, including 2-deoxyglucose and α-

methylglucoside. These results demonstrate that *S. mutans* possesses a glucose transport system that can function independently of the PEP PTS.

Introduction

Carbohydrate metabolism by *Streptococcus mutans* results in the formation of acid end products that can contribute to the demineralization of the tooth enamel leading to dental caries (11, 42). The phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) (29) is the principal transport process in oral streptococci for glucose and a variety of sugars, including mannose, fructose, sucrose, lactose and maltose (2,7,37). In the PTS, phosphate is transferred from PEP via the general PTS proteins HPr and EI to the sugar-specific, membrane-bound protein EII and then to the incoming sugar. Much of the current information on the structure of the PTS has come from work with *Escherichia coli* and *Salmonella typhimurium*, particularly with respect to the various sugar-specific EIIs (29). The arrangement of the domains that make up the EIIs can vary depending on the organism and the sugar to be transported, appearing either (i) as a single membrane-bound protein consisting of three domains (A, B and C), (ii) two or more proteins, one of which is membrane-bound (B and C), while one is soluble (IIA or EIII), (iii) where domains A and B are fused into a single soluble protein and are associated with two membrane components (C and D), or (iv) where domains IIA and IIB can also exist as separate soluble proteins (29,34). While other variations in the domain organization are known, phosphoryl

transfer generally occurs sequentially via EI, HPr, EIIA, and EIIB, with the EIIC and EIID components probably forming a translocation channel in the membrane.

With respect to *S. mutans*, information is currently available on the genetic arrangement of some, but not all, of the PTS and associated components responsible for the transport of sucrose (*scrA*, EII)(35) and mannitol (EIII)(20). More complete information is available on the lactose operon in this organism, including the nucleotide and deduced amino acid sequences of the repressor, the tagatose-6-phosphate pathway and IIA^{lac} (*lacF*) and $IICB^{lac}$ (*lacE*) of the PTS (30). A more recent paper (21) indicates that the gene for phospho-ß-galactosidase (*lacG*), the enzyme that cleaves lactose-P that is generated by the lactose-PTS, is also located in this operon. We have cloned and sequenced the genes for the general PTS proteins HPr and EI in *S. mutans* and shown that they are not associated with other PTS genes (3), a result shown earlier for *S. salivarius* (10).

Several lines of evidence support the notion of a second, non-PTS transport system for glucose in *S. mutans*. Early chemostat studies (8,9,15) with *S. mutans* Ingbritt suggested the existence of such a system in cells grown at high growth rates and low pH and with sucrose and excess glucose, conditions repressing the glucose PTS. Furthermore, a reciprocal relationship was shown to exist between the

activity of the glucose PTS and glucokinase suggesting an uptake system requiring the phosphorylation of free intracellular glucose (12). Later kinetic studies with *S. mutans* DR0001 grown in continuous culture with glucose limitation at growth rates of 0.04 and 0.6 h^{-1} indicated two transport processes, a high affinity system with K_s values of 6.8-8.0 μM, shown to be the PTS, and a lower affinity system with K_s values of 57 to 125 μM (18). A PTS-defective strain, *S. mutans* DR0001/6, on the other hand, possessed only one transport system with K_s values 62 to 133 μM. Subsequent continuous culture studies with *S. mutans* Ingbritt (16,40,41), demonstrated that the repression of the glucose PTS was associated with reduced synthesis of the membrane EII^{glc}, in fact, cells grown at pH 5.0 were completely devoid of EII^{glc} activity (40) confirming earlier results obtained with decryptified cells (15). More recently, membrane vesicles, prepared from chemostat-grown cells of *S. mutans* Ingbritt and devoid of cytoplasmic components, including EI and HPr, demonstrated glucose counterflow, indicating a carrier capable of transporting glucose (4). Early models suggested that the non-PTS glucose transport system was linked to proton motive force in *S. mutans* (18, 22), however, this hypothesis was questioned by more recent research (6).

The discovery by genetic means of a novel binding protein-dependent sugar uptake system (33) in *S. mutans* has also suggested a possible route for glucose uptake. This multiple

sugar metabolism (MSM) system is encoded on an 11 kb multigene region of the *S. mutans* chromosome and is primarily responsible for the transport of raffinose, melibiose, and isomaltosaccharides. One essential gene in this operon is *msmK*, which codes for an ATP-binding protein, the first such protein identified in gram-positive bacteria. A recent report (36) has indicated that unlabelled glucose could effectively disrupt transport of radiolabelled melibiose by *S. mutans*, although this is not conclusive proof of glucose transport by the MSM system.

With the exception of the MSM studies, PTS-independent transport studies with intact cells of *S. mutans* have been hampered by the presence of the PTS and it has not been possible to characterize alternative transport processes. The objective of the current work was to study glucose transport in *S. mutans* in the absence of PTS activity. The preferred mutants would be those defective in the gene for either general PTS protein, EI or HPr, since they are required for the transport of all PTS sugars. We have generated a mutant, *S. mutans* DC10, defective in EI from information obtained during a previous study (3) on the sequence and expression of the genes for these proteins from *S. mutans* NG-5. The mutant contains a truncated *ptsI* gene missing 150 amino acids from the C-terminal end and cannot be phosphorylated by [^{32}P]-PEP or support glucose phosphorylation via PEP. We have used this mutant to study the

characteristics and properties of non-PTS glucose transport in this organism.

Materials and Methods

Bacterial strains, media, and growth conditions. *E. coli* DH5α [Ø80d lac ZΔ M15, end A1, rec A1, hsdR17 (r_{k-}, m_{k+}), sup E44, thi-1, l-, gyrA, relA1, F-, Δ(lacZYA- arg F), U169], used as a host strain for the isolation and maintenance of plasmids in this study, was maintained in LB broth and on plates (28) containing ampicillin (50 μg, ml^{-1}) or erythromycin (750 μg, ml^{-1}). *S. mutans* strain BM71 was utilized as a parent strain in the construction of *ptsI* mutant DC10. Streptococci were grown and maintained in tryptone-yeast extract (TYE) broth or plates (1.0% tryptone, 0.5% yeast extract, 0.35% K_2HPO_4, 0.2% glucose or raffinose). Selective media, used to isolate PTS-defective streptococcal transformants, included (i) TYE containing 1.6% agar, 1% raffinose and 8 μg erythromycin ml^{-1} and (ii) blood agar (Oxoid blood base agar #2, 5% sheeps blood, 0.0005% hemin) containing 8 μg erythromycin ml^{-1}. Mutant DC10 was maintained on TYE-raffinose (0.3%) plates with 8 μg erythromycin ml^{-1}. Sugar fermentation was determined by anaerobic incubation of the test organism on streptococcal sugar agar plates (2.0% proteose peptone, 0.5% yeast extract 0.5% NaCl, 0.1% Na_2HPO_4, 1.5% agar, 0.002% bromocresol purple) containing the various

sugars at a concentration of 1%. The rate of acid production was measured by auto-titration of metabolizing cells with standardized KOH as described previously (19).

Recombinant DNA methodology and bacterial transformations. Agarose gel electrophoresis was carried out in Tris-acetate-EDTA buffer, while Southern hybridizations were carried out in aqueous solutions as described by Maniatis et al. (25). DNA was transferred to Hybond N+ (Amersham) for detection by radiolabelled probes and to Photogene Nylon (Gibco/BRL, Mississauga, Ontario, Canada) for detection by biotin-labelled probes. Radiolabelled DNA probes were prepared by using the nick translation or random primers labelling kits, and biotin-labelled DNA probes were prepared using the Bio-Nick labelling kit (Gibco/BRL). Recombinant plasmids used for sequencing were produced by subcloning specific restriction fragments after purification from agarose gels with the GENECLEAN kit (Bio 101, La Jolla, Calif.). Rapid screening of plasmids was done from 1.5 ml *E. coli* cultures using alkaline lysis (1). *E. coli* were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad, Richmond California) by the method of Dower et al. (7).

Streptococcal integration vector pDC-5 was constructed by first subcloning a 450 bp blunt-ended fragment from an internal region of the *ptsI* gene into pUC9. This fragment

contained an internal *Eco*RV site into which a 1.8 kb *Hinc*II/*Sma*I fragment containing the erythromycin resistance gene from pDP3 (generously supplied by R. Burne, University of Rochester) was inserted. The vector was linearized with *Nde*I and used to transform BM71. Electro-competent cells were prepared for transformation by growing an overnight culture of *S. mutans* BM71 in Todd-Hewitt broth supplemented with 10% heat-inactivated horse serum (Sigma Chemical Co, St. Louis, MO) with 125 µl of this culture used to inoculate 5 ml of prewarmed Todd-Hewitt broth-10% heat-inactivated horse serum. The culture was incubated anaerobically until it reached an optical density of 0.25 at 600 nm when the cells were cooled on ice, centrifuged at 15,000 x g for 5 min, washed three times in ice-cold 300 mM sucrose and finally resuspended in 100 µl of 300 mM sucrose. The DNA was added to the cell suspension and transferred to a pre-chilled 0.1 cm cuvette after 1 min on ice, and pulsed at 2.5 kV, 200 Ω and 25 µF in a Biorad Gene Pulser. Immediately after pulsing, 1 ml of Todd-Hewitt broth was added to the cuvettes and the contents transfered to a 1.5 ml microfuge tube and incubated at 37°C for 90 min. The cells were centrifuged for 5 min at 15,000 x g and resuspended in 100 µl of THB and plated on the appropriate selective media.

Protein electrophoresis and Western immunoblotting. Cell extracts of *S. mutans* containing HPr and EI for use in Western immunoblotting were prepared as previously described

(41). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Laemmli (23) and transferred to Immobilon-P membranes (Millipore) as described by Towbin et al. (38). After incubation of the membranes with the anti-EI primary antibody (1:500 dilution in Tris-buffered saline), EI and HPr-specific bands were detected by incubation with goat anti-rabbit immunoglobulin G alkaline phosphate conjugate followed by detection with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine (BCIP).

[^{32}P]-PEP phosphorylation of PTS proteins. Phosphorylation of PTS proteins with [^{32}P]-PEP and subsequent autoradiography were done by the method of Bourassa et al. (2) with a slight modification. [^{32}P]-PEP was synthesized by the method of Mattoo and Waygood (27) using purified carboxykinase from *E. coli*, kindly provided by Dr. H. Goldie, University of Saskatchewan, Saskatoon, Canada. The positive control contained 35 µg protein from *S. mutans* BM71 membrane-free cell extract . Samples were incubated at room temperature in a 25 µl reaction mixture containing 0.1 mM [^{32}P]-PEP (180 Ci, mole^{-1}), 5 mM $MgCl_2$, 12.5 mM NaF and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.5). The reactions were stopped after 5 min by the addition of 10 µl of 188 mM Tris-HCl (pH 8.0), which contained 6% (w/v) SDS, 30% (v/v) glycerol, 6% (v/v) 2-mercaptoethanol, and 0.005% bromophenol blue. Samples were

loaded onto a 1.5 mm thick SDS-12.5% polyacrylamide gel which was electrophoresed for 1 h at 200 V in a Biorad Mini Protean II apparatus (Biorad). The dried gel was placed on X-ray film with an intensifying screen at -70°C for 22 h.

Detection of the various forms of HPr. Four separate 500 ml samples (200 mg of cells, sample^{-1}) were rapidly removed during the exponential phase to a stirred solution containing 100 mM Tris-citrate buffer (pH 4.0) with chloramphenicol (50 mg, ml^{-1}) and gramicidin D (1 mM), and the pH of the sample was immediately lowered to 4.5 with 5 N HCl. The cell suspension was centrifuged at 16,000 g for 10 min, resuspended in a minimal amount of the supernatant, re-centrifuged at 27,000 x g for 20 min and frozen at -70°C. The frozen cell pellet was used within 2-3 h to prepare the membrane-free cell extract by a slight modification of the alumina grinding method of Vadeboncoeur et al (39). After grinding, a small volume (1.5 ml) of buffer (10 mM HEPES buffer [pH 7.0]) containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride and 0.1 μM Pepstatin A) was added and the alumina was removed by centrifugation at 3,000 x g for 5 min. The supernatant was then centrifuged at 16,000 x g for 20 min to remove intact cells and cell debris and then subjected to centrifugation at 100,000 x g for 18 hours before being assayed for HPr.

The various forms of HPr were separated by crossed immunoelectrophoresis as described by Vadeboncoeur et al. (39) with minor alterations to the protocol. Agarose (1%) was dissolved in a Tris-barbitone buffer containing (grams, $liter^{-1}$): sodium barbitone (5.01) Tris base (8.86) calcium lactate (0.11) and sodium azide (0.13). The samples were diluted to 2 mg protein, ml^{-1} with 10 mM HEPES buffer (pH 7.0) and 5 µl was deposited at the cathodic end of the gel. Electrophoresis in the first dimension was conducted for 75 minutes at 10 V/cm at 10°C on a LKB Bromma 2117 Multiphor. The second dimension, conducted for 19 hours at 2 V/cm and 4°C, involved electrophoresis against a polyclonal rabbit antibody (8 mg/plate) directed against the purified HPr protein of *S. mutans* DR0001. Following electrophoresis, the gels were dried at room temperature overnight and stained with Coomassie blue.

Quantitation of HPr under each precipitin peak involved scanning each gel in a 300 DPI Apple Scanner (Apple Computer, Inc. Cupertino, Calif.) transferring the image to the Image 1.36 program (National Institute of Health, Bethesda, Md.) and determining the number of pixels under each peak. These values were then compared to a standard curve (0 to 2000 ng of HPr protein) to determine the number of nanograms of the various forms of the HPr protein that existed in the sample. The final values were expressed as micrograms of HPr, milligram of cellular $protein^{-1}$.

Sugar transport and kinetics. Glucose transport was measured on mid-log phase, glucose-grown cells washed three times in 50 mM Na/K phosphate buffer (pH 7.0). The cells (0.5 mg [dry weight], ml^{-1}) were incubated at 37°C in a reaction mixture containing 1 mM [^{14}C]-glucose (9 Ci, $mole^{-1}$) in 50 mM Na-K phosphate buffer (pH 7.0) with a final volume of 5.0 ml. Samples (0.5 ml) were removed at 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 min and filtered through 0.45 µm HA filters (Millipore, Bedford, Mass.) and washed three times with 1 ml of 50 mM Na-K phosphate buffer (pH 7.0) equilibrated at 37°C. The dried filters were then counted in 5.0 ml Aquasol (NEN Research products, Montreal, Quebec, Canada) in a liquid scintillation counter. Kinetics of glucose transport were determined essentially as previously described (12) with the concentrations of glucose ranging from 0.01-10 mM and the reactions being terminated at 0.1 min. Rates were expressed as nanomoles of sugar transported milligram (dry weight) of cell $material^{-1}$, $minute^{-1}$.

Sugar competition. The specificity of non-PTS glucose transport activity was tested by sugar competition experiments in which possible competing sugars were added to glucose transport reactions at a 100-fold excess. Assays were identical to those used for glucose transport with reactions containing 1 mM [^{14}C]-glucose (9 Ci, mol^{-1}) and the competing carbohydrate at 100 mM. The reaction mixtures were

incubated for 2 minutes and then filtered, washed and counted. Samples were taken in quadruplicate and the mean values were used to determine the relative activities.

Glucose phosphorylation. The phosphorylation of glucose via ATP and PEP was carried out as described previously (4). Intact cells were permeabilized with toluene, diluted in buffer to 1 mg, ml^{-1} and assayed in a reaction mixture containing 4 mM PEP or ATP, 50 µg (dry weight) of cells in PTS buffer (50 mM K phosphate buffer [pH 7.0] containing 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 20 mM NaF) in a final volume of 500 µl. The reaction was initiated by the addition of 2 mM [^{14}C]-glucose (9 Ci, $mole^{-1}$) and after a 30 minute incubation at 37°C, the phosphorylated sugar was separated by precipitation with 10 volumes of 30 mM $BaBr_2$ in 90% (vol/vol) ethanol. After 30 min of incubation on ice, the suspensions were filtered through 0.45 µm pore-size HA Millipore filters, rinsed twice with 2 ml of cold 80% ethanol and counted. Activity was expressed as nanomoles of glucose phosphorylated, milligram of dry $cells^{-1}$ $minute^{-1}$.

Chemicals and enzymes. Restriction enzymes, T4 DNA ligase, goat anti-rabbit Immunoglobulin G alkaline phosphatase conjugate,and DNA and protein molecular weight standards were from GIBCO/BRL and were used as directed by the supplier. Radiolabelled nucleotides [γ^{32}P]dATP, [α^{32}P]dCTP and [^{14}C]-glucose were obtained from Dupont New

England Nuclear. All other chemicals were obtained from Sigma Chemical Company, Fisher Scientific Limited, or Difco. Rabbit anti-EI (*S. salivarius*) and anti-HPr (*S. mutans*) polyclonal antibodies were a generous gift from Christian Vadeboncoeur, Laval University, Ste-Foy, Quebec , Canada.

Results

Construction and characterization of *ptsI* mutant DC10. *S. mutans* BM71 was transformed with linearized pDC-5 and two erythromycin-resistant transformants were picked from blood agar plates after 6 days of anaerobic incubation at 37°C. One transformant, DC10, selected for all subsequent experiments, was compared to BM71 for the ability to metabolize and grow on a variety of sugar substrates. As illustrated in Table 1, the wild-type strain BM71 was able to grow and ferment a variety of PTS sugars and the non-PTS substrates, melibiose and raffinose, on streptococcal sugar agar plates. However, growth of DC10 was only apparent after 3 days of incubation on plates containing melibiose, raffinose, or glucose. The failure to ferment or grow on the other PTS substrates suggested that the PTS was non-functional. Growth curves of DC10 in TYE-0.3% glucose broth exhibited a doubling time of 180 minutes compared with a doubling time of 40 min for the parent strain BM71 (Fig. 1). With glucose as the substrate, the rates of acid production by glucose-grown BM71 and mutant DC10 cells were 212 ± 14 and

Table 1

Sugars and sugar alcohols transported by the PEP: PTS and non-PTS systems capable of supporting growth and metabolism of wild-type *S. mutans* BM71 and *ptsI* mutant DC10.

S. mutans strain	Fermentation Substrate [a]	
	PTS	Non-PTS
BM71	glucose maltose sucrose mannitol lactose sucrose fructose	raffinose melibiose
S. mutans DC10	glucose	raffinose melibiose

[a] Tests were conducted on carbohdyrate-indicator agar plates with anaerobic incubation for 3 days and included all of the substrates listed for BM71.

56 ± 8 nmoles of acid neutralized, mg of dry cells^{-1} min^{-1}, respectively, indicating a decrease in acid production of about 75%. Transport assays with [^{14}C]-glucose revealed that the rate of glucose uptake by DC10 (1.4 ± 0.3 nanomole of glucose, mg dry cells^{-1} min^{-1}) was only 1.2 % of that exhibited by the wild-type strain BM71 (115 ± 12 nanomoles, mg dry cells^{-1}, min^{-1}).

In order to confirm integration of pDC-5 into the *ptsI* gene of BM71, chromosomal DNAs from the parent and transformant DC10 were subjected to Southern blot analyses with radiolabelled probes that contained either the erythromycin-resistance gene from pDP-3 or a 3.5 kb *Eco*RI-*Sst*I restriction fragment containing the cloned *ptsI* gene from *S. mutans*. Fig. 2 shows the results of the latter, with the *ptsI* probe hybridizing to a 4.8 kb *Sst*I fragment from BM71 (lane F) and a 6.6 kb SstI fragment from DC10 (lane C). This increase in size corresponds to the size of the erythromycin-resistance gene (1.8 kb). Probing of the same blot with the erythromycin resistance gene also revealed hybridization to a 6.6 kb *Sst*I fragment in DC10, with no hybridization to the parent strain (data not shown). The same blot was also probed with the ampicillin-resistance gene from pUC9 to ensure that ampicillin resistance had not been transferred to DC10 by integration vector pDC-5. No apparent hybridization was observed.

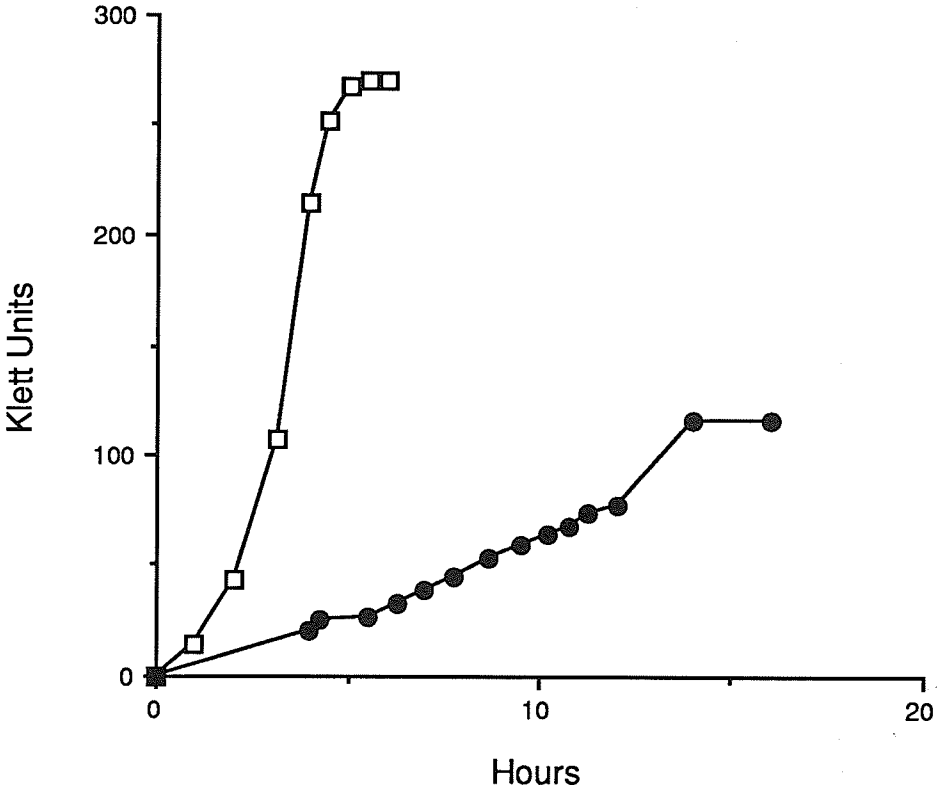


Fig. 1. Growth of the wild-type *S. mutans* BM71 (□) and *ptsI* mutant DC10 (●) in TYE-0.3% glucose broth.

Analysis of the cytoplasmic extracts of BM71 and transformant DC10 by Western blotting with anti-EI antibody from *S. salivarius* revealed that DC10 expressed a truncated protein with an apparent size of 56 kDa. The truncated EI showed less apparent reactivity with the anti-EI antibody than the parent EI as demonstrated by the lower intensity of the 56 kDa band (Fig. 3). This is likely due either to an alteration of the antigenic sites or possibly to the rapid degradation of the truncated protein so that its steady state level was lower than that of the wild-type protein. In comparison with the EI of the parent strain, BM71, which has an apparent size of 67 kD along with the predicted site of integration, we determined that the DC10 EI was missing approximately 150 of a total 577 amino acid residues from the COOH terminus of the protein.

Further study of the mutant DC10 involved examining the cytoplasmic proteins phosphorylated by [^{32}P]-PEP. These experiments revealed that EI and HPr were phosphorylated in the wild-type BM71, as observed previously in our laboratory (3), however, no detectable phosphorylation of the truncated EI or HPr was observed in DC10 cell extracts (Fig. 4), further supporting the concept of a dysfunctional PTS in the mutant. The phosphorylated band running below HPr at 10 kDa

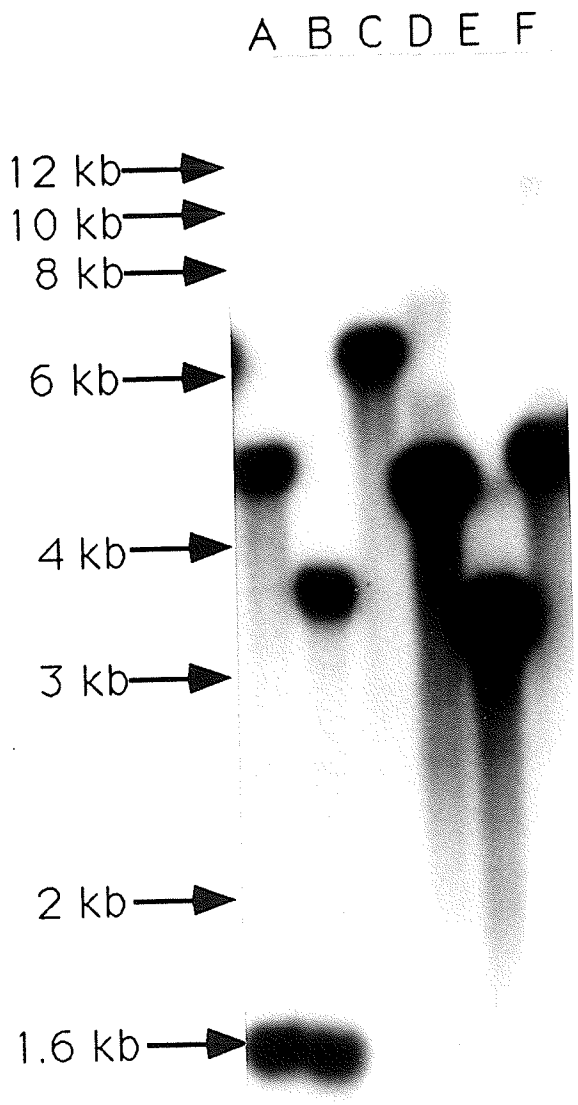


Fig. 2. Southern hybridization of DC10 and BM71 restriction-digested chromosomal DNAs probed with a radiolabelled 3.5 kb *Eco*RI-*Sst*I fragment containing the *ptsI* gene from *S. mutans*. Lanes: A, DC10 *Eco*RI; B, DC10 *Eco*RI-*Sst*I; C, DC10 *Sst*I; D; BM71 *Eco*RI; E, BM71 *Eco*RI-*Sst*I; F, BM71 *Sst*I.

has not been identified, however, it is probably not a PTS protein since it was observed in the mutant.

Detection of the various forms of HPr. Since the possiblity exists that the truncated EI in the phosphorylation experiments was unstable and, therefore, any labelling with [^{32}P]-PEP might be limited and not detected by autoradiography, further confirmation of the absence of PEP-dependent phosphorylation of HPr in the mutant DC10 was undertaken by measuring the concentration of P~(His)-HPr in DC10 and wild-type *S. mutans* BM71. The various forms of HPr can be detected by employing the crossed immunoelectrophoresis technique of Vadeboncoeur et al. (39) with anti-HPr antibody. Normal cells contain four forms of HPr: nonphosphorylated HPr, P~(His)-HPr, P-(Ser)-HPr and the doubly phosphorylated derivative, P~(His)-P-(Ser)-HPr. P~(His)-HPr, generated by phosphotransfer from PEP via EI, and P-(Ser)-HPr, generated by the action of ATP-dependent (Ser)-HPr kinase, migrate to the same position in the first dimension, however, they can be separated by boiling a portion of the cell extract to degrade the phosphoamidate bond of P~(His)-HPr. Since phosphomonoester bonds are heat stable, P-(Ser)-HPr is unaffected by boiling (26), thus, boiling the sample quantitatively converts P~(His)-HPr to free HPr and P~(His)-P-(Ser)-HPr to P-(Ser)-HPr. Comparison of the areas under the peaks of the unboiled and boiled

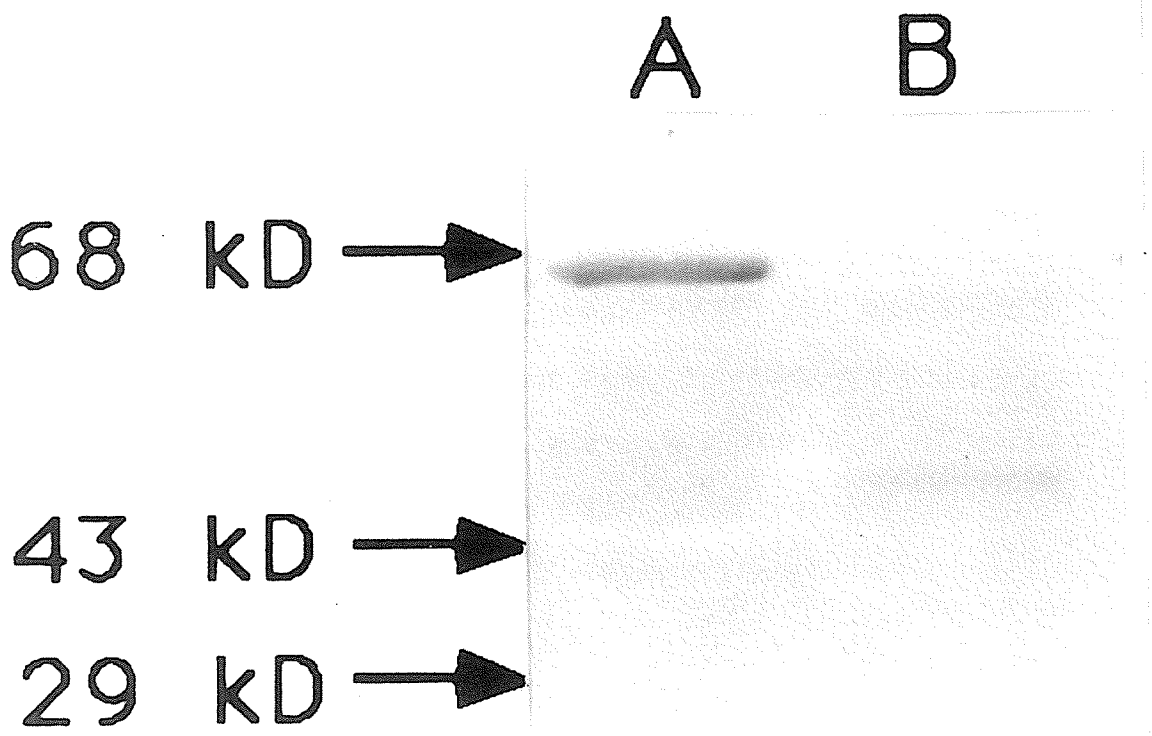


Fig. 3. Western blot of BM71 (lane A) and *ptsI* mutant DC10 (lane B) with anti-EI antibody. Each sample (total protein, 4 μg) was electrophoresed at 200 V for 60 minutes in a 12% polyacrylamide gel and was blotted to polyvinylidene difluoride membranes (Immobilon P, Millipore) for 1 h at 100 V. Immunodetection was performed with a primary antibody against purified *S. salivarius* EI. kD, kilodaltons

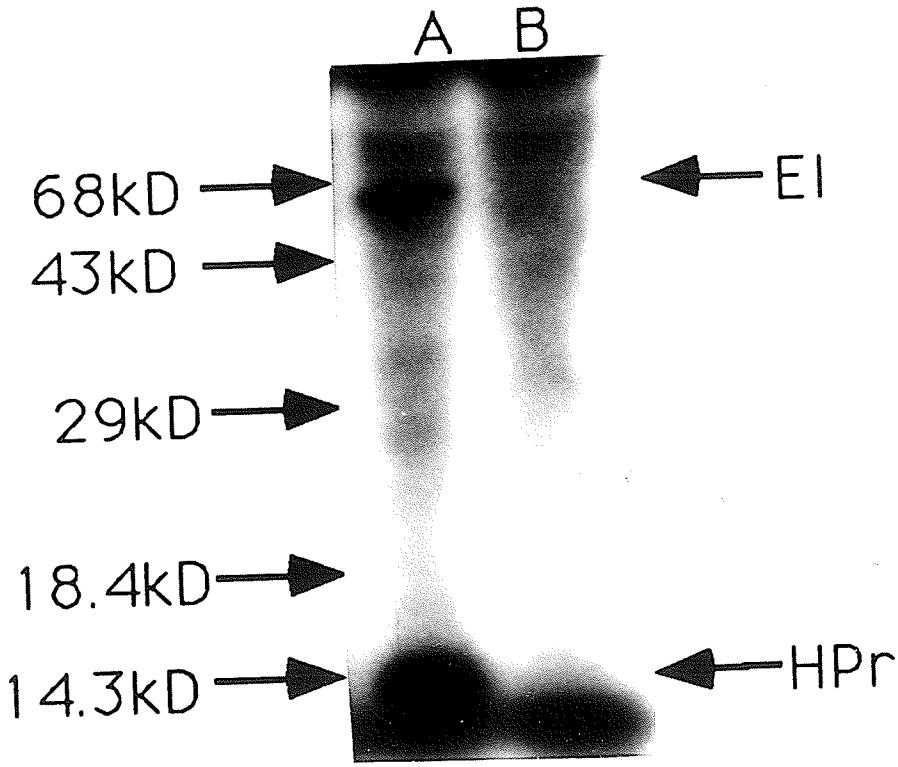


Fig. 4. Polyacrylamide gel electrophoresis of phosphorylated PTS proteins in reactions using [^{32}P]-PEP with cytoplasmic extracts of *S. mutans* parent strain BM71 (lane A) and *ptsI* mutant DC10 (lane B). Each sample (35 µg) was incubated with [^{32}P]-PEP for 5 min and then electrophoresed on a SDS-12% polyacrylamide gel at 200 V for 1 h. The dried gel was placed on X-ray film for 22 h at -70°C. kD, kilodaltons

samples of the same extract permit the estimation of the cellular concentration of the four forms of HPr.

All four forms of HPr could be detected in wild-type strain *S. mutans* BM71 cell extracts, however, only non-phosphorylated HPr and P-(Ser)-HPr could be detected in cell extracts of the mutant DC10 (Fig. 5). Quantitative analysis of the peaks indicated that the total pool of HPr was not the same in both strains with the wild-type strain possessed 98.6 μg of total HPr, mg dry weight of cells^{-1}, while the mutant had only 16.8 μg, mg^{-1}. For the wild-type BM71, the major component was the doubly phosphorylated (P~(His)-P-(Ser)-HPr) fraction (63.0 μg, mg^{-1}), while P~(His)-HPr and free HPr were present at 19.5 and 12.1 μg, mg^{-1}, respectively, and P-(Ser)-HPr at only 4.0 μg, mg^{-1}. In mutant DC10, free HPr was present at 15.0 μg, mg^{-1}, while P-(Ser)-HPr was present at only 1.8 μg, mg^{-1}. The fact that the mutant had no P~(His)-HPr or P~(His)-P-(Ser)-HPr indicated the absence of PEP-dependent phosphorylation typical of PTS activity confirming the [^{32}P]-PEP data. As a consequence, most (90%) of HPr in the mutant was in the form of free HPr.

Characterization of the non-PTS glucose transport system. The kinetics of [^{14}C]-glucose uptake by intact, glucose-grown BM71 and transformant DC10 cells were determined by measuring initial rates of transport at glucose concentrations ranging from 0.01-10 mM. Experiments with

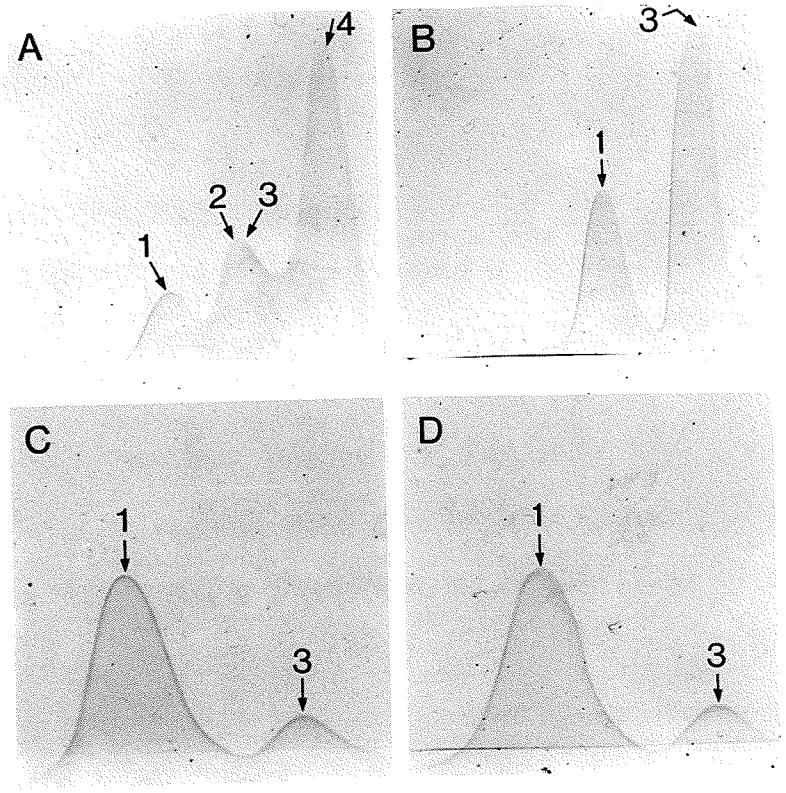


Fig. 5. Crossed immunoelectrophoresis of membrane-free extracts from *S. mutans* BM71 and DC10 cells grown in batch culture. Each sample contained 10 μg of cytoplasmic proteins and was probed with polyclonal anti-HPr rabbbit antibodies, directed against *S. mutans* DR0001. A untreated extract from *S. mutans* BM71; B same as panel A but boiled for 3 minutes prior to electrophoresis; C untreated extract from *S. mutans* DC10; D same as panel C but boiled for 3 minutes prior to electrophoresis. The numbers indicate immunoprecipitate peaks: 1, nonphosphorylated HPr; 2, P~(His)-HPr; 3, P-(Ser)-HPr; 4, P~(His)-P-(Ser)-HPr.

BM71 revealed two apparent K_s values of 17 and 138 μM and Vmax values of 39 and 64 nmol, mg (dry weight) of cells^{-1}, min^{-1}, respectively. Mutant DC10 had only one detectable system with an apparent K_s of 125 μM and a Vmax of 0.87 nmol, mg of dry cells^{-1}, min^{-1}.

Subsequent experiments were directed at determining the intracellular donor employed to phosphorylate glucose in mutant strain DC10 following transport. For this, glucose-grown cells were permeabilized with toluene and incubated with glucose and either ATP or PEP. As shown in Fig. 6, PEP-dependent phosphorylation of glucose was negligible in the mutant DC10 but predominant in the parent strain. Substantial glucose-6-P was formed in the presence of ATP in the mutant and to a lesser extent in the parent. The experiment was repeated with cells pre-incubated with 2-deoxyglucose to deplete the intracellular PEP pool and the level of phosphorylation by PEP and ATP was similar to that seen with non-depleted cells.

To determine the specificity of non-PTS glucose transport, we incubated glucose-grown cells of wild-type *S. mutans* BM71 and mutant DC10 with [^{14}C]-glucose in the presence of a 100-fold excess of various unlabelled sugars. This competition study (Fig. 7A) demonstrated that BM71 was subject to significant inhibition by mannose, a PTS sugar transported at rates similar to those of glucose (41), and moderately by

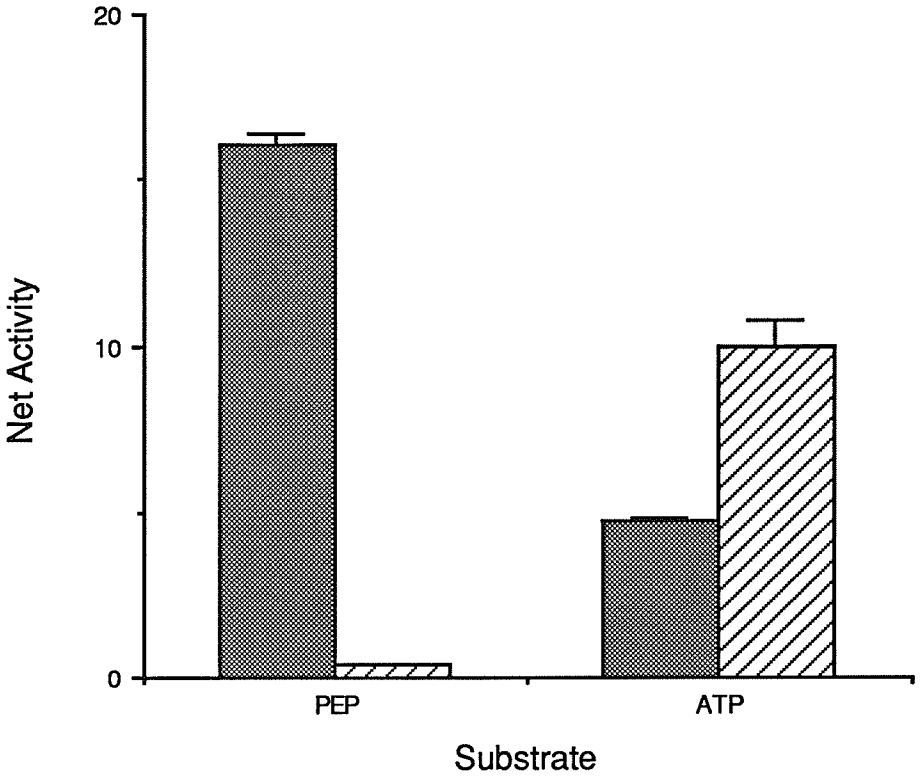


Fig. 6. Glucose phosphorylation by decryptified cells of *S. mutans* BM71 (▩) and *ptsI* mutant DC10 (▨) incubated with PEP or ATP. Net activity is expressed as nanomoles glucose phosphorylated, milligram (dry weight) of cells^{-1}, min^{-1}.

arabinose and fructose. The mutant, however, had a high specificity for glucose, and little inhibition was observed with any of the substrates tested (Fig. 7B). The best competitor for glucose transport via the system was the glucose analog, 2-deoxyglucose, which decreased uptake by nearly 40%. Surprisingly, the glucose analog α-methylglucoside inhibited glucose uptake by only 10%. Lactose, galactose, sucrose, raffinose and the sugar alcohol sorbitol, were also tested, but failed to demonstrate significant inhibition with either the wild-type or mutant strain.

Discussion

Early studies with decryptified cells of *S. mutans* Ingbritt grown in continuous culture showed that the glucose PTS was repressed under a variety of conditions, including growth at low pH (15), at high growth rates, (9) growth with excess glucose (9), and following a transition to growth on sucrose (8). Interestingly, the rates of glucose uptake and glycolysis were reduced, but not to the same degree, suggesting that during PTS repression *S. mutans* employed an alternative, non-PTS glucose transport system (13). Later studies demonstrated that this repression was associated with the synthesis of membrane-bound EII for glucose (EII^{glc}) and mannose (EII^{man})(16,40,41). Of particular interest was the

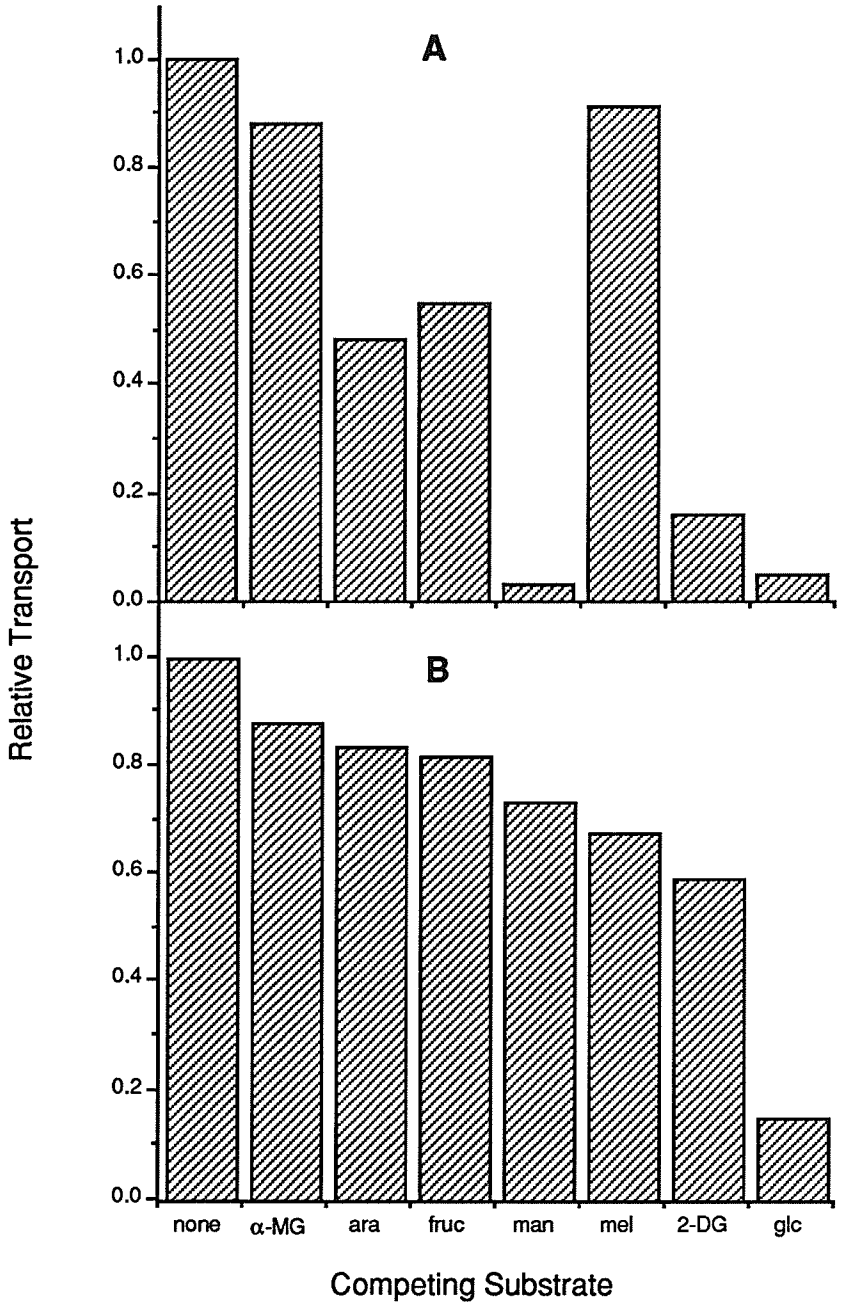


Fig. 7. Sugar competition for [^{14}C]-glucose transport with intact cells of the wild-type *S. mutans* BM71 (A) and the *ptsI* mutant DC10 (B). Competing sugars were used at a concentration 100-fold in excess of that of the labelled glucose. Abbreviations: α-MG, α-methlyglucoside; ara, arabinose; fruc, fructose; man, mannose; mel, melibiose; 2-DG, 2-deoxyglucose; glc, glucose.

concentration-dependent repression of the two activities by glucose itself, with a 40-fold reduction observed with an increase from 3.6 to 271 mM glucose in the growth medium (16). Growth at pH values below 8.0 also reduced the synthesis of EII^{glc} and EII^{man} and cells at pH 5.0 were completely devoid of activity (40). On the other hand, changes in the medium glucose concentration (2.7 to 304 mM), pH (8.0 to 5.0) and growth rate (0.1 to 1.0 h^{-1}) resulted in only four-fold changes in the cellular concentrations of the general PTS proteins HPr and EI (16,40,41).

Prior to the present study, evidence for the hypothetical non-PTS glucose transport system was indirect. Early results (18) suggested that glucose uptake via this system was coupled to proton motive force (PMF), however, uptake studies with 6-deoxyglucose (6) questioned this model. This latter conclusion was supported by measurements of the components of the transmembrane electrochemical proton gradients in cells of *S. mutans* and *S. sobrinus* grown in continuous culture under a variety of conditions (14). Proton motive force values were normally low (< 70 mV) under all conditions, including those that repress the PTS, suggesting that insufficient proton motive force was generated to support significant sugar transport.

More substantial evidence for the non-PTS system was obtained in experiments with *S. mutans* DR0001 and a PTS-

defective mutant, strain DR0001/6, of the organism grown in chemostats at growth rates between 0.04 and 0.6 h^{-1} (18). The wild-type strain possessed two glucose transport processes, one with K_s values for glucose ranging from 6.7 to 8.0 μM and a second with values ranging from 57 to 125 μM, with the high affinity system shown to be the glucose PTS. The mutant, on the other hand, had only the lower-affinity system (62 to 132 μM). More recently (4), membrane vesicles of *S. mutans* Ingbritt, prepared from cells grown in continuous culture under optimum and repressed PTS conditions and devoid of cytoplasmic components and PTS activity exhibited glucose counterflow, indicating the presence of a constitutive transmembrane carrier able to recognize glucose.

Clearly, the characterization of a non-PTS glucose transport system in *S. mutans* requires inactivation of the PTS. Although a mutant of *S. mutans* defective in glucose PTS transport activity has been used in the past, the nature of the mutation was unknown and uptake may have occurred via other PTS permeases (18). The construction of strain DC10 circumvented these problems by eliminating glucose transport and phosphorylation via the PTS since all PTS transport requires a functional EI. The mutant is missing approximately 150 amino acid residues from the COOH terminus of the protein, and while the putative phosphorylation site was present on the truncated protein (3), it was unable to phosphorylate HPr at the histidyl residue as demonstrated by

the [^{32}P]-PEP phosphorylation and immunoelectrophoresis experiments. These results are in agreement with the results obtained with a truncated form of EI from *S. typhimurium* which was not phosphorylated by PEP, although it contained the active His^{15} site (24). Thus, it appears that the COOH-terminal domain of the enzyme is a key factor in both catalysis and regulation.

It has been previously reported that PTS-defective strains of *S. typhimurium* (31) and *E. coli* (32) can accumulate mutations that uncouple transport from phosphorylation in EIIs allowing them to transport glucose via facilitated diffusion. It is unlikely that the ability of *S. mutans* DC10 to grow on glucose is a result of mutations altering the specificity of an EII or uncoupling of an EII from phosphorylation since mutations of this nature have been shown to be selected only at limiting glucose concentrations under the strong selective pressures presented by growth in continuous culture (31). The growth characteristics of strain DC10 with glucose were also stable regardless of whether the organism was cultured with glucose or raffinose, suggesting that the selective pressure of repeated growth on glucose was not required to generate a glucose fermenting phenotype.

Data obtained recently with membrane vesicles of *S. mutans* Ingbritt have indicated that the intracellular product of a

non-PTS glucose transport process would be free glucose (4). The same study demonstrated that ATP-dependent phosphorylation of glucose was predominant in PTS-repressed cells compared with PTS-optimal cells, indicating that the intracellular glucose is phosphorylated by ATP and a glucokinase. The results obtained with *ptsI* mutant DC10 in this study support this, as demonstrated by the phosphorylation of glucose by ATP, but not by PEP in decryptified cells (Fig. 6). Furthermore, the K_s value for glucose of 125 μM obtained with mutant DC10 is similar to that observed with the PTS-defective mutant of *S. mutans* DR0001 reported in a previous study, i.e. 57 to 133 μM (18).

The kinetic studies with the mutant strain revealed that the maximum velocity of glucose uptake was 0.87 nmol, mg (dry weight) of $cells^{-1}$, min^{-1} compared with a V_{max} for wild-type strain BM71 of 64 nmol, mg (dry weight) of $cells^{-1}$, min^{-1} for total glucose transport. The latter value, of course, represents the contribution of both PTS and non-PTS glucose transport processes. This large difference in the maximum rates of glucose transport is difficult to reconcile on the basis of the K_s value for glucose (125 μM) since growing DC10 cells would normally be exposed to glucose concentrations well in excess of this concentration. The data seem to suggest that the PTS has a significant role in regulating the expression or activity of this system. The low growth rate of the mutant on glucose (Fig. 1) tends to support this

hypothesis. Thus, it appears that the non-PTS transport process is a secondary system supplementing the PTS and, from the earlier continuous culture data (12,13), is essential for the organism under conditions, of low pH, high glucose concentrations, and high growth rates.

An interesting question is whether the non-PTS mechanism is related to the MSM system recently discovered by Russell and coworkers in *S. mutans* (33). The suggestion has been made that glucose may be transported via the MSM system because glucose was able to inhibit uptake of radiolabelled melibiose when present in a 100-fold excess concentration (35). These experiments did not consider the possibility that glucose was acting as a catabolic repressor of uptake via the *Msm* system, a phenomenon that has recently been demonstrated in our laboratory (5). The fact that the MSM system must be induced by one of its substrates (33) also supports the hypothesis that the glucose uptake observed in DC10 occurred via an independent mechanism since the cells were grown in the absence of an MSM system inducer and were also unable to transport radiolabelled raffinose (data not shown). Also, raffinose and melibiose were not efficient inhibitors of glucose transport by DC10 (Fig. 7B) suggesting that glucose entry occurred via an alternate route.

The high degree of specificity of the non-PTS system for glucose is amply demonstrated by the competition experiment

depicted in Fig. 7B. The observation that 2-deoxyglucose and, particularly, α-methylglucoside, are not effective inhibitors of glucose transport at 100-fold excess concentrations suggests that the permease is probably restricted to glucose uptake alone. The 40% inhibition by 2-deoxyglucose, a mannose analog, probably reflects the close stereospecificity of the mannose and glucose molecules, a factor evident in the competition experiment with wild-type BM71 (Fig. 7A). Mannose and glucose exhibit similar uptake rates via the PTS in cells of *S. mutans* and other oral streptococci (16,40,41).

These results confirm the existence of a non-PTS glucose transport system in *S. mutans* possessing an affinity for glucose lower than that of the glucose PTS. The mutant strain *S. mutans* DC10 will be utilized for further study of the regulation of the two systems and should give insight into the various ways that *S. mutans* can transport other non-PTS substrates. Results obtained recently with this strain and the parent BM71 (5) demonstrated that the PTS controls uptake of sugars via the MSM system. This mutant will undoubtably be useful in the study of other non-PTS sugar transport processes, as well as the role of some PTS components, such as HPr, in cellular metabolism.

Acknowledgement

This research was supported by an operating grant from the Medical Research Council of Canada (MT-3546).

References

1. Birnboim, H. C. 1979. A rapid alkaline extraction method for isolation of plasmid DNA. Methods. Enzymol. **100**:243-255.
2. Bourassa, S., L. Gauthier, R. Giguere, and C. Vadeboncoeur. 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. Oral Microbiol. Immunol. **5**: 288-297.
3. Boyd, D. A., D. G. Cvitkovitch, and I. R. Hamilton. 1994. Sequence and expression of the genes for HPr (*ptsH*) and Enzyme I (*ptsI*) of the phosphoenolpyruvate-dependent phosphotransferase transport system from *Streptococcus mutans*. Infect. Immun. **62**: 1156-1165.
4. Buckley, N. D., and I. R. Hamilton. 1994. Vesicles prepared from *Streptococcus mutans* demonstrate the presence of a second glucose transport system. Microbiology **140**: 2639-2648.
5. Cvitkovitch, D. G., D. A. Boyd, and I. R. Hamilton. 1995. Inhibition of sugar uptake via the multiple sugar metabolism operon by the phosphoenolpyruvate-dependent sugar phosphotransferase transport system of *Streptococcus mutans*, *In*: J. J. Ferretti (ed.), Genetics of streptococci, enterococci and lactococci. In press. National Bureau Standards, Washington, D. C.
6. Dashper, S. G., and E. C. Reynolds. 1990. Characterization of transmembrane movement of glucose

and glucose analogs in *Streptococcus mutans* Ingbritt. *J. Bacteriol.* **172**: 556-563.

7 Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**: 6127-6145.

8. Ellwood, D. C., and I. R. Hamilton. 1982. Properties of *Streptococcus mutans* Ingbritt growing in limiting sucrose in a chemostat: repression of the phosphoenolpyruvate phosphotransferase transport system. Infect. Immun. **36**: 576-581.

9. Ellwood, D. C., P. J. Phipps, and I. R. Hamilton. 1979. Effect of growth rate and glucose concentration on the activity of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans* Ingbritt grown in continuous culture. Infect. Immun. **23**: 224-231.

10. Gagnon, G., C. Vadeboncoeur, R. C. Levesque, and M. Frenette. 1992, Cloning, sequencing and expression in *Escherichia coli* of the *ptsI* gene encoding enzyme I of the phosphoenolpyruvate phosphotransferase transport system from *Streptococcus salivarius*. Gene **121**: 71-78.

11. Hamada, S., and H. D. Slade. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331-384.

12. Hamilton, I. R. 1984. Growth of the oral "pathogen", *Streptococcus mutans*, in continuous culture reveals two glucose transport systems, p. 58-71. *In* A. C. R. Dean, D. C. Ellwood and C. G. T. Evans (eds), Continuous culture 8: biotechnology, medicine and the environment. Ellis Harwood, Chichester, England.

13. Hamilton, I. R. 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94-133. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.

14. Hamilton, I. R. 1990. Maintenance of proton motive force by *Streptococcus mutans* and *Streptococcus sobrinus* during growth in continuous culture. Oral Microbiol. Immunol. **5**: 280-287.

15. Hamilton, I. R., and D. C. Ellwood. 1978. Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various pH values in a chemostat. Infect. Immun. **19**: 434-442.

16. Hamilton, I. R., L. Gauthier, B. Desjardins, and C. Vadeboncoeur. 1989. Concentration-dependent repression of the soluble and membrane components of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus mutans* by glucose. J. Bacteriol. **171**: 2942-2948.

17. Hamilton, I., R., and G. C. Y. Lo. 1978. Co-induction of ß-galactosidase and the lactose P-enolpyruvate phosphotransferase system in *Streptococcus mutans* and *Streptococcus salivarius*. J. Bacteriol. **136**: 900-908.

18. Hamilton, I. R., and E. J. St. Martin. 1982. Evidence for the involvement of proton-motive force in the transport of glucose by a mutant of *Streptococcus mutans* strain DR0001 defective in glucose phosphoenolpyruvate phosphotransferase activity. Infect. Immun. **36**: 567-575.

19. Hamilton, I. R., and G. Svensater. 1991. Sorbitol inhibition of glucose metabolism by *Streptococcus sanguis* 160. Oral Microbiol. Immunol. **6**:151-159.14.

20. Honeyman, A. L., and R. Curtiss III. 1992. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* mannitol-phosphate dehydrogenase gene and the mannitol-specific factor III gene of the phosphoenolpyruvate phosphotransferase system. Infect. Immun. **60**: 3369-3375.

21. Honeyman, A. L., and R. Curtiss III. 1993. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* lactose-phosphate Enzyme II (*lacE*)

gene of the PTS and the phospho-ß-galactosidase (*lacG*) gene. J. Gen. Microbiol. **139**: 2685-2694.

22. Keevil, C. W., A. S. McDermid, P. D. Marsh and D. C. Ellwood. 1986. Protonmotive force driven 6-deoxyglucose uptake by the oral pathogen, *Streptococcus mutans* Ingbritt. Arch. Microbiol. **146**: 118-124.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**: 680-685.
24. LiCalsi, C., T. S. Crocenzi, E. Freire and S. Roseman. 1991. Sugar transport by the bacterial phosphotransferase system. Structural and thermodynamic domains of enzyme I of *Salmonella typhimurium*. J. Biol. Chem. **266**: 19519-19527.
25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
26. Martensen, T.M. 1984. Chemical properties, isolation and analysis of O-phosphates in proteins. Methods Enzymol. **107**: 3-23.
27. Mattoo, R. L., and E. B. Waygood. 1983. An enzymatic method for [^{32}P]phosphoenolpyruvate synthesis. Anal. Biochem. **128**: 245-249.
28. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
29. Postma, P. W, J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **57**: 543-594.
30. Rosey, E. L., and G. C. Stewart. 1992. Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans* . J. Bacteriol. **174**: 6159-6170.
31. Ruijter, G. J. G., P. W. Postma, and K. van Dam. 1990. Adaptation of *Salmonella typhimurium* mutants containing

uncoupled enzyme IIglc to glucose-limited conditions. J. Bacteriol. **172**:4783-4789.

32. Ruijter, G. J. G., G. van Meurs, M. A. Verwey, P. W. Postma, and K. van Dam. 1992. Analysis of mutations that uncouple transport from phosphorylation in enzyme II^{glc} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system. J. Bacteriol. **174**:2843-2850.
33. Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao, and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J. Biol. Chem. **267**: 4631-4637.
34. Saier, M. H., Jr., and J. Reizer. 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. **174**: 1433-1438.
35. Sato, Y., F. Poy, G. R. Jacobson, and H. K. Kuramitsu. 1989. Characterization and sequence analysis of the *scrA* gene encoding enzyme II^{Scr} of the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system. J. Bacteriol. **171**: 263-271.
36. Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti. 1993. Transport of sugars, including sucrose, by the *msm* transport system of *Streptococcus mutans*. J. Dent. Res. **267**: 4631-4637.
37. Thompson, J. 1987. Sugar transport in the lactic acid bacteria, p. 13-38, *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.
38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.

39. Vadeboncoeur, C., D. Brochu, and J. Reizer. 1991. Quantitative determination of the intracellular concentration of the various forms of HPr, a phosphocarrier protein of the phosphoenolpyruvate: sugar phosphotransferase system in growing cells of oral streptococci. Anal. Biochem. **196**: 24-30.

40. Vadeboncoeur, C., S. St. Martin, D. Brochu, and I. R. Hamilton. 1991. Effect of growth rate and pH on intracellular levels and activities of the components of the phosphoenolpyruvate: sugar phosphotransferase system in *Streptococcus mutans* Ingbritt. Infect. Immun. **59**: 900-906.

41. Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson, and I. R. Hamilton. 1987. Effect of growth conditions on levels of components of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus sobrinus* grown in continuous culture. J. Bacteriol. **169**: 5686-5691.

42. van Houte, J. 1986. Bacterial specificity in the etiology of caries. Int. Dent. J. **30**: 305-326.

Chapter 6

Regulation of Sugar Transport via the Multiple Sugar Metabolism Operon of *Streptococcus mutans* by the Phosphoenolpyruvate Phosphotransferase System

Dennis G. Cvitkovitch, David A. Boyd and Ian R. Hamilton*

Department of Oral Biology
Faculty of Dentistry,
University of Manitoba,
Winnipeg, Canada R3E 0W2

Submitted for publication to the Journal of Bacteriology

Abstract

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is the primary high affinity sugar transport system in *Streptococcus mutans*, the principal etiological agent of dental caries. In other bacteria it has been demonstrated that the PTS is also a central regulator of sugar transport, being involved in controlling uptake via a variety of non-PTS transport processes. Strains of *S. mutans* are also known to possess the multiple sugar metabolism (*msm*) operon, a non-PTS transport system that is utilized for the transport of a number of sugars, including raffinose and melibiose. Previous work has demonstrated that wild-type *S. mutans* BM71 exhibited diauxic growth when grown in a mixture of glucose and melibiose with glucose used preferentially, suggesting catabolite repression of the *msm* operon by the PTS substrate glucose. To further study the relationship of the *msm* and the PTS, a *ptsI* defective mutant (DC10) was constructed. When grown on raffinose, the mutant exhibited a doubling time of 150 ± 4 min. compared to the parent strain which doubled in 76 ± 4 min. The rates of [^{3}H]-raffinose transport and acid production with raffinose as a substrate were also decreased by 90% and 93% respectively, further suggesting PTS control of the *msm* operon. Inhibition of [^{3}H]-raffinose uptake was also observed in both BM71 and DC10 with increasing concentrations of glucose and the non-metabolizable glucose analogs, α-methyl glucoside and 2-

deoxyglucose. In vitro phosphorylation experiments with [^{32}P]-PEP and extracts of *S. mutans* BM71 prepared from cells grown on glucose, raffinose and melibiose revealed a 59 kDa phosphorylated protein in extracts of the cells grown on the *msm* substrates, but not with the PTS substrate glucose. Since phosphorylation occurred via the PTS, this suggested that the 59 kDa phospho-protein is a PTS-dependent regulator of the *msm* operon. Furthermore, [^{32}P]-PEP phosphorylation experiments with cell extracts from *E. coli* clones harboring the *msm* operon, or the *gtfA* gene of the operon, failed to produce this 59 kDa protein, suggesting that the gene encoding this potential *msm* regulator is encoded outside the *msm* operon.

Introduction

Streptococcus mutans is an important etiological agent in the initiation and progression of dental caries (4,16,21). This organism is capable of generating acid end-products during carbohydrate metabolism which contributes to the demineralization of tooth enamel. The primary sugar transport system utilized by *S. mutans* is the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). The PTS is a group translocation process which derives its energy from PEP and catalyzes the sequential phosphorylation of the general, non-sugar specific proteins Enzyme I (EI) and HPr, required for the uptake of all PTS substrates, and subsequently a sugar-specific, membrane-bound Enzyme II complex (EII), which catalyzes the transport and phosphorylation of the specific carbohydrate (26). The EII complex consists of three functional domains: (a) IIA domain (also referred to as Enzyme III) possessing the first phosphorylation site, (b) IIB domain bears the second phosphorylation site, and (c) IIC domain forms the transmembrane channel and provides the sugar-binding site (34). In *S. mutans*, the PTS is known to transport glucose, mannitol, sorbitol, lactose, sucrose, mannose, fructose, maltose, N-acetylglucosamine, and trehalose (19,20,30,43).

In addition to its role in sugar transport, the PTS is also involved in the regulation of non-PTS transport and

metabolism in both Gram-positive and Gram-negative bacteria either by direct phosphorylation of target systems or by protein-protein interactions of target systems with components of the PTS (26,27). We were interested in non-PTS sugar transport processes in *S. mutans* since early results had indicated an alternative mechanism for the uptake of glucose in the organism (17). In order to study such transport independently of the phosphotransferase system, we constructed a mutant of *S. mutans* BM71 defective in the gene for Enzyme I, *ptsI*, of the PTS (7). This mutant, designated DC10, was unable to phosphorylate EI and HPr with [^{32}P]-PEP and could not grow or transport PTS sugars, except glucose, indicating the presence of non-PTS glucose transport system in *S. mutans* (7).

The multiple sugar metabolism operon (*msm*) in *S. mutans* is a non-PTS sugar uptake system involved in the transport and metabolism of several sugars, including melibiose, raffinose and isomaltosaccharides (31). The operon shares homology with the osmotic shock-sensitive transport systems in enteric bacteria (31) and contains eight characterized genes including: *aga* (α-galactosidase)(1), *msmE* (sugar-binding lipoprotein)(37), *msmF* and *msmG* (membrane-bound proteins), *gtfA* (sucrose phosphorylase)(12,33), *msmK* (an ATP-binding protein), and *dexB* (dextran glucosidase)(6,32). These genes appear to be under the control of a positive regulator encoded by *msmR*.

We have previously demonstrated diauxic growth of *S. mutans* BM71 on a mixture of glucose and melibiose with inhibition of melibiose utilization by glucose suggesting that the PTS may be involved in repression of the *msm* operon (8). In the present study, we examine the effects of glucose and glucose analogs on raffinose transport in BM71 and the *ptsI* mutant, DC10, demonstrating the direct effect of the PTS on sugar transport via the *msm* system. In vitro phosphorylation experiments with [^{32}P]-PEP and cell extracts prepared from cells of BM71 grown on PTS and *msm* substrates provide evidence for a PTS-dependent phosphoprotein that is specific for transport via the *msm* system, but not present during uptake via the PTS.

Materials and Methods

Bacterial strains, plasmids and growth conditions. The *S. mutans* and *E. coli* strains, and plasmids used in this study are listed in Table 1. Streptococci were grown in TYE broth (1% tryptone, 0.5% yeast extract, 17 mM K_2PO_4) with the addition of the appropriate sugar at 0.25%. *S. mutans* strain DC10 was maintained on TYE-raffinose plates containing 10 µg/ml erythromycin (7). *E. coli* strains were maintained on LB plates (23) supplemented when needed with the appropriate

Table 1

Bacterial strains and plasmids

Strains	Relevant Phenotype	Reference or Source
S. mutans		
BM71	Wild-type parental strain	G. Bowden
DC10	PTS^-	7
E. coli		
DH5α	Cloning host	Gibco/BRL
DH120	Expresses *msm* operon	This study
DHGTA	Expresses *gtfA*	This study
CHE11	PTS^-	15
CHE-GTA	PTS^- expresses *gtfA*	This study
Plasmids		
pSUCR1	Contains *gtfA*	6
pVA891	Cm^r Em^r in *E. coli*	22
pSF120	Contains *msm* operon	41
pDCGT	Contains *gtfA*	This study

antibiotics at the following concentrations: 100 μg/ml ampicillin, 500 μg/ml erythromycin, 20 μg/ml chloramphenicol.

Molecular cloning and bacterial transformations. Agarose gel electrophoresis, Southern hybridizations, ligations and transformations were carried out as previously described (2). Plasmid pDC-GT was constructed by subcloning a 2.5 kb *Eco*RI fragment containing the entire *gtfA* gene (product is sucrose phosphorylase [SucP]) from plasmid pSUCR1 (generously provided by Dr. R. Burne, University of Rochester) into pVA891. Recombinants were selected for SP activity on M9 minimal media (23) containing sucrose as a sole source of carbon. *E. coli* DH5α was transformed with plasmid DNA by electroporation (11). *S. mutans ptsI* mutant DC10 was constructed as previously described (7).

Growth and acid production. Growth rates by the test strains were determined anaerobically by inoculating 5 ml TYE media, containing either 0.3% raffinose or glucose with 0.2 ml of an overnight culture grown with the same sugar at 0.1%. Growth was monitored in a Klett Summerson colorimeter with a red filter. The rate of acid production (glycolytic rate) from glucose was measured with washed intact cells incubated in a pH stat at pH 7.0 as previously described (18). Glycolytic rates are expressed as nanomoles acid neutralized, mg (dry weight) $cells^{-1}$, min^{-1}.

Sugar transport. Sugar transport was measured using mid-log phase cells harvested by centrifugation at 10,000 x g for 15 min and washed three times in 50 mM Na/K phosphate buffer (pH 7.0)(PB). The cells (0.5 mg [dry weight]$^{-1}$, ml) were incubated in buffer with 1 mM [^{14}C]-glucose (9 μCi, μmole^{-1}) or 250 μM [^{3}H]-raffinose (450 μCi, μmole^{-1}) in a final volume of 5.0 ml at 37°C. Samples (0.5 ml) were removed periodically over a 2.0 min period, filtered through 0.45 μm HA filters (Millipore, Bedford, MA) and washed three times with 1 ml of PB equilibrated at 37°C. The dried filters were then counted in 5.0 ml Aquasol (NEN Research products, Montreal, Quebec) in a liquid scintillation counter. The effect of glucose, 2-deoxyglucose (2-DG) or α-methylglucoside (α-MG) on raffinose uptake was determined by adding these PTS substrates at various concentrations just prior to the addition of the radiolabelled raffinose. Transport rates are expressed as nanomoles sugar transported, mg (dry weight) cells^{-1}, min^{-1}.

[^{32}P]-PEP phosphorylation. Phosphorylation of PTS proteins with [^{32}P]-PEP and subsequent autoradiography was performed as previously described (2). *S. mutans* cell extracts, membrane and cytoplasmic fractions were prepared by alumina grinding followed by ultracentrifugation at 200,000 x g for 16 h (42), while *E.coli* cell extracts were prepared by passage of a thick cell suspension through a French pressure cell (2). Before ultracentrifugation, all extracts were

dialyzed overnight against two changes of 10 mM potassium phosphate buffer [pH 7.5] containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 μM pepstatin A and 0.1 mM phenylmethlysulfonyl fluoride.

[^{32}P]-PEP was synthesized by the method of Mattoo and Waygood (24) with purified carboxykinase from *E. coli*, kindly provided by Dr. H. Goldie, University of Saskatchewan, Saskatoon. Samples were incubated at room temperature in a 25 μl reaction mixture containing 0.1 mM [^{32}P]-PEP (180 μCi, μmole^{-1}), 5 mM $MgCl_2$, 12.5 mM NaF and 10 mM HEPES (pH 7.5). The reactions were stopped after 5 min by the addition of 10 μl of 188 mM Tris-HCl (pH 8.0), containing 6% (w/v) SDS, 30% (v/v) glycerol, 6% (v/v) 2-mercaptoethanol, and 0.005% bromophenol blue. Parallel reactions were also performed with 0.1 mM [γ^{32}P]-ATP (1800 μCi, μmole^{-1}) replacing the [^{32}P]-PEP. Samples were loaded without boiling onto 10% or 12.5% 1.5 mm thick SDS-polyacrylamide gels and electrophoresed for 1 h at 200 V in a BioRad Mini Protean II apparatus (BioRad Laboratories, Richmond, CA). The dried gels were placed on X-ray film with an intensifying screen at -70°C for 22 h.

Chemicals and enzymes. Restriction enzymes, T4 DNA ligase, DNA and protein molecular weight standards were from GIBCO/BRL and were used as directed by the supplier. [^{14}C]-glucose, [^{3}H]-raffinose and [γ^{32}P]-ATP were obtained from

Dupont New England Nuclear. All other chemicals were reagent grade.

Results

Growth and sugar transport. The *ptsI* mutant DC10, constructed from *S. mutans* BM71, has been shown to be defective in the utilization of all PTS sugars except glucose, lacking the ability for phosphoryl transfer from PEP to the incoming PTS sugar via the various components of the system (7). However, since the mutant was able to utilize glucose and the *msm* substrates, raffinose and melibiose, it was anticipated that growth with raffinose would be similar to that of the parental organism *S. mutans* BM71 since the *msm* system and the PTS were believed to be independent transport systems. In order to confirm this, we first examined the growth of BM71 and DC10 with glucose and raffinose as carbon sources. As seen in Fig. 1, the mutant grew more slowly on glucose than BM71 as expected (7), however, growth on raffinose was also significantly slower than the parental strain. Calculation of the doubling times (t_D) for the two strains revealed that the t_D values for DC10 were 4 and 2-fold higher with glucose and raffinose, respectively, as the carbon source compared to that of the parental strain (Table 2). In addition, transport activity exhibited by intact washed cells of DC10 with glucose and raffinose was only 1% and 13% that of BM71, respectively, and similar differences

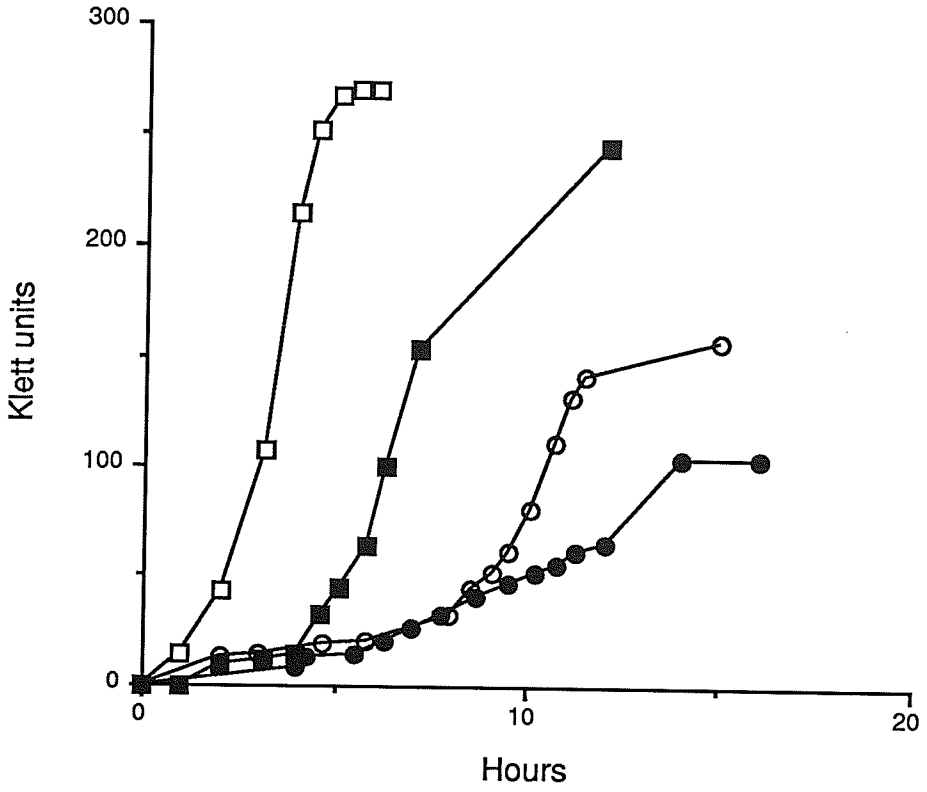


Fig. 1. Growth of the wild-type *S. mutans* BM71 and the *ptsI* mutant, DC10, on glucose and raffinose. BM71-glucose □; BM71-raffinose ■; DC10-glucose ●; and DC10-raffinose ○. Cells were grown in TYE broth with 0.3% sugar.

Table 2

Growth and sugar transport rates generated by washed cells of the wild-type *S. mutans* BM71 and the *ptsI* mutant, DC10

	BM71	DC10
Growth rate		
glucose	50 ± 5 [a]	199 ± 5 [a]
raffinose	76 ± 4	150 ± 4
Sugar transport		
glucose	115 ± 12 [b]	1.4 ± 0.3 [b]
raffinose	9 ± 1.5	1.2 ± 0.1
Glycolytic rate		
glucose	418 ± 33 [c]	56 ± 8 [c]
raffinose	475 ± 33	35 ± 5

[a] Growth measured as the doubling time in TYE media with 0.3% sugar.
[b] Nanomoles sugar transported, mg dry cells^{-1}, min^{-1}.
[c] Nanomoles acid neutralized, mg dry cells^{-1}, min^{-1} at pH 7.0.

were also observed for the glycolytic rates with the two substrates (Table 2). These substantial differences in growth, transport and acid production between the wild-type strain and the mutant with raffinose as the substrate indicated an impairment of *msm* transport and metabolism suggesting that the PTS was involved in the regulation of the *msm* system. We undertook to examine this phenomenon in more detail.

Effect of glucose on *msm* transport. Since the PTS is the primary sugar transport system in *S. mutans* (17), it was hypothesized that an increase in PTS transport would likely lead to inhibition of uptake via any non-PTS secondary system, including the *msm* system. To study the effects of PTS substrates on raffinose transport, glucose at concentrations between 0.1 and 1 mM were added to reaction mixtures containing [^{3}H]-raffinose and washed raffinose-grown cells of the parental strain BM71 and the mutant, DC10. As illustrated in Fig. 2, BM71 typically demonstrated a two to three-fold higher rate of raffinose transport compared to the *ptsI* mutant and glucose concentrations as low as 0.1 mM had a pronounced inhibitory effect on its raffinose uptake. Conversely, similar transport by the mutant was essentially unaffected by glucose at concentrations as high as 1 mM. Similar effects were observed with the glucose analogs, 2-deoxyglucose and α-methylglucoside in both the parent and mutant.

[^{32}P]-PEP phosphorylation. Although catabolite repression in Gram-positive bacteria is poorly understood, it has been demonstrated that the phosphotransferase system can regulate the uptake of non-PTS sugars via PTS-mediated phosphorylation of specific components of the non-PTS system (27-29). As a consequence, we carried out phosphorylation reactions with crude extracts, as well as membrane and cytoplasmic fractions obtained from the wild-type strain and the *ptsI* mutant grown on raffinose and glucose, and incubated with [^{32}P]-PEP. The proteins were separated by SDS-PAGE and those phosphorylated by [^{32}P]-PEP were identified by autoradiography. As seen in Fig. 3, reactions with crude extracts derived from cells of *S. mutans* BM71 revealed a phosphoprotein formed with extracts from raffinose-grown, but not glucose-grown cells that migrated at a slightly lower molecular weight than Enzyme I (68 kDa). The phosphorylation of this 59 kDa protein was dependent on a functional PTS since no detectable phosphoproteins were observed in extracts prepared from raffinose or melibiose-grown cells of the *ptsI* mutant DC10 (data not shown). Parallel experiments with [γ^{32}P]-ATP showed no difference in the profiles of glucose, raffinose or melibiose-grown cells (data not shown) indicating that the protein is phosphorylated via PEP and not ATP.

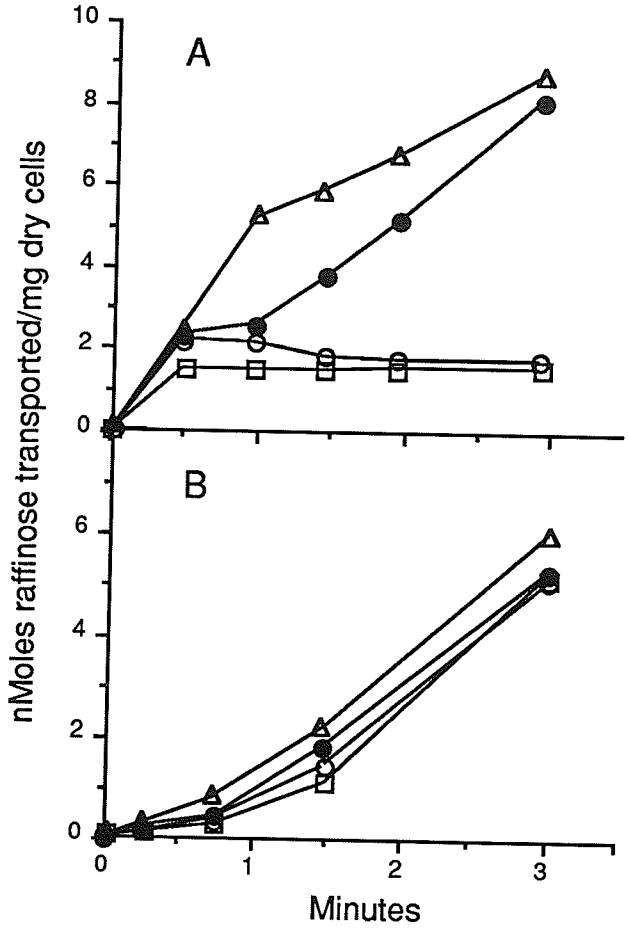


Fig. 2. The effect of glucose on the transport of [^{3}H]-raffinose in raffinose-grown *S. mutans* BM71 (A) and *ptsI* mutant DC10 (B). The concentrations of glucose added to the reactions are as follows: 0 (control) Δ; 0.1 mM ●; 0.5 mM O; and 1.0 mM □.

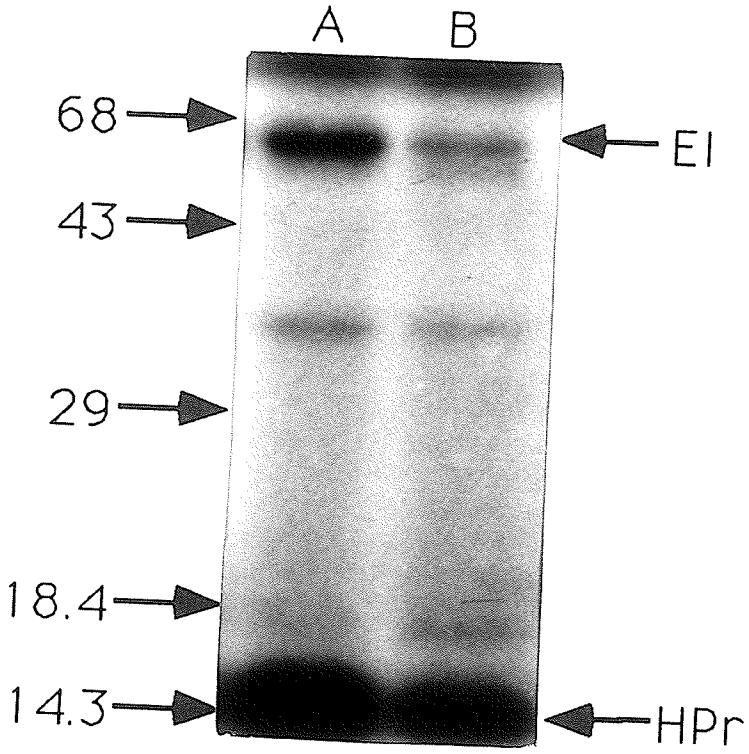


Fig. 3. Autoradiogram of a 12.5% SDS-PAGE gel of proteins present in extracts of *S. mutans* BM71 grown on glucose (A) and raffinose (B) phosphorylated by [^{32}P]-PEP.

As raffinose can be cleaved extracellularly by fructosyltransferase, fructanase and invertase, to generate the PTS substrate, fructose, and the *msm* substrate, melibiose (31), we repeated the experiment with melibiose-grown cells to eliminate the possibility that we were observing the phosphorylated Enzyme II for fructose (EII^{fru}) in raffinose-grown cells. Fig. 4 illustrates an autoradiogram of a 10% SDS-PAGE gel of the PTS-phosphorylated proteins in crude extract, membrane and cytoplasmic fractions prepared from melibiose-grown cells of *S. mutans* BM71. This photograph clearly demonstrates the presence of a 59 kDa phosphoprotein in the crude extract (lane A) and in the membrane fraction (lane B), however, this phosphoprotein was not detected in the cytoplasmic fraction (lane C).

The role of the 59 kDa phosphoprotein is unknown, however, we postulated that it would likely be part of the *msm* operon and be involved in activation of transport via this system. To test this hypothesis, [^{32}P]-PEP phosphorylation experiments were repeated on cell extracts of *E. coli* clones expressing the entire *msm* operon (strain DH120) and the *gtfA* gene (strain DHGTA) (Fig. 5). The latter gene product, sucrose phosphorylase, was suspected of being the phosphoprotein since it has a calculated MW of 55,565 kDa (12), quite close to that of the observed 59 kDa phosphoprotein. Cell extracts of glucose-grown *S. mutans*

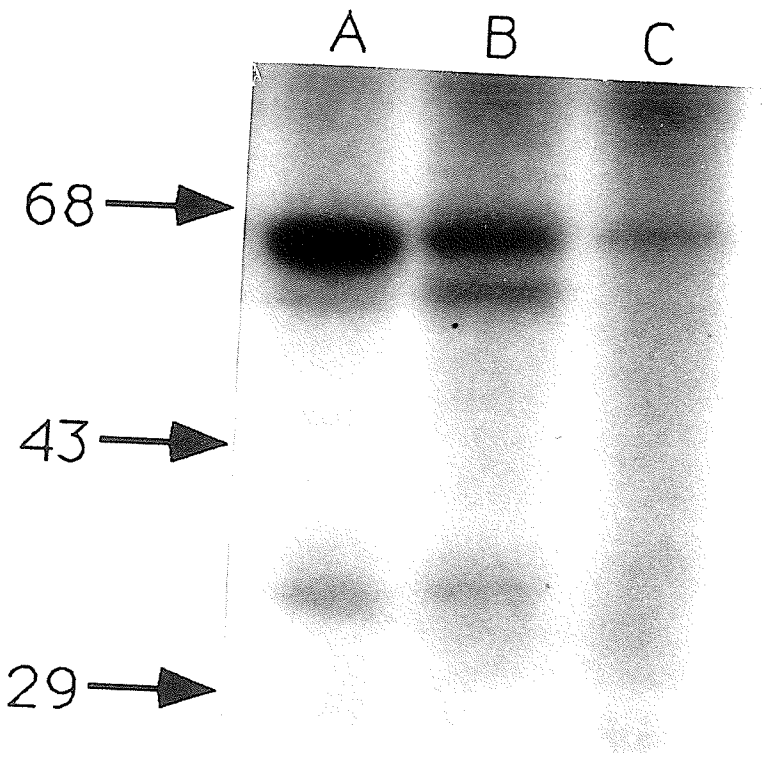


Fig. 4. Autoradiogram of a 10% SDS-PAGE gel of proteins present in various fractions of melibiose-grown *S. mutans* BM71 phosphorylated by [^{32}P]-PEP: (A) crude extract, (B) membrane fraction, and (C) soluble fraction.

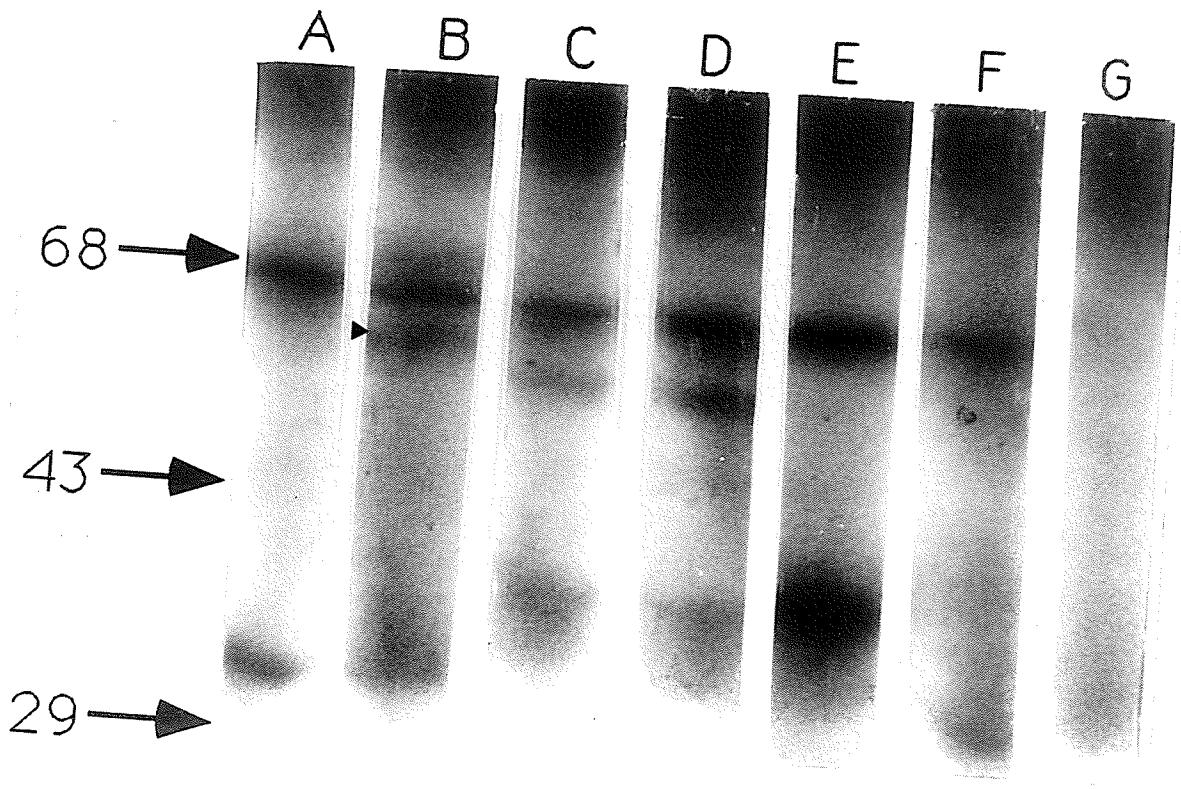


Fig. 5. Autoradiogram of a 10% SDS-PAGE gel of proteins present in *E. coli* and *S. mutans* BM71 phosphorylated by [^{32}P]-PEP: Lane A: glucose-grown BM71, lane B: melibiose-grown BM71, lane C: *E. coli* DH5α containing pVA891, lane D: *E. coli* DH120, lane E: *E. coli* DHGTA, lane F: glucose-grown BM71 + CHE-GTA, and lane G: *E. coli* CHE-GTA. Arrow indicates 59 kDa phosphoprotein.

BM71 were also combined with the *E. coli* extracts to alleviate the possibility that the *E. coli* PTS might not phosphorylate the 59 kDa protein. Fig. 5 shows the relative position of EI at 68 kDa in lane A, which contains the crude extract from glucose-grown BM71. Lane B shows EI and the 59 kDa phosphoprotein (arrow) in melibiose-grown BM71, while lane C contains *E. coli* DH5α harboring pVA891 without the insert as a negative control. Lanes D and E, containing the extracts of the *E. coli* strains expressing the entire *msm* operon (DH120) and *gtfA* (DHGTA), respectively, demonstrate no 59 kDa phosphoprotein. An *E. coli* phosphoprotein migrating lower than the 59 kDa *S. mutans* phosphoprotein is visible at 52 kDa in both the control lane C and in lane D. This protein migrates significantly faster then the *S. mutans* 59 kDa phosphoprotein as seen in lane B.

In order to circumvent the possibility that the negative results in lanes D and E were not due to the inability of the *E. coli* PTS to phosphorylate SucP, the reaction in lane F was included. This profile resulted from the incubation of an extract from an *E. coli ptsI* mutant expressing SucP (CHEGTA) in combination with glucose-grown BM71 extract, containing EI and HPr of the PTS. Lane G contains the extract from the *E. coli ptsI* mutant alone to demonstrate the absence of PEP phosphorylation. A similar reaction with the *E. coli ptsI* mutant expressing the *msm* operon in combination with glucose-grown *S. mutans* BM71 extract was also included (not shown).

These experiments were unable to demonstrate phosphorylation of the sucrose phosphorylase or other components of the *msm* operon under the conditions tested, suggesting that the protein is likely encoded outside the region of the *msm* operon.

Discussion

The construction of the *S. mutans ptsI* mutant DC10 has allowed us to study non-PTS sugar transport in this organism without interference from PTS transport. In previous work (7), we were able to demonstrate that glucose is transported in *S. mutans* by a non-PTS process. The present study utilized this mutant to examine the regulation of sugar transport via the *msm* system in *S. mutans*. The *msm* system is the best characterized of the non-PTS sugar transport systems in *S. mutans* and is utilized to transport a variety of sugar substrates including melibiose, raffinose and isomaltosaccharides. Molecular genetic analysis reveals that eight genes comprise the *msm* operon and have homology to some of the components of the osmotic-shock-sensitive sugar transport systems of enteric bacteria (31). Although the nucleotide sequence of the *msm* genes has been completed and the function of the individual components is partially characterized, little is known about the transport process and how it is regulated.

mutations produced a phenotype which is able to utilize lactose, a non-PTS substrate for this organism (13).

To further examine the ability of the PTS to control uptake via the *msm*, we tested the inhibitory effect of the PTS substrates glucose and the non-metabolizable glucose analogs, 2-deoxyglucose and α-methylglucoside, on raffinose transport. In the *S. mutans* wild-type strains, glucose and 2-DG are transported via EII^{man} and EII^{glc}, while α-MG is transported exclusively via EII^{glc} (25,44) with both analogs accumulating in the cell as non-metabolizable phosphorylated forms. Since neither of the PTS systems were pre-induced, the increased inhibitory effect of 2-DG and glucose over α-MG was likely due to the residual levels of the two permeases versus the single route of entry for α-MG. It has also been suggested that the *msm* itself is capable of transporting glucose, since a 200-fold concentration of glucose over melibiose was shown to inhibit transport of the latter by nearly 90% (40). The interpretation of these results did not, however, consider the possibility that the PTS may be modulating transport via the *msm*.

The inhibitory effects of the PTS substrates with the *ptsI* mutant DC10 is apparent but the inhibition was not as strong as that observed with most *ptsI* mutants of enteric bacteria (35). In these bacteria, enzyme I is believed to perform a catalytic role in relieving inhibition of non-PTS

transport by a PTS-mediated mechanism by decreasing the ratio of P-IIA^{glc}:IIA^{glc} which in turn modulates the activity of non-PTS transport. The transport of 2-DG and α-MG by the mutant DC10 likely occurred via the non-PTS glucose permease (7) with the substrates entering the cell in a non-phosphorylated form, but phosphorylated by ATP rather than PEP (5). The inhibitory effect of these non-metabolizable analogs is likely due to their utilization of the energy source and intracellular accumulation.

The involvement of the PTS in regulating non-PTS transport in Gram-negative bacteria is well characterized and involves interactions of IIA^{glc} of the PTS with either the non-PTS permease, or an enzyme involved in metabolism of the non-PTS substrate, as well as modulating the activity of adenylate cyclase and, consequently, cyclic AMP formation (26,35). A IIA^{glc}-like domain has been characterized in *S. mutans* as part of the II^{suc} domain utilized for sucrose transport, but no regulatory role for this region has been suggested (36). A more likely candidate for a PTS regulatory protein is III^{man}, a component involved in glucose and mannose transport in *S. mutans, S. salivarius* and *S. lactis* (3). This protein has been shown to be involved in the control of sugar transport and catabolite repression in *S. salivarius*, but the regulatory mechanism has not be fully elucidated (13,14).

Catabolite repression in Gram-positive bacteria in general is not yet well understood, but it is believed that PTS-mediated repression involves phosphorylation of non-PTS target proteins. This phenomenon has been demonstrated in the regulation of glycerol utilization in *Enterococcus faecalis* (9,10). This bacterium is able to regulate glycerol utilization by direct phosphorylation of glycerol kinase via EI and HPr increasing the activity of the enzyme 9-fold over the unphosphorylated form (9). The phosphorylation of glycerol kinase occurs in vivo in the absence of PTS substrates, a condition allowing for the intracellular accumulation of phospho-HPr, which in turn drives phosphoryl transfer to the constitutively synthesized glycerol kinase.

In order to determine if a similar reaction was taking place to activate transport via the *msm* in *S. mutans*, we examined the phosphoprotein profiles of cell extracts of raffinose, melibiose and glucose-grown cells. Our results suggest that a similar mechanism may be taking place in *S. mutans*, as indicated by the presence of the 59 kDa membrane-associated phosphoprotein in cell extracts of raffinose or melibiose-grown, wild-type cells. The phosphorylation of this protein is dependent upon a functional PTS since no phosphorylation via PEP was observed in cell extracts of raffinose or melibiose-grown cells of the *ptsI* mutant DC10. The function and route by which this protein is phosphorylated by PEP has yet to be elucidated, but it is

possibly involved in activation of one or more of the *msm* components.

The *msm* operon is known to be induced by growth of *S. mutans* on melibiose or raffinose. The *msm*R gene is divergently transcribed from the rest of the operon and it is believed that the *msm*R protein product is a positive regulator of the operon as its deduced amino acid sequence has strong homology to the MelR protein of *E. coli*, which is a positive effector of melibiose transport and metabolism (31). Recent experiments, using gel-retardation assays to study the effect of a variety of potential inducers on the binding of MsmR protein to its target sequence, suggest that the true inducers of the operon are not melibiose or raffinose, but are likely the metabolic breakdown products of these sugars with enhanced binding of MsmR observed with glucose-6-P and glucose-1-P (39). Glucose-1-P seems the more likely candidate, since it is produced, along with fructose, by the action of sucrose phosphorylase on sucrose (33), which is generated intracellularly by the breakdown of raffinose by α-galactosidase (*aga* product) (1).

The fact that non-PTS transport is usually regulated at the inducer-generating step of the system (35), and the fact that SucP has a similar molecular mass (55,665 Da) as the observed 59 kDa phosphoprotein, led us to investigate the possibility that sucrose phosphorylase was the observed

phosphoprotein. The experiments with *E. coli* clones expressing either the entire *msm* operon or *gtfA* failed to demonstrate that this was the case. Thus, it is likely that the 59 kDa phosphoprotein is involved in the regulation of transport or metabolism by the *msm*, but further study and characterization of this phenomenon is necessary before a definitive role is established for this protein.

Acknowledgements

This research was supported by an operating grant from the Medical Research Council of Canada (MT-3546).

References

1. Aduse-Opoku, J., L. Tao, J. J. Ferretti, and R. R. B. Russell. 1991. Biochemical and genetic analysis of *Streptococcus mutans* α-galactosidase. J. Gen. Microbiol. **137**: 757-764.
2. Boyd, D. A., D. G. Cvitkovitch, and I. R. Hamilton. 1994. Sequence and expression of the genes for HPr (*ptsH*) and Enzyme I (*ptsI*) of the phosphoenolpyruvate-dependent phosphotransferase transport system from *Streptococcus mutans*. Infect. Immun. **62**: 1156-1165.
3. Bourassa, S., L. Gauthier, R. Giguere, and C. Vadeboncoeur. 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. Oral Microbiol. Immunol. **5**: 288-297.
4. Bowden, G. H. 1990. Which bacteria are cariogenic in man? p. 266-286. *In* N. W. Johnson (ed.), Dental caries,

Vol 1, Markers of high and low risk groups and individuals. Academic Press, London.

5. Buckley, N. D., and I. R. Hamilton. 1994. Vesicles prepared from *Streptococcus mutans* demonstrate the presence of a second glucose transport system. Microbiol. **140**: 2639-2648.
6. Burne, R. A., B. Rubinfeld, W. H. Bowen, and R. E. Yasbin. 1986. Tight genetic linkage of a glucosyltransferase and dextranase of *Streptococcus mutans* GS-5. J. Dent. Res. **65**: 1392-1401.
7. Cvitkovitch, D. G, D. A. Boyd, T. T. Thevenot, and I. R. Hamilton. 1995. Glucose transport by a mutant of *Streptococcus mutans* unable to transport sugars via the phosphoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. 177: 2251-2258.
8. Cvitkovitch, D. G., Boyd, D. A., and Hamilton, I. R. 1994. Regulation of sugar uptake via the multiple sugar metabolism operon by the phosphoenolpyruvate-dependent sugar phosphotransferase transport system of *Streptococcus mutans, In* J. J. Ferretti, M. S. Gilmore and T. R. Klaenhammer (ed.), Streptococcal genetics, Third edition. Int'l Assoc. Biol. Stand. (In press).
9. Deutscher, J., B. Bauer, and H. Sauerwald. 1993. Regulation of glycerol metabolism in *Enterococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation of glycerol kinase catalyzed by enzyme I and HPr of the phosphotransferase system. J. Bacteriol. **175**: 3730-3733.
10. Deutcher, J., and H. Sauerwald. 1986. Stimulation of dihydroxyacetone and glycerol kinase activity in *Streptococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation catalyzed by enzyme I and HPr of the phosphotransferase system. J. Bacteriol. **166**: 829-836.
11. Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high

voltage electroporation. Nucleic Acids Res. **16**: 6127-6145.

12. Ferretti, J. J., T. T. Huang, and R. R. B. Russell. 1988. Sequence analysis of the glucosyltransferase A gene (*gtfA*) from *Streptococcus mutans* Ingbritt. Infect. Immun. **56**: 1585-1588.
13. Gauthier, L., S. Thomas, G. Gagnon, M. Frenette, L. Trahan, and C. Vadeboncoeur. 1994. Positive selection for resistance to 2-deoxyglucose gives rise, in *Streptococcus salivarius*, to seven classes of pleiotropic mutants, including *ptsH* and *ptsI* missense mutants. Mol. Microbiol. **13**: 1101-1109.
14. Gauthier, L., S. Bourassa, D. Brochu, and C. Vadeboncoeur. 1990. Control of sugar utilization in oral streptococci. Properties of phenotypically distinct 2-deoxyglucose-resistant mutants of *Streptococcus salivarius*. Oral Microbiol. Immunol. **5**: 352-359.
15. Gottesman, M., M. Hicks, and M. Gellert. 1973. Genetics and function of DNA ligase in *Escherichia coli*. J. Mol. Biol. **77**: 531-547.
16. Hamada, S., and H. D. Slade. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331-284.
17. Hamilton, I. R. 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94-133. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.
18. Hamilton, I. R., and G. Svensater. 1991. Sorbitol inhibition of glucose metabolism by *Streptococcus sanguis* 160. Oral Microbiol. Immunol. **6**: 151-159.
19. Honeyman, A. L., and R. Curtiss III. 1992. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* mannitol-phosphate dehydrogenase gene and the mannitol-specific Factor III gene of the

phosphoenolpyruvate phosphotransferase system. Infect. Immun. **60**: 3369-3375.

20. Jacobson, G. R., J. Lodge, and F. Poy. 1989. Carbohydrate uptake in the oral pathogen *Streptococcus mutans*: mechanism and regulation by protein phosphorylation. Biochimie **71**: 997-1004

21. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. Micobiol. Rev. **50**: 353-380.

22. Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones. 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgenic cloning. Gene **25**: 145-150.

23. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

24. Mattoo, R. L., and E, B. Waygood. 1983. An enzymatic method for [^{32}P]phosphoenolpyruvate synthesis. Anal. Biochem. **128**: 245-249.

25. Neron S., and C. Vadeboncoeur. 1987. Evidence for the presence of two distinct phosphoenolpyruvate: mannose phosphotransferase systems in *Streptococcus mutans* GS5-S. FEMS Microbiol. Lett. **42**: 7-11.

26. Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57**: 543-594.

27. Reizer, J., A. H. Romano, and J. Deutscher. 1993. The role of phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, in the regulation of carbon metabolism in Gram-positive bacteria. J. Cell. Biochem. **51**: 19-24.

28. Reizer, J., M. J. Novotny, I. Stuiver, and M. H. Saier. 1984. Regulation of glycerol uptake by the phosphoenolpyruvate-sugar phosphotransferase system in *Bacillus subtilis*. J. Bacteriol. **159**: 243-250.

29. Romano, A. H., M. H. Saier, O. T. Harriot, and J. Reizer. 1990. Physiological studies on regulation of glycerol utilization by the phosphoenolpyruvate: sugar phosphotransferase system in *E. faecalis*. J. Bacteriol. **172**: 6741-6748.

30. Rosey, E. L. and G. C. Stewart. 1992. Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans*. J. Bacteriol. **174**: 6159-6170.

31. Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J. Biol. Chem. **267**: 4631-4637.

32. Russell, R. R. B., and J. J. Ferretti. 1990. Nucleotide sequence of the dextran glucosidase (*dexB*) gene of *Streptococcus mutans*. J. Gen. Microbiol. **136**: 803-810.

33. Russell, R. R. B., H. Mukasa, A. Shimamura, and J. J. Ferretti. 1988. *Streptococcus mutans gtfA* gene specifies sucrose phosphorylase. Infect. Immun. **56**: 2763-2765.

34. Saier, M.H. Jr., and J. Reizer. 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphatase system. J. Bacteriol. **174**: 1433-1438.

35. Saier, M. H. 1989. Protein phosphorylation and allosteric control of inducer exclusion by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol. Rev. **53**: 109-120.

36. Sato, Y., F. Poy, G. R. Jacobson, and H. K. Kuramitsu. 1989. Characterization and sequence analysis of the scrA gene encoding enzyme II^{scr} of the *Streptococcus*

mutans phosphoenolpyruvate-dependent sucrose phosphotransferase system. J. Bacteriol. **171**: 263-271.

37. Sutcliffe, I. C., L. Tao, J. J. Ferretti, and R. R. B. Russell. 1993. MsmE, a lipoprotein involved in sugar transport in *Streptococcus mutans*. J. Bacteriol. **175**: 1853-1855.
38. Tangney, M., C. J. Buchanan, F. G. Priest, and W. J. Mitchell 1992. Maltose uptake and its regulation in *Bacillus subtilis*. FEMS Microbiol. Lett. **97**: 191-196.
39. Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti. 1994. Regulation of the multiple sugar metabolism operon in *S. mutans*, *In* J. J. Ferretti, M. S. Gilmore and T. R. Klaenhammer (ed.), Streptococcal genetics, Third edition. Int'l Assoc. Biol. Stand. (In press).
40. Tao, L., I. C. Sutcliffe, R. R. B. Russell, and J. J. Ferretti. 1993. Transport of sugars, including sucrose, by the *msm* transport system of *Streptococcus mutans* . J. Dent. Res. **267**: 4631-4637.
41. Tao, L., I. C. Sutcliffe, R. R. B., Russell, and J. J. Ferretti. 1993. Cloning and expression of the multiple sugar metabolism operon *msm* in heterologous streptococcal hosts. Infect. Immun. **61**: 1121-1125.
42. Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson, and I. R. Hamilton. 1987. Effect of growth conditions on levels of components of the phosphoenolpyruvate: sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus sobrinus* grown in continuous culture. J. Bacteriol. **169**: 5686-5691.
43. Vadeboncoeur, C. 1984. Structure and properties of the phosphoenolpyruvate: glucose phosphotransferase system of oral streptococci. Can. J. Microbiol. **30**: 495-502.
44 Vadeboncoeur, C., and L. Trahan. 1982. Glucose transport in *Streptococcus salivarius*. Evidence for the presense of a distinct phosphoenolpyruvate: glucose phosphotransferase system which catalyzes the

phosphorylation of α-methylglucoside. Can. J. Microbiol. **28**: 190-199.

Chapter 7

Summary and Conclusions

The overall goal of this thesis was to utilize molecular genetic techniques to generate a PTS-defective mutant of *S. mutans* and examine non-PTS sugar transport in this organism. The project began with the cloning of the genes coding for EI and HPr, the general proteins of the PTS. The cloned EI gene allowed the construction of an integration vector that effectively inactivated the PTS. The resultant mutant was used to examine the non-PTS glucose transport process in *S. mutans* and also to demonstrate PTS-mediated regulation of transport via the MSM transport system.

The project was initiated with the cloning of the *ptsH* and *ptsI* genes which code for HPr and EI, respectively. In the pursuit of these genes, we first attempted to screen a genomic library of *S. mutans* constructed in the expression vector λGT11 with antibodies directed against purified EI. These initial attempts were unsuccessful and led us to utilize a nucleic acid probe constructed from the *ptsI* gene from *S. salivarius*. After successfully cloning the gene into a λEMBL3 library, we experienced difficulty in subcloning fragments containing the *ptsH* gene. This was overcome by utilizing an *E. coli* host defective in *ptsI*, since we believed that a functional *S. mutans* HPr was toxic to an *E. coli* host with a functional PTS. This assumption was most likely correct since the *ptsH* gene was successfully cloned using this technique.

The cloning of these genes allowed us to examine the expression of the *S. mutans* EI and HPr proteins in an *E coli* host demonstrating complementation of the *E. coli* PTS. The subsequent sequence analysis of the genes revealed homology with the *ptsH* and *ptsI* genes from other bacteria. With the cloning of these genes I was able to proceed with their inactivation to generate a PTS-defective mutant.

The initial attempts to generate a PTS-defective mutant employed an integration vector constructed from the *ptsI* gene of *S. salivarius*. This approach was initiated before the cloning of the *S. mutans* genes, since it was believed that this vector would have sufficient homology to recombine at an internal region of the *ptsI* gene. Several attempts at transforming *S. mutans* were unsuccessful, although in one case several transformants were isolated that had the vector successfully integrated into the chromosome. Analysis of these transformants revealed that, while retaining an intact *ptsI* gene, they were unable to ferment mannose, a PTS substrate.

Subsequent biochemical and genetic analysis of these transformants revealed that the plasmid had inserted into the *msm* operon, which encodes a non-PTS sugar transport system. Attempts to reproduce this phenotype with the the *S. salivarius-ptsI*-containing integration vector and other *msm*-specific integration vectors were unsuccessful. It is

possible that the mannose-negative phenotype may have been the result of a secondary mutation that occurred elsewhere in the genome. The possibility does, however, exist that the integration event may be responsible for the mannose-negative phenotype, since we later demonstrated interaction between the PTS and the MSM. As the regulatory mechanisms controlling these two systems becomes resolved, this mutant may provide a useful tool for further characterization of the interaction between them.

The pursuit for a PTS-defective mutant resumed with the construction of integration vectors harboring fragments of the cloned *ptsI* gene from *S. mutans*. One of these vectors was successful in generating a PTS-defective mutant and was useful in the examination of non-PTS glucose transport in the organism. Although strong biochemical evidence for the non-PTS system existed (4,5), the experiments with this mutant provided the first direct evidence supporting its existence. The results of this study confirmed recent experiments conducted by Buckley and Hamilton (1) utilizing membrane vesicles of *S. mutans* grown under PTS repressed and active conditions. The mode of transport proposed by Dr. Buckley as the result of the vesicle experiments suggested a phosphate-bond driven carrier with specificity for glucose, probably utilizing ATP as an energy source. This hypothesis is difficult to prove since this mode of transport has not yet been demonstrated in bacteria. To test this theory, one

would need to demonstrate concentrative uptake in membrane vesicles loaded with ATP alone. It would also be necessary to demonstrate that glucose was entering the cell in an unaltered form and not as a phosphoester due to residual glucokinase activity. In addition, the transport could only occur in one direction across the membrane.

The work presented in this thesis does not discount this theory, and in light of our results and recent insights into PTS-mediated regulation of non-PTS system, I would agree that the mode of glucose transport involves a carrier with high specificity for glucose. I would not, however, rule out the possibility that an ABC-type transport system was responsible for glucose transport in this organism. Since the non-PTS system is believed to function primarily under conditions of low pH, high glucose concentrations and high growth rates, the energy requirement for the cells under these conditions would likely be lower than with limiting sugar. Under these conditions, the organism could probably afford the extra ATP required for the transport of a single, unphosphorylated glucose molecule. In fact, at high concentrations, glucose poses a metabolic threat to the organism and it is known that *S. mutans* possesses mechanisms that quickly deplete the surrounding glucose concentration (2). One may even speculate that the ability of the acidogenic streptococci to rapidly convert sugar into acid, although metabolically inefficient, gives them a competitive advantage by rapidly

lowering the surrounding pH thereby killing off other acid-sensitive bacteria.

Although previous results had demonstrated that the glucose PTS is repressed under conditions of low pH (4), high growth rates, and high sugar concentrations (5), there is no evidence suggesting that transport via the non-PTS secondary system is regulated. Our work with the mutant, however, strongly suggests that the PTS is required for the non-PTS system to function optimally. This is indicated by the observation that the mutant transported glucose at only about 1% of the rate of the parent using the low-affinity system for glucose transport (Chapter 5). This dependence on the PTS was also reflected with the mutant and the parent having Vmax's of 0.87 and 64 nmoles mg^{-1} (dry weight) glucose transported, respectively, while the mutant retained the same K_s value (125-138 μM). This suggests that a functional PTS is necessary for the non-PTS system to be activated and that the PTS controls transport via the non-PTS system. Further study of this transport process at both the cellular and genetic level will clarify the mechanism utilized by this system.

At present, attempts to clone the genes coding for the glucose permease and the glucokinase are underway. The strategy involves the use of an *E. coli* host defective in EII^{glc}, EII^{man}, HPr, and EI which is, therefore, unable to transport glucose (6). An *S. mutans* genomic library

constructed in a plasmid expression vector will be used to transform this *E. coli* host and the transformants capable of glucose fermentation on MacConkey plates will be selected and these should harbor plasmids containing a functional permease gene.

Since the glucose permease is most possibly arranged in an operon with glucokinase, transformations will also be performed with an *E. coli* host with the same PTS defects but also with a defective glucokinase gene. To select for transformants harboring a functional *S. mutans* glucokinase gene, selection with this host will be performed on MacConkey plates supplemented with fucose, which is a gratuitous inducer of the galactose permease, that facilitates glucose transport. The cloning and subsequent nucleotide sequencing of the glucose permease and the glucokinase genes will allow comparison of the deduced amino acid sequence against databanks containing sequences of several transporters and will give insight into the mechanism of non-PTS glucose transport in *S. mutans*. The cloned genes will also be useful in the construction of isogenic mutants of *S. mutans* that would be useful in the examination of glucose transport and its regulation in this organism.

Another interesting observation was that the mutant DC10 possessed only about 1/6 of the total amount of HPr as determined by quantitative crossed immunoelectrophoresis. This result suggests that the level of HPr may be regulated

by a functional EI. It is possible that phosphorylation of an antiterminator or activator of HPr synthesis is the modulator of this mechanism. To test this hypothesis one could introduce the *ptsI* gene into DC10 on a low copy number replicative plasmid or integrate it into the chromosome with a suicide vector. If the re-introduction of an intact *ptsI* gene (and functional EI) restores the level of HPr in mutant DC10 to wild-type levels the regulation of HPr expression by EI would be demonstrated.

The final section of the thesis deals with the regulation of transport via the MSM system by the PTS in *S. mutans*. I demonstrated diauxic growth in the presence of glucose and the *msm* substrate melibiose suggesting that glucose repressed expression of the *msm* operon (3). I further demonstrated that growth, transport, and acid production with raffinose as the substrate was greatly reduced in the PTS-defective mutant DC10 relative to the parent strain (Table 2, Chapter 6). A further examination of raffinose transport demonstrated that glucose and glucose analogs could inhibit transport via the MSM system, indicating that the PTS was able to modulate MSM transport .

The mechanism of regulation was suspected to involve PEP-mediated phosphorylation. Experiments utilizing [^{32}P]-PEP indicated that a 59 kDa protein was phosphorylated in this manner when cells were grown on the MSM substrates melibiose and raffinose. It was hypothesized that this protein might

be the sucrose phosphorylase encoded by *gtfA* of the *msm* operon (7). Experiments utilizing *E. coli* extracts harboring the sucrose phosphorylase on a multi-copy plasmid failed to demonstrate phosphorylation of this protein via PEP. The experiments did not eliminate sucrose phosphorylase as the target of phosphorylation, since the conditions to mediate this reaction may have not been present under the tested conditions. However it is unlikely that the phosphoprotein is sucrose phosphorylase since this protein is known to be a soluble enzyme, while the 59 kDa phosphoprotein was membrane-associated. Experiments that would conclusively eliminate sucrose phosphorylase could include the use of antibodies against the sucrose phosphorylase to evaluate their inhibitory effect on phosphorylation of the 59 kDa protein and to determine if the location of the phosphorylase on Western blots correspond to the location of the phosphoprotein on duplicate [^{32}P]-PEP autoradiograms.

It was suggested to us that the 59 kDa phosphoprotein may be a sugar-specific EII, which is expressed due to induction by an intracellular breakdown product of melibiose or raffinose. Since the phosphoprotein is present with both raffinose and melibiose, the intracellular breakdown product that may act as an inducer would likely be a product generated during the hydrolysis of both sugars. The only common sugars generated by the action of the MSM are glucose and galactose. It is unlikely that the phosphoprotein would

be a glucose-specific EII since this protein would be expected to be observed with growth on glucose. The possibility of it being the galactose permease still exists, however, the intracellular inducer of a galactose-specific EII would likely be phosphorylated galactose, since PTS-mediated transport of this sugar would result in this molecule entering the cell. To test if the 59 kDa phosphoprotein is a galactose-specific EII phosphorylation experiments could be repeated with extracts of galatose-grown cells.

The work presented in this thesis is an example of the application of molecular genetic techniques to study the physiology of sugar transport in *S. mutans*. The relatively recent use of this technology has been extremely useful in unravelling the mechanisms and regulation of both PTS and non-PTS sugar transport in this organism. The ability of the microbial physiologist to construct defined mutants and to evaluate their properties will continue to provide insight into a variety of physiological processes. Gene cloning and site-directed mutagenesis are also powerful techniques that are combined to explore these problems. Work of this nature will undoubtedly open many doors to expand our knowledge of the intricate machinery used by bacteria to survive in a multitude of environments.

References

1. Buckley, N. D., and I. R. Hamilton. 1994. Vesicles prepared from *Streptococcus mutans* demonstrate the presence of a second glucose transport system. Microbiology **140**: 2639-2648.
2. Carlsson, J., and C. J. Griffith. 1974. Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci. Archs. Oral Biol. **19**: 1105-1109.
3. Cvitkovitch, D. G., D. A. Boyd, and I. R. Hamilton. 1995. Inhibition of sugar uptake via the multiple sugar metabolism operon by the phosphoenolpyruvate-dependent sugar phosphotransferase transport system of *Streptococcus mutans*, *In*: J. J. Ferretti (ed.), Genetics of streptococci, enterococci and lactococci. In press. National Bureau Standards, Washington, D. C.
4. Ellwood, D. C., and I. R. Hamilton. 1982. Properties of *Streptococcus mutans* Ingbritt growing in limiting sucrose in a chemostat: Repression of the phosphoenolpyruvate phosphotransferase transport system. Infect. Immun. **36**: 576-581.
5. Ellwood, D. C., P. J. Phipps, and I. R. Hamilton. 1979. Effect of growth rate and glucose concentration on the activity of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans* Ingbritt grown in continuous culture. Infect. Immun. **23**: 224-231.
6. Ruijter, G. J., G. van Meurs, M. A. Verwey, P. W. Postma, and K. van Dam. 1992. Analysis of mutations that uncouple transport from phosphorylation in enzyme II^{Glc} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system. J. Bacteriol. **174**: 2843-2850.
7. Russell, R. R. B., H. Mukasa, A. Shimamura, and J. J. Ferretti. 1988. *Streptococcus mutans gtfA* gene

specifies sucrose phosphorylase. Infect. Immun. **56:** 2763-2765.