Aspects of Sugar Transport via the Phosphoenolpyruvate:Sugar Phosphotransferase System of *Streptococcus mutans*

by

Tracy Lynn Thevenot

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment for the degree of

Doctor of Philosophy

Department of Oral Biology
The University of Manitoba

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TRACY LYNN THEVENOT

A Thesis/Practicum 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

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To my Family

Denys, Joshua, and Tyler.

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Acknowledgements

I would like to thank my supervisor Dr. Ian Hamilton for all his patience, support, and guidance throughout the years and especially for encouraging me to transition into the PhD program. I would also like to thank the members of my committee Dr. Bowden, Dr. Bhullar, and Dr. Worobec for their time to review my thesis, as well as, their recommendations and direction over the years.

I would like to acknowledge Dave Boyd for teaching me many molecular biology techniques and for all his insight and helpful discussions. Elke Greif for her assistance, especially during the time I was on maternity leave, and for her positive attitude and encouragement. Dennis Cvitkovitch for all his help, from proof-reading essays in the first year of graduate studies to writing reference letters for scholarship applications. Nicole Buckley for her guidance while I learnt the continuous culture technique and Beverly Grimshire for introducing me to the world of chemostats.

I would like to thank my husband, Denys, my mother, Celia, my brother Darrell, my father Mike Thorvaldson, as well as, my in-laws Ellen and Romeo Thevenot for their support and encouragement over the years. A special thank-you to Ellen for taking care of Joshua and Tyler.

Abstract

Carbohydrate metabolism by the oral pathogen, *Streptococcus mutans*, produces acid end-products that can promote dental caries. The phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) is the principal sugar transport system of oral streptococci. Using PEP as a phosphoryl donor, the PTS catalyzes the sequential phosphorylation of the general proteins, Enzyme I and HPr. P~(His)-HPr, phosphorylated at histidine 15, then transfers the phosphate group to a sugar-specific, membrane-bound Enzyme II complex that catalyzes the transport and phosphorylation of the specific carbohydrate. HPr protein of Gram-positive bacteria can also be phosphorylated at a serine residue (46) by a fructose-1,6-bisphosphate (FBP)-activated, ATP-dependent (Ser)HPr kinase. HPr from oral streptococci can exist in four phosphorylated forms; free HPr, P~(His)-HPr, P-(Ser)-HPr and P~(His)-P-(Ser)-HPr.

In the first phase of my research, I was interested in the activity and regulation of (Ser)HPr kinase in whole cells and cell preparations. Early work with membrane preparations of *S. mutans* and *S. salivarius* indicated that (Ser)HPr kinase activity increased 2-fold with 5 mM FBP and 0.5 mM ATP, and the enzyme was slightly inhibited by 1.0 mM ATP. To complement this work, the level of the four forms of HPr were assessed in steady state cells of *S. mutans* Ingbritt grown in

continuous culture with limiting (10 and 50 mM) and excess glucose (100 and 200 mM) at a constant pH (7.0) and growth rate (dilution rate=0.1 h^{-1}). Crossed immunoelectrophoresis detected all four forms of HPr in the four chemostat-grown cultures. The relative amount of P-(Ser)-HPr (50%) was highest for 100 mM glucose-grown and the level of FBP (1,114 µM) and ATP (1,416 µM) was highest for 200-mM glucose excess cells. The results indicated that the intracellular level of FBP was not high enough to promote activation of (Ser)HPr kinase and the level of ATP could be considered inhibitory. We conclude that FBP in *S. mutans* is not a critical factor in the activation of (Ser)HPr kinase.

The second phase of my research involved altering the amino acid sequence of HPr by converting glycine-67, an amino acid which may be important for the recognition of P~(His)-HPr by the EII complexes, to an aspartate residue by site-directed mutagenesis of the *ptsH* gene. The gene from *S. mutans* NG5 was successfully mutated using single-stranded site-directed mutagenesis. The plasmid carrying the mutated gene also carried the gene for EI (*ptsI*) and the 5' portion of the glyceraldehyde-3-phosphate dehydrogenase gene (*gapN*). A kanamycin-resistance gene was integrated between the *ptsI* and *gapN* genes to provide convenient selection of mutants. Transformations into *S. mutans* produced kanamycin-resistant colonies and Southern hybridization analyses verified integration of the kanamycin gene. Potential HPr mutants

fermented PTS sugars indicating unsuccessful integration of the mutated gene. The inability to obtain HPr mutants defective in glycine-67 may have been due to the structure of the mutant plasmid or the possibility that the amino acid alteration would not support the growth of transformants.

The third phase of my research focused on the genetics of sorbitol transport in *S. mutans*. A plasmid carrying Tn*4001* was transformed into *S. mutans* LT11 generating the sorbitol-defective strain, *S. mutans* BH96. Examination of carbohydrate utilization indicated that *S. mutans* LT11 utilized glucose first, verifying catabolite repression by glucose. A long lag period was observed prior to growth by *S. mutans* LT11 on sorbitol, confirming the induction of sorbitol-related genes; *S. mutans* BH96 only grew only in the presence of glucose. The integrative plasmid pΩIS was used to recover a transposon/genome junction from *S. mutans* BH96. A 948-bp chromosomal fragment carried on pΩ-SB was shown to consist of two partial open reading frames (ORFs). ORF1 codes for the carboxy-terminal 158 amino acids of a protein demonstrating homology to transcription regulatory proteins. ORF2 codes for the N-terminal 101 amino acids of a protein demonstrating homology with various sugar-specific EIIA proteins or domains of the PTS. We suggest that *S. mutans* BH96 carries Tn*4001* within a gene coding for a transcriptional regulator and this gene is part of an operon responsible for sorbitol metabolism.

Chapter 1

Literature Review

(A) The oral environment and dental caries

1. General characteristics of the oral cavity. One important feature in a natural environment is the availability of different surfaces which facilitate the adherence and colonization of bacteria. In the oral cavity, the two major surfaces are mucosal soft tissues and mineralized tooth enamel (Hamilton and Bowden, 1992). Bacterial colonization on oral mucosal surfaces is limited, due to the continual loss of surface epithelial cells and the slow growth rate of bacteria, however, the tooth surface supports complex layers of bacteria comprising dental plaque. Oral bacteria colonize the tooth surface through adherence to a proteinaceous film, the acquired pellicle (Nyvad and Fejerskov, 1994), the major components of which are salivary glycoproteins, phosphoproteins, lipids and constituents from the gingival crevicular fluid (Levine *et al.*, 1985). Salivary proteins, such as, statherin and proline-rich proteins, that accumulate in the acquired pellicle inhibit calcium phosphate precipitation, fostering supersaturation of these salts in saliva, promoting both protective and reparative mechanisms to maintain tooth enamel integrity (Tenovuo and Lagerlof, 1994).

Saliva serves to regulate bacterial colonization through various anti-bacterial activities (Ericson and Makinen, 1986; Hay, 1990). Secretory immunoglobulins and mucous glycoproteins (mucin) interfere with oral bacterial adherence

through interaction with adhesions on the bacterial surface, promoting aggregation of bacteria and their subsequent removal from the oral cavity by saliva (Hay, 1990; Tenovuo and Lagerlof, 1994). Other components of saliva possess specific anti-bacterial activities interfering with the metabolic processes of the organism or causing cell death. Lactoferrin chelates ferric ion, an important nutrient for oral bacteria, while lysozyme causes lysis of bacterial cells by hydrolyzing cell wall mucopeptide (Hay, 1990; Tenovuo and Lagerlof, 1994). Salivary peroxidase catalyzes the oxidation of salivary thiocyanate by hydrogen peroxide to hypothiocyanite and hypothiocyanous acid, toxic compounds which interfere with glycolysis and sugar transport (Tenovuo *et al.*, 1981). The antimicrobial factors described above provide the oral cavity with a defence system against colonizing bacteria, particularly invading pathogenic organisms, yet permit colonization of commensal organisms not normally pathogenic in a healthy oral environment (van der Hoeven, 1990). The anti-bacterial effects of saliva are exerted in the presence of other specific salivary components which can serve as nutrient resources for oral bacteria.

Saliva has been shown to support the growth of many oral microorganisms (De Jong *et al.*, 1984; De Jong *et al.*, 1986), including oral streptococci (De Jong *et al.*, 1987). Glycoproteins in saliva can be degraded by glycosidases, which cleave the oligosaccharide side chain from the protein

backbone, and proteases generating peptides and amino acids (van der Hoeven, 1990). The degradation of salivary glycoproteins is important in terms of the survival of the oral flora during periods of low carbohydrate supply because the levels of glucose in saliva (5-40 μM) are too low to promote growth (Carlsson, 1986a; van der Hoeven, 1990; Carlsson and Hamilton, 1994). The levels of other simple nutrients in saliva are also low and include: pyruvate (17-70 mM), lactate (200-400 mM), urea (2 mM), amino acids (5-150 mM), as well as, water-soluble vitamins (Carlsson and Hamilton, 1994). The gingival crevicular fluid, an exudate from between the teeth and gums, also provides plasma components rich in protein, vitamins and growth factors, favoring the growth of the anaerobic and asaccharolytic organisms important components of the microflora of the gingival margin (Bowden and Edwardsson, 1994; Hamilton and Bowden, 1992).

2. Dental plaque microbial flora. Initial colonization and subsequent shifts in the composition of the bacterial community characterize stages in the development of the complex, heterogeneous bacterial biofilm community of dental plaque. Bacteria initially colonizing the acquired pellicle include, *Streptococcus sanguis*, *Streptococcus oralis*, and *Streptococcus mitis* (Nyvad and Kilian, 1990), as well as, minor proportions of Gram-negative bacteria and species of *Actinomyces* (Bowden and Edwardsson, 1994). These initial

colonizers, or pioneer bacteria, are able to adhere to the pellicle through binding between adhesins on the bacterial surface and complementary components in the acquired pellicle. For example, *S. sanguis* and *S. oralis* attach to sialic acid residues on salivary glycoproteins (Nyvad and Fejerskov, 1994).

Colonization is characterized by interactions between bacteria with the environment, as well as, interspecies bacterial interactions. For example, early plaque development is attributed to the growth of adherent streptococci which can coaggregate with filamentous microorganisms to form corncob structures (DiRienzo *et al.*, 1985; Kolenbrander and London, 1992). Specifically, *in vitro* studies have examined the corncob formation between the early colonizer *S. sanguis* and the filamentous microorganisms *Bacterionema matruchotii* (Lancy *et al.*, 1980) and *Fusobacterium nucleatum* (Lancy *et al.*, 1983). Different types of receptors on the surface of the filamentous bacteria have been suggested to interact with *S. sanguis* and in one model, membrane-bound lipoteichoic acid on the surface of *S. sanguis* binds to a loosely-bound surface protein on the surface of *Fusobacterium nucleatum* (DiRienzo *et al.*, 1985). *Actinomyces* can colonize the acquired pellicle by binding to galactosyl residues on glycoproteins exposed by bacterial neuraminadase activity (Nyvad and Fejerskov, 1994). In addition, the early colonizers *S. sanguis* and *S. mitis* have

been shown to coaggregate with *A. viscosus* and *A. naeslundii* through lectin-carbohydrate interactions (McIntire, 1985).

The first substantial change in the bacterial community involves a shift from a plaque dominated by *Streptococcus* species to one dominated by *Actinomyces* species (Syed and Loesche, 1978). Successful colonization of any bacterial population may involve competition, for example, through inhibition of other populations by bacteriocin production (Rogers *et al.*, 1978), or by taking advantage of an environmental feature. For example, the ability of *S. mutans* to adapt and grow in a low pH environment provides an ecological advantage to this species (Bowden and Hamilton, 1987). One important aspect of bacterial succession in dental plaque is the progression from predominantly aerobic populations to facultative anaerobic populations, and then to a community composed of facultative and obligate anaerobic species, microbial shifts characterized by a decrease in available oxygen supply in the expanding biofilm (Ritz, 1967). Succession of bacterial populations continues until the bacterial community is stable and, consequently, in balance with the conditions of the oral environment, with all organisms possessing a specific niche or functional role in the community. If left undisturbed, supragingival dental plaque matures over time to a complex, diverse bacterial community comprised of over 30 genera and more than 300

species of bacteria (Bowden *et al.*, 1979; Bowden and Edwardsson, 1994).

3. Development of dental caries. Dental caries is caused by the demineralization of tooth enamel by acid-end products produced by dental plaque bacteria metabolizing dietary carbohydrates (Stephan, 1940, 1944). The development of dental caries involves the simultaneous presence of a high carbohydrate diet, cariogenic microorganisms, and a susceptible tooth surface (Keyes and Jordan, 1963). In addition, other factors, such as diminished saliva flow and poor oral hygiene, increase the potential for caries development.

The extent of plaque acid production can be significantly influenced by the types of food in the diet and the frequency of intake. Various carbohydrates commonly found in the diet including, glucose, sucrose, lactose, fructose, maltose and galactose, are readily fermented by plaque bacteria to produce acid end-products (Schachtele and Harlander, 1984). Sucrose is the major sugar in the average diet (Johansson and Birkhed, 1994) and is generally considered the most cariogenic sugar, as it is also the substrate for extracellular polysaccharide production, which promotes colonization of plaque bacteria (Newbrun, 1982). Starch can also contribute to the level of plaque acid as this polysaccharide can be degraded to its glucose components by

salivary amylase (Jensen and Schachtele, 1982). The consumption of fermentable sugars results in an immediate reduction in plaque pH to values between 5.2-4.7, followed by a gradual return to neutral pH values, the two major phases in plaque pH vs time curves, characterized originally by Stephan (Stephan, 1940, 1944). The first phase of the Stephan curve characterized by a dramatic decrease in pH is due primarily to the formation of lactic acid by plaque bacteria (Geddes, 1975). Over time, the return to neutral plaque pH values, or 'resting' plaque pH, is characterized by the formation of acetic, formic, propionic and butyric acids (Gilmour *et al.*, 1976). Generally, the frequent intake of sugar or the consumption of a high sugar diet increases the length of time the plaque pH is below the critical level, generally thought to be between pH 5.0 and 5.5 (Larsen, 1990). Below this critical pH, tooth enamel solubility increases and demineralization occurs.

The chemical composition and physical properties of food can affect the extent of plaque acid production and the length of time required to return to neutral pH values. The cariogenic potential of food is now normally determined by pH telemetry (Muhleman and Imfeld, 1978), a technique that continuously monitors plaque pH *in situ* in subjects during food consumption, thereby, assessing the length of time the tooth enamel is exposed to the critical pH with certain food groups. For example, foods such as a sugar cookie or milk

chocolate produced sufficient acid to drop the pH close to 4.0 and the pH could remain at that level for up to 2 hours (Schachtele and Harlander, 1984). In addition to the chemical composition, the physical properties of food including, solubility, adhesiveness and texture, can influence the acidogenic potential of food (Johansson and Birkhed, 1994). These factors can increase the contact time between plaque bacteria and the component sugars resulting in higher levels of acid production over longer periods of time.

Prolonged acid production by plaque bacteria promotes dominance by aciduric opportunistic pathogens associated with the altered environment. The cariogenic potential of bacteria can be measured by their ability to rapidly produce acid end-products (acidogenic), as well as, grow and metabolize in the presence of a low pH environment (aciduric) (Hamilton and Bowden, 1992). Significant cariogenic bacteria possessing acidogenic and aciduric properties include members of the 'mutans streptococcus' group and the genus, *Lactobacillus*. The members of the mutans streptococcus group include: *S. mutans, S. ferus, S. rattus, S. cricetus, S. sobrinus, S. macacae* and *S. downei* (Hamilton and Bowden, 1992). Only two of these species, *S. mutans* and *S. sobrinus*, are currently associated with dental caries in humans and the former species has been studied in the most detail (Bowden and Edwardsson, 1994).

The microbiological stages of dental caries development are initiated by a variation in the environmental conditions, such as, the introduction of a high carbohydrate diet or conditions that lead to low salivary flow rate promoting a shift in the community favoring *S. mutans* and the concomitant initial demineralization of enamel (Bowden, 1991). *Lactobacillus* colonizes demineralized tooth enamel after *S. mutans* (Milnes and Bowden, 1985), emphasizing the primary role of *S. mutans* in the dental caries process. Two members of the normal resident oral flora, *S. mitis* and *A. viscosus*, can also increase in numbers during caries development in association with *S. mutans* and *Lactobacillus* (Bowden, 1991). This association has been described in root-surface and nursing caries studies that examined the capability of *S. mitis* and *A. viscosus* strains to compete and survive in a community dominated by *S. mutans* and *Lactobacillus* (Milnes, 1987; Bowden *et al.*, 1990). Microbial succession leading to dental caries is also characterized by a decrease in the numbers of *A. naeslundii*, another member of the resident flora (Bowden, 1991). Overall, a caries-conducive environment will favor colonization by *S. mutans* and *Lactobacillus* along with other potentially cariogenic bacteria on a caries-susceptible area.

Interproximal and subgingival, as well as, pits and fissures are more susceptible to dental caries development than smooth tooth surfaces, as these areas are predisposed to

caries-promoting factors, such as, plaque accumulation, prolonged food retention and reduced salivary flow (Bowden and Edwardsson, 1994; van Houte, 1994). Additionally, root surfaces exposed by receding gingiva are more sensitive to acid compared to enamel surfaces and, as a consequence, are more susceptible to caries development (Hamilton and Bowden, 1992). Overall, tooth surface areas continually exposed to carbohydrates and reduced salivary flow are highly susceptible to caries formation.

Sufficient salivary flow is important in terms of the rinsing effect to increase the rate of oral carbohydrate clearance (Dawes, 1990). In fact, higher unstimulated flow rates and lower volumes of saliva before and after swallowing have been suggested to decrease the time required for carbohydrate clearance resulting in a lower level of acid produced by plaque bacteria (Dawes, 1983). In addition, as the flow rate of saliva increases there is a concomitant increase in the concentration of bicarbonate, the most important buffering system in saliva contributing to the neutralization of plaque acids (Tenovuo and Lagerlof, 1994). Unstimulated saliva is considered to be very poorly buffered due to the low level of bicarbonate ion (Dawes, 1990).

Saliva is present in the mouth as a slow-moving film and plaque acid is removed by diffusion into this overlying film (Dawes, 1993). If the salivary film velocity is low, acid

tends to accumulate in the salivary film, thereby increasing the extent and duration of the Stephan Curve and suggests that areas with slower moving saliva will be more susceptible to caries development. In fact, higher caries susceptibility has been correlated to lower salivary film velocity on the buccal surfaces of teeth compared to the lingual side where the salivary film velocity is much higher (Dawes, 1993).

A reduction in the flow rate of saliva may be caused by salivary gland dysfunction and the major causes of 'dry mouth', or xerostomia, include reactions to medications, autoimmune diseases, malnutrition, or irradiation to the head and neck area (Tenovuo and Lagerlof, 1994). The increased risk for caries development in patients with xerostomia can be attributed to reduced salivary flow and the concomitant reduction in the rate of carbohydrate and acid clearance resulting in increased plaque acid levels that promotes the proliferation of cariogenic bacteria, such as, *Lactobacillus* species and *S. mutans* (Dreizen *et al.*, 1977).

4. Virulence properties of *Streptococcus mutans*. *S. mutans*, originally isolated in 1924 from human carious lesions by Clarke, possesses a variety of virulence characteristics that can provide the organism with a competitive advantage over other plaque bacteria and contribute to caries progression (Hamilton, 1987). These include: high rates of sugar transport and glycolytic

activity, high levels of endogenous metabolism, the synthesis of abundant extracellular polysaccharides in the presence of sucrose, and the capacity to adapt to acidurance. In addition to providing the organism with the capacity to utilize exogenous sugar efficiently, these characteristics also provide *S. mutans* with the ability to survive adverse environmental conditions including low exogenous nutrient supply and low pH environments.

(a) Sugar transport. The major transport system in saccharolytic oral bacteria, including streptococci, is the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) (Kanapka and Hamilton, 1971; Dills *et al.*, 1980). The PTS has been referred to as a group translocation system differing only from active transport in that the carbohydrate transported is chemically modified during transport (Thompson, 1987; Saier and Reizer, 1992; Postma *et al.*, 1993). This system catalyzes the transport and phosphorylation of monosaccharides, disaccharides and sugar alcohols by deriving energy from the phosphoryl transfer from PEP to the incoming sugar. The *S. mutans* glucose-PTS (Figure 1-1) is a high affinity system (Ks = 10 μM) operating in the presence of low glucose concentrations, while a low affinity

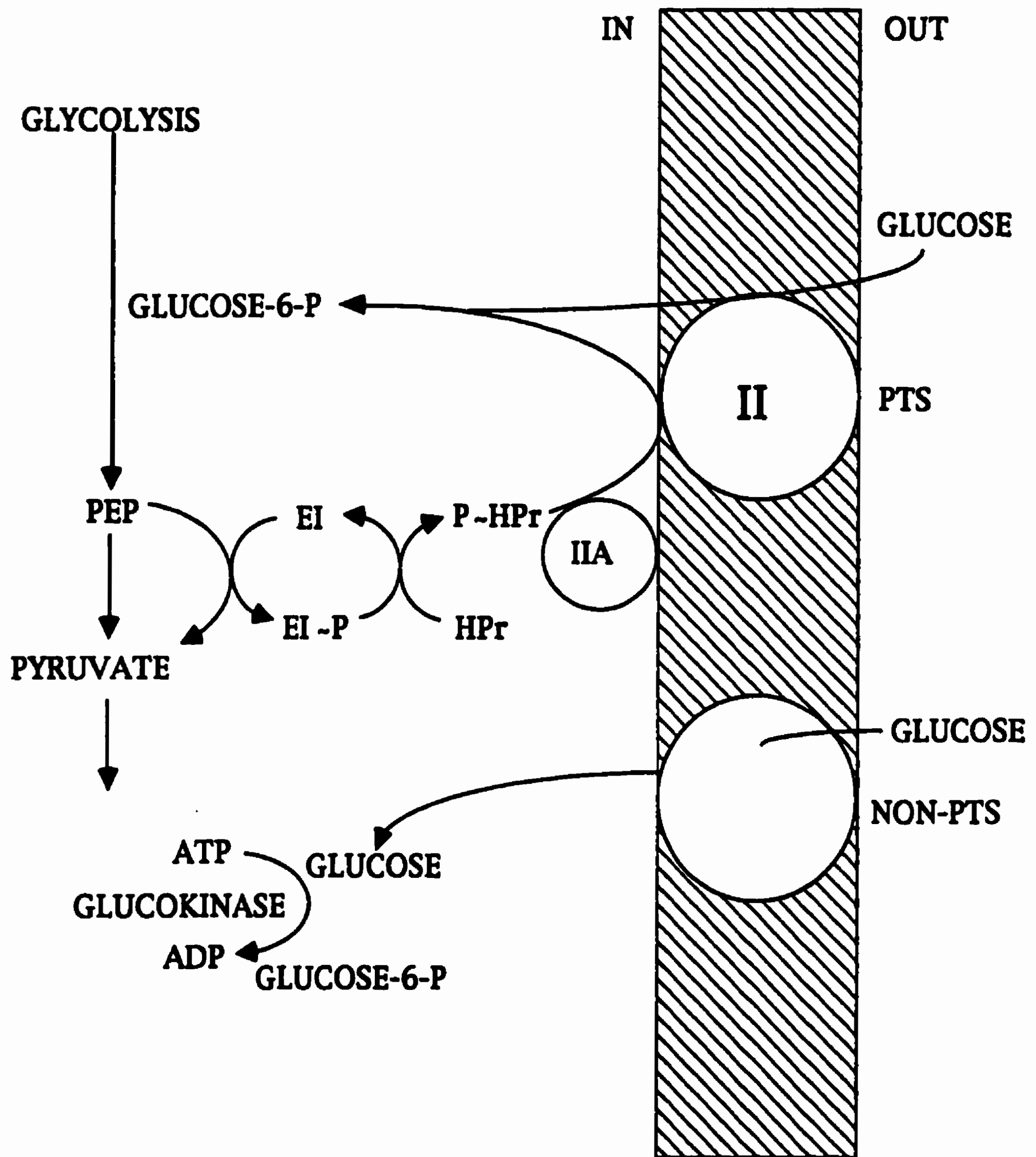

Figure 1-1: The glucose-PEP phosphotransferase system (PTS) of oral streptococci.

non-PTS transport system (Ks = 100-200 μM) is the main transport system in the presence of excess glucose concentrations (Ellwood *et al.*, 1979).

Continuous culture studies have shown that the glucose-PTS in *S. mutans* is repressed under various conditions including, low pH (Hamilton and Ellwood, 1978), high growth rates, excess glucose (Ellwood *et al.*, 1979) and with sucrose (Ellwood and Hamilton, 1982). Although PTS repression was observed under these conditions, glycolytic rates were not inhibited to the same degree suggesting the existence of an alternative, non-PTS glucose transport system. Kinetic studies have shown that the wild-type *S. mutans* strain DR0001, grown in continuous culture, possessed both a low affinity (K_s = 57-125 μM) and a high affinity glucose transport system (K_s = 6.7-8.0 μM), whereas the PTS-deficient *S. mutans* strain, DR0001/6, possessed only the low affinity system (K_s = 62-133 μM) (Hamilton and St. Martin, 1982). Early studies suggested that the alternative, non-PTS glucose transport system in *S. mutans* was linked to proton-motive force (Hamilton and St. Martin, 1982; Keevil *et al.*, 1986), while more recent studies using 6-deoxyglucose have proposed uptake via simple, or passive diffusion (Dashper and Reynolds, 1990). However, more recently, counterflow experiments using membrane vesicles prepared from cells of *S. mutans* grown in continuous culture under PTS-repressed and PTS-optimal conditions demonstrated that free glucose is

transported via a constitutive transmembrane carrier and phosphorylated by ATP and a glucokinase (Buckley and Hamilton, 1994). This physiological study has recently been verified genetically through research generating a PTS-defective *S. mutans* strain (Cvitkovitch *et al.*, 1995b). The mutant strain possessed an EI protein that was truncated by approximately 150 amino acids and could not phosphorylate glucose with PEP, but used ATP to generate glucose-6-P. In addition, kinetic studies of this mutant demonstrated a Ks of 125 μM for glucose, well within the range determined for the PTS-defective *S. mutans* strain DR0001/6 (Ks= 62-133 μM) (Hamilton and St. Martin, 1982).

In addition to PTS and non-PTS systems, indirect evidence has indicated that glucose can be transported in *S. mutans* via the sugar-binding protein-dependent transport system responsible for multiple sugar metabolism (Msm) (Russell *et al.*, 1992; Tao *et al.*, 1993). The Msm system involves eight genes located on an 11-kilobase (kb) gene region and positively regulated by the gene, *msmR* (Russell *et al.*, 1992). Originally, only raffinose, melibiose and isomaltosaccharides were thought to be transported by this system as they are substrates for the *aga* and *dexB* gene products, α-galactosidase and dextran glucosidase, respectively. However, competition studies using unlabelled sugars and radiolabelled melibiose demonstrated the ability of glucose, as well as isomaltose, panose, sucrose, and

fructose to effectively inhibit the transport of melibiose (Tao *et al.*, 1993).

(b) Glycolytic activity. Oral streptococci, including *S. mutans*, possess specific mechanisms for regulating the glycolytic rate in response to nutrient limitation and nutrient excess (Carlsson, 1983). During carbohydrate limitation, pyruvate kinase is inhibited by the cellular level of inorganic phosphate (Abbe and Yamada, 1982) and, as a result, accumulated PEP is available for sugar transport by the PTS. Consumption of food can result in a 10,000-fold increase in the sugar level in the oral cavity and cause 'substrate-accelerated death' through the accumulation of toxic glycolytic intermediates (Carlsson and Hamilton, 1994). Oral streptococci, including *S. mutans*, possess specific mechanisms that reduce the level of intracellular glycolytic intermediates (Carlsson, 1983). High levels of fructose-1,6-bisphosphate (FBP) activate lactate dehydrogenase resulting in the production of high concentrations of lactic acid (Yamada and Carlsson, 1975a). Excess glucose-6-phosphate activates pyruvate kinase (Yamada and Carlsson, 1975b) which, together with the activated lactate dehydrogenase, increases the overall glycolytic rate. In addition, an excess of fructose-1,6-bisphosphate activates ADP-glucose phosphorylase promoting the synthesis of intracellular glycogen (Mattingly *et al.*, 1977).

(c) **Endogenous metabolism.** *S. mutans* has been shown to produce an intracellular glycogen-like glucan (Critchley *et al.*, 1976; Hamilton, 1976) through the activity of two enzymes, adenosine diphosphate (ADP)-glucose pyrophosphorylase and ADP-glucose-glycogen glucosyltransferase (Birkhed and Tanzer, 1979). The former enzyme catalyzes the formation of ADP-glucose from glucose-1-phosphate at the expense of ATP, while the latter enzyme uses ADP-glucose to produce glycogen. Intracellular glycogen can be used as an endogenous energy supply as glycogen phosphorylase converts glycogen to glucose-1-phosphate which, following conversion to glucose-6-phosphate, can be metabolized via the Embden-Meyerhof pathway to generate formic and acetic acids as well as ethanol (Huis in't Veld and Backer Dirks, 1978). Nitrosoguanidine-generated mutants of *S. mutans* demonstrated the importance of intracellular polysaccaride formation in the caries process, as these mutants were determined to be less cariogenic than wild-type *S. mutans* (Tanzer *et al.*, 1976). More recently, Tn*916* transposon mutagenesis was used to facilitate cloning of genes involved in glycogen synthesis in *S. mutans* (Spatafora-Harris *et al.*, 1992). A deletion mutant was generated in *E. coli* and subsequent introduction into *S. mutans* through allelic exchange produced a glycogen-synthesis-deficient strain which demonstrated less cariogenic potential than the glycogen-proficient, wild-type strain in gnotobiotic rats.

(d) Synthesis of extracellular polysaccharides. *S. mutans* has been shown to synthesize extracellular glucans and fructans from sucrose through the activity of extracellular glucosyltransferases and fructosyltransferases (Hamada and Slade 1980; Loesche, 1986). The glucans produced by *S. mutans* can be of two types, water soluble and water insoluble, with the latter thought to promote dental plaque formation through enhanced adherence and aggregation (Hamada and Slade, 1980). Extracellular glucan production has been proposed to enhance the pH-lowering ability of plaque by increasing the spacing between bacterial cells (van Houte et *al.*, 1989). A high glucan content increased the volume of the bacterial plaque mass promoting diffusion channel formation, thereby, permitting sugars to penetrate deep into the bacterial cell mass. The generation of glucosyltransferase and fructosyltransferase *S. mutans* mutants (Munro *et al.*, 1991) confirmed the importance of glucan and fructan production in the cariogenic process as these extracellular polysaccarides can serve as endogenous food sources when the exogenous nutrient supply has been exhausted promoting continued acid production. The fructans and glucans are degraded to their fructose and glucose components, respectively, and these simple sugars can then be readily transported and metabolized by plaque bacteria (Carlsson and Hamilton, 1994).

(e) Adaptation to acidurance. Acid tolerance (acidurance) is a virulence factor exhibited by cariogenic organisms, such as *S. mutans*, for survival in low pH environments typical of pits, fissures and caries lesions (Harper and Loesche, 1984; Carlsson, 1986b). Continuous culture studies have demonstrated the capability of *S. mutans* to adapt to growth at pH 4.8 following a natural pH drop from pH 7.0 (Hamilton and Bowden, 1982). Growth in an acidic environment was shown to promote changes in specific aspects of cell physiology, such as, decreases in the pH optimum for glucose uptake and glycolysis, as well as, the pH optimum for the minimum permeability to protons (Hamilton and Buckley, 1991). In many bacteria, such as *E. coli*, the intracellular pH is maintained near neutrality (Padan *et al.*, 1981) and, as a result, cells expend considerable energy to maintain the pH of the cytoplasmic compartment in low pH environments. On the other hand, oral streptococci maintain a relatively constant transmembrane pH gradient allowing their intracellular pH to decrease in response to a declining environmental pH, thereby, conserving energy (Hamilton, 1990; Hamilton and Buckley, 1991). Central to the regulation of the transmembrane gradient is the activity and rate of synthesis of the membrane-bound, proton-translocating ATPase (Bender *et al.*, 1986, Kobayashi *et al.*, 1986). In many bacteria, the movement of protons out of the cell by the ATPase serves to protect the acid-sensitive glycolytic

enzymes, although glycolysis by *S. mutans* can occur at pH values as low as 4.0 (Bender *et al.*, 1986).

The virulence of *S. mutans* can be partially attributed to its acidogenicity or its ability to rapidly ferment sugars to lactic acid in the presence of a low pH environment (Loesche, 1986). Lactate efflux was suggested to involve lactate excretion through a membrane carrier in association with more than one proton, generating a proton motive force which provides energy to the cell (Michels *et al.*, 1979). More recently, the mechanism of lactate efflux by *S. mutans* has been shown to involve a membrane carrier for lactic acid (Carlsson and Hamilton, 1996), as well as, the membrane-bound, proton-translocating ATPase (Dashper and Reynolds, 1996). In the latter study, lactic acid efflux only occurred as the intracellular concentration of lactic acid approached 100 mM and transport across the membrane was an electroneutral process. The role of the proton-translocating ATPase was to maintain the transmembrane gradient through the extrusion of protons, thereby, allowing the accumulation of lactate necessary for the generation of a lactate gradient to drive the efflux of lactic acid through the carrier (Dashper and Reynolds, 1996).

Elucidation of acid tolerance from a genetic standpoint has been initiated through generation of a mutant strain of *S. mutans* strain defective in aciduricity using the conjugative

transposon Tn*916* (Yamashita *et al.*, 1993). Physiologically, this mutant was unable to grow below pH 5.5 and demonstrated sensitivity to high osmolarity and elevated temperatures. Genetic characterization indicated that Tn*916* inserted at the 3' end of an open reading frame (ORF1) and the deduced amino acid sequence of ORF1 showed significant homology to the diacylglyceral kinase (DGKase) from *E. coli* (Lightner *et al.*, 1983; Loomis *et al.*, 1985). The *E. coli* DGKase catalyzes the ATP-dependent phosphorylation of *sn*-1,2-diacylglycerols to generate phosphatidic acid and the authors propose that this system may be involved in the transmission of extracellular environmental signals in *S. mutans* (Yamashita *et al.*, 1993).

More recently, insertional mutagenesis using the transposon Tn*917*, delivered on a replication-conditional (temperature-sensitive) vector generated three acid sensitive mutants of *S. mutans* (Gutierrez *et al.*, 1996). Two of these strains, AS5 (*fhs*::Tn*917*) and AS17 (*sat*::Tn*917*) displayed a tight acid-sensitivity phenotype and experiments to further define and characterize the origin of acid sensitivity are currently being conducted. In other work, the gene encoding the *S. mutans* stress protein, DnaK, has been sequenced and physiological studies have shown an increase in DnaK protein levels in response to acid shock (Jayaraman and Burne, 1996). It has been proposed that DnaK is one of several proteins involved in assisting other polypeptides achieve their active state (LaRossa and Van Dyk, 1991). Considering this role in

polypeptide folding, DnaK may be used as a molecular probe to identify the proteins expressed by *S. mutans* in response to acid (Jayaraman and Burne, 1996).

(B) The phosphoenolpyruvate: sugar phosphotransferase transport system (PTS)

1. Components of the PTS. Since the PTS was first described (Kundig *et al.*, 1964) this system, which utilizes PEP as a phosphoryl donor for sugar phosphorylation and transport, has been studied in both Gram-negative and Gram-positive bacteria to characterize and establish the functions of the various components (Figure 1-2). The two soluble, cytoplasmic proteins of the PTS are the heat-stable protein, HPr, and Enzyme I (EI), involved in the phosphorylation of all PTS sugars (Postma and Lengeler, 1985; Postma *et al.*, 1993). The genes encoding enzyme I (*ptsI*) and HPr (*ptsH*) have been cloned and sequenced for *E. coli* (Saffen *et al.*, 1987; De Reuse and Danchin, 1988), *Salmonella typhimurium* (Byrne *et al.*, 1988; Schnierow *et al.*, 1989), *Staphylococcus carnosus* (Eisermann *et al.*, 1991; Kohlbrecher *et al.*, 1992), as well as, *S. salivarius* (Gagnon *et al.*, 1992; Gagnon *et al.*, 1993) and *S. mutans* (Boyd *et al.*, 1994). In all of these cases, *ptsI* has been located immediately downstream of *ptsH* forming a *pts* operon co-transcribed from a promoter upstream of *ptsH*. Three transcripts of the *S. salivarius pts* operon have been identified, two long transcripts of the *ptsH* and *ptsI* genes and a short transcript specific for *ptsH*

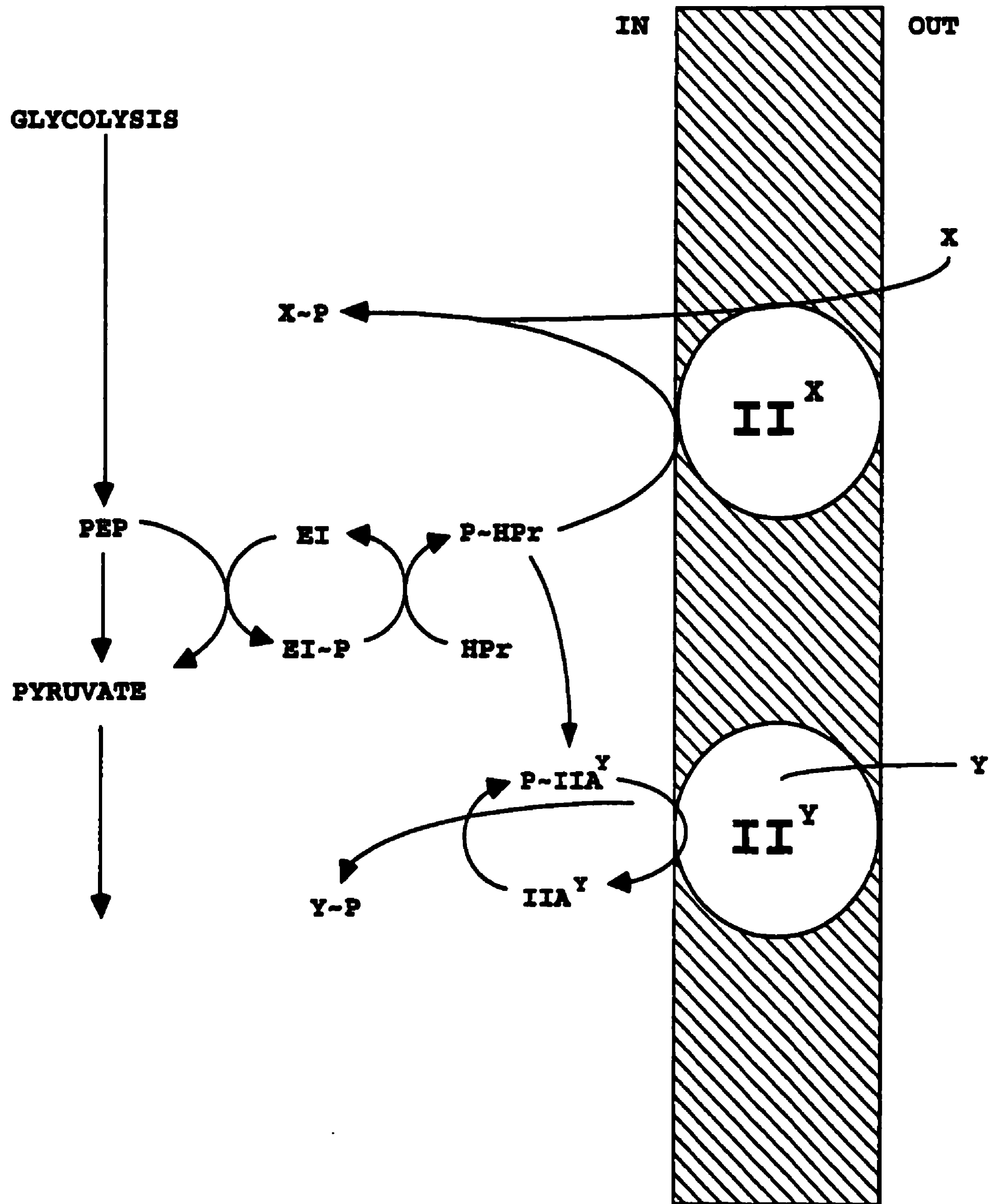

Figure 1-2: The phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) of *S. mutans* consists of two general cytoplasmic proteins EI and HPr, as well as, membrane-associated sugar-specific EIIs. EIIs can exist as a single entity II^X or may require a sugar-specific cytoplasmic-associated IIA^Y, where X and Y represent the specific sugar, such as glucose, lactose, mannitol or sorbitol (see text for details).

(Gagnon *et al.*, 1995). The three mRNA species were transcribed from the promoter located upstream of the *ptsH* gene and transcription of the short transcript terminated at a high-energy stem-loop structure located at the 5' end of the *ptsI* gene. The long transcripts terminated at the two terminator structures located at the 3' end of the *ptsI* gene. In addition, a second promoter was located at the 3' end of the *ptsH* gene and was suggested to uncouple the synthesis of HPr and EI (Gagnon *et al.*, 1995). Similar sequences corresponding to the second promoter and high-energy, stem-loop structure are present at the same position within the sequence of the *S. mutans pts* operon (Boyd *et al.*, 1994). Unlike all other bacterial species characterized thus far the *ptsH* and *ptsI* genes from *Mycoplasma capricolum* are not contiguous on the chromosome (Zhu *et al.*, 1993). In this organism the *ptsH* gene is part of a monocistronic operon that is located between two open reading frames unrelated to the PTS.

EI has been purified to homogeneity for several organisms (Postma *et al.*, 1993) and research has demonstrated that phosphorylation of EI not only requires a dimeric form of the protein (Kukuruzinska *et al.*, 1982; Misset *et al.*, 1980), but also divalent cations, such as, Mg^{++} or Mn^{++} (Weigel *et al.*, 1982a). In addition, examinations of EI from *Sal. typhimurium* (Weigel *et al.*, 1982c) and *E. coli* (Waygood, 1986) have indicated that there are two phosphorylation sites

per dimer and phosphorylation by PEP occurs at the N-3 position of a histidyl residue (Weigel *et al.*, 1982c). The monomer molecular weights have been precisely determined, or estimated, for several bacteria including *E. coli* (Waygood and Steeves, 1980) and *Sal. typhimurium* (Weigel *et al.*, 1982c), as well as, the oral streptococci, *S. salivarius* (Vadeboncoeur *et al.*, 1983) and *S. mutans* (Thibault and Vadeboncoeur, 1985). The sequences of enzyme I have shown homology with both pyruvate:phosphate dikinases of plants and bacteria and PEP synthase of *E. coli* suggesting that the individual components of the PTS evolved independently and then came together to produce a functional sugar transport system (Saier and Reizer, 1994).

HPr is a small protein with a molecular weight ranging from 8,901 to 9,418 daltons (Vadeboncoeur, 1995) and has been shown to be phosphorylated at the N-1 position (Weigel *et al.*, 1982b) of a histidine residue (His-15), presumably because the N3 position is protonated at physiological pH (Van Dijk *et al.*, 1990). HPr from Gram-positive bacteria can be phosphorylated at a serine residue (Ser-46) by a specific HPr kinase at the expense of ATP and dephosphorylated by an inorganic phosphate-dependent phosphatase (Reizer *et al.*, 1993). In addition, P-(Ser)-HPr has been shown to be phosphorylated by EI at the expense of PEP to produce a doubly-phosphorylated form, P~(His)-P-(Ser)-HPr (Deutscher *et al.*, 1984). As a consequence, HPr can be found in four

different forms: free HPr, P~(His)-HPr, P-(Ser)-HPr and P~(His)-P-(Ser)-HPr. All four forms have been detected in *S. mutans* and *S. salivarius* using crossed immunoelectrophoresis (Vadeboncoeur *et al.*, 1991a) with exponentially growing cells shown to possess P-(Ser)-HPr and P~(His)-P-(Ser)-HPr, whereas stationary phase cells contained free HPr and P~(His)-HPr.

Free HPr from various streptococci and *Lactococcus lactis* has been shown to exist in two forms differing only in the presence of an N-terminal methionine (Robitaille *et al.*, 1991; Vadeboncoeur *et al.*, 1991b), which is cleaved by streptococcal methionine aminopeptidase (MAP) (Ben-Basset, 1987). In *S. salivarius*, the two forms of HPr, HPr-1 (without methionine) and HPr-2 (with methionine), can be phosphorylated by ATP and PEP, and the ratio of HPr-1/HPr-2 differs with the growth conditions (Robitaille *et al.*, 1991). For example, the ratio of HPr-1/HPr-2 for *S. salivarius* grown in continuous culture was highest with glucose limitation at a low dilution rate and was decreased at high glucose concentrations and dilution rates indicating that MAP activity was optimal during cell growth with low carbohydrate (Vadeboncoeur *et al.*, 1993). Recently, originally identified as antigen D, HPr-1 from *S. mutans* has been shown to exist on the cell surface as a wall-associated protein (Sutcliffe *et al.*, 1993) and immunogold microscopy has also identified HPr-1 on the external surface of *S. suis* (Dubreuil *et al.*, 1996.)

HPr from *S. mutans* contains 86 (HPr-1) or 87 (HPr-2) amino acids and shares considerable sequence homology with other Gram-positive HPr proteins, specifically at the phosphorylation sites (His-15 and Ser-46) and the area around these sites (Dashper *et al.*, 1994). In addition, the tertiary structures of HPr from Gram-positive and Gram-negative bacteria have been examined using NMR spectroscopy and X-ray crystallography (van Nuland *et al.*, 1994) and are composed of an "open-faced β-sandwich consisting of two or three α-helices located on top of a four stranded anti-parallel β-sheet" (Dashper *et al.*, 1994). Using comparative molecular modeling, the *S. mutans* HPr has been predicted to have the same three-dimensional structure and possessing two α-helices (Dashper *et al.*, 1994). The amino acid arginine-17 (Arg-17) is conserved in all HPr proteins (Meadow *et al.*, 1990) and three-dimensional structural examinations of HPr have led to the proposal that Arg-17 has an important role in stabilizing P~(His)-HPr, probably via interaction with the phosphate group (Herzberg *et al.*, 1992).

The original nomenclature of the Enzyme II complexes (permeases) of the PTS referred to Enzymes II (EII) and factor III, describing the sugar-specific membrane-bound proteins and the separate soluble protein, respectively (Postma *et al.*, 1993). In other cases, however, the soluble factor III was not a separate protein but a cytoplasmic domain of EII (Saier *et al.*, 1988). Recently, Saier and

Reizer (1992) have proposed new nomenclature taking into consideration that the enzyme II complexes are comprised of three (or four) structural domains consisting of a single, fused protein with three domains or several proteins, with at least one protein bound to the membrane. The term, 'IIA', has replaced 'factor III' describing the hydrophilic domain possessing the first phosphorylation site, whereas 'EII' has been divided to describe the hydrophobic domain bearing the second phosphorylation site as 'IIB', and the hydrophobic domain responsible for sugar binding and transport as 'IIC'. In some cases, the permeases may include an additional protein, referred to as 'IID' (Erni *et al.*, 1987) which has been suggested, like IIC, to be involved in membrane channel formation (Saier and Reizer, 1992). EII complexes consisting of fused domains are connected through short amino acid 'linkers' and the domains may be arranged in different sequences (Postma *et al.*, 1993).

Sequence comparisons have led to the classification of the various EII complexes into 4 main groups referred to as the glucose, mannitol, lactose and mannose classes, each class consisting of permeases possessing more than 20% amino acid homology (Postma *et al.*, 1993). The permeases in these classes consist of three domains, IIA, IIB, and IIC, with the exception of the mannose class that also includes the IID protein. This latter class has been referred to as a 'splinter group' (Reizer *et al.*, 1991) as these mannose

complexes have been demonstrated to have little sequence homology with the other classes of EII complexes. Recently, the *N*-acetylgalactosamine EII complex, coded within the *aga* gene cluster of *E. coli*, has been shown to have sequence similarities to the mannose class of enzymes II (Reizer *et al.*, 1996b). The *N*-acetylgalactosamine EII complex is unusual being comprised of IID, two IIC proteins, one full-length and one truncated, two IIB proteins, and without a IIA protein (Reizer *et al.*, 1996b).

Variation in the domain structure of EII complexes has led to the general rule stating that if the IIA domain is part of a polypeptide EII, it is connected to the C-terminal end of the chain (Saier and Reizer, 1994). For example, within the glucose class, the EII for sucrose transport in *S. mutans* consists of a single polypeptide including fused IIA, IIB and IIC domains joined by linkers resulting in the domain structure IIBCA (Sato *et al.*, 1989). On the other hand, the glucose EII for *Bacillus subtilis* has the domain structure of IICBA (Gonzy-Treboul *et al.*, 1991). The mannitol IIA in Gram-positive bacteria including, *S. mutans* (Honeyman and Curtiss, 1992), *Enterococcus faecalis* (Fischer *et al.*, 1991) and *Staph. carnosus* (Fischer and Hengstenberg, 1992) exists as a separate protein, whereas the mannitol EII in *E. coli* consists of a single polypeptide chain, IICBA (Lee and Saier, 1983). In addition, the glucitol EII for *E. coli* consists of a IIB domain between two hydrophobic domains, IIC and IIC',

each half the size of a normal IIC domain (Yamada and Saier, 1987). This is of particular interest, since glucitol (sorbitol) is a sugar alcohol structurally related to mannitol, however the mannitol and glucitol EII complexes are significantly different resulting in separate classification of the glucitol EII (Postma *et al.*, 1993).

Many EII complexes are capable of binding several different substrates, however, the relative affinities for the substrates differ, resulting in preferential transport of one sugar over another when present in equal concentrations (Postma and Lengeler, 1985). This competition between substrates for transport results in diauxic growth as seen in wild-type *E. coli*, which transports mannitol preferentially over glucitol (Postma and Lengeler, 1985). The diauxic effect is reversed in a mutant *E. coli* strain lacking the mannitol Enzyme II (EII^{Mtl}) as the glucitol Enzyme II (EII^{Gut}) has lower affinity for mannitol compared to glucitol (Lengeler and Lin, 1972) resulting in preferential transport of glucitol.

2. PTS-mediated transport and phosphorylation. Transport of carbohydrates into the cell by the PTS is coupled to phosphorylation although the two processes are mechanistically separate. The primary role of the general proteins, HPr and EI, is phosphoryl donation and the EII complex is directly involved in translocation and

phosphorylation of the carbohydrate across the membrane. The first phosphorylation reaction involves the transfer of a phosphoryl group from PEP to EI producing phospho-enzyme I and pyruvate (Postma and Lengeler, 1985). It has been proposed that a carboxy-terminal domain of EI is required for both dimer formation and interaction with phosphoenolpyruvate, while an amino-terminal domain possessing the phosphorylated histidine residue is involved in the phosphoryl exchange with HPr (LaCalsi *et al.*, 1991). In addition, unlike EI, the phosphorylation of HPr does not require divalent metal ions (Postma and Lengeler, 1985). Following transfer from EI to form P~(His-15)-HPr, the phosphoryl group is transferred to a histidine residue on IIA, then to a histidine or cysteine residue on the IIB domain (Reizer *et al.*, 1993). Upon phosphorylation of IIB, a conformational change occurs in the protein resulting in translocation of the bound carbohydrate across the membrane to face the cytoplasm. The last stages of the PTS cascade include phosphorylation of the bound substrate by P~IIB followed by release of the sugar phosphate into the cytoplasm (Postma *et al.*, 1993).

Constitutive PTS systems have been identified in *S. mutans*, including systems for glucose (Schachtele and Mayo, 1973) and mannose (Vadeboncoeur, 1984), as well as, systems induced by the presence of the specific sugar. In *S. mutans*, inducible systems are known for lactose (Calmes, 1978), sucrose (Slee

and Tanzer, 1979a; Slee and Tanzer, 1979b), sorbitol and mannitol (Maryanski and Wittenberger, 1975; Brown and Wittenberger, 1973). Fructose, however, has two PTS transport systems (Gauthier *et al.*, 1984), one which is inducible and the other constitutive. In addition, many of the inducible PTS systems are subject to catabolite repression by glucose (Carlsson and Hamilton, 1994). Oral streptococci other than *S. mutans* possess similar constitutive and inducible phosphotransferase systems (Thompson, 1987).

Sucrose metabolism by *S. mutans* plays a significant role in its cariogenicity and this disaccharide has been shown to be transported by several different mechanisms. While recent research has demonstrated that sucrose can be transported by the multiple sugar metabolism system (Msm) in *S. mutans* (Tao *et al.*, 1993), earlier studies have demonstrated transport through both an inducible, high affinity (Slee and Tanzer, 1979b) and low affinity PTS systems (Poy and Jacobson, 1990). The high-affinity sucrose PTS has a K_m for sucrose of 70 μM (Lodge and Jacobson, 1988), while the K_m for the low-affinity system is 350 μM (Jacobson *et al.*, 1989). This latter system has been reported to be a high-affinity trehalose system that is capable of recognizing sucrose (Poy and Jacobson, 1990). The intact disaccharide transported by the high-affinity sucrose-PTS is cleaved by intracellular sucrose-6-phosphate hydrolase to glucose-6-phosphate and fructose (Chassy and

Porter, 1982; St. Martin and Wittenberger, 1979). The gene encoding sucrose-6-phosphate hydrolase in *S. mutans*, *scrB*, has been cloned and a *scrB*-defective mutant was generated by allelic exchange (Lunsford and Macrina, 1986) and shown to be sensitive to sucrose at concentrations above 0.05 mM, presumably due to the accumulation of toxic levels of sucrose-6-P (St. Martin and Wittenberger, 1979).

(C) Regulation and the PTS

1. Regulation by P-(Ser)-HPr. In 1983, research investigating the regulatory processes of inducer expulsion in *S. pyogenes* led to the discovery of the phosphorylation of HPr at the serine-46 residue, a reaction catalyzed by an ATP-dependent (Ser)HPr kinase (Reizer *et al.*, 1983; Deutscher and Saier, 1983). Subsequently, ATP-dependent phosphorylation of HPr has been established for many Gram-positive bacteria, however, Gram-negative bacteria do not possess a corresponding HPr kinase (Reizer *et al.*, 1993). ATP-dependent (Ser)HPr kinases for several Gram-positive bacteria have been shown to be activated by different metabolites including, fructose-1,6-bisphosphate (FBP), gluconate-6-phosphate, and 2-P-glycerate, and inhibited by inorganic phosphate (Pi) (Reizer *et al.*, 1993). Alternatively, P-(Ser)-HPr phosphatase catalyzes the formation of free HPr from P-(Ser)-HPr and is stimulated by Pi (Deutscher *et al.*, 1985).

In vitro studies with Gram-positive bacteria have led to the proposal that ATP-dependent phosphorylation of HPr serves to regulate PTS activity. P-(Ser)-HPr can not replace P~(His)-HPr as a phosphoryl donor in the PTS cascade (Deutscher and Saier, 1983; Reizer *et al.*, 1984), therefore, P-(Ser)-HPr formation has been suggested to repress PTS activity. In the presence of excess exogenous glucose, the level of intracellular glycolytic intermediates, such as fructose-1,6-bisphosphate, is high thereby stimulating (Ser)HPr kinase activity and promoting the formation of P-(Ser)-HPr. Consequently, the level of free HPr available for phosphorylation by EI and PEP is reduced, inhibiting PTS activity. Formation of the doubly phosphorylated form of HPr (P~(His)-P-(Ser)-HPr) has been shown to occur slowly, as phosphorylation of P-(Ser)-HPr by EI at the expense of PEP occurs 5000-fold slower than the phosphorylation of free HPr (Deutscher *et al.*, 1984). In addition, a similar inhibition was demonstrated for phosphorylation of P~(His)-HPr by the ATP-dependent kinase (Reizer *et al.*, 1984). The three dimensional structure of HPr has shown that the Ser-46 residue is in close proximity to His-15 (Chen *et al.*, 1993a), and as a consequence, prior HPr phosphorylation of the active site, His-15, or the regulatory site, Ser-46, significantly inhibits subsequent phosphorylation, either through electrostatic repulsion or by blocked binding of the secondary enzyme (Reizer *et al.*, 1993).

Regulation of sugar uptake via the PTS by P-(Ser)-HPr has been demonstrated in vesicles of *L. lactis* (Ye *et al.*, 1994c,d). To demonstrate this, wild-type *B. subtilis* HPr was replaced by two mutant forms of HPr, S46A and S46D, where the serine residue was replaced by alanine and aspartate residues, respectively. The mutant *B. subtilis* proteins were electroporated into *L. lactis* vesicles and the uptake of the glucose analogue, 2-deoxyglucose (2-DG) was monitored in the presence and absence of FBP. In the presence of the S46A mutant HPr protein, uptake of 2-DG was comparable to uptake in the presence of the wild-type HPr, however, FBP inhibition of uptake was insignificant (Ye *et al.*, 1994d). On the other hand, in the presence of the P-(Ser)-HPr analogue, S46D HPr, inhibition of 2-DG uptake was observed in the presence and absence of FBP. Similar results were obtained for the uptake of the lactose analogue, thiomethyl-β-galactoside (TMG), by the same *L. lactis* vesicles incubated in the presence and absence of glucose (Ye *et al.*, 1994c). Overall, the results from these studies indicated that P-(Ser)-HPr serves to regulate the lactose and glucose PTS systems in *L. lactis* vesicles.

P-(Ser)-HPr-mediated regulation of the PTS has been demonstrated by a chromosomal mutant of *B. subtilis* (GM1222) carrying the S46A HPr mutation (*ptsH1*) (Ye and Saier, 1996). The uptake of fructose by the wild-type and mutant strains of *B. subtilis* was monitored in the presence and absence of

glucose. The presence of glucose strongly inhibited fructose uptake in the wild-type strain, however, weak inhibition was observed with the mutant strain. The authors proposed that P-(Ser)-HPr-mediated regulation may provide a feedback mechanism to control the rate of PTS sugar uptake or possibly a mechanism for the preferential use of some PTS sugars over others, with glucose at the top of the hierarchy (Ye and Saier 1996). HPr, (Ser)HPr kinase, as well as P-(Ser)-HPr phosphatase have been demonstrated in heterofermentative lactobaccilli lacking a functional PTS (Reizer *et al.*, 1988a) suggesting a non-PTS regulatory role for P-(Ser)-HPr. This will be discussed in detail in the last section of this review.

2. Factors affecting synthesis of PTS components. While some environmental factors, such as presence of a PTS substrate and anaerobic conditions, promote expression of the PTS components (Postma and Lengeler, 1985; Postma *et al.*, 1993) others, including low pH and high growth rates, serve to repress synthesis (Vadeboncoeur *et al.*, 1991c). As mentioned previously, early continuous culture studies with *S. mutans* had indicated that the activity of the glucose-PTS was regulated by environmental factors (Hamilton and Ellwood, 1978; Ellwood *et al.*, 1979; Ellwood and Hamilton, 1982). Later studies were designed to determine the component(s) of the PTS affected by the various environmental parameters (Vadeboncoeur *et al.*, 1987; Hamilton *et al.*, 1989;

Vadeboncoeur *et al.*, 1991c). In these studies, EII activity was assayed by measuring the PEP-dependent phosphorylation of radiolabelled sugars in the presence of excess HPr and EI, while the intracellular levels of HPr and EI were assayed using rocket immunoelectrophoresis. Synthesis of the general proteins, EI and HPr, were repressed 4-fold in the presence of excess glucose (304 mM) compared to glucose-limiting conditions (2.6 mM) (Hamilton *et al.*, 1989). Similarly, only a three-fold decrease in the expression of these proteins was observed following changes in growth pH (pH 8.0 to 5.0) and growth rate (D = 0.1 to 1.0 h^{-1}) (Vadeboncoeur *et al.*, 1991c). These results indicated that environmental factors that serve to repress glucose-PTS activity did not significantly repress the synthesis of HPr or EI, but acted on the synthesis of the EII component. Specifically, during growth in the presence of excess glucose (304 mM), a 27-fold repression in the level of activity was observed for EII^{Glc} and EII^{Man} (Hamilton *et al.*, 1989). Similarly, EII activity for glucose, mannose and 2-deoxyglucose was repressed 24 to 27-fold at pH 5.5 and D = 0.4 h^{-1} compared with growth at pH 7.0 and D = 0.1 h^{-1} (Vadeboncoeur *et al.*, 1987). Higher growth rates (0.5-1.0 h^{-1}) resulted in negligible levels of EII activity for these sugars, while growth at pH 5.0 completely abolished activity (Vadeboncoeur *et al.*, 1991c).

The ability of intracellular metabolites, specifically carbohydrate phosphates, to regulate EII activity negatively

has been established for over two decades (Reizer *et al.*, 1988b). This inhibitory effect is not observed upon the initial presence of the particular carbohydrate phosphate in the cell, but the extent of inhibition is proportional to the intracellular levels of the carbohydrate phosphate (Postma and Lengeler, 1985). For example, methyl α-glucoside phosphate inhibited *Sal. typhimurium* II^{Glc} at concentrations above 0.2 mM (Liu and Roseman, 1983). In addition, the synthesis of the xylitol EII complex (EII^{Xtl}) in *Lactobacillus casei* has been demonstrated to be repressed in the presence of increased levels of xylitol-5-P (London and Hausman, 1982), while accumulation of 2-deoxyglucose-6-P repressed the synthesis of EII^{Man} in *S. lactis* (*L. lactis*) (Thompson and Chassy, 1983). The mechanism involved in carbohydrate phosphate inhibition of EII activities has not been elucidated, however, it has been speculated that inhibition is due to binding of the carbohydrate phosphate to an allosteric site on the protein (Postma and Lengeler, 1985; Reizer *et al.*, 1988b)

3. Regulation of inducible PTS operons. Mechanisms regulating the expression of inducible PTS systems have not been elucidated for oral streptococci, therefore, regulatory mechanisms established for Gram-negative and other Gram-positive bacteria will be examined in this review (Table 1-1).

Table 1-1

List of inducible PTS sugar systems and corresponding regulatory proteins.

PTS System	Organism	Regulatory Proteins
Sucrose	*Sal. typhimurium*	Repressor (ScrR)
	B. subtilis	Antiterminators (SacT/SacY)
Mannitol	*E. coli*	Repressor (MtlR)
Glucitol	*E. coli*	Repressor/Activator (GutR/GutM)
	B. subtilis	Activator(GutR)
β-glucoside	*E. coli*	Antiterminator (BglG)
	B. subtilis	Antiterminator (LicT)

(a) Regulation by repressor and/or activator proteins. In many bacteria, sucrose has been demonstrated to be transported and phosphorylated by EII^{Scr}, followed by cleavage by a sucrose-6-phosphate hydrolase to fructose and glucose-6-P (Chassy and Porter, 1982). The genes encoding EII^{Scr} (*scrA*) and sucrose-6-phosphate hydrolase (*scrB*) have been sequenced for several bacteria, including the Gram-positive bacteria, *B. subtilis* (Fouet *et al.*, 1986; Fouet *et al.*, 1987), *S. mutans* (Sato and Kuramitsu, 1988; Sato *et al.*, 1989) and *S. sobrinus* (Chen *et al.*, 1993b). *Klebsiella pneumoniae* has been shown to possess a sucrose regulon (Titgemeyer *et al.*, 1996), however, most enteric bacteria, including *E. coli*, are unable to ferment sucrose unless they harbor the plasmid pUR400, which possesses the sucrose PTS regulon from *Sal. typhimurium* (Schmid *et al.*, 1982). The sucrose regulon from enteric bacteria comprises the *scrA* and *scrB* genes together with the genes *scrK*, *scrY* and *scrR*, encoding fructokinase, a sucrose-specific porin and a repressor protein, respectively (Titgemeyer *et al.*, 1996). The *scrR* gene is transcribed independently from the other *scr* genes (Schmid *et al.*, 1988; Jahreis and Lengeler, 1993) and sucrose PTS regulons have been shown to possess a characteristic operator region in the *scrK* promoter (Aulkemeyer *et al.*, 1991) and in the promoter for the *scrYAB* operon (Cowan *et al.*, 1991). Overall, sucrose regulons from enteric bacteria have been shown to be regulated by the repressor protein, ScrR, and free intracellular fructose and

fructose-1-phosphate have been identified as inducers of ScrB (Schmid *et al.*, 1988; Jahreis and Lengeler, 1993).

In *E. coli*, expression of the mannitol (*mtl*) operon has been shown to involve a repressor (Figge *et al.*, 1994), while expression of the glucitol (*gut*) operon has been shown to be regulated by a repressor protein (*gutR*) and an activator protein (*gutM*) (Yamada and Saier, 1988). The gene order of the *mtl* operon has been determined to be *mtlOPADR*, where *mtlA* codes for the EII, *mtlD* codes for mannitol-phosphate dehydrogenase and *mtlR* codes for the repressor protein (Figge *et al.*, 1994). Free cytoplasmic mannitol has been proposed to be the inducer of the operon and it is of interest that the operon has been shown to be induced in the absence of exogenous mannitol in *pts* mutants, an observation not clearly understood. In *E. coli*, the gene order of the *gut* operon is *gutOPABDMR*, where the *gutA*, *gutB* and *gutD* genes code for EII^{Gut}, $EIIA^{Gut}$, and glucitol-6-phosphate dehydrogenase, respectively (Yamada and Saier, 1988). The GutR and GutM proteins have been proposed to either compete for a binding site in the operator or impose their antagonistic effects by binding to two separate sites (Yamada and Saier, 1988). Physiological experiments have led to the proposal that glucitol is the inducer binding to the GutR protein causing dissociation from the DNA (Lengeler and Steinberger, 1978). In *B. subtilis*, the genes for glucitol metabolism have been determined to be *gutR*, *gutA*, *gutB*, coding for a regulatory

factor, permease, and a dehydrogenase, respectively (Ye *et al.*, 1994e). Although these genes have been determined to be clustered together in the order *gutR-gutB-gutA* (Gay *et al.*, 1983), *gutR* is not in the same operon as it is transcribed in the opposite direction (Ye *et al.*, 1994e). The regulatory mechanism for glucitol metabolism has been proposed to involve two conformations of GutR, an inactive form in the absence of glucitol and an active form in the presence of glucitol leading to induction of *gutB* transcription.

(b) Regulation by transcriptional antitermination. In *E. coli*, the *bgl* operon involved in β-glucoside utilization has been determined to consist of the genes *bglG*, *bglF* and *bglB* (Schnetz *et al.*, 1987). The first gene of the operon *(bglG)*, which codes for the transcriptional antiterminator regulatory protein, has been shown to be flanked by rho-independent terminator-coding sequences (Mahadevan and Wright, 1987: Schnetz *et al.*, 1987). The *bglF* gene codes for EII^{Bgl}, while the last gene of the operon, *bglB*, encodes a phospho-β-glucosidase (Amster-Choder and Wright, 1993). Central to the regulation of the *bgl* operon is the phosphorylated state of the BglG protein, as active BglG has been shown to be a non-phosphorylated dimer, while inactive BglG has been shown to be a phosphorylated monomer (Amster-Choder and Wright, 1992). In the absence of β-glucosides, transcription of the *bgl* operon has been shown to be constitutive with transcription termination at the first

rho-independent terminator (Mahadevan and Wright, 1987), as the formation of a terminator structure results in dissociation of RNA polymerase. In the absence of β-glucosides, BglF has been shown to act as a protein kinase to phosphorylate BglG (Amster-Choder *et al.*, 1989) producing the inactive monomeric form of the protein. In the presence of β-glucosides, BglG-P becomes dephosphorylated as BglF-P (EII^{Bgl}-P) phosphorylates the incoming β-glucoside. As a result, non-phosphorylated BglG can dimerize and bind to a 32-nucleotide sequence in the leader region of the mRNA preventing formation of the terminator structure and permitting transcription of the operon (Houman *et al.*, 1990).

In *B. subtilis*, the expression of genes involved in sucrose metabolism has been shown to occur by a mechanism similar to that established for *bgl* operon expression in *E. coli* (Amster-Choder and Wright, 1993). The *sacPA* operon and the *sacB* gene are regulated by the proteins encoded by the *sacT* gene and *sacXY* operon, respectively (Arnaud *et al.*, 1992). The *sacY* gene product has been determined to be fully activated only in the presence of sucrose concentrations above 1% (Crutz *et al.*, 1990). The genes *sacP* and *sacA* code for an endocellular sucrase (Fouet *et al.*, 1986) and EII^{Scr} (Fouet *et al.*, 1987), respectively, while *sacB* encodes a second sucrose-hydrolyzing enzyme, levansucrase (Steinmetz *et al.*, 1985). SacY and SacT have been shown to have homology to BglG (Debarbouille *et al.*, 1990), and SacX has been

identified as a membrane-bound PTS protein similar to BglF, which regulates SacY through phosphorylation and dephosphorylation (Arnaud *et al.*, 1992; Amster-Choder and Wright, 1993). SacT activity has been proposed to be regulated at two phosphorylation sites (Arnaud *et al.*, 1992). In one model, SacT has been considered inactive if phosphorylated both at the activation site by a soluble component of the PTS and at the inactivation site in the absence of sucrose. Upon transport of sucrose, the inactivation site becomes dephosphorylated and SacT becomes active.

In *B. subtilis*, expression of the *bglPH* operon for β-glucoside utilization (Le Coq *et al.*, 1995) has been proposed to be regulated by the antiterminator protein encoded by the *licT* gene (Kruger and Hecker, 1995). LicT belongs to the BglG-SacY family of transcriptional antiterminators and it has been proposed that the general PTS components are involved in promoting LicT dimer formation (Kruger and Hecker, 1995). Similarly, β-glucoside utilization by *L. lactis* has been shown to involve the product of the *bglR* gene (Bardowski *et al.*, 1994). The BglR protein has been identified as a regulatory protein homologous to the BglG family of antiterminators as the expression of *bglR* was shown to be positively regulated by the presence of β-glucosides and disruption of the gene resulted in impaired growth on β-glucosides (Bardowski *et al.*, 1994).

(D) Regulation of carbon metabolism in Gram negative and Gram positive bacteria.

1. Catabolite repression and inducer control in enteric bacteria. Early research with *E. coli* demonstrated the inhibitory effect of exogenous glucose on the transport of various non-PTS carbohydrates (Adhya and Echols, 1966; Winkler and Wilson, 1967), as well as, the activity of the biosynthetic enzyme, adenylate cyclase (Makman and Sutherland, 1965). A prerequisite for transcription of many inducible carbohydrate regulons in enteric bacteria is the presence of the specific carbohydrate inducer and cyclic AMP (cAMP) produced by adenylate cyclase in the presence of ATP (Saier, 1993). Catabolite repression describes the decreased expression of many catabolic genes in the presence of a readily metabolizable carbon source, such as glucose, mediated through a reduction in the cellular level of cAMP (Paigen and Williams, 1970; Neidhart *et al.*, 1990). Cyclic AMP is formed by the membrane-bound enzyme, adenylate cyclase, which together with the catabolite gene activator protein (CAP), positively regulates the expression of a variety of catabolic genes (Postma *et al.*, 1993). The phosphorylated form of $EIIA^{Glc}$ has been shown to activate adenylate cyclase (Saier, 1989). During glucose transport, the level of P-$EIIA^{Glc}$ would be low due to the continual phosphate group transfer to the incoming glucose molecules. Upon exhaustion of exogenous glucose, the concentration of P-

$EIIA^{Glc}$ would increase, thereby activating adenylate cyclase. An increase in the intracellular concentration of the cAMP-CAP complex, coupled with the presence of sugar inducer, provides the cell with the capability to express inducible, non-PTS operons (Neidhart *et al.*, 1990).

Inducer exclusion describes the inhibitory effect of glucose on the transport of sugar inducers through inactivation of their permeases (Magasanik, 1970). In enteric bacteria, the $EIIA^{Glc}$ component of the PTS mediates inducer exclusion of the non-PTS sugars: lactose, melibiose, maltose, and glycerol (Saier and Reizer, 1992). Exclusion has been postulated to occur through direct binding of $EIIA^{Glc}$ to the lactose and melibiose permeases (Osumi and Saier, 1982; Saier *et al.*, 1983), the MalK component of the maltose permease (Postma *et al.*, 1988) and the glycerol catabolic enzyme, glycerol kinase, respectively (De Boer *et al.*, 1986; Novotny *et al.*, 1985; Postma *et al.*, 1984). Specifically, unphosphorylated $EIIA^{Glc}$ binds to an allosteric site on the specific permease or enzyme to inhibit transport activity (Saier, 1993). Mutations in the gene encoding $EIIA^{Glc}$, *crr*, (Zeng *et al.*, 1992), as well as, the *lacY* (Wilson *et al.*, 1990) and *malK* genes (Dean *et al.*, 1990; Kuhnau *et al.*, 1991) have been sequenced and specific residues have been implicated in the binding process. More recently, the plasmid-encoded raffinose permease (RafB) in *E. coli* has shown sequence similarity with LacY and MalK within the

regions suggested to be involved in binding to $EIIA^{Glc}$, supporting allosteric regulation of RafB by $EIIA^{Glc}$ (Titgemeyer *et al.*, 1994).

A model has been proposed by several investigators describing the mediation of adenylate cyclase and permease activity by the phosphorylated and unphosphorylated states of $EIIA^{Glc}$, respectively (Saier and Feucht, 1975; Postma and Roseman, 1976; Gonzalez and Peterkofsky, 1977). In the absence of a PTS sugar, EI and HPr, as well as $EIIA^{Glc}$, would be phosphorylated and the non-PTS permeases and adenylate cylase would be active, promoting inducer transport, cAMP synthesis and subsequent catabolic enzyme synthesis (Saier, 1989). Any PTS sugar will promote dephosphorylation of $EIIA^{Glc}$, as phosphorylation of $EIIA^{Glc}$ by P~(His)-HPr is a reversible process (Postma *et al.*, 1993). As a consequence, the phosphorylation of any EII complex during the transport of a PTS sugar would result in dephosphorylation of P~(His)-HPr and the consequent dephosphorylation of $EIIA^{Glc}$ (Saier, 1989). The unphosphorylated state of $EIIA^{Glc}$ would promote inactivation of non-PTS permeases and adenylate cyclase activity inhibiting expression of the inducible non-PTS operons.

2. Regulation by the PTS in Gram-positive bacteria. As described above, the activity of glycerol kinase in enteric bacteria was regulated by the allosteric interaction

with $EIIA^{Glc}$ (De Boer *et al.*, 1986), however, the kinase from *Enterococcus faecalis* has been shown to be regulated by PEP-dependent phosphorylation catalyzed by EI and HPr of the PTS (Deutscher, 1985). In the older literature, the glycerol kinase was referred to as DHA/glycerol kinase due to its role in glycerol dissimilation (Reizer *et al.*, 1988b). Following uptake by a facilitator membrane protein, glycerol can be phosphorylated by glycerol kinase and subsequently oxidized by glycerol-3-phosphate dehydrogenase to dihydroxyacetone phosphate (DHA-P), or oxidized to DHA first, followed by phosphorylation by DHA kinase (Lin, 1976). Overall, DHA/glycerol kinase (glycerol kinase) has been shown to phosphorylate DHA or glycerol and, in *E. faecalis*, glycerol was phosphorylated six times slower than DHA (Deutscher and Sauerwald, 1986). PEP-dependent phosphorylation of glycerol kinase from *E. faecalis* was shown to be a reversible reaction catalyzed by EI and HPr (Deutscher, 1985) and the phosphorylated kinase demonstrated a 10-fold increase in activity (Deutscher and Sauerwald, 1986).

It has been suggested that EII complexes of the PTS compete with glycerol kinase for the phosphate group from P~(His)-HPr and, in the absence of a PTS sugar, glycerol metabolism would increase 10-fold reflecting the phosphorylated state of the kinase (Deutscher and Sauerwald, 1986). Subsequent transport of a PTS sugar has been proposed to lead to dephosphorylation of the kinase by phosphoryl transfer to HPr. In addition,

generation of an *E. faecalis* EI (*ptsI*) mutant (Romano *et al.*, 1990) further defined the role of the general PTS proteins in the regulation of glycerol kinase activity as this mutant demonstrated an inability to transport any PTS sugar and showed reduced uptake of glycerol. Accordingly, it has been proposed that a defect in EI promotes reduced phosphorylation of glycerol kinase and, as a result, glycerol is not retained within the cell following transport via the glycerol facilitator (Deutscher *et al.*, 1993).

Early studies have demonstrated that glucose and glycerol-grown *E. faecalis* cells possessed similar levels of glycerol kinase (Deutscher and Sauerwald, 1986), however more recently, glycerol kinase expression has been shown to increase during growth on glycerol (Deutscher *et al.*, 1993). In the latter study, glycerol kinase synthesis was repressed in the presence of the PTS sugars, glucose and fructose, and the sugar alcohol, mannitol. In glycerol-grown cells, approximately 85% of the glycerol kinase existed in the phosphorylated form, while in glycerol and glucose-grown cells the level of glycerol kinase decreased 95% compared to glycerol-grown cells and the unphosphorylated form predominated. Overall, this study confirmed that phosphorylated glycerol kinase is the active form of the enzyme and exists primarily in the absence of a PTS sugar.

In *S. salivarius*, the mannose PTS transports glucose, mannose, fructose, and 2-deoxyglucose, and the EII complex consists of EII^{Man} and two forms of III^{Man}, differentiated by their molecular masses, 38.9 kDa ($III^{Man}{}_H$) and 35.2 kDa ($III^{Man}{}_L$) (Bourassa *et al.*, 1990). The isolation and characterization of two classes of spontaneous 2-deoxyglucose resistant mutants led to the proposal that the III^{Man} proteins may be involved in the regulation of gene expression (Gauthier *et al.*, 1990). Class I mutants lacking $III^{Man}{}_L$ were unable to grow on mannose and exhibited abnormal growth in mixed-sugar cultures, such that lactose inhibited growth on glucose. Class II mutants possessed both forms of III^{Man}, however, this class of mutants grew at a slower rate than the wild-type strain in the presence of mannose and exhibited a loss of catabolite repression by glucose since glucose and lactose were used concurrently (Gauthier *et al.*, 1990). Further characterization of the class I mutants identified an altered sensitivity to toxic compounds that normally interfere with the cell envelope and analysis of the membrane proteins by two-dimensional electrophoresis identified the presence of five new proteins and alterations in the levels of two other proteins (Brochu *et al.*, 1993). Although further research is required to identify the genes mutated in these spontaneous mutants, it has been suggested that $III^{Man}{}_L$, or a global regulatory gene controlling its synthesis, is responsible for the regulation of various physiological

functions, including the properties of the cellular envelope (Brochu *et al.*, 1993).

The PTS-defective *S. mutans* strain used to identify a non-PTS glucose transport system (Cvitkovitch *et al*, 1995b) has more recently been employed to provide evidence indicating that the PTS regulates Msm-mediated sugar transport (Cvitkovitch *et al.*, 1995a). The *ptsI* mutant (DC-10) demonstrated a reduced rate of raffinose transport compared to the parental strain indicating that a functional PTS was required for sugar transport by the Msm. In addition, inhibition of raffinose uptake by glucose and two glucose analogues in the parental strain and DC-10 further demonstrated the regulation of Msm transport by the PTS (Cvitkovitch *et al.*, 1995a). Inducer control mechanisms (Saier *et al.*, 1996) have been suggested to explain PTS regulation of Msm transport in *S. mutans*, however, more research is required to address this potential mechanism.

3. The regulatory role of P-(Ser)-HPr in Gram-positive bacteria. Generally, inducer expulsion is a process whereby pre-accumulated sugars are rapidly expelled from the cell upon the addition of a metabolizable substrate, such as glucose (Ye *et al.*, 1996). In *L. lactis* and *S. pyogenes*, the inducer expulsion phenomenon is a two step process whereby intracellular sugar phosphate is hydrolyzed to free sugar and inorganic phosphate and then the free sugar

is expelled from the cell (Reizer *et al.*, 1983). In these organisms, lactose is transported by the PTS and its non-metabolizable analogue, thiomethyl-β-galactoside (TMG), accumulates in the cell in the phosphorylated form (Reizer and Panos, 1980; Thompson and Saier, 1981). Using permeabilized vesicles of *L. lactis* cells, electroporation of the *B. subtilis* mutant protein S46D HPr, possessing the serine to aspartate substitution, (Reizer *et al.*, 1989), stimulated TMG-P phosphatase activity (Ye *et al.*, 1994c). Furthermore, TMG-P phosphatase activity was stimulated upon electroporation of ATP, FBP and HPr, conditions which activate the (Ser)HPr kinase. Similar results have been observed for *E. faecalis* vesicles as electroporation of HPr and FBP or the P-(Ser)-HPr analogue, S46D HPr, promoted rapid efflux of pre-accumulated TMG-P (Ye *et al.*, 1996).

In *L. lactis*, a small membrane-associated phosphatase enzyme (Pase II) has been purified and characterization studies have shown 10-fold stimulation in the presence of wild-type P-(Ser)-HPr or its analogue, S46D mutant HPr (Ye and Saier, 1995c). Stimulation of Pase II was attributed to an increase in the V_{max} for sugar phosphate hydrolysis and a decrease in the K_m for sugar phosphate. P-(Ser)-HPr-activated Pase II has been identified in *E. faecalis* and *S. pyogenes*, however, Pase II was absent in *Staph. aureus, S. mutans, S. salivarius,* or *B. subtilis*, organisms which do not exhibit the inducer expulsion phenomenon (Ye *et al.*, 1996).

Lactobacillus brevis also lacked Pase II (Ye *et al.*, 1996), confirming earlier research demonstrating that inducer expulsion results from the control of non-PTS permease activity (Ye *et al.*, 1994a, b).

In *L. brevis*, lactose is transported by permease-catalyzed proton symport and its non-metabolizable analogue, TMG, accumulates as the non-phosphorylated sugar (Romano *et al.*, 1987). Recently, experiments employing vesicles demonstrated that pre-accumulated TMG was lost from the vesicles upon the addition of glucose as P-(Ser)-HPr converted the lactose:H+ symporter (permease) to a facilitated diffusion system (Ye *et al.*, 1994b). Similarly, the efflux of the glucose analogue, 2-deoxyglucose, transported by the glucose/H^+ symporter was shown to be regulated by P-(Ser)-HPr (Ye *et al.*, 1994a). More recent evidence has led to the suggestion that P-(Ser)-HPr binds to an allosteric site on the lactose (Ye *et al.*, 1995a) and glucose (Ye *et al.*, 1995b) permeases. In fact, it has been proposed that binding of the sugar on the outer surface of the permease promotes a conformational change resulting in an increased affinity for P-(Ser)-HPr binding to the allosteric site on the cytoplasmic side of the permease. Overall, the allosteric regulation of the glucose and lactose permeases by P-(Ser)-HPr serves to uncouple sugar transport and, as a result, sugars cannot be accumulated against a concentration gradient. In this way, the rate of sugar

transport is regulated to meet the energy requirements of the cell.

Although *B. subtilis* does not mediate inducer expulsion, substantial evidence supports the role of P-(Ser)-HPr in catabolite repression (Deutscher *et al.*, 1994; Deutscher *et al.*, 1995; Fujita *et al.*, 1995). A *B. subtilis* mutant strain has been generated in which phosphorylation at Ser-46 was obviated by the substitution of an alanine residue (*ptsH1* mutation) (Deutscher *et al.*, 1994). In the *ptsH1* mutant strain (SA003), the activities of gluconate kinase, glucitol dehydrogenase, mannitol-1-P dehydrogenase, and the mannitol PTS permease were relieved from catabolite repression in the presence of glucose, fructose or mannitol. In addition, inositol dehydrogenase activity was partially repressed by glucose when the *ptsH1* mutant was grown in minimal medium, whereas when grown in rich Luria Bertani medium (LB) this enzyme was almost completely repressed. Similar results were found with a *B. subtilis ccpA* mutant (GM1225), defective in a transcription factor homologous to the LacI-GalR family of repressors/activators (Weickert and Adhya, 1992). The same enzymes were relieved from catabolite repression in the *ccpA* mutant strain as the *ptsH1* mutant, however inositol dehydrogenase was completely relieved from catabolite repression when the *ccpA* mutant was grown in LB or minimal media.

The similar phenotypes of the *ptsH1* and *ccpA* mutant strains suggested an association between CcpA and P-(Ser)-HPr during catabolite repression prompting an investigation that demonstrated specific binding between P-(Ser)-HPr and CcpA (Deutscher *et al.*, 1995). Using affinity chromotography columns with immobilized CcpA, retardation experiments were conducted with the different forms of HPr. The CcpA protein was not bound by unphosphorylated HPr, P~(His)-HPr, nor the doubly phosphorylated form of HPr, (P~(His)-P-(Ser)-HPr), and the interaction between P-(Ser)-HPr and CcpA was strengthened in the presence of FBP (Deutscher *et al.*, 1995). The CcpA/P-(Ser)-HPr complex promotes CcpA binding to the cis-acting catabolite response element (CRE) in the regulatory regions of catabolite-repressible operons as recently demonstrated for the *gnt* operon (Fujita *et al.*, 1995) and, as a result, this nucleoprotein complex interferes with transcription initiation. The overall mechanism proposed for catabolite repression in *B. subtilis* is illustrated in Figure 1-3.

More recently, another HPr mutation in which His-15 was replaced by an alanine residue was shown to inhibit carbon catabolite repression of the *B. subtilis gnt* operon (Reizer *et al.*, 1996a). The *ptsH2* gene, encoding H15A mutant HPr, or the wild-type *ptsH* gene was integrated into the *amyE* locus of the *ptsH1* mutant strain SA003 generating SA063 and SA053, respectively. The synthesis of the wild-type HPr by SA053 restored catabolite repression of the *gnt* operon as gluconate

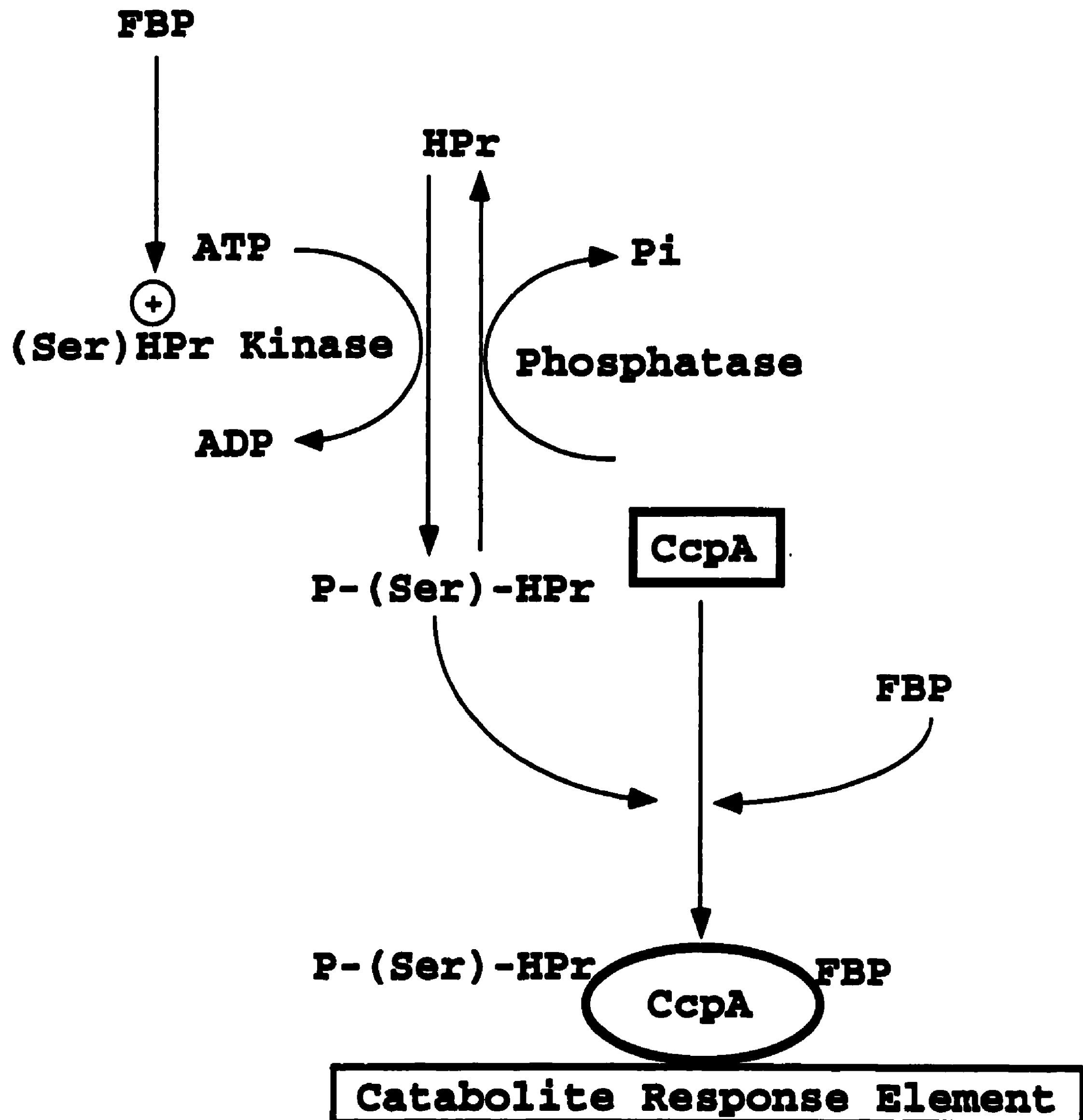

Figure 1-3: The proposed mechanism for catabolite repression in *B. subtilis*. The plus symbol indicates activation and the FBP-dependent formation of the P-(Ser)-HPr(CcpA) complex is shown.

kinase activity was reduced to wild-type levels in the presence of gluconate and glucose. In comparison, the level of gluconate kinase activity in SA063 carrying the H15A mutant HPr was eight-fold higher under the same growth conditions. Since H15A mutant HPr can be phosphorylated at Ser-46, it was suggested that the mutation at His-15 was responsible for preventing catabolite repression in SA063. Moreover, this study demonstrated that His-15 is necessary for P-(Ser)-HPr interaction with CcpA as wild-type P-(Ser)-HPr was strongly retarded on affinity chromotography columns with immobilized CcpA, while the seryl-phosphorylated H15A mutant HPr was only weakly retarded. Overall, this study concluded that His-15 is required for catabolite repression as alterations at this site inhibited P-(Ser)-HPr/CcpA complex formation (Figure 1-3).

Recently, the *B. subtilis* CcpB DNA-binding protein has been shown to be involved in catabolite repression and sequence similarity to the CcpA transcription factor has been observed specifically within the DNA-binding domains (Saier *et al.*, 1996). Like CcpA, CcpB functions as a transcription factor in association with P-(Ser)-HPr and its contribution to catabolite repression appears to depend on the environmental conditions. Specifically, catabolite repression was mediated by both CcpA and CcpB when *B. subtilis* was grown in liquid media, with low agitation, or on solid agar plates, while

only CcpA mediated catabolite repression upon growth in liquid media with high agitation (Saier *et al.*, 1996).

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Chapter 2

Background to the Research

My research can be divided into three phases:

(1) Regulation of ATP-dependent P-(Ser)-HPr formation in *Streptococcus mutans*.

(2) Site-directed mutagenesis of the HPr gene of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans*.

(3) Isolation of genes important to sorbitol metabolism by *Streptococcus mutans*.

Phase 1: Regulation of ATP-dependent P-(Ser)-HPr formation in *Streptococcus mutans*.

The initial phase of my research program, comprising Chapter 4, was designed to examine the relationship between the intracellular levels of the four forms of HPr and the concentration of specific metabolites in *S. mutans* cells grown in continuous culture. The goal of this research was to gain a better understanding of (Ser)HPr kinase activity in *S. mutans* and to possibly define the specific role of (Ser)-HPr phosphorylation in carbohydrate transport.

Initially, *S. mutans* was grown in continuous culture under PTS optimal (glucose-limited) and PTS repressive (glucose-excess) conditions to compare the steady state levels of the four forms of HPr. Continuous culture is an effective technique used to grow cells under defined environmental conditions or growth parameters in a highly reproducible

manner (Herbert, 1958; Tempest, 1970). The growth pH, carbohydrate concentration, gaseous phase and growth rate can be regulated in continuous culture, and this system permits the researcher to vary one growth parameter while the other chosen growth conditions remain constant. In a medium with one limiting nutrient and all other components in excess, the growth rate of the organism is dependent on the dilution rate, which represents the number of culture volumes that pass through the growth chamber in one hour (Herbert, 1958). In general, the continuous culture technique offers one of the best methods to study the adaptability of an organism in response to a changing environment.

The continuous culture technique has been used extensively in our laboratory to examine the effects of various environmental conditions on the repression of the PTS (Hamilton and Ellwood, 1978; Ellwood *et al.*, 1979; Ellwood and Hamilton, 1982) and the levels and activities of the components of the PTS (Hamilton *et al.*, 1989; Vadeboncoeur *et al.*, 1987; Vadeboncoeur *et al.*, 1991b). These continuous culture studies and studies with batch-grown cells of *S. mutans* (Thibault and Vadeboncoeur, 1985) did not detect a significant variation in the intracellular levels of HPr during growth under the various conditions. However, a closer examination of the intracellular levels of the four forms of HPr from oral streptococci cells grown in batch culture detected a correlation between the stages of growth

and the levels of the different forms of HPr (Vadeboncoeur *et al.*, 1991a). Specifically, exponential-phase cells of *S. mutans* Ingbritt and *S. salivarius* ATCC 25975 possessed mainly P-(Ser)-HPr and P-(His)-P-(Ser)-HPr, while stationary-phase cells possessed only non-phosphorylated HPr and P-(His)-HPr. With this information, our laboratory was interested in a quantitative assessment of the various forms of HPr from *S. mutans* cells grown under defined environmental conditions of continuous culture which promoted repression of the PTS.

In conjunction with the quantitative determination of the four forms of HPr, we were interested in determining whether the levels of specific glycolytic intermediates correlated with the activity of the ATP-dependent (Ser)HPr kinase. The activity of this enzyme in cell extracts from *S. mutans* V843 (GS-5, serotype c) has been shown to be activated by fructose-1,6-bisphosphate (FBP) and inhibited by inorganic phosphate (Mimura *et al.*, 1987). Therefore, our intent was to determine the intracellular levels of FBP, as well as, glucose-6-phosphate and ATP from cells of *S. mutans* Ingbritt grown in continuous culture using the same growth parameters employed for the HPr determinations.

Our working hypothesis at the beginning of this phase was based on the theory that FBP regulated the *S. mutans* (Ser)HPr kinase and that the intracellular concentration of P-(Ser)-HPr would directly reflect the level of glucose in the

medium. In the presence of excess glucose (PTS repressive conditions), we predicted that the level of FBP would be high, activating the (Ser)HPr kinase and resulting in elevated levels of P-(Ser)-HPr. Conversely, in the presence of minimal glucose (PTS optimal conditions), we expected low levels of P-(Ser)-HPr indicating reduced (Ser)HPr kinase activity and HPr was predicted to exist predominantly as P-(His)-HPr and free HPr.

Phase 2: Site-directed mutagenesis of the HPr gene of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans*.

As mutant studies with other organisms have identified residues within the HPr protein that are important for several physiological processes, the next phase of my research (Chapter 5) focussed on the generation of a mutant strain of *S. mutans* altered within the *ptsH* gene. A *B. subtilis* chromosomal *ptsH* mutant GM1222, altered by changing serine-46 to an alanine residue, has demonstrated the role of the HPr protein in the regulation of PTS uptake (Ye and Saier, 1996) and catabolite repression (Deutscher *et al.*, 1994). In *S. salivarius*, phosphorylation of HPr at serine 46 was inhibited when methionine-48 was replaced by a valine residue (Vadeboncoeur *et al.*, 1994). In addition, spontaneous *S. salivarius* chromosomal mutants have been generated following growth in the presence of the glucose

analogue, 2-deoxyglucose (Gauthier *et al.*, 1994). The class of *ptsH* mutants in this study included a strain in which glycine-67 was substituted by an aspartate residue. This mutant was unable to grow on the PTS sugars mannose, glucose and fructose, and the authors proposed that this residue plays a role in the recognition of HPr by EI, or P~(His)-HPr by the EII complexes (Gauthier *et al.*, 1994).

The chromosomal *ptsH* mutant studies described above provided the basis for our examination of the physiological effects of a similar mutation in the *ptsH* gene of *S. mutans*. At the beginning of these genetic studies, we intended to create a mutant *S. mutans* strain possessing an altered HPr protein at serine-46, histidine-15, or glycine-67 residue. The generation of such mutants would be followed by a series of characterization studies to establish the effect of the mutation on growth in the presence of PTS and non-PTS sugars. Moreover, the effect of the mutation on phosphorylation at histidine-15 and serine-46 would be examined following growth of the mutant strain in continuous culture using the same growth conditions as employed in the first phase of this research. The project was initiated by an attempt to isolate a mutant of *S. mutans* BM71 with glycine-67 substituted by an aspartate residue.

Phase 3: Isolation of genes important to sorbitol metabolism by *Streptococcus mutans*.

The final stage of my research (Chapter 6) was aimed at characterizing a *S. mutans* mutant strain defective in sorbitol metabolism. Sorbitol transport by *S. mutans* has been shown to involve an inducible PTS system with the resultant intracellular sorbitol-6-phosphate converted to fructose-6-P by an inducible sorbitol-6-P dehydrogenase (Brown and Wittenberger, 1973; Dills and Seno, 1983; Maryanski and Wittenberger, 1975; Slee and Tanzer, 1983). A long range plan was initiated to identify and clone the *S. mutans* genes encoding the proteins of the sorbitol EII (EII^{Sor}) complex and the sorbitol-6-P dehydrogenase in order to characterize the sorbitol system.

The first step of this process was the generation of a sorbitol-defective mutant by transposon mutagenesis. This was accomplished by Mr. Dave Boyd in our laboratory using *S. mutans* LT11 and resulted in a strain unable to grow on sorbitol, but able to grow on many PTS and non-PTS sugars. Preliminary characterization of the mutant indicated a single insertion of the transposon and subculturing the mutant in the absence of drug selection promoted reversion of the mutant phenotype. Therefore, at the earliest stages of this research our laboratory possessed a *S. mutans* strain that would possibly facilitate the identification of the genes

coding for the sorbitol-PTS, or possibly other genes important to sorbitol metabolism, such as the sorbitol-6-P dehydrogenase.

My contribution to this research focussed on further characterization of the *S. mutans* sorbitol-defective strain. My studies were designed to examine physiological aspects of the mutant through growth comparisons between the mutant and wild-type strains, while genetic studies were aimed at defining the precise insertion point of the transposon. Cloning the gene interrupted by the transposon would permit the rescue and identification of other genes involved in sorbitol metabolism provided they exist in the same operon.

Relatively little is currently known regarding the sorbitol-PTS in oral streptococci, therefore, cloning of genes involved in sorbitol metabolism in *S. mutans* would permit the identification of homologous genes in other organisms such as the sorbitol-fermenting strain *S. sanguis* 160. Collaborative studies between our lab and Dr. Gunnel Svensater in Sweden have addressed sorbitol transport by *S. sanguis* 160 (Hamilton and Svensater, 1991; Svensater and Hamilton, 1991), as *S. sanguis* was recognized to be a dominant member of the dental plaque microflora following frequent exposure to sorbitol (Kalfas and Edwardsson, 1990). Sequencing of the *S. mutans* genes involved in sorbitol metabolism would lead to the development of probes to locate

and clone homologous genes in *S. sanguis* 160. It is anticipated that the cloned *S. sanguis* gene would then be used to generate a mutant through interruption of the gene by insertional inactivation. The *S. sanguis* sorbitol mutant strain would then be characterized using the continuous culture growth conditions previously employed to examine sorbitol transport by the wild-type strain (Hamilton and Svensater, 1991; Svensater and Hamilton, 1991).

Author's contribution. The continuous culture and crossed immunoelectrophoresis experiments were conducted by myself with some assistance of Ms. Elke Greif. The (Ser)HPr kinase assays of Chapter 4 were completed by Mr. Denis Brochu, a member of Dr. Christian Vadeboncoeur's laboratory at Laval University, Ste-Foy, Quebec, a collaborator in the overall project. The experiments designed to create the *S. mutans ptsH* mutant in Chapter 5 and the isolation of the sorbitol-defective mutant of *S. mutans* LT11 (Chapter 6) involved the technical assistance of Mr. Boyd. Finally, the Appendix consists of a published paper elucidating the characteristics of a *S. mutans ptsI* mutant unable to transport sugars via the PTS. Dr. Dennis Cvitkovitch was responsible for most of the work and my contribution involved confirming by crossed immunoelectrophoresis the inability of the mutant to phosphorylate HPr in the *ptsI* mutant.

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Chapter 3

Materials and Methods

General bacteriology

The streptococcal strains used in this study are listed in Table 3-1, while the *Escherichia coli* strains are shown in Table 3-2. The *E. coli* strains were maintained on LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.8% agar) in the presence of 13 µg of tetracycline (XL1-Blue), 50 µg tetracycline (strain BMH 71-18 *mutS*) or 30 µg chloramphenicol (strain CJ236) per ml. All streptococcal strains were maintained on blood agar 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, (pH 7.0)]. For transformation experiments, *S. mutans* BM71 was grown in Todd-Hewitt broth. Sugar fermentation was tested 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 (pH 7.0) with 1.0% sugar concentration. *S. salivarius* ATCC 25975, and the *S. mutans* strains Ingbritt, GS-5, and 10449, were grown in batch culture containing (per litre) 10 g tryptone (Difco), 5 g yeast extract (Difco), 2.5 g NaCl, 2.5 g Na_2HPO_4 and 50 g glucose. Growth was stopped during the mid-exponential phase (OD_{660nm} = 0.4) by the addition of chloramphenicol (50 µg/ml). The cells were harvested by centrifugation at 4°C and kept frozen at -40°C for not more than three days.

Table 3-1

Streptococal strains used in this study.

Strain	Source
S. mutans Ingbritt	J. Sandham, University of Toronto
S. mutans GS-5	A. S. Bleiweis, University of Florida
S. mutans NCTC 10449	R. Linzer, SUNY, Buffalo
S. mutans BH96	This laboratory
S. mutans BM71	G. Bowden, University of Manitoba
S. mutans LT11	Roy Russell, Newcastle-Upon-Tyne
S. salivarius ATCC 25975	This laboratory

Table 3-2

Escherichia coli strains used in this study.

Strain	Genotype	Source
XL1-Blue	{*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' proAB, lacIqZ*ΔM15 Tn10(TetR)]}	Stratagene
DH5α	[Ø80d Δ*lacZ* M15, *endA1, recA1, hsdR17* (r_{k-}, m_{k+}), *supE44 thi-1* λ- *gyrA relA1 F-* Δ(*lacZYA-argF*), *U169*]	GIBCO/BRL
GM2163	[*ara-14, leuB6, fhuA13, lacy1, tsx-78, glnV44(AS), galT22, galK2(OC), LAM-, mcrAO, dcm-6, hisG4(Oc), rfbD1, rpsL136(strR), dam-13::*Tn9, *xylA5, mtl-1, thi-1, mcrB9999, hsdR2*]	New England Biolabs
DM1	[F- *dam-13*::Tn*9* (Cm^r) *dcm-, mcrB, hsdR-M+, gal1, gal2, ara-, lac-, thr-, leu-, tonR, tsxR Su*^{0}]	GIBCO/BRL
CJ236	[*dut, ung, thi, rel*A; pCJ105 (Cm^r)]	M. McGavin, Univ. Manitoba
BMH 71-18 *mutS*	*thi, supE,* Δ*(lac-proAB)*, [*mutS*::Tn10][F'*proAB, lacIqZ*ΔM15]}	Clontech

S. mutans LT11 was maintained on Todd-Hewitt plates and was grown for transformation experiments and DNA isolation in Todd-Hewitt and Todd-Hewitt-glucose (0.2%) broth. The *S. mutans* sorbitol-negative mutant, BH96 was maintained on Todd-Hewitt plates containing 300 µg/ml kanamycin to maintain integration of the transposon Tn*4001*. For growth experiments, these strains were grown in batch culture in the semi-defined media used in the continuous culture studies (described below) with 5 mM glucose, 5 mM sorbitol, or both glucose and sorbitol (5 mM each), with 50 mM phosphate buffer (Na_2HPO_4/KH_2PO_4). In addition, the growth bottles for the mutant strain, BH96, also contained kanamycin (300 µg/ml).

Studies on the regulation of P-(Ser)-HPr formation in intact cells

Continuous culture. Anaerobic growth of *S. mutans* Ingbritt in continuous culture was carried out in LH 500 Series III chemostats (L.H. Engineering, Stoke Poges, Buckinghamshire, United Kingdom) with a working volume of 700 ml. Glucose and phosphate salts were autoclaved separately and added to a semi-defined medium (Bowden *et al.*, 1976). The glucose concentrations in the medium were 10, 50, 100 or 200 mM with the concentrations at 10 mM and 50 mM being limiting for glucose, while the cultures at 100 and 200 mM were nitrogen limited or excess with respect to glucose. In all cases, the pH of the culture was maintained automatically at 7.0 by the addition of 2M KOH and the dilution rate (D) was held at 0.1

h^{-1} (doubling time of 7 hours) with a gas phase 5% CO_2 in nitrogen.

Daily routine maintenance of the chemostats included optical density readings in a Klett-Summerson spectrometer with a red filter (640-700 nm), pH measurements, and dilution rate determinations. Cell dry-weight determinations were carried out by filtering 5 ml of culture through four pre-weighed 0.45-µm filters. The glucose concentration in each batch of media and in culture fitrates was determined using the glucose oxidase method of Kingsley and Getchell (1960), described below. The cultures were grown at each glucose concentration for at least 10 mean generations before steady state was considered to have been established and the cells removed from the growth chamber for the appropriate assay.

HPr determinations. Cells for the quantitative analysis of the various forms of HPr were rapidly collected directly from the chemostat under vacuum into a stirred solution containing 100 mM Tris-citrate buffer (pH 4.0), chloramphenicol (50 µg/ml) and gramicidin D (1 µM), and the pH of the sample was immediately lowered to 4.5 with 5 N HCl. The cell suspension was centrifuged at 16,000xg for 10 min, resuspended in a minimal amount of the supernatant to consolidate the cells, re-centrifuged at 27,000xg for 20 min and frozen at -70°C. The frozen-cell suspension was used within 2-3 hours to prepare the membrane-free cellular extract by a slightly

modified method of Vadeboncoeur and coworkers (1991). Our method involved a reduction in the volume of the buffer from 3.0 ml to 1.5 ml. The buffer was comprised of 10 mM Hepes buffer (pH 7.0), 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF and 10^{-7} M Pepstatin A. In addition, the amount of cells ground with alumina was held relatively constant at 0.60 g per sample. The HPr determinations were repeated at least 4 and as many as nine times for each condition.

The various forms of HPr were separated by crossed immunoelectrophoresis as described previously (Vadeboncoeur *et al.*, 1991) with minor alterations to the protocol. Agarose (1%) was dissolved in a Tris-barbitone buffer containing (grams, liter^{-1}): 5.01 sodium barbitone, 8.86 Tris base, 0.11 calcium lactate, and 0.13 sodium azide. The samples were diluted to 2 mg of 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 (Pharmacia, Baie d' Urfe, Quebec). The second dimension, conducted for 19 hours at 2 V/cm at 4°C, involved electrophoresis against a rabbit polyclonal antiserum (8 mg total protein, plate^{-1}) directed against HPr of *S. mutans* DR0001. Following electrophoresis, the gels were washed three times using 0.1 M NaCl and once with distilled water, left to dry at room temperature overnight and then stained with Coomassie blue.

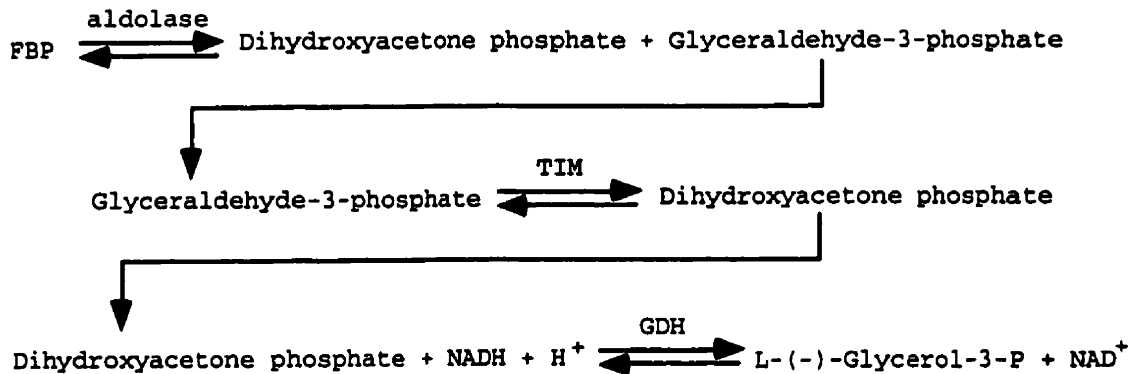
Quantitation of HPr in the various peaks was obtained by scanning each gel in a 300 DPI Apple Scanner (Apple Computers Inc, Cupertino, Calif.) followed by transferring the image to the Image 1.37 program (NIMH, Bethesda, Maryland) and determining the number of pixels under each peak. These values were then compared to a standard curve (100 to 2000 ng of protein) to determine the number of nanograms of each HPr form present in each cell sample. The final values were expressed as micrograms of HPr, mg of cellular protein^{-1} .

Cellular metabolites. The intracellular concentrations of glucose-6-phosphate and fructose-1,6-bisphosphate in steady state cells were determined following a modified sampling method originally described by Iwami *et al.* (1975). A total of approximately 40-60 mg of chemostat-grown cells were collected directly from the chemostat with the amount never exceeding 10% of the chamber volume to ensure maintenance of steady-state conditions for the remaining culture. To obtain sufficient cells, six sequential samples (6-8 ml) were rapidly collected directly from the growth chamber and filtered through 0.4 μ Acropor filters. The filters were rapidly washed with 5 ml of cold 10 mM Tris-HCl buffer pH 7.0 and immediately placed into ice-cold 0.5 M perchloric acid. Cellular ATP, on the other hand, was determined in an equivalent amount of cells removed directly from the chemostat into perchloric acid (1 M). This method was faster

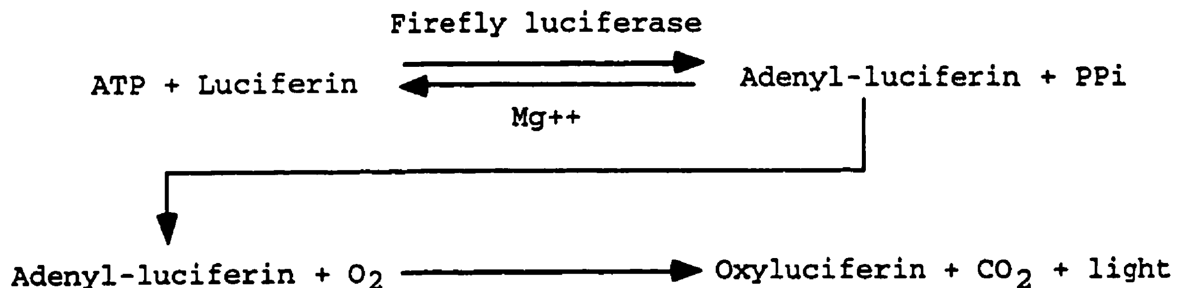
(~6 sec) and was made possible since the extracellular level of ATP was less than 1.3 pmoles, ml^{-1} and the intra- to extracellular ATP ratios were always greater than 1000-fold. In both cases, cells were extracted for 30 minutes at room temperature, the samples neutralized to pH 7.6 with 10 mM K_2CO_3 and cooled in ice. The potassium perchlorate was removed by centrifugation and any further precipitate formed during incubation on ice (30 min) was removed by filtration through a 0.2 μ filter. On the day of sampling, the cell biomass (mg dry weight per ml) in the chemostat was determined and cell-free medium was collected by filtration.

Glucose-6-phosphate was assayed with glucose-6-phosphate dehydrogenase (G6P-DH) by following the reduction of NADP at 340 nm (Lang and Michal, 1974) :

Fructose-1,6-bisphosphate was assayed with aldolase, triosephosphate isomerase (TIM), and glycerol-3-phosphate dehydrogenase (GDH) by following the oxidation of NADH at 340 nm as indicated below (Michal and Beutler, 1974):



ATP was measured by the luciferin-luciferase assay (Strehler and McElroy, 1957) using a Model 2000 Integrating Photometer (JRB Inc.) according to the following reactions:



Prior to the ATP determinations all glassware was acid washed (1 N HCl) and autoclaved. The total reaction volume was 1.0 ml and contained the following (final concentrations): 8 mM Tris-acetate buffer, 5 mM $MgCl_2$, 0.06 mM dithiothreitol, 0.1 mM EDTA, 0.28 mM luciferin and 0.14 µg/ml luciferase. All unknowns were assayed in triplicate with and without an internal standard (2.5 pmoles) to correct for sample quenching. In order to correct for the ATP concentration in the extracellular medium, ATP was assayed in the cell-free

medium filtrate obtained on the day of sampling by filtering (0.2 μ filter) an aliquot of the culture.

All final concentrations (μM) for all three intermediates are reported on the basis of the intracellular water volumes using the cell dry weight and actual internal water volume determined for each condition as described below. All intermediate assays were repeated a minimum of six times and at most 15 times.

Estimation of internal water volumes. The internal water volume (V_i) of the cells was estimated by subtracting the extracellular water volume determined with [^3H]-polyethylene glycol (PEG) from the total aqueous space measured using ^3H_2O (Kashket and Barker, 1977). Four ml of culture suspension received either 40 μl of ^3H_2O (20 μCi/ml) or [^3H]-PEG (15 μCi/ml) and following a 5 min incubation period, ten 0.4 ml samples were removed and centrifuged through silicone oil as previously described (Hamilton, 1990). The V_i values were determined for each growth condition along with cell dry weight determinations and represented the average (± SE) of six determinations. The V_i values for the chemostat cultures were 2.01 ± 0.10 and 1.98 ± 0.05 μl per mg (dry weight) cells for the 10 and 50 mM glucose-limited cells and 2.00 ± 0.03 and 1.99 ± 0.09 μl per mg cells for the 100 and 200 mM glucose-excess cells, respectively.

Assay for (Ser)HPr kinase. Cells were washed twice with 50 mM Tris-HCl (pH 7.5) buffer containing 50 µg/ml chloramphenicol to eliminate phosphate since phosphate interferes with the HPr kinase activity (Vadeboncoeur *et al.*, 1991). The pellet was suspended (20% wet w/v) in 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 M PMSF, 0.1 µM Pepstatin A, 0.1 µM Leupeptin and 0.5 g alumina per ml of the cell suspension. The cells were disrupted in a sonicator (Heat Systems-Ultrasonic, Inc., Model W350) in the pulse mode for three periods of 4 min during which the suspension was maintained in a dry ice-ethanol-water (80:20, v/v) mixture. Alumina was removed by centrifugation (2,500 g for 5 min) and the cells and cell debris were sedimented at 20,000 g for 20 min at 4°C. Membrane fragments in the supernatant containing the (Ser)HPr kinase activity were collected following centrifugation at 140,000 g for 24 hours at 4°C, resuspended in 50 mM Tris-HCl (pH 7.5) buffer and stored in 200 µl aliquots at -40°C. Protein concentrations were estimated by the method of Lowry (1951).

(Ser)HPr kinase activity was assayed in a mixture (50 µl) containing 100 mM Tris-acetate (pH 7.0 for *S. mutans* and pH 7.5 for *S. salivarius*), 2 mM $MgCl_2$, 12.5 mM NaF, 10 µM HPr, 0.05, 0.1 or 1.0 mM ($\gamma^{32}P$) ATP (0.1 µCi/nmole) and 3 to 30 µg of membrane protein depending on the strain tested. Although the reaction was linear over a 5 min period with up to 40 µg

protein, incubation was carried out at 37°C for 4 min. The reaction was stopped by the addition of 25 µl of denaturing buffer (187.5 mM Tris-HCl pH 6.8), 6% sodium dodecyl sulfate (SDS), 15% 2-mercaptoethanol, 30% glycerol and 0.003% bromophenol blue) followed by heating at 100°C for 5 min. The product, ^{32}P-HPr, was isolated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) by loading 20 µl samples onto a polyacrylamide gel (1 mm, 5 x 8 cm) with a 15% resolving gel and running the gel at 200 V at room temperature until the bromophenol blue reached the bottom of the gel. The ^{32}P-HPr band was located by autoradiography following incubation of the dried gel with Kodak X-ray film (X-Omat AR) for 16 h at room temperature. The labelled gel band was excised and counted in 5 ml of Cytoscint ES (ICN) in a liquid scintillation counter. Preliminary experiments had indicated that no other phosphoprotein migrated in the same position as P~HPr under identical conditions and the formation of 3-P~(His)-HPr was less than 5% (Waygood *et al.*, 1986)

Assays testing the effect of glycolytic intermediates on kinase activity were carried out with 1.0 mM ATP and included the following: glucose-6-phosphate (0.2, 0.5 and 2.0 mM), fructose-6-phosphate (0.01, 0.05 and 2.0 mM), dihydroxyacetone phosphate (0.5, 1.0 and 2.0 mM), glyceraldehyde-3-phosphate (0.05, 0.2 and 0.5 mM), 2-phosphoglycerate (0.1, 0.25 and 0.5 mM), 3-phosphoglycerate (0.5, 2.0 and 5.0 mM), 2,3-diphosphoglycerate (0.2, 2.0 and

10.0 mM) and pyruvate (0.5, 1.0 and 2.0 mM). The effects of fructose-1,6-bisphosphate (FBP) were assayed with the *S. salivarius* enzyme at various concentrations up to 10 mM with 0.05, 0.1 and 1.0 mM ATP, while *S. mutans* enzyme was assayed with 1.0 mM ATP at FBP concentrations to 15 mM. Units of (Ser)HPr kinase activity were pmoles P-(Ser)-HPr formed, µg membrane protein^{-1}, min^{-1}.

General DNA methodology

Isolation of DNA. *S. mutans* genomic DNA was prepared as described by Boyd *et al.*, (1994). Cells from overnight cultures were centrifuged, washed in TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0) and resuspended in TE buffer. The cell suspension was heated at 60°C for 20 minutes, cooled to 37°C, lysozyme (20 mg/ml) and mutanolysin (200 U/ml) were added, and the suspension was incubated for 1 hour at 37°C. The cells were lysed by the addition of sodium dodecyl sulfate (SDS) to 2% with gentle inversion of the samples. The NaCl concentration was adjusted to 0.7 M, 1/10 volume of 0.7 M NaCl-10% cetyltrimethylammonium bromide was added and the mixture was incubated at 65°C for 20 minutes before extraction with an equal amount of chloroform. The aqueous phase was collected and extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and once with chloroform. The DNA was precipitated with 2 volumes of cold ethanol, washed with cold 70% ethanol and resuspended in TE buffer.

Plasmid DNA was isolated using a modified alkaline lysis method (Feliciello and Chinali, 1993). For rapid screening, mini-preps were prepared from 1.5 ml overnight cultures with the initial nucleic acid pellet being washed once with 70% ethanol before storage and use. Large scale preparations of plasmid DNA were from 1 litre cultures of *E. coli* using the complete modified alkaline lysis method. Plasmids used for recombinant DNA methodology and sequencing were isolated in a highly purified form from CsCl gradients as described by Maniatis *et al.,* (1982). The plasmid DNA from a large scale preparation was applied onto a CsCl/ethidium bromide gradient prepared in 4.9 ml OptiSeal tubes and run in a NvTi65.2 rotor in a L8-70M ultracentrifuge (Beckman) at 275,000 x g for 16-20 hrs. The lower band in the gradient corresponding to supercoiled plasmid DNA was collected and the ethidium bromide removed by several extractions with an equal amount of isoamyl alcohol. Two volumes of TE buffer were then added and the DNA precipitated by addition of 2 vol of ethanol. After 15-20 minutes at room temperature, the DNA was collected by centrifugation and then washed with cold 70% ethanol. The pellet was dried at room temperature and then resuspended in TE buffer.

All DNA preparations were analyzed by agarose gel electrophoresis carried out in 40 mM Tris-acetate, 1 mM EDTA (pH 8.3) buffer. Recombinant plasmids were generated by

subcloning specific restriction fragments following purification from agarose gels using the Geneclean kit (Bio 101, La Jolla, Calif.). Sequencing was based on the dideoxy termination method of Sanger *et al.* (1977) using [α-^{35}S]dATP and the Sequenase version 2.0 kit (Amersham). Additional sequencing was completed by the University of Calgary Core DNA Services. DNA sequence comparisons were conducted via BLAST searches (Altschul *et al.*, 1990) of the GenBank Database.

Transformations. *E. coli* were made electro-competent and transformed with DNA by electroporation as described by Dower *et al.* (1988). Briefly, 1 L of mid-exponential phase cells (OD_{600nm} = 0.5-1.0 in LB medium) were harvested by centrifugation at 4000g for 15 minutes, washed three times, first in 1 vol sterile ice-cold dH_2O followed by 0.5 vol dH_2O, and finally in 1/50 vol sterile ice-cold 10% glycerol. The final cell pellet was resuspended in a total volume of 3 ml using 10% glycerol and stored in 100 µl aliquots in sterile microcentrifuge tubes at -70°C.

For transformation, plasmid DNA was added to a 30 µl aliquot of electro-competent cells and the mixture pulsed in a pre-chilled 0.1 cm cuvette at 1.25 kV, 400 ohms and 25 µF using a Bio-Rad Gene Pulser and a pulse controller. Immediately after pulsing, the cells were resuspended in 1 ml of SOC media (0.5% yeast extract, 2% tryptone, 10 mM NaCl,

2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) and then transferred to a Falcon 2059 tube and incubated at 37°C for one hour. Aliquots were then plated on LB medium with the appropriate antibiotics.

Cells of *S. mutans* were made competent for transformation essentially as described by Perry *et al.*, (1983). Briefly, 125 µl of an overnight culture grown in Todd-Hewitt broth supplemented with 5% heat-inactivated horse serum (THS) was used to inoculate 5 ml of pre-warmed THS and the culture incubated anaerobically until it reached an OD_{600nm} of 0.2-0.25. Then 0.5 ml of these cells were transferred to a sterile microcentrifuge tube containing the plasmid to be transformed and incubated anaerobically for 30 minutes at which time 0.75 ml of fresh THS was added and the incubation continued for another 90 minutes. The cells were centrifuged at 15,000 x g, resuspended in 100 µl of the supernatant and plated on the appropriate selective media.

Southern hybridizations. DNA was transferred to Photogene Nylon (GIBCO/BRL) for the Southern hybridizations and the biotin-labelled DNA probes were prepared using the Bio-Nick Labelling Kit (GIBCO/BRL). DNA transfer was based on the alkaline-denatured downward DNA transfer as described by Chomczynski (1992). A 0.8% agarose gel was run overnight at 35 V and the following day the gel was cut 1 cm above and below the highest and lowest DNA bands and a photo was taken

of the gel with rulers along each outer lane beside the 1 Kb ladders. The gel was then placed in denaturing buffer (0.4 M NaOH, 1.5 M NaCl) for 30-45 minutes and then neutralizing buffer [0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl] for 30 minutes. Eight filter papers and one nylon membrane were cut to the same size of the gel and five filter papers were saturated with 10X SSC (1.5 M NaCl, 150 mM Na citrate, pH 7.0) and placed on top of a 4-5 inch stack of paper towels. The nylon membrane, presoaked for 5 minutes in 10X SSC, was placed on top of these filter papers followed by the gel and then three additional filter papers also saturated with 10X SSC with care being taken to exclude all air bubbles between each layer. Filter papers were cut to the same width of the gel and to a length that would reach an elevated container containing 1 L of 10X SSC. This Southern blot transfer stack set-up was left overnight and the next day the membrane was washed for 5 minutes in 2X SSC and then dried for 1 hour at 80°C.

The dried nylon membrane was then placed in 100 ml pre-hybridization solution consisting of 6X SSC, 5X Denhardt's reagent (0.5 g Ficoll, 0.5 g polyvinylpyrrolione, 0.5 g BSA), 0.5% SDS, 100 µg/ml salmon sperm DNA and incubated at 65°C for 2-4 hours. The Denhardt's reagent was prepared as described in Maniatis (1982), and the salmon sperm DNA was boiled for 5 minutes and immediately cooled on ice prior to addition to the pre-hybridization solution. Following pre-

hybridization, the volume was decreased to approximately 30 ml and the biotin-labelled probe was added subsequent to boiling and immediate cooling on ice. The hybridization step was carried out at 65°C overnight with gentle agitation. The following day the Photogene Nucleic Acid Detection Kit (Bethesda Research Laboratories) was employed for non-radioactive detection of the hybridized probe.

Site-directed mutagenesis of the *ptsH* gene

Initial double-stranded mutagenesis experiments (Chapter 5) employed the mutagenic primers S46D (5'CAC CCA TGA TAT CCT TAA GGT TTA3'), H15A (5'TGG GCG TGC AGC AAT TCC TGT TTC3') and G67D (5'CAT CTG CAT CTG CAT CTT CAG CAG TGA TTG3'). The selection primer SP1-SH (5'AGG GCA TCG GTT AAC GCT CTC CCT3'), which converts a *Sal*I restriction site to a *Hpa*I site, was also used in these studies. The second series of experiments (Chapter 5) employed single-stranded mutagenesis and the mutagenic primer G67D. The *ptsH* G67D mutation and the *EcoR*I site in the *ptsH* gene were sequenced using the primers H-SP (5'TGA GCC TTG GTG TTG GTC AA3') and HPr (5'TGT AAT ATC TGA GGC A3'), respectively. All these primers were obtained from Oligo Express Custom Oligonucleotide Synthesis Service (BIO/CAN Scientific Inc.)

The site-directed mutagenesis studies were conducted using plasmids previously generated in our laboratory by Mr. Dave Boyd. The plasmid pDB201, consisting of a 3.5 kb *Nco*I

fragment of *S. mutans* NG5 genomic DNA and carrying the entire *ptsH* gene and most of the *ptsI* gene, was cloned into the vector pVA891 (Boyd *et al.*, 1994). This plasmid was used during mutagenesis studies employing Clontech's Transformer Site-Directed Mutagenesis kit that is based on the method of Deng and Nickoloff (1992). Prior to the annealing reaction, the primers were phosphorylated in a reaction mixture consisting of 2.0 µl 10x kinase buffer [500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 50 mM DTT, and 10 mM ATP], 1.0 µl T4 polynucleotide kinase (10 U/µl) and the volume was adjusted to 20 µl with sterile dH_2O. The reaction was incubated at 37°C for 60 minutes and stopped by heating at 65°C for 10 minutes.

All experimental steps, including the annealing reactions and the synthesis of the mutant DNA strand, were conducted as described in the manual that accompanied the kit using the selection primer SP1-SH, which converted the *Sal*I to a *Hpa*I restriction site, and the three different mutagenic primers. Primary selection of the mutated double-stranded DNA was accomplished by linearization of the parental DNA using the restriction enzyme *Sal*I. The DNA was then transformed by electroporation into *E. coli* BMH 71-18 *mutS* and the mixed plasmid pool was prepared using the alkaline-lysis method. Parental plasmids were then linearized using the restriction enzyme *Sal*I and the mutated plasmid was once again transformed into *E. coli* BMH 71-18 *mutS*. Transformants were

characterized by digestion with the restriction enzyme *Hpa*I, as the theory behind this protocol suggests that the plasmids successfully mutated in the *ptsH* gene would also possess the site created by the selection primer. Unfortunately, this method was not successful, however, the procedure has been included for the sake of completeness.

The site-directed mutagenesis studies employing the plasmid pDB102 were carried with the Muta-gene Phagemid In Vitro Mutagenesis kit Version 2 (Bio-Rad) that is based on the method described by Kunkel (Kunkel, 1985; Kunkel *et al.*, 1987). This plasmid consists of a 2.5 *Eco*RI-*Nsp*V fragment of *S. mutans* NG5 genomic DNA cloned into pBluescript II KS (Boyd *et al.*, 1994). The pDB102 plasmid was transformed into CJ236 by electroporation and single-stranded uracil-containing plasmid DNA was generated as described in the kit manual.

Phosphorylated G67D mutagenic primer was annealed to the single stranded DNA (ssDNA) using the primer to template ratio 30:1 and 1.2 µg (1.8 pmoles) of DNA. The primer/plasmid annealing reaction was set up in a 0.5 ml microcentrifuge tube in a total volume of 20 µl including 2 µl of 10X annealing buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$ and 500 mM NaCl]. Prior to the annealing reaction, the primer was placed into a boiling water bath for 2 minutes, followed by immediate placement into ice. The annealing reaction mixtures were placed into a 85°C water bath and

allowed to cool to 40°C at a rate of approximately 1 degree per minute and then placed on ice. With the 20 µl reaction still on ice, 2 µl synthesis buffer [100 mM Tris-HCl (pH 7.5), 5 mM each dATP, dCTP, dGTP, and dTTP, 10 mM ATP, 20 mM DTT] was added, along with 6 U T4 ligase and 1 U T7 DNA polymerase. The reactions were left on ice for 5 minutes to stabilize the primer and then at room temperature for 5 minutes followed by 90 minutes at 37°C. The reaction products were then examined on a 1% agarose gel and the double-stranded DNA (dsDNA) band, presumed to carry the *ptsH* mutation and corresponding with the double-stranded pDB102 control, was purified from the gel using the Geneclean kit and subsequently transformed into the *E. coli* strain BMH 71-18 *mutS*. DNA from several transformants were isolated and sequenced to verify the glycine 67 to aspartate substitution within the *ptsH* gene.

Generation of plasmid pHIK-2. Plasmid pDB102-G67D was used in a multi-step subcloning procedure in order to place the *ptsH* G67D mutation on a fragment containing the complete mutated *ptsH* gene, the complete *ptsI* gene, a drug resistance marker, and additional flanking DNA (Figure 5-1). First the *ptsI/gapN* intergenic region was isolated by removing the 5'-end of the *ptsI* gene and the *ptsH* gene sequences from pDB102 by *Sma*I/*Nco*I digestion, filling-in the *Nco*I site with T4 DNA polymerase, and religation of the plasmid to yield pNcN1. Next, the *Apo*I sites in the vector part of pNcN1 were removed

by deleting a non-essential 765 bp *Ssp*I/*Ecl136*II fragment to yield pNcN1-ΔSS. A kanamycin resistance gene marker isolated from pECK1 on a 2.3 kb *Mun*I fragment was then cloned into the *Apo*I sites located in the *ptsI/gapN* intergenic region in pNcN1-ΔSS, to yield pIKG. The intergenic region with the marker was isolated from pIKG by digestion with *Bcl*I and *Sal*I and cloned into the same sites of pDB102-G67D to yield pHIK-1 thus reconstructing the complete *ptsI* gene. Plasmid pEco1.2ΔET7 was then utilized to construct a complete mutated *ptsH* gene. Plasmid pEco1.2ΔET7 is pBluescript carrying a 1.2 kb *Eco*RI fragment of *S. mutans* DNA comprised of the 5'-end of the *ptsH* gene at one end and in which the *Eco*RI site distal to this had been destroyed by filling-in. The procedure was completed by cloning the *Eco*RI/*Hinc*II fragment from pHIK-1 into *Eco*RI/*Sma*I cut pEco1.2ΔET7 to yield pHIK-2.

The plasmid pΩIS, kindly provided by R. D. Lunsford, Bethesda, Maryland, carries a truncated IS*256* sequence from Tn*4001* cloned into the streptococcal integration plasmid pΩ (Lunsford, 1995). This plasmid was used for Tn*4001* junction rescue in the *S. mutans* sorbitol mutant BH96 as follows (Figure 6-4). Plasmid pΩIS was transformed into *S. mutans* BH96 and transformants were selected on medium containing erythromycin. Genomic DNA isolated from a transformant was cut with *Sst*I and ligated at dilute DNA concentrations in order to promote intragenic ligation, used to transform *E. coli* DH5α, and colonies were selected on erythromycin. All *E.*

coli transformants screened were found to contain a plasmid with the same rescued junction of about 1 kb of flanking DNA. This plasmid, pΩIS-SB, was used as a probe in Southern hybridizations of *S. mutans* LT11 genomic DNA to confirm its origin of the LT11 DNA on the plasmid. Restriction mapping of pΩIS-SB revealed a *Hind*III site very near the site of the original transposon integration, thus allowing removal of the IS*256* sequences in pΩIS-SB by a *Hind*III digestion followed by religation to yield pΩ-SB, facilitating sequencing of the insert.

***S. mutans* BH96 characterization.** *S. mutans* LT11 and the sorbitol-negative mutant, BH96, were grown in the presence of glucose, sorbitol and both glucose and sorbitol to examine growth characteristics and carbohydrate utilization. Samples were taken immediately following inoculation and at various time points throughout the stages of growth for a period of 12 hours. At each time point, approximately 5 ml of culture was removed from each of the six bottles and 3 ml was used to measure the growth using a Klett-Summerson spectrometer with a red filter (640-700 nm). The remaining 2 ml of culture was added to 200 µl of 25% $ZnSO_4$ to precipitate and inactivate the cells and, after 30 minutes, the samples were neutralized using 2 N NaOH and the precipitate was removed following a 3 minute centrifugation in a clinical centrifuge. The supernatants were stored at -20°C until analyzed for glucose using the glucose oxidase method (Kingsley and Getchell,

1960) and for sorbitol using an enzymatic-colorimetric assay (Boehringer, Mannheim). The procedure for the sorbitol analyses were carried out precisely as described in the instructions accompanying the kit.

Materials.

Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), polyethylene glycol 3350, gramicidin D and chloramphenicol were from Sigma Chemical Co. (St. Louis, Mo.). Agarose was from Bio-Rad and sodium barbitone was from BDH (Toronto, Canada). Restriction enzymes, T4 DNA ligase and DNA molecular weight standards were from GIBCO/BRL and were used as directed. Radiolabelled nucleotides [α-^{35}S]dATP and [γ-^{32}P]ATP were obtained from Dupont New England Nuclear. The rabbit polyclonal anti-*S. mutans* HPr antiserum was a generous gift from Dr. Christian Vadeboncoeur, Laval University (Ste-Foy, Quebec, Canada). All other chemicals were obtained from Sigma, Fisher Scientific Limited, Difco, or GIBCO/BRL.

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Chapter 4

Regulation of ATP-dependent P-(Ser)-HPr formation in *Streptococcus mutans* and *Streptococcus salivarius*

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Journal of Bacteriology 177:2751-2759 (1995).

Abstract

Sugar transport via the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) involves the PEP-dependent phosphorylation of the general PTS protein, HPr, at histidine 15. However, gram-positive bacteria can also carry out the ATP-dependent phosphorylation of HPr at serine 46 by (Ser)HPr kinase. In this study, we demonstrate that (Ser)HPr kinase in crude preparations of *Streptococcus mutans* Ingbritt and *S. salivarius* ATCC 25975 is membrane associated with pH optima of 7.0 and 7.5, respectively. The latter organism possessed 7 to 27-fold higher activity than *S. mutans* strains NCTC 10449, GS-5 and Ingbritt. The enzyme in *S. salivarius* was activated by fructose-1,6-bisphosphate (FBP) 2-fold at 0.05 mM ATP, but this intermediate was slightly inhibitory at 1.0 mM ATP at concentrations up to 10 mM FBP. Similar inhibition was observed with the enzyme from *S. mutans* Ingbritt. A variety of other glycolytic intermediates had no effect on kinase activity under these conditions. The activity and regulation of (Ser)HPr kinase was assessed *in vivo* by monitoring P-(Ser)-HPr formation in steady state cells of *S. mutans* Ingbritt grown in continuous culture with limiting glucose (10 mM and 50 mM) and with excess glucose (100 mM and 200 mM). All four forms of HPr: [free HPr, P~(His)-HPr, P-(Ser)-HPr and P~(His)-P-(Ser)-HPr] could be detected in the cells, however, significant differences in the intracellular levels of the forms was apparent during growth at the

different glucose concentrations. The total HPr pool increased with increasing medium glucose with significant increases in the P-(Ser)-HPr and P~(His)-P-(Ser)-HPr concentrations. For example, while the total PEP-dependent phosphorylation (P~(His)-HPr + P~(His)-P-(Ser)-HPr) varied only from 21.5 to 52.5 μg, mg cell protein^{-1} in cells grown at the four glucose concentrations, the total ATP-dependent phosphorylation (P-(Ser)-HPr + P~(His)-P-(Ser)-HPr) increased 12-fold from the 10 mM glucose cells (9.1 μg, mg cell protein^{-1}) to 106 and 105 μg, mg^{-1} in the 100 and 200 mM glucose cultures, respectively. (Ser)HPr kinase activity in membrane preparations of the cells varied little between the 10, 50 and 100 mM glucose-grown cells, but increased 3-fold in the 200 mM glucose cells. The intracellular level of ATP, G6P and FBP increased with the external glucose concentration, with the level of FBP 3.8-fold higher for cells grown with 200 mM glucose compared to 10 mM glucose. However, the variation in the intracellular level of FBP, particularly between cells grown with 100 and 200 mM glucose, did not correlate with the extent of P-(Ser)-HPr formation suggesting that the activity of the (Ser)HPr kinase is not critically dependent on the availability of intracellular FBP.

Introduction

The principal sugar transport system in the oral streptococci is the phosphoenolpyruvate (PEP):sugar phosphotransferase transport system (PTS) (Thompson, 1987). The PTS is a group translocation process which utilizes PEP for the phosphorylation of incoming sugars via a phosphoryl-transfer process involving the general, non-sugar specific proteins, enzyme I (EI) and HPr, and subsequently a sugar-specific, membrane-bound Enzyme II complex (EII), which catalyzes the transport and phosphorylation of the specific carbohydrate (Postma *et al.*, 1993). During this process, HPr is transiently phosphorylated by P-EI on histidyl-residue 15 (His-15) and the phosphate group from phospho-HPr [P~(His)-HPr] is then transferred to the membrane-bound Enzyme II complex. 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 (Saier and Reizer, 1992).

In 1983, Deutscher and Saier (Deutscher and Saier, 1983) showed that the HPr of *Streptococcus pyogenes* could also be phosphorylated on a serine residue (Ser-46) by a specific HPr kinase at the expense of ATP. This phosphorylation reaction was subsequently found to be widespread amongst the Gram-

positive, but not Gram-negative bacteria (Postma *et al.*, 1993; Reizer *et al.*, 1993), and has even been shown to occur in some species lacking a functional PTS (Reizer *et al.*, 1993). Whereas several observations over the past ten years have suggested that P-(Ser)-HPr possesses regulatory properties, only recently have some of the physiological functions of this phosphoprotein been determined. These include the regulation of glucose and lactose permease activity in *Lactobacillus brevis* (Ye *et al.*, 1994a; Ye *et al.*, 1994b), regulation of inducer expulsion in *Lactococcus lactis* (Ye *et al.*, 1994c) and involvement in catabolite repression in *Bacillus subtilis* (Deutscher *et al.*, 1994).

In vitro cell extract studies have suggested that the intracellular concentration of P-(Ser)-HPr is controlled by the conjugated action of a metabolite-activated, ATP-dependent protein kinase [(Ser)HPr kinase] and a Pi-dependent P-(Ser)-HPr phosphatase (Postma *et al.*, 1993; Reizer *et al.*, 1989a; Reizer *et al.*, 1988; Reizer *et al.*, 1984). Phosphorylation by purified (Ser)HPr kinases from *S. pyogenes*, *Enterococcus faecalis*, *B. subtilis* and *L. brevis* (Reizer *et al.*, 1989a; Reizer *et al.*, 1988) is stimulated by fructose-1,6-bisphosphate (FBP) and inhibited by inorganic phosphate (Pi). (Ser)HPr kinase activity has been observed in the oral species, *S. salivarius* (Vadeboncoeur *et al.*, 1991; Waygood *et al.*, 1986) and *S. mutans* (Mimura *et al.*, 1987; Vadeboncoeur *et al.*, 1991) with the activity of the

enzyme in the latter organism shown to be activated by FBP and inhibited by Pi and some glycolytic intermediates (Mimura *et al.*, 1987).

Studies with intact whole cells have confirmed the phosphorylation of HPr on a serine residue and shown that the activity of both the ATP-dependent (Ser)HPr kinase and the PEP-dependent phosphoryl cascade in cells results in the formation of four different forms of HPr in Gram-positive bacteria: free HPr, P~(His)-HPr, P-(Ser)-HPr and a doubly phosphorylated form, P~(His)-P-(Ser)-HPr. All four forms have been detected in *S. salivarius* and *S. mutans* by crossed immunoelectrophoresis after rapid acidification of the cytoplasm followed by grinding a frozen pellet of the cells to extract cytoplasmic proteins (Vadeboncoeur *et al.*, 1991). Batch-grown, exponential-phase cells have been shown to contain only P-(Ser)-HPr and P~(His)-P-(Ser)-HPr, whereas stationary cells contained predominantly HPr and P~(His)-HPr. These results imply a change in the levels of the HPr forms in response to the availability of the carbon source which was the limiting nutrient, supporting earlier observations describing the dephosphorylation of P-(Ser)-HPr during starvation (Lodge and Jacobson, 1988).

The *in vitro* and whole cell studies have clearly established the phosphorylation of HPr on Ser-46 by a regulated ATP-dependent protein kinase in Gram-positive

bacteria. Nevertheless, none of these studies have demonstrated a correlation between the cellular levels of the putative effectors of (Ser)HPr kinase and the extent of P-(Ser)-HPr formation. With this in mind, we have undertaken to examine (Ser)HPr kinase activity in *S. salivarius* and various strains of *S. mutans*, and to examine the regulation of the enzyme in more detail. Early results with crude membrane preparations indicated that, unlike other studies with *S. mutans* (Mimura *et al.*, 1987), (Ser)HPr kinase was not activated by fructose-1,6-bisphosphate. Consequently, we undertook to monitor the formation of P-(Ser)-HPr *in vivo* by assaying the four forms of HPr in steady state cells of *S. mutans* Ingbritt growing in continuous culture with increasing concentrations of glucose in order to generate increased levels of the key glycolytic intermediates.

Results

***In vitro* (Ser)HPr kinase activity.** In order to compare the intracellular level of (Ser)HPr kinase activity in oral streptococci, we assayed the enzyme in membrane and cytoplasmic fractions of glucose-grown, exponential-phase cells of *S. salivarius* ATCC 25975, and *S. mutans* strains, Ingbritt, GS-5 and 10449. In all cases, (Ser)HPr kinase was associated with the cell membrane with less than 1% of the activity appearing in the cytoplasmic fraction. The pH optimum for the enzyme in *S. salivarius* was 7.5, while that for *S. mutans* Ingbritt was 7.0 (Figure 4-1) and the latter enzyme was more acid tolerant with half-maximum activities at pH 5.5 and 8.0 compared to pH 6.3 and 8.8 for the *S. salivarius* enzyme. Comparative (Ser)HPr kinase assays with the four strains indicated that *S. salivarius* possessed 7 to 27-fold higher activity than the three *S. mutans* strains. This could simply be due to inherent differences in the specific activities of the enzymes from these different bacteria or suggests that either the *S. salivarius* enzyme was produced at a higher level or the enzyme in the various strains was dependent on different effectors.

As previous research has demonstrated the importance of glycolytic intermediates in the regulation of (Ser)HPr kinase activity, we employed the ^{32}P~HPr/gel-isolation assay to examine this regulation in *S. salivarius* 25975 and *S. mutans*

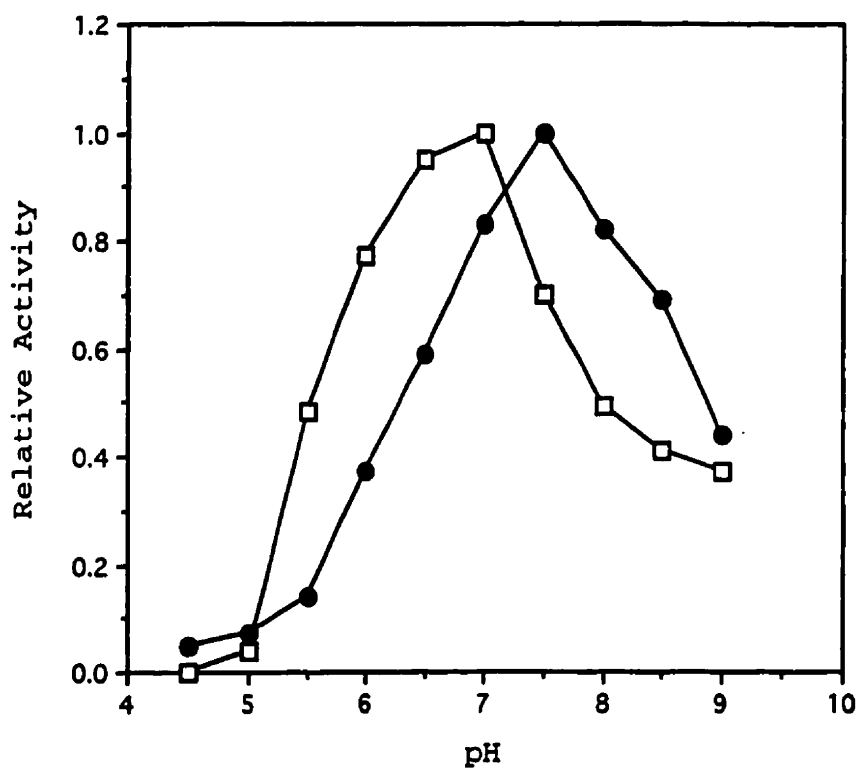

Figure 4-1: Effect of pH on the (Ser)HPr kinase activity in membrane preparations of *S. mutans* Ingbritt (□) and *S. salivarius* ATCC 25975 (●).

Ingbritt. For this, enzyme activity in membrane preparations from each organism was measured at the pH optimum in the presence of various intermediates at three concentrations typical of that in the cytoplasmic compartment of oral streptococci at pH 7.0 (Iwami *et al.*, 1975). At 0.05 mM ATP, the activity of the *S. salivarius* enzyme was stimulated 2-fold by fructose-1,6-bisphosphate with maximum activation at 5 mM FBP (Figure 4-2). However, if the ATP concentration was increased to 0.1 mM in the assay, FBP activation was less than 20% at 4 mM FBP and a further increase to 1.0 mM ATP resulted in slight inhibition increasing with the FBP concentration. At 1.0 mM ATP, the activity of the *S. mutans* enzyme was also progressively inhibited and to a slightly greater extent by FBP with the reduction in activity 45% at 15 mM FBP (Figure 4-2). The intermediates glucose-6-phosphate, fructose-6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, 2,3-phosphoglycerate, and pyruvate had no effect on (Ser)HPr kinase activity (data not shown). These results suggest that the level of P-(Ser)-HPr in these streptococci is not regulated by the intracellular concentration of the major glycolytic intermediates, including FBP, contrary to studies with *S. mutans* GS5 (Mimura *et al.*, 1987), *S. pyogenes* (Reizer *et al.*, 1988) and *E. faecalis* (Deutscher and Engelmann, 1984).

Glucose concentration and intracellular HPr. The *in vitro* assay results have raised questions as to the extent of

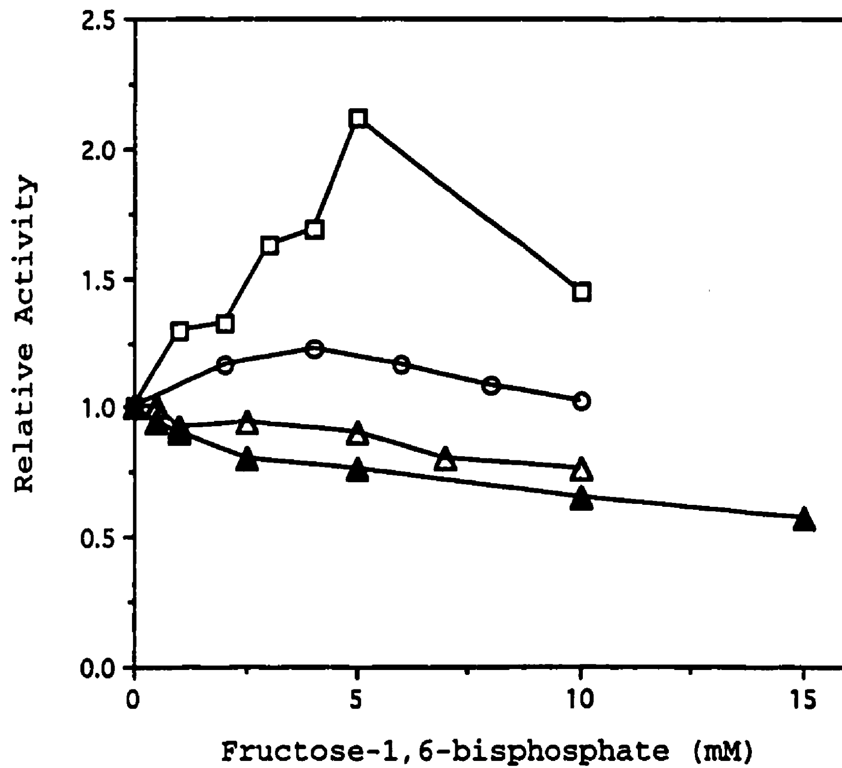

Figure 4-2: Effect of fructose-1,6-bisphosphate on (Ser)HPr kinase activity by membrane preparations of *S. mutans* Ingbritt (solid symbol) and *S. salivarius* ATCC 25975 (open symbols). ATP concentrations (mM): 0.05 (□), 0.1 (○) and 1.0 (△, ▲).

(Ser)HPr kinase activity in cells of *S. mutans* and the nature of the intracellular regulation of the enzyme. To determine whether the activity of the (Ser)HPr kinase was influenced by environmental factors and governed by the energetic status of the cell, we measured the intracellular levels of the various forms of HPr in cells of *S. mutans* Ingbritt grown at various glucose concentrations in continuous culture. Growth was at pH 7.0, (D=0.1 h^{-1}) with four glucose concentrations (10, 50, 100 and 200 mM). As seen in Table 4-1, growth at 10 and 50 mM glucose resulted in the complete utilization of the energy source in the growth chamber and, therefore, the cultures were glucose limited. On the other hand, significant residual glucose was present with the 100 and 200 mM glucose chemostats and these cultures were nitrogen-limited or excess with respect to glucose.

Inspection of the yields and the rate of glucose utilization by the cells in each culture (Table 4-1) clearly indicated differences in the physiological status of the cells at each glucose concentration. As expected, the cell yields decreased with the increasing medium glucose concentration and, although both the 10 and 50 mM cultures were glucose limited, there were significant physiological differences in these cultures as would be expected from the different concentrations of glucose utilized by each culture, i.e. 8.2 vs 46.4 mM (Table 4-1). This resulted in a 2-fold higher cell yield for the 10 mM culture (53.7 g [dry weight]

Table 4-1

Effect of glucose concentration on the yield and glucose transport by cells of *Streptococcus mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 and a dilution rate of 0.1 h^{-1}.

Nominal Glucose (mM)	Actual Initial Glucose (mM)	Residual Glucose (mM)	Glucose Utilized (mM)	Dry Weight Cells (mg/ml)	Cell Yield [a]	Glucose Transport in Chemostat [b]
10	8.2	0	8.2	0.39	53.7	32.8
50	46.4	0	46.4	1.34	25.7	57.7
100	82.3	22.7	59.6	1.44	23.6	69.0
200	227	106	121	1.30	16.1	155.1

a Grams (dry weight) of cells per mole of glucose utilized.
b Nanomoles of glucose per milligram (dry weight) of cells per minute.

cells, mole of glucose^{-1}) compared to the 50 mM culture (25.7 g, mole^{-1}). Similarly, the 100 and 200 mM cultures were glucose excess, however, the 200 mM culture utilized twice the amount of glucose (121 mM) compared to the 100 mM culture (59.6 mM) and this was reflected in a significantly lower cell yield for the 200 mM culture (16.1 g, mole^{-1}) compared to the 100 mM culture (23.6 g, mole^{-1}). Calculation of the actual rate of glucose uptake in the chemostat (Herbert and Kornberg, 1976) revealed increases from 32 nmoles, mg cells^{-1}, min^{-1} at 10 mM glucose to a maximum (155 nmoles, mg cells^{-1}, min^{-1}) at 200 mM (Table 4-1). The progressive increase observed in the rate of glucose transport with respect to the initial glucose concentration was consistent with the gradual change observed in the amount of glucose utilized during cellular growth, being minimum for cells having the lowest rate of glucose transport and maximum for cells that exhibited the highest rate.

The different forms of HPr were quantitatively estimated in steady state cells growing in the continuous cultures under the four conditions described above. As shown previously (Vadeboncoeur *et al.*, 1991), four forms of HPr are possible in steady-state cells of oral streptococci: free HPr, P~(His)-HPr, P-(Ser)-HPr and the doubly phosphorylated product, P~(His)-P-(Ser)-HPr. Although P~(His)-HPr and P-(Ser)-HPr run in the same location on crossed immunoelectrophoresis, they can be separated by boiling a

portion of the cell extract to degrade the phosphoamidate bond of P~(His)-HPr since the phosphomonoester bond of P-(Ser)-HPr is heat stable (Martensen, 1984). Thus, boiling the sample quantitatively converted P~(His)-HPr to free HPr and the doubly phosphorylated form to heat-stable P-(Ser)-HPr (Figure 4-3, A and B). Subsequent quantitative comparison of the peaks resulting from the boiled sample with an unboiled sample of the same cell extract permitted the estimation of the cellular concentration of each of the four forms of HPr (Table 4-2). In addition, the actual total PEP-dependent phosphorylating activity is represented by the sum of P~(His)-HPr and P~(His)-P-(Ser)-HPr, while total ATP-dependent phosphorylation is represented by the sum of P-(Ser)-HPr and the doubly phosphorylated form.

All four forms of HPr could be detected in the four samples of chemostat-grown cells of *S. mutans* Ingbritt, however, continuous growth with the various glucose concentrations resulted in differences in both the concentration of the total 'pool' of HPr in the cells and in the intracellular levels of the various forms of HPr (Table 4-2 and Figure 4-3, A, C, D). The total 'pool' of HPr, expressed as µg, mg of cell protein^{-1}, almost doubled when comparing the 10 mM glucose-starved cells (91.2 µg, mg^{-1}) with those grown at 200 mM (171 µg, mg^{-1}).

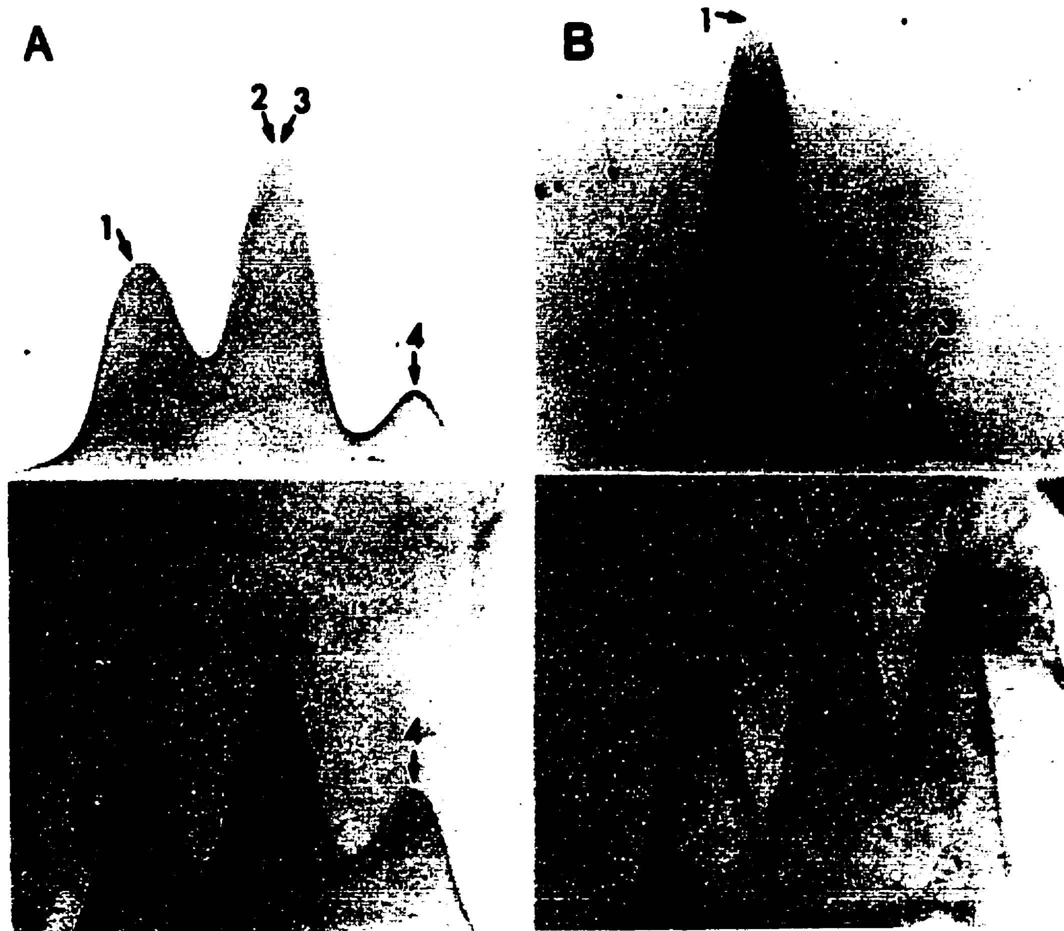

Figure 4-3: Crossed immunoelectrophoresis of membrane-free cellular extracts of cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (D = 0.1 h^{-1}) with 10, 50 and 200 mM glucose. Each sample (5 µl) contained 10 µg of cytoplasmic proteins and was probed with polyclonal anti-HPr rabbit antibodies, directed against *S. mutans* DR0001. (A) Cells grown with 10 mM glucose; (B) cells grown as for panel A incubated at 100°C for 3 minutes prior to electrophoresis; (C) cells grown with 50 mM glucose; (D) cells grown with 200 mM glucose. Numbers indicate immunoprecipitate peaks which correspond to; nonphosphorylated HPr (1), P~(His)-HPr (2), P-(Ser)-HPr (3), P~(His)-P-(Ser)-HPr (4).

Table 4-2

Effects of glucose concentration on the concentrations of the four forms of HPr in cells of *Streptococcus mutans* Ingbritt grown in continuous culture at a dilution rate of 0.1 h^{-1} and pH 7.0.[a]

(mM)	Cellular Concentration [b]						
	HPr	P-(His)-HPr	P-(Ser)-HPr	P-(His)-P-(Ser)-HPr	Total HPr	Total PEP-dependent Phosphorylation	Total ATP-dependent Phosphorylation
10	63.9 ± 5.8	18.2 ± 6.8	5.8 ± 1.4	3.3 ± 1.4	91.2 ± 5.7	21.5 ± 8.0	9.1 ± 1.7
50	59.2 ± 11.2	31.8 ± 12.4	28.6 ± 11.5	14.3 ± 3.1	134 ± 11.3	46.1 ± 12.7	42.9 ± 10.6
100	52.1 ± 8.0	11.2 ± 3.7	84.7 ± 13.8	21.5 ± 8.1	170 ± 19.0	32.7 ± 11.2	106 ± 9.9
200	52.5 ± 2.9	13.5 ± 6.2	66.4 ± 4.9	39.0 ± 11.9	171 ± 13.5	52.5 ± 14.6	105 ± 8.9

[a] Determinations were carried out by crossed immunoelectrophoresis and the values represent the mean of at least 4 determinations.

[b] Micrograms per milligram of cell protein ± SE.

The cellular concentrations of P~(His)-HPr and P-(Ser)-HPr were of particular interest in these experiments. As seen in Table 4-2, the P~(His)-HPr levels alone varied only from 11.2 to 31.8 μg, mg cell protein^{-1} in cells grown between 10 and 200 mM with the highest concentration in the 50 mM glucose-grown cells. The cellular levels of the doubly-phosphorylated form, P~(His)-P-(Ser)-HPr, increased 12-fold over the complete range of glucose concentrations. Thus, the total PEP-dependent phosphorylation of HPr (i.e., P~(His)-HPr + P~(His)-P-(Ser)-HPr) changed from 21.5 to 52.5 μg, mg cell protein^{-1} from 10 to 200 mM glucose. P-(Ser)-HPr, on the other hand, increased 15-fold from the 10 mM glucose cells (5.8 μg, mg cell protein^{-1}) to a high at 100 mM glucose of 84.7 μg and declined at 200 mM glucose (66.4 μg). As a consequence, the total ATP-dependent phosphorylation of HPr (P-(Ser)-HPr + P~(His)-P-(Ser)-HPr) increased progressively with respect to the medium glucose level from 9.1 μg at 10 mM to reaching a maximum value 106 μg, mg protein^{-1} in cells grown at 100 mM glucose. Increasing the glucose concentration to 200 mM did not increase the level of the total P-(Ser)-HPr, or ATP-dependent phosphorylation.

Since the total cellular HPr concentration changes with increasing glucose in the medium, a more informative method of analyzing the differences in the levels of the four forms of HPr is to calculate the relative proportions of the various forms within each growth condition. As seen in

Figure 4-4, the relative amount of free, non-phosphorylated HPr in *S. mutans* Ingbritt cells grown in continuous culture was the highest for cells grown at 10 mM glucose (70%) and this declined to 31% in the cells grown with 100 and 200 mM glucose. As for P~(His)-HPr, the 10 and 50 mM glucose-limited cultures had the highest levels of this form (20 and 24%, respectively) with the 100 and 200 mM glucose-excess cells exhibiting the lowest relative amounts (6.6 and 7.9%, respectively). On the other hand, P-(Ser)-HPr, increased from 6% with the 10 mM cells to a maximum of 50% with the 100 mM glucose-grown cells. The doubly phosphorylated form, P~(His)-P-(Ser)-HPr, increased progressively from 3.6 to 23% as the medium glucose was increased from 10 to 200 mM.

Further analysis of the data in Table 4-2 reveals the extent of the PEP- and ATP-dependent phosphorylating activity as a fraction of total HPr phosphorylation. This is obtained by combining the relative values for P~(His)-HPr and P-(Ser)-HPr peaks with those in the doubly phosphorylated form, P~(His)-P-(Ser)-HPr. From these calculations it can be determined that PEP-dependent phosphorylation ranged from 70% at 10 mM to 24% at 100 mM, while that dependent on ATP was 30% at 10 mM glucose increasing to 76% at 100 mM reflecting the increased uptake of glucose into the cell (Table 4-1).

(Ser)HPr kinase activity and medium glucose. In association with the estimation of the various forms of HPr

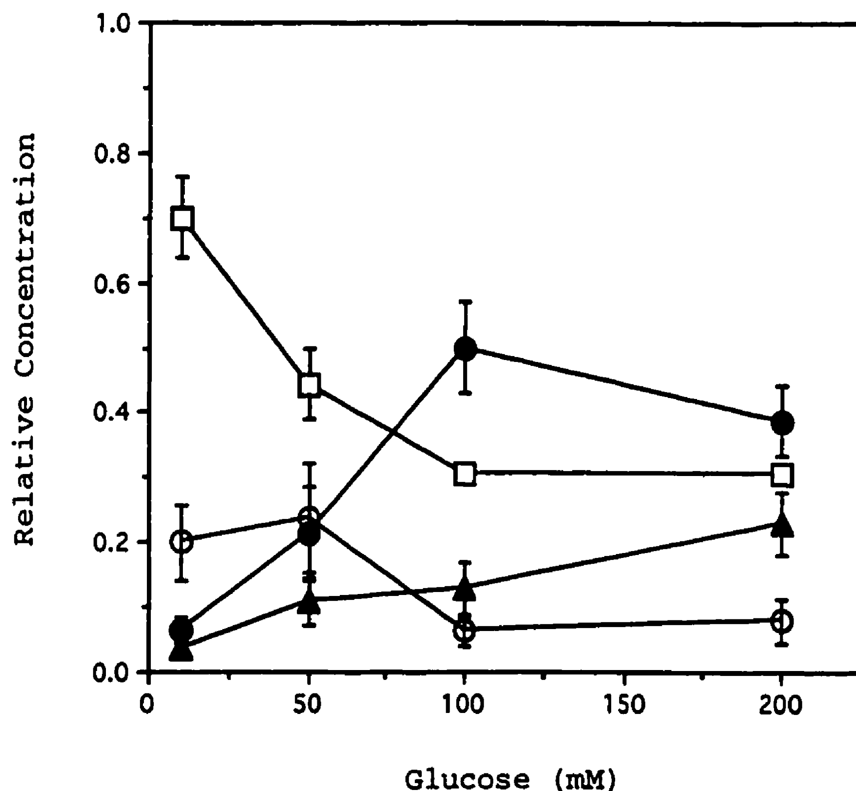

Figure 4-4: Comparison of the relative amounts of the four forms of HPr in cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (D=0.1 h^{-1}) with concentrations of glucose ranging from 10 mM to 200 mM. Symbols correspond to: nonphosphorylated HPr (□), P~(His)-HPr (○), P-(Ser)-HPr (●), and P~(His)-P-(Ser)-HPr (▲). The results (in micrograms per milligram of protein) represent the mean of at least four determinations and bars represent the standard error.

in vivo, (Ser)HPr kinase activity was measured in membrane preparations from the same cells grown in chemostats at the four concentrations of glucose. As seen in Figure 4-5, enzyme activity was very similar in the cells grown at 10, 50 and 100 mM glucose, varying from 2.7 to 3.5 units, µg membrane protein^{-1}, min^{-1}. Growth at 200 mM glucose, however, resulted in a 3-fold increase in (Ser)HPr kinase activity to 10.6 units suggesting a significant change in enzyme synthesis. Nevertheless, this increase in the cellular amount of the enzyme did not result in an increase in the intracellular concentration of P-(Ser)-HPr (Table 4-2, Figure 4-4).

Cellular concentrations of key glycolytic intermediates. The earlier *in vitro* (Ser)HPr kinase assays have suggested that, contrary to earlier studies (Mimura *et al.*, 1987), the enzyme in *S. mutans* and *S. salivarius* was not stimulated by fructose-1,6-bisphosphate (Figure 4-2) and other glycolytic intermediates. In view of this and the data for enzyme activity in the chemostat-grown cells (Figure 4-5), we undertook to determine whether there was a relationship between the intracellular concentrations of FBP and G6P, as well as the substrate, ATP, and enzyme activity in whole cells grown in continuous culture under the same conditions as that for the HPr determinations.

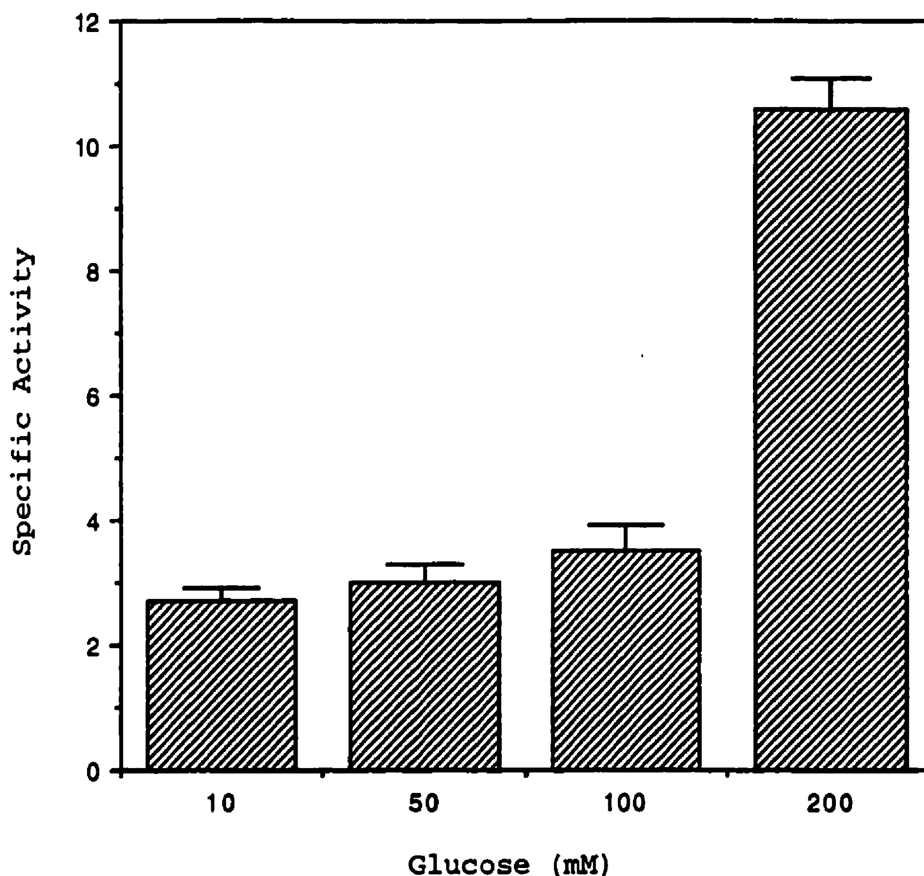

Figure 4-5: (Ser)HPr kinase activity in membrane preparations derived from cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (D=0.1 h^{-1}) with 10, 50, 100, and 200 mM glucose. Activity units are expressed in picomoles of P-(Ser)-HPr formed per microgram of membrane protein per minute.

Table 4-3

Effect of glucose concentration on the concentration of the fructose-1,6-bisphosphate, glucose-6-phosphate and ATP in cells of *Streptococcus mutans* Ingbritt grown in continuous culture at a dilution rate of 0.1 h^{-1} and pH 7.0.

Glucose (mM)	Intracellular Concentration (µM) ± SE [a]		
	Fructose-1,6-bisphosphate	Glucose-6-phosphate	ATP
10	290 ± 10	801± 56	415 ± 46
50	637 ± 22	497 ± 47	1,004 ± 101
100	976 ± 109	662 ± 56	1,494 ± 80
200	1,114 ± 67	1,227 ± 39	1,416 ± 86

[a] Determinations were carried out enzymatically (see Methods) and the values represent the mean of at least 5 determinations.

As seen in Table 4-3, with the exception of the G6P concentration in cells from the 10 mM chemostat culture, the glycolytic intermediate levels increased with the medium glucose concentration in a predictable manner. In particular, the intracellular levels of FBP increased in the chemostat-grown cells such that the 200 mM cells contained 3.8-fold higher level than the 10 mM glucose-grown cells. The cellular concentration of ATP increased from 415 μM at 10 mM glucose and reached a relatively stable level in the 100 mM and 200 mM glucose cells (1416-1494 μM).

Discussion

Steady-state chemostat-grown cells of *S. mutans* Ingbritt possessed all four forms of HPr during growth at four different glucose concentrations. A notable effect of increasing the glucose from 10 to 200 mM was the increase in the total cellular content of all forms of HPr from 91.2 to 171 µg, mg cell protein^{-1}, an observation contrary to our previous results (Hamilton *et al.*, 1989). In the latter study, the cellular HPr concentration decreased 4-fold from medium glucose concentrations of 6.8 to 304 mM confirming an earlier observation (Vadeboncoeur *et al.*, 1987) that changes from glucose to nitrogen limitation (glucose excess) resulted in a small decrease in the synthesis of HPr. The reason for this difference in results is not immediately apparent since the current experiments were carried out with the same organism in the same medium and under similar conditions (pH 7.0, D=0.1 h^{-1}). A notable difference between the two studies, however, was the fact that the HPr concentrations in the earlier studies were estimated by rocket immunoelectrophoresis, a technique that does not allow separation of the chemically-different forms of HPr. It is reasonable to assume that the antibodies present in the polyclonal antiserum were directed against epitopes common to the HPr molecule. This suggestion is supported by the generation of a single peak by P~(His)-HPr and P-(Ser)-HPr in unboiled extracts. Differentiation is based on

electrophoretic separation and not on the specific epitopes among the different forms of HPr. With rocket electrophoresis, the peak area would be influenced by the form of HPr in the highest concentration and this peak might not reflect accurately the sum of all forms of HPr. Assay of the different forms of HPr after electrophoretic separation, as was done in the current study, should provide a more accurate estimate of the total HPr in the cells.

The most notable effect of glucose on the intracellular forms of HPr in *S. mutans* Ingbritt was a progressive increase in the concentration of the doubly phosphorylated form with the medium glucose concentration, accompanied by a small decrease in the amount of P~(His)-HPr (Table 4-2). The concentration of P~(His)-P-(Ser)-HPr was minimal in cells grown under conditions of glucose limitation (10 mM) when P~(His)-HPr predominated, and maximum in cells grown with excess glucose (200 mM), being 3-fold higher than P~(His)-HPr. Consequently, the total ATP-dependent phosphorylation of HPr, calculated from the P-(Ser)-HPr and the P~(His)-P-(Ser)-HPr concentrations, increased 12-fold from 12.4 to 144 µg, mg cell protein^{-1} with increases in the medium glucose from 10 to 200 mM, while the PEP-dependent phosphorylation varied only 2-fold from a low of 21.5 µg at 10 mM glucose to a high of 52.5 µg, mg cell protein^{-1} at 200 mM glucose.

These results are consistent with those obtained with batch-grown cells of *S. mutans* DR0001 and *S. salivarius* 25975 (Vadeboncoeur *et al.*, 1991). In the latter study, using the same methods, exponential-phase cells possessed no free HPr and no or very low amounts of P~(His)-HPr, with most of the HPr in the form of P-(Ser)-HPr with lesser amounts in the doubly phosphorylated form, P~(His)-P-(Ser)-HPr. On the other hand, the dominant forms in stationary-phase cells were free HPr and P~(His)-HPr with the cells devoid of P-(Ser)-HPr and the doubly phosphorylated form. Globally, these results suggest that in energy-deprived cells, phosphate is transferred to the EII complexes of the PTS mainly by P~(His)-HPr, whereas under conditions of an abundant energy source, this function is taken over by the doubly phosphorylated product. Although our results are consistent with this hypothesis, further research is needed to determine whether the doubly phosphorylated form of streptococcal HPr is efficient at transferring phosphate groups to the EII complexes.

Physiologically, the replacement of the P~(His)-HPr by the doubly phosphorylated protein as the phosphocarrier in the PTS phosphoryl cascade is obviously linked to the function of P-(Ser)-HPr. The results reported here, as well as those obtained with batch-grown cells (Vadeboncoeur *et al.*, 1991), indicate that the assigning of regulatory functions to P-(Ser)-HPr must take into account the existence of the doubly

phosphorylated product. Recent studies have suggested that P-(Ser)-HPr is involved in regulating non-PTS transport systems in *L. brevis* (Ye *et al.*, 1994a; Ye *et al.*, 1994b), a bacterium that apparently lacks EI and EII complexes of the PTS. Such a role for P-(Ser)-HPr is compatible with the presence of the doubly-phosphorylated protein growing in cells possessing a PTS. Hence, when cells are growing in the presence of glucose excess, P-(Ser)-HPr would be produced to prevent utilization of other less rapidly degraded non-PTS sugars, whereas the doubly phosphorylated protein would be synthesized to maintain the transfer of phosphate from PEP to the EII complex of the glucose PTS at an optimal rate. Under conditions of energy deprivation, the cells would produce very low levels of P-(Ser)-HPr permitting the concomitant utilization of several sugars.

Several years ago, it was suggested that phosphorylation of HPr on Ser-46 might serve to regulate the activity of the PTS (Deutscher *et al.*, 1984). This hypothesis was supported by the observation that P-(Ser)-HPr was phosphorylated *in vitro* at a very much slower rate by PEP than non-phosphorylated HPr. However, the data reported in this study, as well as those obtained with batch-grown cells (Vadeboncoeur *et al.*, 1991), are not fully consistent with this proposal as the amount of the doubly phosphorylated protein found in growing cells suggested that the phosphorylation of P-(Ser)-HPr by EI of the PTS or, conversely, the phosphorylation P~(His)-HPr by

the HPr kinase, are not limiting steps *in vivo*. Other cellular studies also failed to provide evidence supporting the hypothesis suggesting that P-(Ser)-HPr controls PTS activity (Reizer and Peterkofsky, 1987; Reizer *et al.*, 1992; Reizer *et al.*, 1989b; Sutrina *et al.*, 1990). Nevertheless, this latter hypothesis has recently reappeared by way of the suggestion that P-(Ser)-HPr is involved in the control of lactose expulsion in *L. lactis* by controlling the activity of both a sugar-P phosphatase and the lactose EII (Ye *et al.*, 1994c). Although the control of the phosphatase by P-(Ser)-HPr is compatible with the presence of a doubly phosphorylated HPr in the cell, the control of the lactose permease raises some questions as it would imply that this PTS permease could not be phosphorylated by P~(His)-P-(Ser)-HPr and would be inhibited by P-(Ser)-HPr, whereas the glucose PTS would be immune to the inhibitory effect of P-(Ser)-HPr and be able to react with the doubly phosphorylated protein. This is unlikely as it has already been reported that the IIA^{Lac} and IIA^{Mtl} enzymes from *B. subtilis* could catalyze the *in vitro* phosphorylation of lactose or mannitol, respectively, using as a phosphocarrier a mutated HPr in which the serine at position 46 was replaced by an aspartate resulting in a protein having properties similar to P-(Ser)-HPr (Reizer *et al.*, 1989b). Therefore, the control of PTS activity by P-(Ser)-HPr is not consistent with the presence of high amounts of the doubly phosphorylated HPr in rapidly growing cells or in cells

growing in the presence of excess glucose. It should be pointed out that even though the presence of the doubly phosphorylated HPr has been confirmed in streptococci (this study, Vadeboncoeur *et al.*, 1991), it remains to be established in other genera, including lactococci.

Notwithstanding the *L. lactis* results, our data, together with previously published kinetic data (Reizer *et al.*, 1992), provide further evidence suggesting that phosphorylation of HPr on Ser-46 has no physiological consequences with respect to activity of the PTS. Assuming that 0.5 mg protein is equivalent to 1 mg of cell dry weight, simple calculations reveal that the cellular concentrations of free HPr and P~(His)-HPr under all growth conditions were approximately 1.5 mM and 0.3-0.75 mM, respectively. These concentrations are substantially higher than the apparent Km values of the relevant enzymes for these substrates, e.g. the Km of streptococcal EI for HPr being 25 µM (Vadeboncoeur *et al.*, 1983) and that of *Bacillus* IIA^{Glu} for P~(His)-HPr being 0.5 µM (Reizer *et al.*, 1992). Similar calculations indicate that the cellular concentrations of P-(Ser)-HPr are also higher than that required for saturation of EI and IIA^{Glu} of *B. subtilis* (Reizer *et al.*, 1992; Reizer *et al.*, 1989b). We are aware that determination of these kinetic parameters with protein from streptococcal origin is required to substantiate this conclusion. However, the data reported in this study clearly indicate that phosphorylation of HPr on Ser-46 is not

dedicated to reducing the entry of glucose into these cells since the rate of glucose transport by growing cells increases with respect to the cellular level of the total P-(Ser)-HPr (Tables 4-1 and 4-2).

Recent observations also suggest that (Ser)-HPr phosphorylation plays a role in repression of several catabolite genes in *B. subtilis* (Deutscher *et al.*, 1994). A form of catabolite repression has been observed with chemostat-grown cells of *S. mutans* Ingbritt growing on glucose. In this case, the glucose-PTS is subjected to concentration-dependent repression by glucose itself (Ellwood *et al.*, 1979), a process known to inhibit the synthesis of EII^{Glc} and EII^{Man} in membrane preparations of the organism (Hamilton *et al.*, 1989). In these latter experiments, the synthesis of EII^{Glc} and EII^{Man} was repressed during growth at all glucose concentrations above 2.6 mM with 33- and 39-fold inhibition observed at 271 mM glucose. The EII for fructose (EII^{Fru}) was also repressed 4-fold, although activity for the latter enzyme was low in the glucose-grown cells. In view of the data with *B. subtilis* (Deutscher *et al.*, 1994) and the concomitant effects seen with the formation of P-(Ser)-HPr and EII^{Glc} synthesis in the presence of high concentrations of glucose, it is tempting to speculate that P-(Ser)-HPr is involved in the catabolite repression of the glucose-PTS in what would, in fact, be a form of autorepression by the substrate itself. Obviously, much more work is required in

order to ascertain whether this mechanism is functional in oral streptococci.

Comparison of the observed cellular P-(Ser)-HPr levels (Table 4-2, Figure 4-4) and the (Ser)HPr kinase assays (Figure 4-5) from chemostat-grown cells at the four glucose concentrations indicated two separate effects on the kinase in *S. mutans* Ingbritt. Between 10 and 100 mM glucose, there was no increase in enzyme specific activity (Figure 4-5), whereas the intracellular concentration of total P-(Ser)-HPr increased progressively from 9.1 to 106 µg, mg cell protein^{-1}, indicating a control of the enzyme at the protein level. On the other hand, specific enzyme activity increased 3-fold between 100 and 200 mM glucose suggesting a stimulation of kinase synthesis, presumably at the transcriptional level, however, this increase in enzyme synthesis did not result in higher levels of P-(Ser)-HPr (Table 4-2). A plausible explanation for this would be that at 200 mM glucose, cells accumulate one or more metabolites that down-regulate the (Ser)HPr kinase. To keep the cellular level of total P-(Ser)-HPr at the appropriate level, the cells compensate by increasing enzyme synthesis. This hypothesis is consistent with the fact that (a) the rate of glucose transport increased significantly between 100 and 200 mM glucose (Table 1), which increases the cellular concentration of metabolites (Iwami *et al.*, 1975, Table 4-3), and (b) the kinase was inhibited *in vitro* by high concentrations of FBP (Figure 4-

2). These effects will undoubtedly be influenced by the nature of the enzyme in the particular strain, the effects of FBP (Figure 4-2) and other metabolites, as well as subtle differences in the pH profile of kinase activity. For example, the difference in the (Ser)HPr kinase pH profile between *S. mutans* Ingbritt and *S. salivarius* (Figure 4-1) is in keeping with the acid-tolerant characteristics of the *S. mutans* strain (Hamilton and Buckley, 1991) and will be a factor at pH values below 6.0.

The effect of glycolytic intermediates on the activity of (Ser)HPr kinase in membrane preparations of *S. salivarius* and *S. mutans* suggests differences from the results reported by other workers. FBP-activation of (Ser)HPr kinase has been a consistent feature of the studies with *S. pyogenes* (Reizer *et al.*, 1988), *E. faecalis* (Deutscher and Engelman, 1984), *L. brevis* (Reizer *et al.*, 1993) and *B. subtilis* (Reizer *et al.*, 1989a), as well as *S. mutans* GS-5 (Mimura *et al.*, 1987). Our *in vitro* and intact cell results suggest that the intracellular concentration of glucose-6-phosphate or fructose-1,6-bisphosphate are not critical factors in the stimulation of (Ser)HPr kinase activity. In the *in vitro* assay, only at 0.05 mM ATP was FBP slightly stimulatory with membrane preparations of the enzyme from *S. salivarius*. When the concentration of ATP was increased to 1.0 mM, the *S. salivarius* and *S. mutans* enzymes were either not affected or

inhibited by FBP, as well as being unaffected by other intermediates.

Correlation of the concentration of FBP from the intact cells studies seen in Table 4-3 and the level of the total P-(Ser)-HPr reported in Table 4-2 showed that both concentrations increased with respect to the medium glucose concentration. This is apparently consistent with the suggestion that FBP activates the (Ser)HPr kinase. However, the concentration of FBP observed in the intact cell studies is far below the concentration that activated the kinase of other organisms *in vitro* (~5 mM) (Reizer *et al.*, 1984), as well as, the kinase in *S. salivarius* even in the presence of 0.05 mM ATP (Figure 4-2). As we have seen in Table 4-3, other than the 10 mM cells, the cellular ATP levels were at or above 1.0 mM suggesting the cellular ATP levels would not be low enough to promote the FBP activation as that seen for *S. salivarius* in Figure 4-2. Moreover, in spite of the fact that the 200 mM glucose cells contained the highest cellular level of FBP (1.1 mM), as well as, a 10-fold higher level of the enzyme, the total intracellular concentration of P-(Ser)-HPr did not change from that at 100 mM glucose. Considering the *in vitro* and cellular results, we suggest that the intracellular concentration of FBP and P-(Ser)-HPr are not related in *S. salivarius* and *S. mutans*. Obviously, increased levels of other metabolites, alone or in combination, may contribute to the increase in (Ser)HPr

kinase activity *in vivo* and may also be involved in the increased synthesis of the enzyme in the 200 mM cells.

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Chapter 5

Isolation of a mutant *Streptococcus mutans* strain altered at the glycine 67 residue of the HPr protein.

Abstract

The HPr protein functions as a non-sugar specific phosphocarrier in the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) in both Gram-positive and Gram-negative bacteria. HPr is phosphorylated at the histidine-15 residue during the PTS cascade, however, in Gram-positive bacteria, HPr can also be phosphorylated at the serine-46 residue. Site-directed mutagenesis studies have identified the role of seryl-phosphorylated HPr in catabolite repression, inducer expulsion, and the regulation of PTS and non-PTS transport in several Gram-positive bacteria. This study intended to examine the role of the HPr protein from the oral streptococcus strain *Streptococcus mutans* following site-directed mutagenesis of the plasmid-encoded *ptsH* gene. The HPr residues histidine-15, serine-46 and glycine-67 were the target for substitutions during double-stranded DNA mutagenesis. The lack of success of the double-stranded technique prompted the use of single-stranded mutagenesis which resulted in successful replacement of glycine-67 by an aspartate residue. The plasmid carrying the mutated *ptsH* gene also carried a kanamycin-resistance gene between the *ptsI* and *gapN* genes encoding the non-sugar-specific PTS protein enzyme I and glyceraldehyde-3-phosphate dehydrogenase, respectively. This plasmid was transformed into *S. mutans* and replacement of the wild-type gene by the mutated gene was expected to occur via a double cross-over

event and selection of potential mutants was based on kanamycin resistance. Southern hybridization analysis verified the integration of the kanamycin gene, however, the potential mutants readily fermented PTS sugars, a phenotypic characteristic we anticipated would be lost upon successful integration of the mutated *ptsH* gene. The inability to successfully integrate the mutated *ptsH* gene in the *S. mutans* BM71 genome could have been due to the structural features of the plasmid, or the integration of the mutated HPr may have been a lethal event. Nevertheless, further work was not undertaken as part of the thesis research project, however, alternative integration protocols have been suggested along with experiments designed to examine the effect of the mutation employing the plasmid-encoded gene.

Introduction

The heat-stable histidine-containing protein (HPr) is a component of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) that catalyzes the concomitant transport and phosphorylation of carbohydrates in both Gram-positive and Gram-negative bacteria (Postma *et al.*, 1993; Saier and Reizer, 1994). The proteins, HPr and enzyme I (EI), are soluble, cytoplasmic proteins common to all PTS systems, whereas, the enzymes II (EIIs) are membrane-bound and carbohydrate specific (Saier and Reizer, 1992). The PTS cascade is characterized by a series of phosphorylated intermediates with the phosphoryl group initially transferred from the glycolytic intermediate PEP to EI which, in turn, transfers it to the histidine-15 residue of HPr. The phosphate group is then transferred from P~(His)-HPr to the membrane-bound permease (Postma *et al.*, 1993) (see Figure 1-2).

The HPr protein of Gram-positive bacteria, unlike that of Gram-negative bacteria, can also be phosphorylated at a serine residue at position 46 by a membrane-associated, metabolite-activated ATP-dependent (Ser)HPr kinase (Cozzone, 1993; Deutscher and Saier, 1983; Reizer *et al.*, 1983; reviewed in Reizer *et al.*, 1993). *In vitro* studies demonstrated that P-(Ser)-HPr from *S. pyogenes* could not replace P~(His)-HPr as a phosphoryl donor in the PTS cascade

(Deutscher and Saier, 1983) and PEP-dependent phosphorylation of P-(Ser)-HPr from *S. lactis* occurred 5000-fold slower than the phosphorylation of free HPr (Deutscher *et al.*, 1984). The inhibition of PEP-dependent phosphorylation of HPr, due to prior HPr serine phosphorylation, was completely relieved upon interaction of P-(Ser)-HPr with the *S. faecalis* gluconate-specific EIIA ($EIIA^{Gct}$), whereas the effect in the presence of the *Staph. aureus* lactose-specific EIIA ($EIIA^{Lac}$) was slightly lower (Deutscher *et al.*, 1984). Overall, these studies suggested that (a) P-(Ser)-HPr formation may serve to regulate carbohydrate uptake by the PTS and (b) complex formation between P-(Ser)-HPr and various EIIAs may determine the hierarchy of PTS sugar utilization.

Several studies employing site-directed mutagenesis have examined the role of seryl-phosphorylation of HPr in the preferential use of some PTS sugars and the results have been somewhat conflicting. An *in vitro* experiment was conducted to examine the EI-catalyzed phosphorylation of wild-type *B. subtilis* HPr, or the mutant form of HPr in which serine 46 (Ser-46) was replaced by an aspartate residue (S46D), in the presence of the glucose-specific EIIA ($EIIA^{Glc}$) or mannitol-specific EIIA ($EIIA^{Mtl}$) (Reizer *et al.*, 1992). The rate of EI-catalyzed phosphorylation of the S46D mutant HPr protein was inhibited to a similar extent as wild-type HPr in the presence of $EIIA^{Glc}$ or $EIIA^{Mtl}$. This indicates that complex formation between $EIIA^{Glc}$ and S46D mutant HPr, which

conformationally resembles P-(Ser)-HPr (Wittekind *et al.*, 1990), does not increase EI-catalyzed phosphorylation compared to when the mutant HPr protein is complexed to $EIIA^{Mtl}$. The results from this study suggested that neither the phosphorylation of HPr at Ser-46, nor a preferential complexation of P-(Ser)-HPr with $EIIA^{Glc}$, contribute to the hierarchical use of PTS sugars with glucose at the top of the list.

More recently, PTS sugar uptake studies using a HPr chromosomal mutant of *B. subtilis* (GM1222), in which Ser-46 was replaced by an alanine residue, indicated that ATP-dependent phosphorylation of HPr may be responsible for the exclusion of one PTS sugar by another (Ye and Saier, 1996). Fructose uptake was strongly inhibited by glucose in the wild-type strain, whereas fructose uptake was only weakly inhibited in the mutant strain. As the only difference between the two strains was the S46A HPr mutation, this result suggested that phosphorylation at Ser-46 promotes the preferential use of one PTS sugar over another. In addition, in the presence of a high concentration of glucose, fructose or mannitol, the rate of sugar uptake was considerably less for the wild-type strain compared to the S46A *ptsH* mutant strain. These latter results suggested that P-(Ser)-HPr promotes a feedback mechanism whereby the metabolism of any PTS sugar in turn inhibits its own transport (Ye and Saier, 1996).

Studies employing site-directed mutagenesis have also identified the role of ATP-dependent HPr phosphorylation in the regulation of catabolite repression. In a *B. subtilis* *ptsH* chromosomal mutant (SA003) carrying the Ser-46 to alanine mutation, the synthesis of gluconate kinase, glucitol dehydrogenase, mannitol-1-P dehydrogenase and the mannitol-specific permease, were shown to be completely relieved from the catabolite repressive effects of glucose, fructose or mannitol (Deutscher *et al.*, 1994). In a wild-type revertant (GM808), gluconate kinase and glucitol dehydrogenase synthesis were sensitive to catabolite repression in the presence of the PTS sugars glucose and mannitol. These results indicated that P-(Ser)-HPr is involved in the mechanism regulating catabolite repression in *B. subtilis*.

HPr site-specific mutagenesis studies directed at residues other than Ser-46 have led to the identification of residues important for the interaction of HPr with sugar-specific EII complexes (Koch *et al.*, 1996). Along with histidine-15 and Ser-46, glycine-13 (Gly-13), arginine-17 (Arg-17), and glycine-67 (Gly-67) are fully conserved residues, while aspartate-69 (Asp-69) and glutamine-70 (Glu-70) are well conserved residues among both Gram-positive and Gram-negative HPr proteins. The *E. coli* HPr residue Asp-69 was replaced by a glutamine residue resulting in a decrease (increased K_m values) in the affinities of EIIs specific for mannitol, *N*-

acetylglucosamine and β-glucosides for P~(His)-HPr without effecting the maximal reaction rates (V_{max}) (Koch *et al.*, 1996). Alternatively, replacing (Glu-70) with a lysine residue resulted in unchanged K_m values and decreased V_{max} values for the EII-catalyzed interaction with HPr. The authors suggested that residues Asp-69 and Glu-70 play an important role in the interaction between HPr and EII complexes through their potential influence on the conformation of the HPr protein.

Along with site-directed mutagenesis, HPr mutant strains have been generated spontaneously following growth in the presence of the non-metabolizable glucose analogue, 2-deoxyglucose (Gauthier *et al.*, 1994; Vadeboncoeur *et al.*, 1994). A spontaneous of mutant (A66) of *S. salivarius*, in which methionine-48 (Met-48) was replaced by a valine residue, demonstrated reduced growth on PTS sugars and non-PTS sugars (Vadeboncoeur *et al.*, 1994). Met-48 has been shown to be part of a hydrophobic patch located between His-15 and Ser-46 (Herzberg *et al.*, 1992) postulated to be involved in HPr interactions with EI, EII complexes and (Ser)HPr kinase. The results from this study demonstrated that this mutation prevented ATP-dependent phosphorylation and decreased the rate of PEP-dependent phosphorylation, during a PTS assay measuring the rate of phosphorylation of fructose. Moreover, *S. salivarius* spontaneous mutants in which Gly-67 and phenylalanine-29 (Phe-29) were replaced by

aspartate and valine residues, respectively, were unable to grow on PTS sugars (Gauthier *et al.*, 1994). Therefore, along with Met-48, Gly-67 and Phe-29 appear to influence the interaction of HPr with EI and P~(His)-HPr with the EII complexes.

The three-dimensional structure of HPr from *E. coli* and several Gram-positive bacteria has been elucidated by both X-ray and 2D NMR, and the overall folding topology involves two or three α-helices on top of four anti-parallel β-strands (βαββαβα) (reviewed in Jia *et al.*, 1994). Similarly, model building by protein homology has predicted a three-dimensional structure of HPr from *S. mutans* containing two α-helices and a four-stranded anti-parallel β-sheet (Dasper *et al.*, 1994). In all structures, the site of PEP-dependent phosphorylation (His-15) and ATP-dependent phosphorylation (Ser-46) are located on the surface of the protein as these residues cap the N-termini of the first and second α-helices, respectively (Chen *et al.*, 1993). Another common structural feature is the Gly-67 to Glu-70 reverse turn, linking the β-strand to the C-terminal α-helix, which may be important for structural integrity (Jia *et al.*, 1993). The side-chain of Glu-70 is involved in ion-pairing with Met-1 and hydrogen-bonding with Gly-67, while Asp-69 is involved in hydrogen-bonding with Ser-31 and asparagine-30 (Asn-30). According to the secondary structure of HPr (Jia *et al.*, 1993), the

distance between His-15, Ser-46 and Gly-67 does not permit direct interaction between the residues.

Overall, studies on the modification of specific amino acid residues in the HPr molecule have played a vital role in elucidating the role of ATP-dependent phosphorylation Gram-positive bacteria. Furthermore, HPr mutants generated via site-directed mutagenesis or spontaneously in the presence of 2-DG have identified HPr residues which influence the protein interactions characterizing the PTS cascade. The role of HPr in cellular mechanisms other than PEP-dependent sugar transport has not been elucidated in *S. mutans*, nor the effect of specific amino acid substitutions on the sugar transport process. In this report, HPr site-directed mutagenesis studies were designed to alter His-15, Ser-46, and Gly-67 in the HPr of *S. mutans* BM71 in order to study the influence of these changes on PTS sugar utilization in the organism. The *S. mutans* mutant strain would also be used to characterize the effect of the mutation on other cell parameters through growth under various environmental conditions in continuous culture. For example, the effect of the mutation on growth in the presence of limiting nitrogen could be examined under glucose-excess conditions in continuous culture. The mutant strain could also be grown in continuous culture under various conditions to examine the effect of the mutation on HPr phosphorylation.

Results and Discussion

Double-stranded mutagenesis. Prior to *ptsH* mutagenesis, several experiments were conducted using Clontech's Transformer Site-directed Mutagenesis kit and the control plasmid and primers accompanying kit. The experimental protocol provided with the kit was suggested to be 70-90% efficient, however, this result was not achieved by following the manufacturer's guidelines. The control experiment was used to optimize the efficiency by introducing alterations to the protocol and to become familiar with the experimental procedures. The control plasmid, pUC19M, carried a stop codon in the *lacZ* gene and produced white colonies on LB plates containing isopropyl β-D-thiogalactopyranoside (IPTG) and 5-bromo, 4-cloro, 3-indolyl β-D-galactoside (X-gal). The selection primer was designed to change the unique *Nde*I restriction site to a *Nco*I site and the mutagenic primer reverted the stop codon back to a tryptophan codon. Mutagenesis using the control plasmid and primers was completed and no blue colonies were generated. The plasmid preparation used for the final transformation was re-digested with *Nde*I, ethanol precipitated, re-transformed and approximately 30% efficiency was observed, however, with a low number of colonies overall. In the subsequent attempt, the synthesis/ligation reaction was permitted to incubate at 37°C for five hours and then overnight at 16°C instead of the recommended one to two hours at 37°C. Approximately 50%

mutagenesis was observed, once again with an overall low number of colonies, however, increasing the amount of plasmid in the synthesis/ligation reaction did not increase mutagenesis efficiency.

Although the optimal mutagenesis efficiency was not observed with the control experiment, the minor alterations to the protocol were applied during HPr mutagenesis using the plasmid pDB201. Mutagenesis with the three different mutagenic primers, G67D, S46D and H15A, as well as, the selection primer SP1-SH was conducted using a 100:1 primer to plasmid ratio instead of the 1:1 ratio used in the control experiment, as the higher ratio was postulated to promote successful primer binding. Several plasmid preparations from each mutagenesis attempt were screened via restriction-enzyme digestion and the recovered plasmids were considerably smaller than the original 9.0 kb plasmid. The apparent deletions were an unexpected result and mutagenesis employing only the selection primer produced similar results. In order to identify inherent problems with the pDB201 plasmid, mutagenesis was attempted using the vector pVA891 and only the selection primer. Unfortunately, only false positives were recovered as no DNA was observed during electrophoresis.

The lack of success during the double-stranded mutagenesis experiments may be attributed, in part, to the design of the selection primer, as well as, the mutagenic primers. The

manual accompanying the Transformer Site-directed Mutagenesis kit suggests that the mismatch bases of the primer should be located in the center of the primer, with at least 10 nucleotides flanking either side of the mismatch site. It is also recommended that the GC content is at least 50%, that the primers start and end with a G or a C and that the annealing strength of the mutagenic primer be equal or greater than the selection primer.

The selection primer, SP1-SH, carried two base mismatches near the middle of the primer with 11 and 12 bases flanking the mismatches and the GC content was 56%, however, the sequence started with an A and ended with a T. The mutagenic primer H15A carried two mismatches in the center of primer with 10 and 12 bases flanking the mismatches, the GC content was 54% and the sequence started with a T and ended with a C. The mutagenic primer S46D carried four mismatches, three of which contributed to the amino acid change and the fourth, along with the other mismatches, created an *EcoRV* site. These mismatches were flanked by only seven bases on one side and 11 on the other side, the GC content was only 38% and, although the sequence began with a C, it ended with an A. The mutagenic primer G67D closely met the primer design requirements as one base mismatch was flanked by 14 and 15 bases, the GC content was 47% and the sequence began with a C and ended with a G.

Single-stranded mutagenesis. Clontech's kit afforded the convenience of using double-stranded vectors, although the initial mutagenesis step involved denaturation of the plasmid to a single-stranded form. The main difference between double-stranded and single-stranded mutagenesis, as described by Kunkel (1985; 1987), is the selection procedure for the mutagenized DNA and we anticipated that the latter procedure would provide a stronger selection against the non-mutagenized DNA. Single-stranded DNA was generated by transforming the plasmid pDB102 into the *E. coli* strain CJ236 carrying the *dut* and *ung* mutations, which inactivate the enzymes dUTPase and uracil N-glycosylase, respectively. The vector pBluescript carries the f1 filamentous origin of replication, thereby, permitting pDB102 to replicate in the single-stranded form upon superinfection with the helper phage. Single-stranded, uracil-containing DNA was used as a template for mutagenesis and the double-stranded DNA was transformed into an *E. coli* strain with a proficient uracil N-glycosylase. Therefore, the uracil-containing, parental DNA was inactivated and only the mutant strand replicated.

As described above, the mutagenic primer S46D was not employed in these studies as the inherent design problems may have contributed to the instability of primer binding. The mutagenic primer, H15A, was also not applied as the pDB102 plasmid did not carry the first 39 base pairs of the *ptsH* gene and the primer was designed to bind to base pair 31

through base pair 54. Although the primer would have bound to the region of desired mutation, the lack of the 5' end of the gene would have contributed to unstable primer binding.

Single-stranded uracil-containing DNA was generated and used as a template for *in vitro* mutagenesis with the mutagenic primer G67D. The resultant double-stranded DNA was transformed into the *E. coli* strain BMH 71-18 carrying a DNA mismatch repair deficiency mutation (*muts*) and a proficient uracil N-glycosylase. Manual DNA sequence results indicated that the mutation encoding the glycine to aspartate substitution was present, however, since a DNA mismatch repair-deficient *E. coli* strain was used in this study, there was an opportunity for other mutations to be introduced into the *ptsH* gene. Therefore, it was confirmed that the complete sequence of the *ptsH* gene did not contain any other nucleotide mismatches.

Cloning and transformations. Upon successful introduction of the G67D mutation into the *S. mutans ptsH* gene, a kanamycin gene was inserted between the *ptsI* and *gapN* genes, generating the clone pHIK-1 prior to transformation (Figure 5-1). This plasmid was linearized with the restriction enzyme *Kpn*I and, upon transformation into *S. mutans* BM71, integration of the kanamycin gene and the desired mutation was to occur via double cross-over. The initial transformation attempt generated 10 kanamycin-

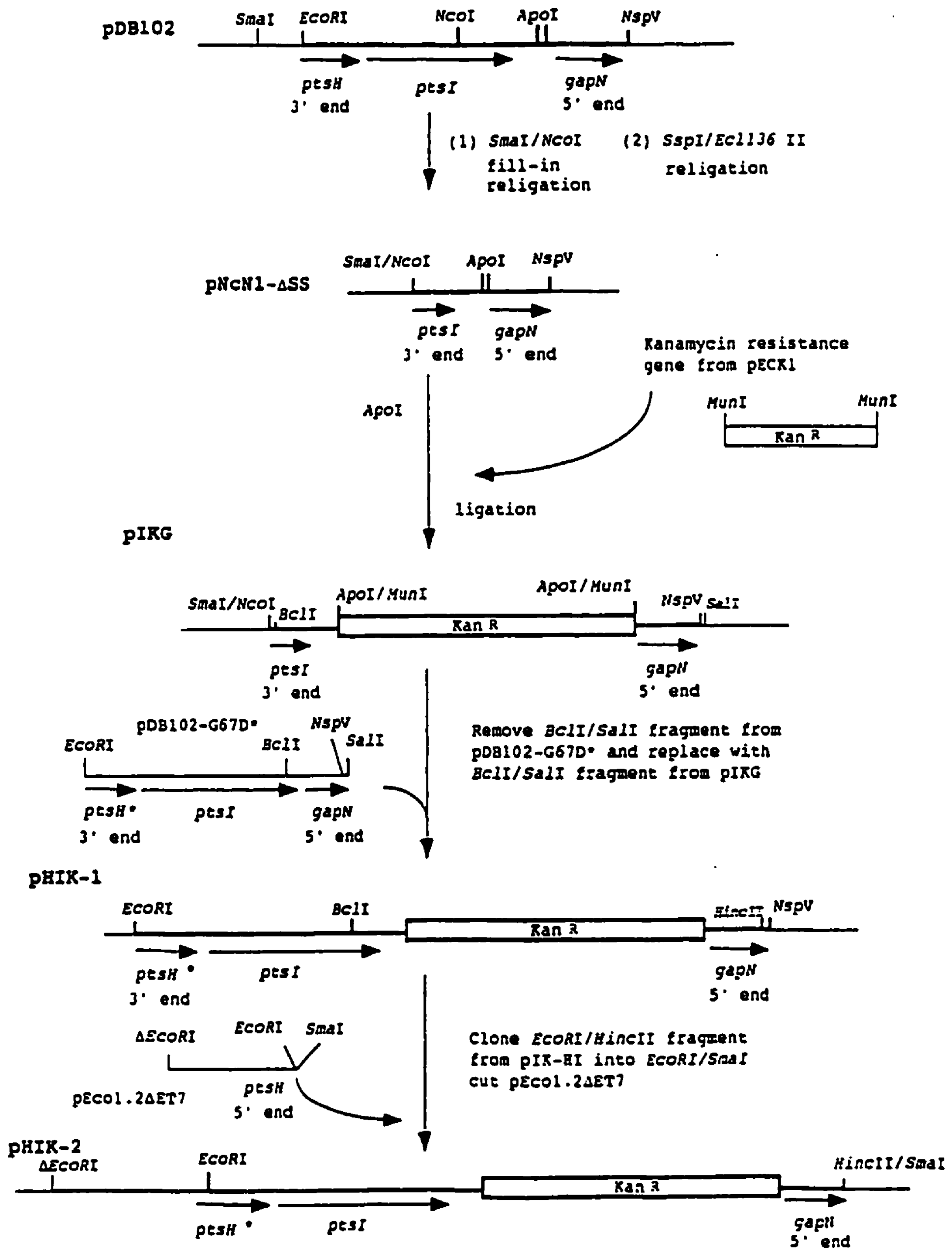

Figure 5-1: Sequence of cloning steps to generate pHIK-2

resistant colonies on Todd-Hewitt plates containing kanamycin and genomic preparations were restricted with *Sst*I and *Eco*RI for Southern blot analysis. The *Eco*RI/*Hinc*II fragment derived from pDB102 was used as a probe and for each of the lanes possessing digested DNA from different potential mutants, one band was shown to hybridize to the probe and one band hybridized to probe in the lane carrying the wild-type control. For six of the 10 potential mutants, the size difference between the wild-type band and the mutant bands was the size of the kanamycin gene, indicating successful integration of the kanamycin gene into the chromosome (Figure 5-2). Although six mutants possessed the kanamycin gene indicating successful double-crossover, all of these potential mutants were capable of utilizing PTS sugars by growth and fermentation on sugar agar plates. If the double-cross over event had included the G67D *ptsH* mutation, we would anticipate that the mutants would not be capable of growing on PTS sugars as was the case for a *S. salivarius* strain carrying the same mutation (Gauthier *et al.*, 1994).

In the pHIK-1 plasmid, there was approximately only 160 base pairs upstream of the G67D mutation, therefore, we suspected that the double cross-over event to include the desired mutation would occur more readily if the clone possessed more upstream flanking DNA. A clone carrying a 1.2 kb *S. mutans* fragment including the missing 39 base pairs from the *ptsH* gene and upstream flanking DNA, was used to

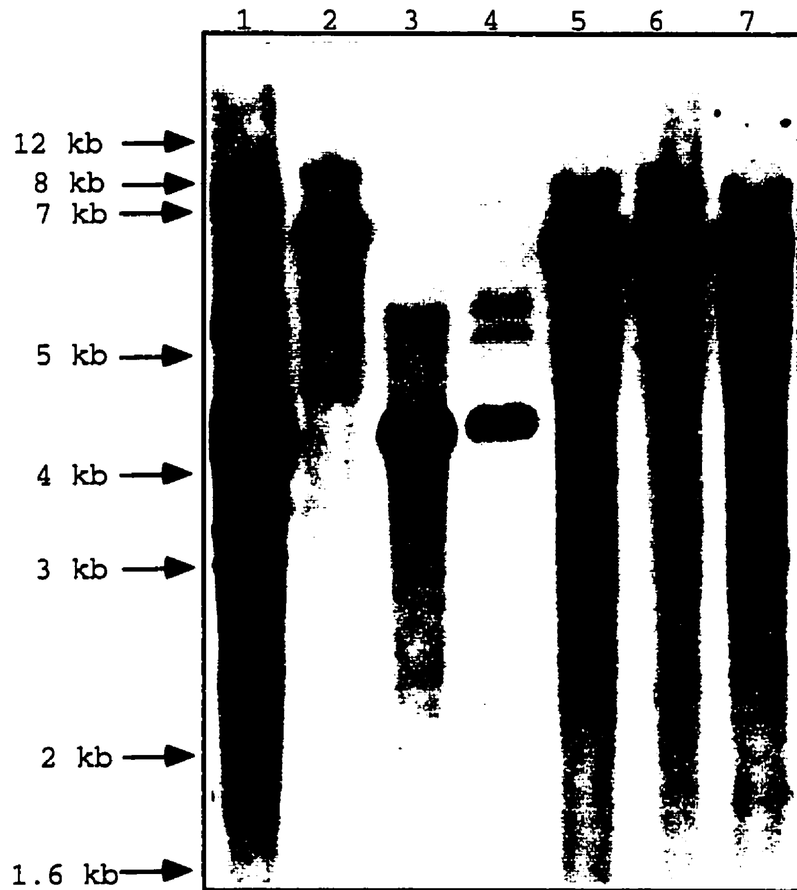

Figure 5-2: Southern hybridization of *S. mutans* BM71 and potential HPr mutants following transformation of pHIK-1. Genomic DNA was digested with *Sst*I and *EcoR*I and probed with a biotinylated *EcoR*I/*Hinc*II fragment carrying the 3' end of the *ptsH* gene, the *ptsI* gene, and the 5' end of *gapN*. Lane 1: Wild-type *S. mutans* BM71; Lanes 2-7: Kanamycin-resistant *S. mutans* BM71 transformants.

generate the clone pHIK-2. The truncated *ptsH* gene of pHIK-1 was joined to the 1.2 kb fragment via an *EcoR*I site and the sequencing primer, HPr, was used to verify the sequence through this site and upstream to ensure that the newly incorporated *ptsH* sequence was correct. The linearized plasmid pHIK-2 was transformed into *S. mutans* LT11 and 58 transformants were generated on tryptone-yeast extract plates containing kanamycin and the non-PTS sugar melibiose. By Southern blot analysis, sixteen transformants were shown to possess the kanamycin gene indicating successful double cross-over, unfortunately, all transformants fermented the PTS sugars tested. We speculate that the desired mutation carried on the *ptsH* gene may be located too far upstream from the kanamycin gene, thereby, affecting the frequency at which it would be included in the double cross-over event. In addition, we cannot exclude the possibility that integration of the mutated HPr gene into the genome was a lethal event.

Subsequent to this work, we employed an alternative gene replacement technique to integrate the mutated *ptsH* gene into the *S. mutans* BM71 genome. This method involved cloning the mutated gene into the replication-thermosensitive plasmid pVE6004 (Maguin *et al.*, 1992). The erythromycin-resistant pVE6004 plasmid can replicate in Gram-positive and Gram-negative bacteria at 30°C, however, above 35°C replication ceases and the entire plasmid is integrated into the host chromosome. The cointegrated clones identified at 37°C are

grown at 30°C promoting a second recombination event that regenerates the free plasmid in the cell. Erythromycin-sensitive colonies at 37°C would no longer have plasmid DNA integrated into the chromosome and, if successful gene replacement occurred, the *S. mutans* chromosome would possess the mutated *ptsH* gene and the wild-type gene would be located on the plasmid. Once again, successful gene replacement would be tested by the ability to grow on PTS sugars and the presence of the mutation would be verified by PCR. Cloning attempts using this method were conducted by Mr. Dave Boyd and myself, however, these were unsuccessful. At this point, it was decided that because of time constraints, my remaining time would best be spent characterizing a recently-isolated sorbitol-negative mutant of *S. mutans* LT11 and this research is outlined in Chapter 6.

Some observations on the HPr-site directed mutagenesis project. Future work with temperature-sensitive vectors should result in the successful resolution of this project and Mr. Boyd is currently attempting to clone the mutated *ptsH* gene using a different temperature-sensitive vector, pGh9:ISS1 (Maguin *et al.*, 1996). The lack of success in the project was due, in part, to the lack of a discernible selection procedure for mutants. The selection procedure available to test for the presence of the G67D HPr mutation was to grow the potential mutants on plates containing PTS sugars. A selection procedure based on erythromycin

sensitivity has been described for the generation of the *B. subtilis* mutant strain (GM3291), carrying the S46A (Ser-46 to Ala) *ptsH* mutation (Eisermann *et al.*, 1988). Briefly, the plasmid-encoded wild-type *ptsH* gene was replaced with an erythromycin gene and integrated into the *B. subtilis* chromosome via a double cross-over event generating the erythromycin-resistant strain GM329. This strain possessed a PTS-negative phenotype and was transformed with a plasmid carrying the desired *ptsH* mutation and a double cross-over event generated the erythromycin-sensitive, PTS-positive strain GM3291 carrying the *ptsH* gene coding for the S46A mutant HPr.

A similar selection procedure could be applied to generate the *S. mutans* G67D *ptsH* mutant strain. Mr. Boyd has successfully integrated an erythromycin gene into the *EcoRI* site of the *ptsH* gene and, upon transformation into *S. mutans* LT11, integration took place via Campbell-type single cross-over rather than double cross-over. If the transformation experiment were to produce a *S. mutans* erythromycin-resistant mutant strain then it could be used to transform a plasmid carrying the G67D *ptsH* mutation. A double cross-over event would replace the antibiotic-interrupted *ptsH* gene with the *ptsH* gene carrying the mutation and selection would be based upon the loss of antibiotic resistance. The strain carrying the G67D HPr mutation would not be capable of growing on PTS

sugars, but could be selected with the non-PTS sugar, melibiose.

Many studies characterizing *ptsH* mutants have been based on the transformation of *ptsH*-negative strains (Reizer *et al.*, 1989; Koch *et al.*, 1996). For example, a plasmid carrying specific *E. coli ptsH* mutations was transformed into the *ptsH*-negative *E. coli* strain LBG1650 and characterization identified specific HPr residues involved in the interaction between HPr and the EII complexes (Koch *et al.*, 1996). Furthermore, plasmids carrying mutant *ptsH* genes from *B. subtilis* have been transformed into the *ptsH*-negative *E. coli* strain, LBG1650, as well as, the *Staph. aureus ptsH*-negative strain, S797A (Reizer *et al.*, 1989). Transformation of the mutant *E. coli* strain permitted an examination of the effect of the *ptsH* mutation on growth and fermentation in the presence of several different carbohydrates. On the other hand, a transformed *Staph. aureus* mutant strain was employed to characterize the uptake rate of the PTS sugars mannitol, fructose, glucose and the non-PTS substrate, maltose.

Similarly, my plasmid, pHIK-2, carrying the entire mutated *ptsH* gene could be transformed into the *E. coli ptsH*-negative strain, 1101. In this way, the growth and fermentation responses to a variety of carbohydrates could be monitored using sugar agar plates. Since an *E. coli* (Ser)HPr kinase has not been identified, this type of experiment would

examine the effect of the G67D mutation on PEP-dependent, EI-catalyzed phosphorylation and, consequently, the effect on PTS transport. In addition, transforming pHIK-2 into a Gram-positive, *ptsH*-negative strain would permit a variety of testing, including the sugar uptake experiments described for the *Staph. aureus* strain (Reizer *et al.*, 1989), as well as, a crossed immunoelectrophoresis examination of the effect of the mutation on PEP and ATP-dependent phosphorylation. To date, a *ptsH*-negative *S. mutans* strain has not been isolated and during a *S. salivarius* experiment designed to create PTS mutants, a mutant entirely lacking HPr was not isolated, while a mutant lacking EI was isolated, suggesting that the former phenotype is not viable (Gauthier *et al.*, 1994). Therefore, at this time, only a heterologous experiment could be conducted using the pHIK-2 plasmid carrying the *S. mutans* mutated *ptsH* gene and an established *ptsH*-negative Gram-positive strain, such as *Staph.* aureus S797A.

PEP and ATP-dependent phosphorylation of the *S. mutans* G67D mutant HPr could be assayed using an experimental protocol similar to that designed for measuring (Ser)HPr kinase activity. The mutated *S. mutans ptsH* gene could be cloned into an expression vector and the mutated HPr protein could be over produced in *E. coli* and purified according to the methods established by Reizer *et al* (1989). *In vitro* (Ser)-HPr phosphorylation could be assayed in the presence of the mutated HPr protein, radio-labelled ATP and membrane protein.

(His)-HPr phosphorylation would be conducted under similar conditions, except ATP and the membrane protein would be replaced by PEP and EI. The proteins could be separated by SDS-polyacrylamide gel electrophoresis and the phosphorylated HPr proteins would be identified by autoradiography or by their change in electrophoretic mobility.

Using a similar protocol, Reizer *et al.* (1989) have demonstrated that some *B. subtilis* mutant HPr proteins, including S46A mutant HPr, are phosphorylated in the presence of PEP and EI as demonstrated by the increase in mobility on SDS-PAGE gels. On the other hand, the S46D and H15A (His-15 to Ala) mutant HPr proteins demonstrated a lack of appreciable mobility indicating non-phosphorylation by PEP and EI. Conversely, the H15A mutant HPr could undergo ATP-dependent phosphorylation while the S46A and S46D HPr proteins were not labelled by radioactive ATP.

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Chapter 6

Partial cloning and sequencing of a regulatory gene involved in sorbitol metabolism by *Streptococcus mutans*.

Abstract

The main sugar transport system of the oral pathogen *Streptococcus mutans* is the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS). The PTS catalyzes the concomitant transport and phosphorylation of a variety of sugars, including monosaccharides and disaccharides, and the sugar alcohols, sorbitol and mannitol. Oral streptococci transport sorbitol via an inducible PTS system and intracellular sorbitol-6-phosphate is converted to fructose-6-P by an inducible sorbitol-6-P dehydrogenase. In this study, the mutant strain *S. mutans* BH96, possessing a sorbitol-negative phenotype, was generated by transposon mutagenesis using the plasmid pα carrying the composite-type transposon Tn*4001*. *S. mutans* BH96, as well as, the wild-type strain, LT11, were grown in media containing glucose, sorbitol or glucose+sorbitol and the carbohydrate-utilization pattern verified catabolite repression by glucose for the wild-type strain. In addition, a long lag period was observed prior to growth on sorbitol by *S. mutans* LT11 confirming induction of the genes required to metabolize sorbitol. Rescue of a transposon/genome junction was initiated by transforming *S. mutans* BH96 with the integration vector pΩIS carrying a truncated Tn*4001* sequence. A 948-bp chromosomal fragment was recovered and DNA sequencing revealed two partial open-reading frames. Protein homology has characterized the gene products as a potential regulatory

protein and the EIIA component of the sorbitol-PTS. Cloning experiments designed to recover all the genes involved in sorbitol metabolism by *S. mutans* have been suggested along with characterization studies to examine the regulation of the operon.

Introduction

Known sorbitol-utilizing bacteria in dental plaque belong to the genera *Streptococcus, Lactobaccillus* and *Actinomyces*, and shifts in the levels of streptococci have been observed following frequent exposure to sorbitol (Kalfas and Edwardsson, 1990). For example, several studies have demonstrated the increased levels of *S. mutans* in saliva and plaque following the use of sorbitol-containing gum demonstrating the capacity of the oral flora to adapt to sorbitol (Loesche *et al.*, 1984; Soderling *et al.*, 1989; Wennerholm *et al.*, 1994). In addition, the sorbitol-fermenting flora in dental plaque has been examined in people with low and normal salivary secretion rates following a period of frequent mouth rinses with a sorbitol solution (Kalfas and Edwardsson, 1990; Kalfas *et al.*, 1990). Following the adaptation period, the numbers of sorbitol-fermenting bacteria increased in both groups, mainly due to an increase in the level of streptococci (Kalfas and Edwardsson, 1990). For the low salivary secretion group, the shift was attributed to an increase in the numbers of *S. mutans*, while an increase in *S. sanguis* was observed for the group with normal salivary secretion. Following the adaptation period, exposure to sorbitol resulted in an overall increase in acid production for both groups, however, the extent and duration of the pH fall was more pronounced in the group with low salivary secretion rates (Kalfas *et al.*,

1990). These studies have clearly established the adaptability of the dental plaque flora following frequent exposure to sorbitol and provide contradictory results to those of an earlier study suggesting that the low levels of glucose in saliva, in conjunction with dietary sugars, represses the sorbitol-PTS (Slee and Tanzer, 1983).

Sorbitol metabolism by *S. mutans* has been attributed to the induction of the sorbitol-PTS, as well as, sorbitol-6-phosphate dehydrogenase which oxidizes intracellular sorbitol-6-P to fructose-6-P (Brown and Wittenberger, 1973; Maryanski and Wittenberger, 1975). Recent research has shown that the synthesis of *S. sanguis* sorbitol-specific EII complex, including a soluble EIIA domain, is induced upon growth in the presence of sorbitol (Svensater and Hamilton, 1991). Sorbitol-6-P dehydrogenase has been purified from *S. mutans, S. sanguis* and *S. mitis* with similarities seen in amino acid composition, pH optima and subunit molecular weight and differences were observed in the chromatographic and electrophoretic patterns of the proteins, as well as, the K_m values for sorbitol-6-P and NAD (Svensater *et al.*, 1992).

Although *S. mutans* and *S. sanguis* transport and metabolize sorbitol via inducible systems, several observations have distinguished these strains with respect to sorbitol metabolism. Sorbitol metabolism by *S. mutans* is subject to catabolite repression as the sorbitol-PTS has been shown to

be induced only upon glucose depletion (Brown and Wittenberger, 1973; Dills and Seno, 1983). Catabolite repression was attributed to inducer exclusion, a process whereby the inhibition of inducer transport by glucose prevents the induction of sorbitol-related enzymes. Alternatively, *S. sanguis* has been shown to transport and metabolize sorbitol and glucose concurrently, although sorbitol was degraded at a slightly slower rate (Hamilton and Svensater, 1991). Since sorbitol utilization by *S. sanguis* requires the induction of the sorbitol-PTS, as well as, sorbitol-6-P dehydrogenase, initial transport in the presence of glucose was suggested to occur via the glucose transport system (Hamilton and Svensater, 1991).

Cells of *S. mutans* and *S. sanguis* adapted to growth on sorbitol have been shown to respond differently to growth in the presence of glucose and sorbitol (Slee and Tanzer, 1983; Hamilton and Svensater, 1991). Upon introduction of glucose in the presence of sorbitol, *S. mutans* sorbitol-adapted cells rapidly repressed the sorbitol-PTS and sorbitol-6-P dehydrogenase activities, while the glucose-PTS was induced (Slee and Tanzer, 1983). When nearly all the glucose was exhausted, the sorbitol-PTS and dehydrogenase activities were de-repressed and the cells once again metabolized sorbitol. Alternatively, in the presence of equal amounts of glucose and sorbitol, glucose transport was inhibited 2-fold by sorbitol-adapted cells of *S. sanguis*, and repression was

shown to be associated with a decrease in the synthesis of the EII complex for glucose (EII^{Glc}) (Hamilton and Svensater, 1991).

B. subtilis has been shown to possess a unique pathway for sorbitol (glucitol) metabolism involving an inducible permease (*gutA*) transporting sorbitol into the cell without chemical modification, as well as, an inducible dehydrogenase (*gutB*), oxidizing sorbitol to fructose (Gay *et al.*, 1983). Intracellular fructose is then converted to fructose-6-phosphate by a constitutive fructokinase and then to fructose-1,6-bisphosphate by the enzyme phosphofructokinase. The *gutR* gene, located upstream of *gutB*, encodes a regulatory protein that is postulated to act as a transcriptional activator since a point mutation within this gene (*gutR1*) resulted in the constitutive expression of both *gutB* and *gutA* (Gay *et al.*, 1983; Ye *et al.*, 1994). The serine-289 to arginine replacement in the GutR1 protein was suggested to promote a conformational change in the protein to an active form, activating transcription of *gutB* in the absence of sorbitol. It has been proposed that the *gutR* gene product exists in active and inactive conformations with the inactive form activated upon the binding of sorbitol. The active GutR protein is suggested to bind to a 78-base pair regulatory sequence located upstream of the *gutB* promoter, thereby, activating transcription (Ye and Wong, 1994).

In this report, two partial open-reading frames have been cloned from *S. mutans* LT11 with sequences homologous to the sorbitol-specific EIIA component of the sorbitol-PTS and a regulatory protein. Although only two sorbitol-related genes were partially cloned and identified in this study, the information in this report could provide the basis for an extensive study designed to clone and identify all the genes involved in sorbitol metabolism by *S. mutans*. The isolation and characterization of genes involved in sorbitol transport and metabolism has not been previously reported for any oral streptococcal strain.

Results

Isolation of a mutant of *Streptococcus mutans* defective in sorbitol metabolism. Initially, a mutant defective in sorbitol metabolism was isolated following transformation of *S. mutans* LT11 with the plasmid pα (Lunsford, 1995). The plasmid pα carries Tn*4001*, a 4.5 kb composite-type transposon consisting of two copies of IS*256* surrounding an aminoglycoside resistance gene, conferring resistance to gentamicin, kanamycin, and tobramycin (Lyon *et al.*, 1984; Lyon *et al.*, 1987; Rouch *et al.*, 1987). An estimated 1000 transformants were isolated on THYE-kanamycin plates and tested for their ability to utilize PTS sugars. One colony was unable to ferment sorbitol upon transfer to TYE plates containing 1% sorbitol and the indicator bromocresol purple. This isolate, referred to as *S. mutans* BH96, was subsequently transferred to several TYE-sugar indicator plates to confirm the ability to ferment glucose, mannose, fructose, galactose, sucrose, lactose, maltose, mannitol, and melibiose. The sorbitol-negative phenotype was maintained in the presence of kanamycin, however, subculturing *S. mutans* BH96 onto TYE-sorbitol without kanamycin resulted in a sorbitol-positive phenotype suggesting that insertion of the transposon produced the sorbitol-negative phenotype.

Growth characteristics with glucose and sorbitol. *S. mutans* BH96 and *S. mutans* LT11 were grown in the presence of glucose, sorbitol, and both glucose and sorbitol to compare growth and carbohydrate utilization. A long lag period was observed following inoculation of the wild-type strain, *S. mutans* LT11, into medium with sorbitol as the sole carbon source indicating that the proteins involved in sorbitol metabolism must be induced prior to exponential growth (Figure 6-1). A short lag period was observed upon inoculation into glucose medium or glucose+sorbitol medium, followed by exponential growth. Analysis of the residual glucose and sorbitol in the latter medium showed that glucose was utilized to exhaustion before sorbitol was consumed (Figure 6-1). A very short lag period was observed during the transition between growth on glucose and sorbitol, again indicating that the enzymes required for sorbitol metabolism were induced prior to complete exhaustion of glucose. These observations indicate that sorbitol metabolism in *S. mutans* LT11 is subject to catabolite repression by glucose as demonstrated by earlier research (Dills and Seno, 1983; Slee and Tanzer, 1983).

Although integration of Tn*4001* eliminated sorbitol transport and metabolism by *S. mutans* BH96, we anticipated that glucose utilization in the mutant would not be affected. As seen in Figure 6-2, exponential growth was initiated almost immediately following inoculation of *S. mutans* BH96

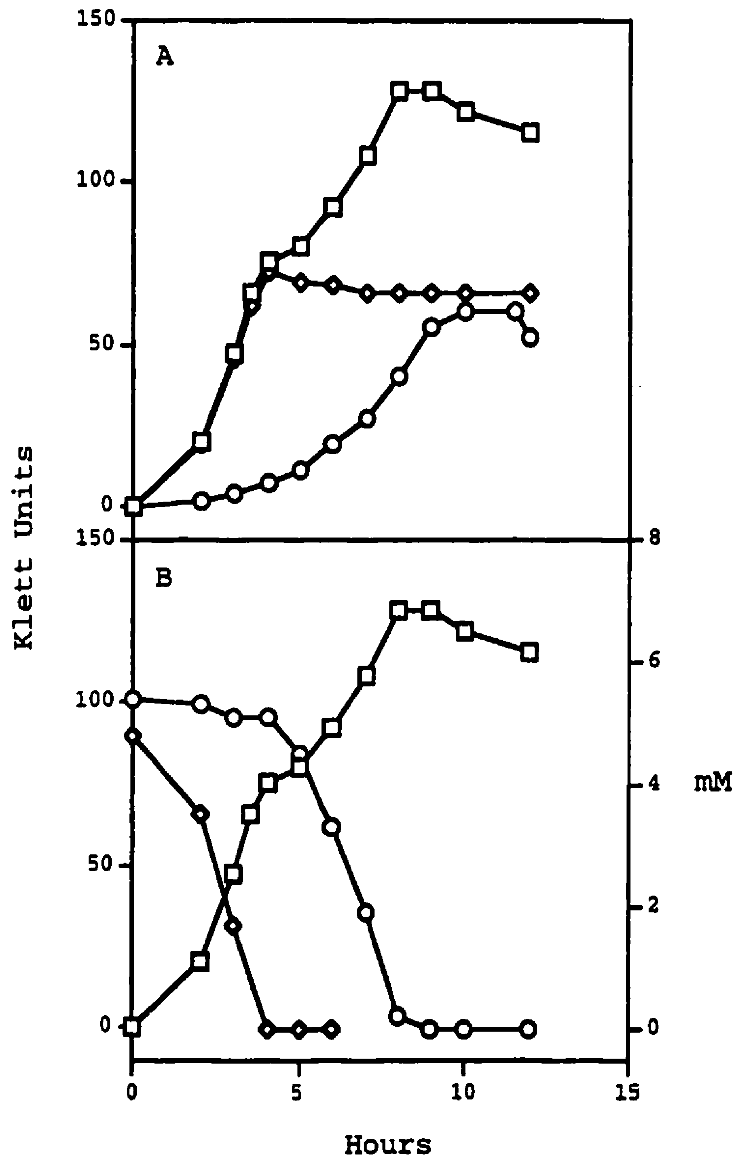

Figure 6-1: (A) Growth of the wild-type *S. mutans* LT11 in medium containing glucose (◆), sorbitol (O), and glucose+sorbitol (□). (B) Glucose (◆) and sorbitol (O) utilization by *S. mutans* LT11 in medium containing glucose+sorbitol (□).

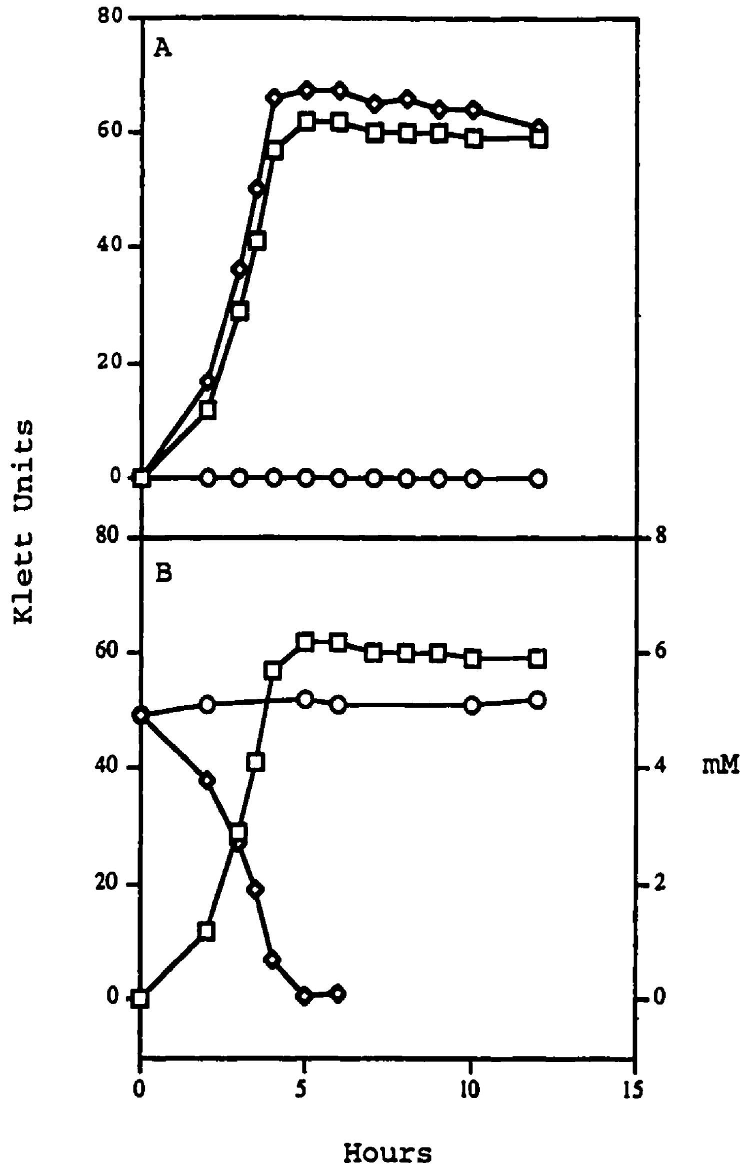

Figure 6-2: (A) Growth of the mutant *S. mutans* BH96 in medium containing glucose (◆), sorbitol (O), and glucose+sorbitol (□). (B) Glucose (◆) and sorbitol (O) utilization by *S. mutans* BH96 in medium containing glucose+sorbitol (□).

into medium with glucose alone or with glucose+sorbitol, although glucose was utilized at a slightly slower rate in the latter medium (data not shown). As expected, *S. mutans* BH96 did not grow in the presence of sorbitol-only medium, and the initial concentration of sorbitol in the medium did not change following growth on glucose.

Characterization of the Tn*4001* insertion site. Southern hybridization experiments using pα as a probe indicated that a single copy of Tn*4001* was inserted at a unique site in the chromosome of *S. mutans* BH96 (Figure 6-3). Labelled pα hybridized to a single 13 kb *Sst*I fragment and a single 13 kb *EcoR*I fragment indicating a single copy of Tn*4001* in the chromosome. In addition, the pα probe hybridized to three *Hind*III fragments corresponding to the 2.4 kb internal fragment of Tn*4001*, and to 1.1 kb and 2.3 kb fragments at the transposon/chromosome junctions. Since the size of Tn*4001* is 4.5 kb, it appeared that Tn*4001* inserted into about 8.5 kb *Sst*I and *EcoR*I fragments in the *S. mutans* BH96 chromosome. The results from the *Hind*III digest suggest that Tn*4001* is inserted very near one end of a 1.1 kb *Hind*III fragment.

The IS*256* integrative plasmid pΩIS was used to rescue a transposon/chromosome junction as follows (Figure 6-4). *S. mutans* BH96 was transformed with pΩIS and fifteen colonies were isolated on Todd-Hewitt/kanamycin/erythromycin plates.

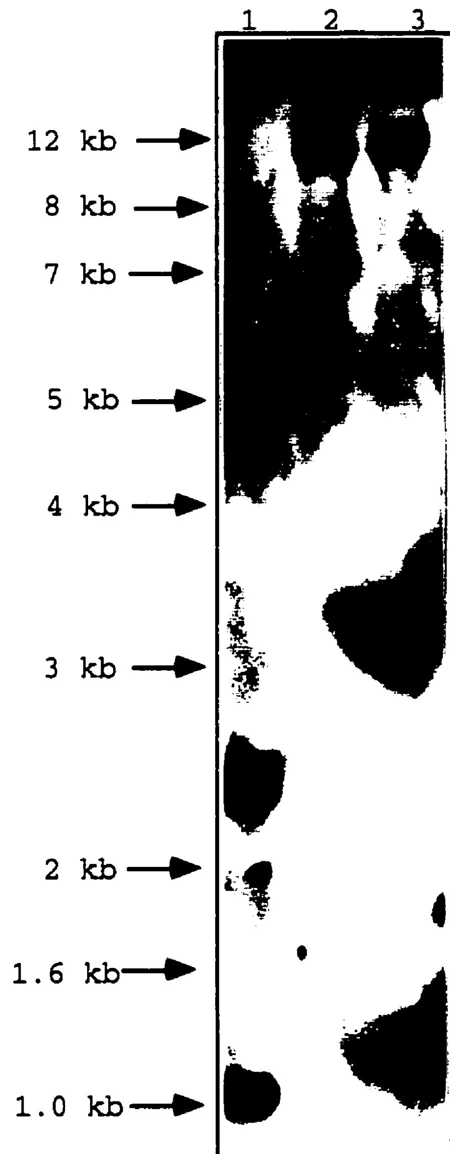

Figure 6-3: Southern hybridization of *S. mutans* BH96 restriction-digested genomic DNA probed with biotinylated pα. Lanes: 1, *Hind*III; 2, *Eco*RI, 3, *Sst*I.

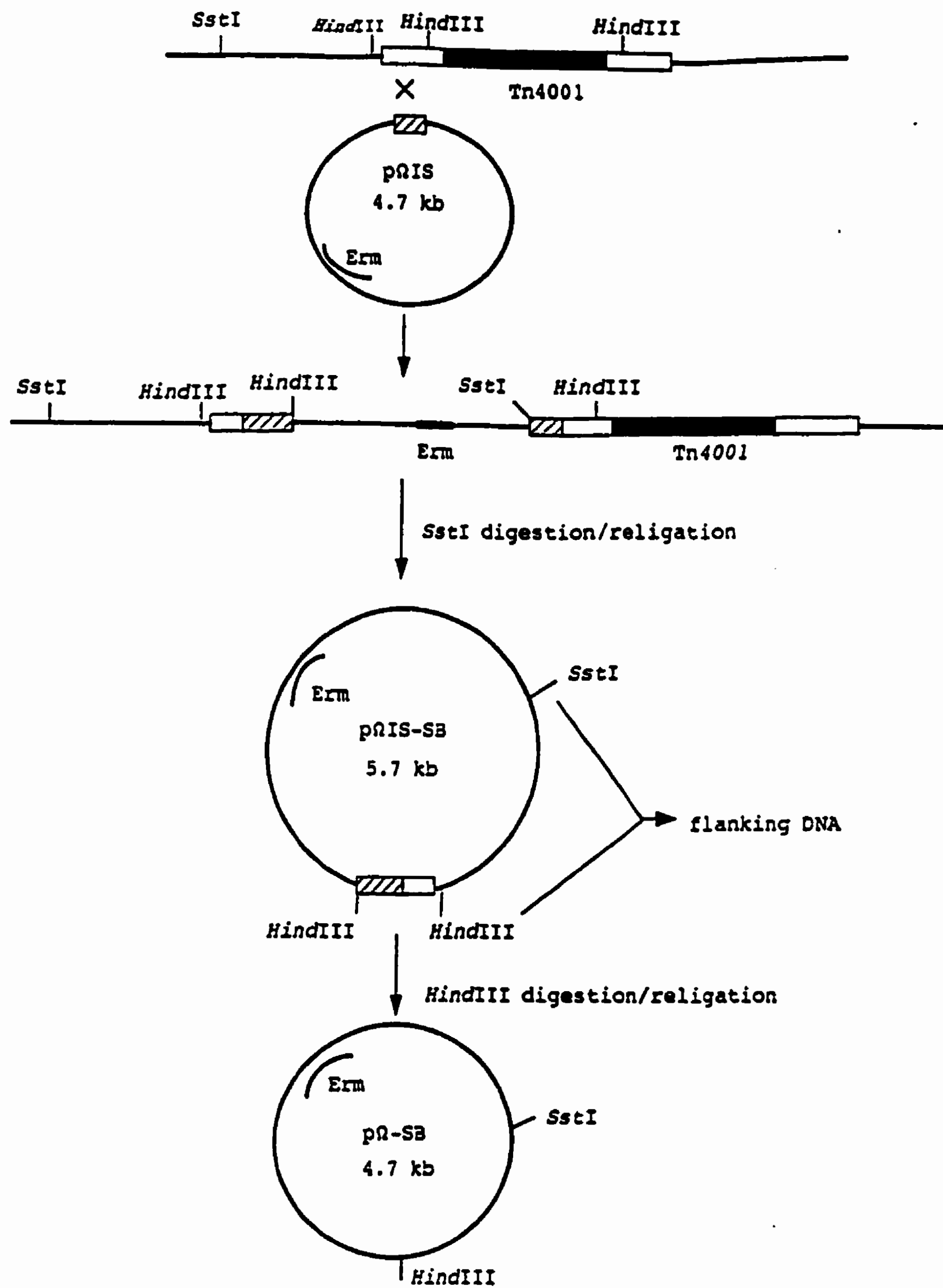

Figure 6-4: Integration of pΩIS via Campbell-type integration and subsequent recovery of 948 bp of *S. mutans* chromosomal DNA carried on pΩ-SB.

Genomic DNA from six transformants was prepared, digested with *Sst*I and screened by Southern blot analysis using pα as a probe (Figure 6-5). Two fragments approximately 5.7 and 12 kb hybridized to the probe in four of the genomic digests corresponding to a fragment carrying pΩIS plus flanking chromosomal DNA, and a fragment carrying the transposon plus flanking chromosomal DNA (Figure 6-4 and Figure 6-5). In two of the *SstI* genomic digests, an additional fragment of about 4.5 kb appeared. This pattern probably resulted from strains in which pΩIS integrated at both ends of Tn*4001*. The 4.5 kb fragment represents a single copy of Tn*4001* located between the two pΩIS/chromosome junction fragments of 5.7 kb and 12 kb in size. One of the *S. mutans* BH96 transformants carrying a single integrated copy of pΩIS was used in a marker rescue procedure in order to obtain DNA flanking the transposon insertion site. Genomic DNA was digested with *Sst*I, treated with T4 DNA ligase at a dilute DNA concentration, and the ligated DNA used to transform *E. coli* DH5α to erythromycin resistance. Approximately 60 colonies were isolated on LB plates containing erythromycin and 16 plasmids were isolated and analyzed by *SstI* digestion. Of these, 14 were 5.7 kb in size consisting of pΩIS carrying about 1 kb of flanking DNA. One of the plasmids was chosen for further study and was given the designation, pΩIS-SB (Figure 6-4).

When *S. mutans* LT11 genomic DNA was analyzed by Southern hybridization with pΩIS-SB as the probe, a 9.0 kb *Sst*I

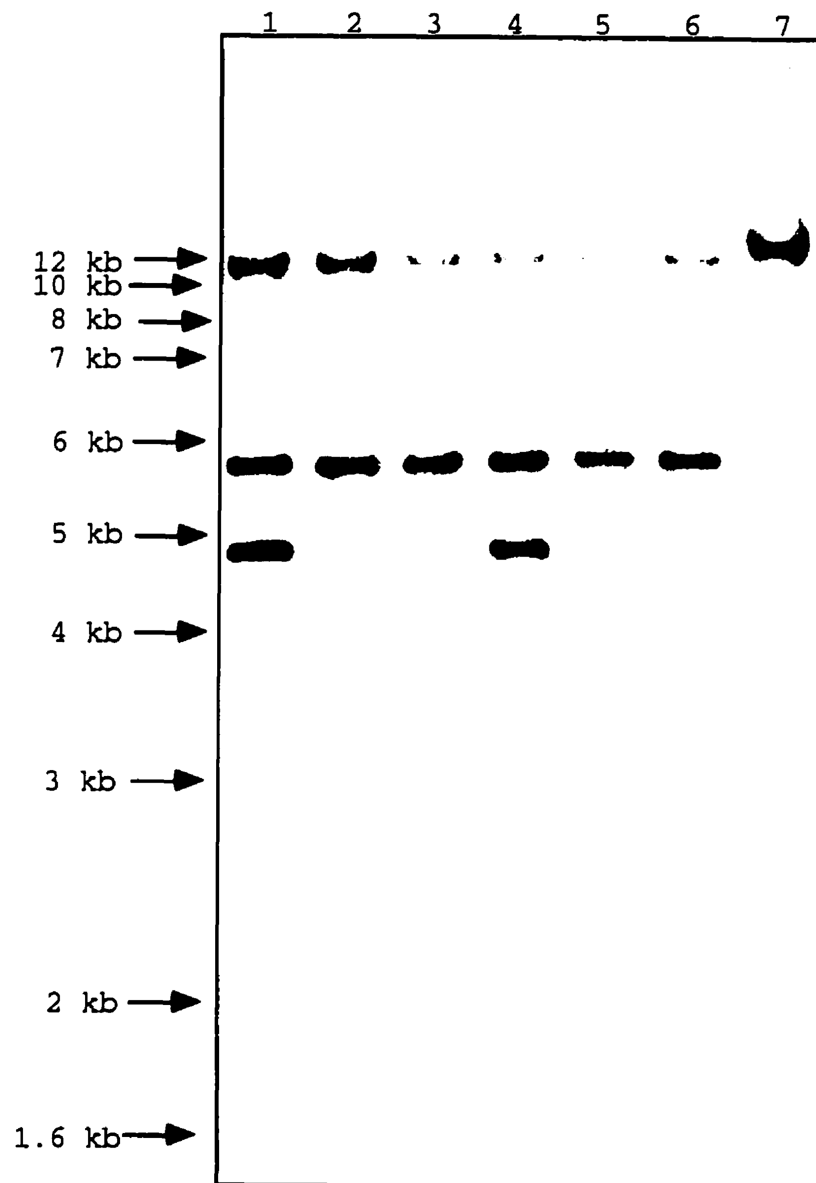

Figure 6-5: Southern hybridization of *S. mutans* BH96 genomic DNA following transformation of pΩIS. *Sst*I-digested genomic DNA was probed with biotinylated pα. Lanes 1-6: Transformants carrying integrated pΩIS; Lane 7: *S. mutans* BH96.

fragment, a 8.5 kb *EcoRI* fragment and a 1.1 kb *Hind*III were detected (Figure 6-6). This data is in agreement with the data generated in the Southern hybridization analysis of *S. mutans* BH96 genomic digests with pα.

Physical mapping of pΩIS-SB confirmed the presence of a *Hind*III site in the *S. mutans* insert DNA very near the junction with the IS*256* sequence of the vector. Thus, to facilitate sequencing from this end of the insert DNA, pΩIS-SB was digested with *Hind*III to remove the IS*256* sequences and the 4.7 kb fragment was isolated and the ends joined with T4 DNA ligase to create pΩ-SB (Figure 6-4).

The *S. mutans* chromosomal fragment carried on pΩ-SB was sequenced and found to consist of 948 bp (Figure 6-7). Analysis of the sequence revealed two open reading frames (ORFs). ORF1 extends from the second A in the *Hind*III site (base 2) to the stop codon TAG at bases 476-478. (Figure 6-7) and codes for the carboxy-terminal 158 amino acids of a putative protein. ORF2 begins with an ATG codon at bases 646-648 and continues to the end of the sequence (Figure 6-7) and codes for the N-terminal 101 amino acids of a putative protein. A putative ribosomal binding site for ORF2, AAGA, was identified at bases 629-632. As well, two regions of dyad symmetry were identified in the intergenic region of ORF1 and ORF2, bases 489-540 has a ΔG of -41.2 kJ and bases 544-622 has a ΔG of -43.7 kJ. In order to identify the

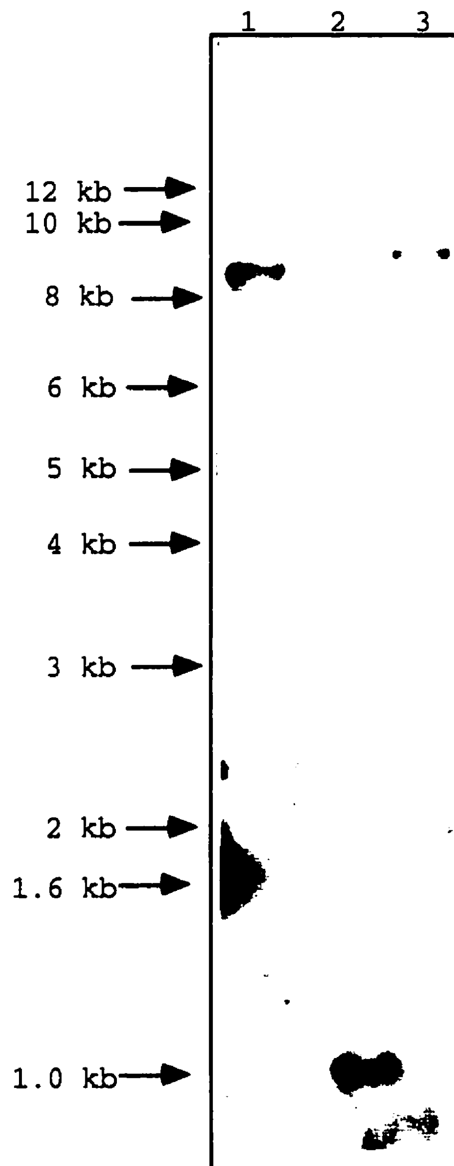

Figure 6-6: Southern hybridization of *S. mutans* LT11 restriction-digested genomic DNA probed with biotinylated pΩIS-SB. Lanes: 1, *Eco*RI; 2, *Hind*III; 3, *Sst*I.

```
 ORF1 -->
AAGC TTT GTA AAG AAG GTT AAG GAT ACA CTC TTG GTT AAC TTT   43
  S   F   V   K   K   V   K   D   T   L   L   V   N   F   14
GAT GAG CAA CGT TTA TTT GCT GAA ATT CAA ACA CAT TTA AAA   85
 D   E   Q   R   L   F   A   E   I   Q   T   H   L   K    28
TTT CTT ATC AAT CGA CTT ATC TTT CAT GTT CAG GCT AAT GAT   127
 F   L   I   N   R   L   I   F   H   V   Q   A   N   D    42
ATT TTC CAT AGA GAA ATT CAA AAT AAG TAT CCC TTG GCC TTT   169
 I   F   H   R   E   I   Q   N   K   Y   P   L   A   F    56
GAA ATG GCA AAA GTC GCT GGT GAT GAT TTA AAA AAT CAT TTT   211
 E   M   A   K   V   A   G   D   D   L   K   N   H   F    70
GGC TGT CAG CTG GAA TTG TCT GAA ATG AGC TAT TTA GCC CTT   253
 G   C   Q   L   E   L   S   E   M   S   Y   L   A   L    84
TAT TTC GAA ATG ATT TTA CAT GAA AAT GGA GCT ATT TTT CAA   295
 Y   F   E   M   I   L   H   E   N   G   A   I   F   Q    98
AAC AAA AAA CGC CGA GTT GCC GTT GTT TGT ACA ACG GGA CGC   337
 N   K   K   R   R   V   A   V   V   C   T   T   G   R    112
GGA ACA GCA CAT ATG ATA AAG CGT CAA TTA AAA CGC GTA CTT   379
 G   T   A   H   M   I   K   R   P   L   K   R   V   L    126
GGC CAT GAT ATC GAA ATC ACT CAA TAT TCA GAA GAA AAC TTT   421
 G   H   D   I   E   I   T   Q   Y   S   E   E   N   F    140
AAT CCT GAC ACT AAT GAT AAT TAC TTT GCT GTC TTT ACA ACC   463
 N   P   D   T   N   D   N   Y   F   A   V   F   T   T    154
                                ------------------------>
ATT CCT TTG AAT TAG GACAGATTAACTCGCCAGTTATTCAAATTAGAAAT   513
 I   P   L   N   *                                        158
  <-------------------     --------------------------
CTTTTTGATGATCAGTGGCTGCGTGAGGAATGGCAACGCGTTAATCATTATCATA   568

----------->  <-------------------------------------
GAAAAAATCTCAAAACAATCATTTTGACCTTTTCGAGATTGGTCAAAGCTGCTAG   623

CTATCAAGATTATTTA GTG TAT ATG ACA CAG TCA CTA GAA GCA TTA   669
      ----
     RBS        ORF2 --> M   T   Q   S   L   E   A   L    10
AGG CTA GTG GAT GAT CAT TTT GAA AAA CGT ATT TTA GAT AGA   711
 R   L   V   D   D   H   F   E   K   R   I   L   D   R    24
GAA AAA GAA CAA TCT ACT ATA TTT GGC AAT GGT ATT GCT TTT   753
 E   K   E   Q   S   T   I   F   G   N   G   I   A   F    38
CCA CAT ACT ATC AAT CAA ACA TTA GAA AAG ATC GTT TTA ATG   795
 P   H   T   I   N   Q   T   L   E   K   I   V   L   M    52
GTA GGA GTT CTT GAG GAG CCC TAC CAT ACA GAT CAT GAA AGT   837
 V   G   V   L   E   E   P   Y   H   T   D   H   G   S    66
GTT GAT CTT ATC TTT TTA GTA GCT ATT CCC AAT AAA ATT GCA   879
 V   D   L   I   F   L   V   A   I   P   N   K   I   A    80
ACA CAA ACA GAG GCA GAA CTT TTA GAA TTG TAT GAT GAT ATT   921
 T   Q   T   E   A   E   L   L   E   L   Y   D   D   I    94
TTT CGA ATT GCT AGC GAT AAA GAG CTC                       948
 F   R   I   A   S   D   K   E   L                        103
```

Figure 6-7: Nucleotide sequence of the *HindIII/SstI S. mutans* DNA insert from pΩ-SB. The amino acid sequences of ORF1 and ORF2 are indicated. Numbers refer to the base or amino acid coordinate of the sequence. A putative ribosomal binding site is underlined. Two regions of dyad symmetry are indicated by arrowheads.

putative proteins coded by ORF1 and ORF2 their amino acid sequences were used as the query sequences in BLAST searches (Altschul *et al.*, 1990) of the GenBank Database (Figures 6-8 and 6-9).

The search with the ORF1 amino acid sequence revealed that it has homology to a number of transcriptional antiterminator proteins, or antiterminator domains of regulatory proteins, belonging to the BglG/SacT family of *E. coli* and *Bacillus subtilis* (Schnetz *et al.*, 1987; Debarbouille *et al.*, 1990) (Figure 6-8). The search with the ORF2 amino acid sequence revealed that it has homology to the PTS IIA family of phosphotransfer proteins, including various sugar-specific IIA proteins or IIA domains and also including IIA^{Ntr} proteins involved in nitrogen assimilation (Figure 6-9). The sequence of the *S. mutans* DNA insert in pΩ-SB thus reveals that the Tn*4001* insertion in the BH96 chromosome disrupts a gene for a transcriptional regulator, probably an antiterminator, which may be a part of a operon involved in the uptake and the metabolism of sorbitol.

```
(a) Score = 94  Identity = 30%, Similarity = 53%

    ORF1      16 EQRLFAEIQTHLKFLINRLIFHVQANDIFHREIQNKYPLAFEMAKVAGDDLKNHF
                 ++ L    + H+K  I+R  + +    +      I+  YPLAFE    +AG  +K
    BsLicR   323 DKELKIGLALHMKPAISRNRYGMNLRNPMLAAIKEHYPLAFEAGIIAGIVIKEQT

                 GCQLELSEMSYLALYF 86
                 G ++   +E+ YLAL+F
                 GIEIHENEIGYLALHF 393

    Score = 60  Identity = 25%, Similarity = 60%

    ORF1      98 QNKKRRVAVVCTTGRGTAHMIKRQLKRVLGHDIEITQYSE 137
                 ++   +R  +VC +G G+A +++ +L+    G  ++I    +E
    BsLicR   403 ESPPKRCIIVCASGAGSAQLLREKLRSHFGKRLDILGTAE 442

(b) Score = 38  Identity = 63%, Similarity = 72%

    ORF1      25 THLKFLINRLI 35
                 THLKF   RL+
    EcArbG   206 THLKFFAQRLL 216

    Score = 71 Identity = 26%, Similarity = 51%

    ORF1      41 NDIFHREIQNKYPLAFEMAKVAGDDLKNHFGCQLELSEMSYLALYFEMILHE 92
                 ++   H  ++ KY LA+  A+    D +  H+    L    E+ +LA++ E +   E
    EcArbG   225 DESLHDVVKEKYTLAYHCAEKIQDHIMLHYDYTLTKEELMFLAIHIERVRSE
276

(c) Score = 36  Identity =33%, Similarity = 73%

    ORF1      20 FAEIQTHLKFLINRL 34
                 +   + THL++ ++RL
    BsSacT   199 YQRLVTHLRYAVSRL 213

    Score = 66  Identity = 33%, Similarity = 52%

    ORF1      48 IQNKYPLAFEMAKVAGDDLKNHFGCQLELSEMSYLALYFEMI 89
                 IQ KY   A++ A      + LKN +    L  SE  Y+ L+ + +
    BsSacT   230 IQKKYSFAYQCALELAEFLKNEYQLHLPESEAGYITLHVQRL 271
```

Figure 6-8: Examples of some local alignments produced by BLAST after searching the GenBank Database using the ORF1 protein as the query sequence. Local alignments of ORF1 protein with (a) *Bacillus subtilis* cellibiose-specific PTS CelR regulatory protein (Tobisch *et al.*, 1997), (b) *Erwinia chrysanthemii* ß-glucoside-specific PTS ArbG protein (Hassouni *et al.*, 1992), (c) *Bacillus subtilis* sucrose-specific PTS SacT protein (Debarbouille *et al.*, 1990). A + indicates chemically similar residues. The BLAST scores, the percentage of shared identical residues, and the percentage of similarity (identical + similar residues) are indicated for each alignment. Residue coordinates for the beginning and end of each region of the proteins in the alignments are shown.

(a) Score = 64 Identity = 70%, Similarity = 70%

```
ORF2        19 ILDREKEQSTIFGNGIAFPH 38
               IL REK  ST  GNGIA PH
EcIIA-Ntr   54 ILTREKMGSTGIGNGIAIPH 73
```

Score = 39 Identity = 42%, Similarity = 78%

```
ORF2        61 DHESVDLIFLVAIP 74
               D++ VDL+F + +P
EcIIA-Ntr  100 DNQPVDLLFALLVP 113
```

(b) Score = 73 Identity = 36%, Similarity = 60%

```
ORF2        11 VDDHFEKRILDREKEQSTIFGNGIAFPHTINQTLEKIV 48
               V + +   +L RE++ ST  GNGIA PH    T ++++
EcFruB      35 VAEGYVNGMLAREQQTSTFLGNGIAIPHGTTDTRDQVL 72
```

(c) Score = 65 Identity = 30%, Similarity = 52%

```
ORF2         3 QSLEALRLVDDHFEKRILDREKEQSTIFGNGIAFPHTINQTLEKIV 48
               Q+L      V + + + + DRE   ST  GNG+A PH  ++     ++
ScIIA-Mtl   28 QALVDAGAVTEDYIQAMKDREAVVSTFMGNGLAIPHGTDEAKSAVL 73
```

Figure 6-9: Examples of some local alignments produced by BLAST after searching the GenBank Database using the ORF2 protein as the query sequence. Local alignments of the ORF2 protein with (a) *Escherichia coli* nitrogen regulatory IIA protein (Powell *et al.*, 1995), (b) *Escherichia coli* fructose-specific PTS diphosphoryl transfer protein (Reizer *et al.*, 1994), and (c) *Staphylococcus carnosus* mannitol-specific PTS IIA protein (Fischer *et al.*, 1989). A + indicates chemically similar residues. The BLAST scores, the percentage of shared identical residues, and the percentage of similarity (identical + similar residues) are indicated for each alignment. Residue coordinates for the beginning and end of each region of the proteins in the alignments are shown.

Discussion

Growth comparison studies. The observations made during the growth comparison studies of *S. mutans* LT11 and BH96 confirm earlier conclusions made by other researchers regarding wild-type growth. The long lag period prior to growth of *S. mutans* LT11 in sorbitol-only medium (Figure 6-1A) indicates that the enzymes involved specifically in sorbitol metabolism, the sorbitol-PTS and sorbitol-6-P dehydrogenase, must be induced prior to exponential growth, confirming earlier research with *S. mutans* (Brown and Wittenberger, 1973; Maryanski and Wittenberger, 1975). Furthermore, in the presence of sorbitol and glucose, sorbitol was only degraded by *S. mutans* LT11 when glucose was depleted (Figure 6-1B) indicating catabolite repression in the presence of glucose, an observation demonstrated previously (Dills and Seno, 1983; Slee and Tanzer, 1983).

S. mutans BH96 did not grow in the presence of sorbitol (Figure 6-2A) and removal of antibiotic selection resulted in reversion of this phenotype confirming that the gene(s) interrupted by the single insertion of Tn*4001* are involved in sorbitol metabolism. A somewhat unexpected observation was the slight inhibitory effect of sorbitol on glucose utilization (Figure 6-2A). Since the inhibitory effect by sorbitol was not observed for the parental, wild-type strain, *S. mutans* LT11 (Figure 6-1A), the observations may be

attributed to the integration of the transposon and the apparent loss of an important element required for sorbitol metabolism. Similarly, glucose uptake has been shown to be inhibited in the presence of sorbitol by *S. sanguis* cells grown under sorbitol limitation in continuous culture (Hamilton and Svensater, 1991). The authors attributed this effect to the cells acquiring a component inhibitory to glucose transport or the loss of a regulatory element necessary for sorbitol transport. A more extensive study regarding glucose transport and glycolytic activity by *S. mutans* BH96 may provide more insight into the mechanism responsible for the observed repression of glucose transport in the presence of sorbitol.

The potential function of ORF1 and ORF2. The nucleotide sequence of the 948-bp chromosomal fragment of pΩ-SB revealed two partial ORFs, ORF1 and ORF2, transcribed in the same direction (Figure 6-7). The sequences of the deduced proteins were compared to known protein sequences in order to determine their potential function (Figures 6-8 and 6-9; summarized in Table 6-1). ORF2 demonstrated homology with a family of EIIA proteins or domains specific for fructose or mannitol through its identity with a signature sequence present in all members of this family (Reizer *et al.*, 1992). Three amino acids in this signature sequence are fully conserved and the corresponding residues in ORF2 are Ala-35, Pro-37 and His-38. This signature sequence has been

Table 6-1

Summary of the various proteins which demonstrated homology with *S. mutans* ORF1 and ORF2 from pΩ-SB.

	Homologous protein	Organism	Function of protein
ORF1	SacT	*B. subtilis*	Antiterminator protein of sucrose utilization
	ArbG	*E. chrysanthemi*	Antiterminator protein of β-glucoside utilization
	LevR	*B. subtilis*	Activator protein of fructose utilization
	LicR	*B. subtilis*	Regulatory protein of β-glucoside utilization
ORF2	$EIIA^{Ntr}$	*E. coli*	Nitrogen-related EIIA
	$EIIA^{Fru}$	*E. coli*	Fructose EIIA
	$EIIA^{Mtl}$	*Staph. carnosus*	Mannitol EIIA

recognized within the *E. coli ptsN* gene product, $EIIA^{Ntr}$, (nitrogen-related Enzyme IIA) (Powell *et al.*, 1995) and 70% identity was demonstrated between $EIIA^{Ntr}$ and ORF2 (Figure 6-9). The signature sequence was also identified within the $EIIA^{Fru}$ domain of the *E. coli* diphosphoryl transfer protein (DTP) (*fruB*) (Reizer *et al.*, 1994) and 36% homology was observed with ORF2 (Figure 6-9). The separate mannitol-specific EIIA ($EIIA^{Mtl}$) component from *Staph. carnosus* demonstrated 30% identity with ORF2 and this homology extended over the signature sequence (Figure 6-9). The *Staph. carnosus* $EIIA^{Mtl}$ component consists of 144 amino acids (Reiche *et al.*, 1988) and this degree of identity indicates that the 101 amino acids derived from ORF2 may constitute most of the sorbitol-specific EIIA component from *S. mutans*.

ORF1 includes 476 bp encoding 158 amino acids of the carboxy-terminus of a protein demonstrating homology to several transcription regulatory proteins (Figures 6-7 and 6-8). The deduced amino acid sequence shares considerable homology with the antiterminator proteins, SacT and ArbG, of the *B. subtilis* sucrose system (Debarbouille *et al.*, 1990) and *E. chrysanthemi* β-glucoside system (Hassouni *et al.*, 1992), respectively. The partial protein sequence of ORF1 is 26% identical to SacT and 33% identical to ArbG.

SacT and ArbG are antiterminator proteins homologous to the well-characterized BlgG protein which regulates the *E. coli*

β-glucoside (*bgl*) operon (Amster-Choder *et al.*, 1989, Houman *et al.*, 1990, Amster-Choder and Wright, 1993). The *E. coli* *bgl* operon consists of the genes *bglG*, *bglF* and *bglB* encoding an antiterminator, Enzyme II^{Bgl} (EII^{Bgl}), and a phospho-β-glucosidase, respectively. In the absence of inducer, most transcripts terminate at a rho-independent terminator upstream of the first gene, *bglG*, and a second rho-independent terminator is located in the region between *bglG* and *bglF*. Under these conditions, BglG is phosphorylated by BglF rendering it inactive as an antiterminator, however, in the presence of an inducer, BglF dephosphorylates BglG and phosphorylates the incoming substrate. In the unphosphorylated state, BglG positively regulates operon expression by binding to the nascent mRNA at the ribonucleic antiterminator (RAT) sequence partially overlapping the palindromic terminator sequence, thereby, inhibiting terminator formation (Aymerich and Steinmetz, 1992).

The *B. subtilis* SacT and *E. chrysanthemi* ArbG proteins consist of 276 and 283 amino acids, respectively, therefore the carboxy-terminal 158 amino acids of ORF1 may represent approximately half of an antiterminator protein regulating sorbitol metabolism. Several other antiterminator characteristics will have to be identified before the protein represented by ORF1 could be classified as an antiterminator. For example, protein sequence comparisons have identified several regions of identity among antiterminator proteins,

including the conserved Asp-His residues at position 100 suggested to be involved in regulation of protein activity (Debarbouille *et al.*, 1990). The complete sequence of the ORF1 gene is needed to determine the presence or absence of the Asp-His residues. The region 5' to ORF1 would have to be sequenced to determine if it contains the characteristic secondary structures common for this region, such as terminator and RAT sequences, with which the the BglG/SacT antiterminators interact.

Analysis of the sequence of the intergenic region of ORF1 and ORF2 detected two regions of dyad symmetry which could also play a role in antitermination regulation of expression of downstream genes, including ORF2. The region of dyad symmetry immediately upstream of the putative ribosome binding site contains two 37-bp sequences centered 63-bp from the translation initiation codon of ORF2. Similarly, the region of dyad symmetry located between the *sacT* gene and the *sacPA* operon contains two 31-bp sequences centered 65 bp upstream of the translation initiation codon for *sacP* (Debarbouille *et al.*, 1990). Further genetic and/or biochemical analysis of this region in both the wild type strain and in BH96 would be needed to determine the role, if any, of these putative terminators. In addition, a putative ribosome-binding site (RBS) was located 13 bases upstream from the translation initiation for ORF2, however, the

typical optimal distance between an RBS and initiation codon is approximately 7 bases.

ORF1 also demonstrated considerable homology to the activator protein LevR responsible for the induction of the levanase operon (*levDEFG sacC*) of *B. subtilis* (data not shown) (Martin-Verstraete *et al.*, 1990; Debarbouille *et al.*, 1991). The genes *levDEFG* encode a fructose-specific PTS while *sacC* encodes levanase, an extracellular enzyme capable of hydrolyzing fructose polymers. The LevR protein consists of 938 amino acids divided into three domains sharing homology with two different families of transcription activators. Domain A is homologous to the NifA/NtrC family of activators functioning with σ^{54}-RNA polymerase to stimulate initiation of transcription (Kustu *et al.*, 1989). Both domain B and domain C are similar to the antiterminator proteins *BglG* of *E. coli* and *SacT* of *B. subtilis* suggesting that domain C is a duplication of domain B (Stulke *et al.*, 1995). LevR has been shown to interact with an activating sequence (UAS) located upstream from the promoter of the levanase operon and a 16-bp palindromic sequence is the target of LevR binding (Martin-Verstraete *et al.*, 1994). Almost the entire sequence of ORF1 demonstrated homology to LevR; amino acids 1-50 demonstrated 20% identity with domain A, amino acids 47-89 were 20% identical to domain B and amino acids 90-119 were 36% identical to the region between domain B and C (data not shown).

Models have been proposed for the regulation of the *sacPA* and levanase operons of *B. subtilis* involving the EII complexes, as well as, the general proteins of the PTS (Arnaud *et al.*, 1992; Stulke *et al.*, 1995). The *sacT30 B. subtilis* mutant (QB6042), which demonstrated constitutive expression of the *sacPA* operon and carries a *sacP-lacZ* fusion, was used to study the role of the general proteins of the PTS in SacT activity (Arnaud *et al.*, 1992). SacT is very similar to the *B. subtilis* SacY antiterminator that regulates the *sacB* gene encoding a second sucrose-hydrolyzing enzyme, levansucrase (Crutz *et al.*, 1990). Since 'cross-talk' has been demonstrated between SacT and SacY (Debarbouille *et al.*, 1990), a deletion inactivating *sacY* and *sacX* (encoding EII^{Scr}) was made in the *sacT30* mutant to prevent the constitutive expression of *sacPA* by SacY, generating the strain (QB6052) (Arnaud *et al.*, 1992). A decrease in expression of the *sacPA* operon was observed following introduction of a point mutation into *ptsI* (QB6053), or an internal deletion of the *ptsH* gene (QB6050), indicating that SacT requires enzyme I (EI) and HPr to be an active antiterminator (Arnaud *et al.*, 1992). The regulation of SacT has been proposed to include two phosphorylation sites: site 1 is the activation site phosphorylated by the general proteins of the PTS, while site 2 is the inactivation site possibly phosphorylated by an EII^{Scr} component. This latter phosphorylation was based on the proposed negative regulation of the *E. coli* BglG (Schnetz

and Rak, 1990) and the *B. subtilis* SacY (Crutz *et al.*, 1990) antiterminators by BglF and SacX, respectively. Overall, in the absence of sucrose, the model proposes that SacT is phosphorylated at both sites and the introduction of sucrose leads to dephosphorylation of the second site and activation of SacT.

Point mutations in the *ptsH* gene and *in vitro* phosphorylation of LevR, involving both EI and HPr, have demonstrated that PEP-dependent phosphorylation of LevR is necessary for its activation (Stulke *et al.*, 1995). Moreover, truncated LevR proteins demonstrated that Domain B is required for activation in the presence of HPr and, in conjunction with a *levE* mutation, it has been shown that the *lev*-PTS may negatively regulate LevR through phosphorylation of Domain C. This latter conclusion is supported by the fact that substituting the conserved His-869 in LevR led to constitutive expression of the levanase operon. Overall, the model proposes that in the absence of fructose, LevD and LevE phosphorylate Domain C causing inactivation. In the presence of fructose, the authors propose that both domains become dephosphorylated leading to transcription of the levanase operon (Stulke *et al.*, 1995).

Although SacT requires both EI and HPr for activity and LevR is positively controlled by HPr, the antiterminator SacY has been shown to be negatively controlled by EI (Crutz *et*

al., 1990). Constitutive expression of *sacB* has been demonstrated in *B. subtilis ptsI* mutants, including a mutant strain carrying a *ptsI*::Tn*917* insertion (Gay *et al.*, 1973; Gonzy-Treboul and Steinmetz, 1987). A deletion including all of *ptsX*, which is homologous to the *crr* gene of *E. coli* (Gonzy-Treboul *et al.*, 1989), *ptsH*, and the 5' end of *ptsI* (Δ*ptsXHI'*) was introduced into a *B. subtilis* strain carrying a *sacB'-'lacZ* fusion (Crutz *et al.*, 1990). The Δ*ptsXHI'* deletion resulted in constitutive expression of the *sacB'-'lacZ* fusion and this constitutivity was removed in a double mutant carrying a *sacY* null mutation. Therefore, EI mutant studies have led to the proposal that EI exhibits a negative effect on SacY activity. The model proposes that in the absence of sucrose, EI phosphorylates SacX, which in turn inhibits SacY. In the presence of sucrose, SacX becomes dephosphorylated as it transports and phosphorylates the incoming sugar, thereby, relieving the inhibition on SacY (Crutz *et al.*, 1990).

ORF1 also demonstrated homology with the *B. subtilis* LicR (CelR) regulatory protein, recently elucidated to be involved in β-glucoside utilization (Figure 6-8) (Tobisch *et al.*, 1997). A promoter precedes *licR* and transcription terminates at a secondary structure located immediately downstream from the reading frame, producing a 641-amino-acid protein. A second transcriptional unit follows the *licR* gene encoding the EII component (*licB* and *licC*), EIIA component (*licA*), and

a possible phospho-β-glucosidase (*licH*). Two regions of the LicR protein sequence, designated Domains C and D, have demonstrated homology with the BglG/SacT family of antiterminator proteins (Schnetz *et al.*, 1987; Debarbouille *et al.*, 1990), as well as, the LevR transcriptional regulator (Martin-Verstraete *et al.*, 1990; Debarbouille *et al.*, 1991). Domain D of LicR extends from residue 315 to 400 and a large portion of this region, amino acids 323-393, demonstrated homology with amino acids 16-86 from ORF1 (Figure 6-8). Moreover, the region immediately downstream of Domain D, residues 403-442, demonstrated homology with residues 98-137 from ORF1 (Figure 6-8).

Future Activity:

Characterization of the sorbitol locus. The isolation of a small region of the *S. mutans* genome (pΩ-SB) that appears to be involved in sorbitol utilization can be exploited to isolate flanking DNA, thus allowing for a complete genetic characterization in this region of the genome. Several methods could be utilized, including PCR-based methods, as well as, the isolation of the region from a wild type genomic library by a marker rescue procedure as was used for the isolation of a transposon/chromosome junction from BH96 (pΩIS-SB). A λ replacement vector, such as λEMBL3, could be used to construct a wild-type genomic DNA library that could then be screened using the insert of pΩ-SB as a

probe to isolate clones of interest carrying large regions (~20 kb) of the genome. Wild-type DNA could be used as a target for use of the cloned region as the integration vehicle for a marker rescue plasmid to be established into the region of interest. Once established in the wild-type chromosome upstream or downstream regions could be rescued using appropriate restriction enzyme digestion/ligation/transformation procedures. This method is analogous to that used for the rescue of pΩIS-SB using the IS*256*-integrative plasmid pΩIS (see above and Figure 6-4). Because the sequence of the insert in pΩ-SB has been obtained it could be used to design primers for use in single specific primer-PCR (SSP-PCR) methods to isolate amplicons from flanking regions which could be directly sequenced and/or cloned into plasmids for further analysis (Arnold and Hodgson, 1991).

Sequencing the upstream region of ORF1 may reveal promoter sequences, as well as a terminator structure, and gene fusion technology could be applied to examine whether the putative antiterminator is autoregulated and if ORF1 gene expression is regulated by components of the PTS. A chromosomal fragment carrying the ORF1 promoter could be cloned into a suicide/promoter probe vector, such as pJG-12, that contains a promoterless *lacZ* gene (Gutierrez *et al.*, 1996). The *ORF1-p::lacZ* fusion carried on an intact plasmid would then be transformed into *S. mutans* LT11 and Campbell-type integration

would generate one intact copy of ORF1, as well as, place the *lacZ* gene in the chromosome under control of the *ORF1* promoter. Integration of the plasmid in the LT11 fusion strain would be verified by Southern hybridization and β-galactosidase activity would monitor expression of ORF1 (Miller, 1972). If the level of β-galactosidase activity decreases with expression of the intact ORF1 gene in the presence of sorbitol, we may conclude that ORF1 is auto-regulated. The *ORF1::lacZ* fusion could also be transformed into *S. mutans* DC10, a mutant strain expressing a defective EI (Cvitkovitch *et al.*, 1995). If the level of β-galactosidase activity is shown to be high, both in the presence and absence of sorbitol, this would indicate constitutive expression and strongly suggest that EI negatively regulates ORF1 gene expression.

The LT11-fusion strain could also be used to monitor the expression of the ORF2 gene under a variety of environmental conditions. Similar to the growth comparison studies illustrated in Figures 6-1 and 6-2, the LT11 fusion strain could be grown in the presence of sorbitol and glucose. Since a very short lag was observed during the transition between glucose and sorbitol (Figure 6-1), we have suggested that the sorbitol operon is activated prior to glucose exhaustion. β-galactosidase activity levels may provide the sensitivity needed to determine the extent of operon expression during growth on glucose and throughout the

transition period. In addition, the LT11 fusion strain could be grown in continuous culture to study the effect of pH and dilution rate on the expression of the operon in the presence of sorbitol.

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Chapter 7

Summary and Discussion

Streptococcus mutans and the regulation of P-(Ser)-HPr formation.

The oral pathogen *Streptococcus mutans* possesses many virulence factors contributing to its cariogenicity, including characteristics which permit growth and survival in adverse environmental conditions, such as low exogenous nutrient supply and low pH environments (Hamada and Slade, 1980; Loesche, 1986). *S. mutans* possesses both high affinity (K_S = 10 μM) and low affinity (K_S = 100-200 μM) sugar transport systems operating in the presence of limiting and excess exogenous sugar, respectively (Ellwood *et al.*, 1979). *S. mutans* responds to nutrient excess by activating specific mechanisms which protect against accumulation of toxic glycolytic intermediates (Carlsson, 1983). For example, high levels of fructose-1,6-bisphosphate activates lactate dehydrogenase producing large amounts of lactic acid (Yamada and Carlsson, 1975). *S. mutans* readily adapts to acidic conditions through its ability to metabolize and grow at pH values, at or below, 5.5 (Harper and Loesche, 1984, Hamilton and Buckley, 1991), thereby providing this organism with a competitive advantage in the dental plaque environment. In addition, *S. mutans* can generate adherent extracellular glucans from sucrose that, along with intracellular glycogen, constitute an endogenous nutrient source when the exogenous nutrient supply has been depleted. In view of the role this organism plays in dental caries formation, considerable

research has focussed on the regulation of sugar transport and the proteins involved in the sugar uptake process.

The main sugar transport system in oral streptococci is the PEP: sugar phosphotransferase system (Postma *et al.*, 1993; Thompson, 1987). Using PEP as a phosphoryl donor, the PTS catalyzes the concomitant transport and phosphorylation of sugars, including monosaccarides and disaccarides, as well as, the sugar alcohols, sorbitol and mannitol. In Gram-positive bacteria, including some lacking a functional PTS, the HPr protein can be phosphorylated at a serine residue (Ser-46) by a metabolite-activated, ATP-dependent (Ser)HPr kinase and dephosphorylated by an inorganic phosphate-dependent phosphatase (Deutscher and Saier, 1983; Reizer *et al.*, 1983; Reizer *et al.*, 1993). Seryl-phosphorylated HPr (P-(Ser)-HPr) can also be phosphorylated at the expense of PEP by EI producing the doubly-phosphorylated form, P~(His)-P-(Ser)-HPr (Deutscher *et al.*, 1984). A study using batch-grown cells (Vadeboncoeur *et al.*, 1991) has established the existence of four forms of HPr in cells of the oral streptococci strains *S. mutans* and *S. salivarius*. With these organisms, the predominant forms in exponential-phase cells were P-(Ser)-HPr and P~(His)-P-(Ser)-HPr, while stationary-phase cells were devoid of these forms carrying only unphosphorylated HPr and P~(His)-HPr.

In the current studies (Chapter 4), *S. mutans* Ingbritt was grown in continuous culture (D = 0.1 h^{-1}, pH 7.0) in the presence of various glucose concentrations and the level of the four forms of HPr were examined using crossed immunoelectrophoresis. All four forms of HPr were detected in the presence of 10 and 50 mM glucose (PTS-optimal conditions), as well as, 100 and 200 mM glucose (PTS-repressive conditions) (Table 4-2 and Figure 4-2). The cellular level of P~(His)-HPr was maximal in the presence of 50 mM glucose, while the level of P-(Ser)-HPr increased 15-fold from 10 mM to a maximum at 100 mM glucose, and increasing the glucose concentration to 200 mM did not result in increased levels of P-(Ser)-HPr. Interestingly, the level of P~(His)-P-(Ser)-HPr increased with increasing glucose concentrations and a 12-fold higher level was observed with the 200 mM compared to the 10 mM glucose-grown cells.

It has been suggested that the formation of P-(Ser)-HPr serves to regulate the PTS as *in vitro* studies demonstrated that P-(Ser)-HPr could not replace P~(His)-HPr as a phosphoryl donor in the PTS cascade (Deutscher and Saier, 1983) and PEP-dependent phosphorylation of P-(Ser)-HPr occurred 5000-fold slower than the phosphorylation of free HPr (Deutscher *et al.*, 1984). The results reported here, however, do not support these conclusions as the level of P~(His)-P-(Ser)-HPr increased with increasing glucose concentrations indicating that P~(His)-P-(Ser)-HPr formation

is not restricted *in vivo*. Moreover, the rate of glucose transport by the chemostat-grown cells increased with increasing glucose concentrations (Table 4-1) indicating that P-(Ser)-HPr formation does not serve to reduce the rate of glucose entry. In light of the increased levels of P~(His)-P-(Ser)-HPr in the presence of excess glucose, we have suggested that P~(His)-P-(Ser)-HPr may replace P~(His)-HPr as a phosphocarrier, thereby, providing phosphate to the EII complex at an optimal rate. Further research is required to substantiate this conclusion.

Studies with *S. pyogenes* (Reizer *et al.*, 1988), *E. faecalis* (Deutscher and Engelmann, 1984), *L. brevis* (Reizer *et al.*, 1993), *B. subtilis* (Reizer *et al.*, 1989) and *S. mutans* GS-5 (Mimura *et al.*, 1987) have demonstrated that fructose-1,6-bisphosphate activates (Ser)HPr kinase. However, our results have shown that FBP, as well as, glucose-6-phosphate are not critical factors for the activity of (Ser)HPr kinase. *In vitro* experiments were conducted to examine the effect of FBP and ATP concentration on the relative activity of (Ser)HPr kinase in membrane preparations from *S. mutans* Ingbritt and *S. salivarius* ATCC 25975. In the presence of 0.05 mM ATP and 5 mM FBP, the activity increased two-fold, compared to activity in the presence of 1.0 mM ATP and a range of FBP concentrations (Figure 4-2). Furthermore, in the presence of 4 mM FBP and 0.1 mM ATP (Ser)HPr kinase activation was less than 20% and a previous *in vitro* study using *S. mutans* GS-5

had demonstrated that these levels of ATP and FBP were sufficient to stimulate phosphorylation (Mimura *et al.*, 1987). The intracellular concentration of ATP and FBP was examined in cells of *S. mutans* Ingbritt grown under the same continuous culture conditions as employed for the HPr determinations in order to observe the level of these metabolites *in vivo*. The intracellular levels of FBP and ATP increased with an increase in glucose concentration, and in the presence of 200 mM glucose, the intracellular level of FBP and ATP was 1.1 and 1.4 mM, respectively (Table 4-3). Relating these *in vivo* results with our intact cell results led us to conclude that the FBP levels in growing cells of *S. mutans* Ingbritt are not sufficiently high to significantly contribute to the activation of the enzyme and the intracellular levels of ATP could be considered inhibitory.

An interesting observation during this study was the level of (Ser)HPr kinase activity in membrane preparations from *S. mutans* Ingbritt grown under the same continuous culture conditions as employed for the HPr and intermediate analyses. Enzyme activities were similar in cells grown in the presence of 10, 50 and 100 mM glucose, however, the level increased approximately 3-fold in cells grown with 200 mM glucose (Figure 4-5), although this increase in (Ser)HPr kinase activity did not result in an increase in P-(Ser)-HPr formation (Table 4-2 and Figure 4-4). Since the rate of glucose transport for 200 mM glucose-grown cells was 2-fold

higher than 100 mM glucose-grown cells (Table 4-1), the former cells would accumulate higher levels of toxic intermediates, which may serve to down-regulate kinase activity. We have proposed that the cells grown at 200 mM glucose increase (Ser)HPr kinase synthesis in order to maintain an appropriate level of P-(Ser)-HPr.

Site-directed mutagenesis of the HPr gene.

In other Gram-positive bacteria, P-(Ser)-HPr has been shown to play a role in catabolite repression (Deutscher *et al.*, 1994), inducer expulsion (Ye and Saier, 1995; Ye *et al.*, 1996), non-PTS transport (Ye *et al.*, 1994a; Ye *et al.*, 1994b) and the exclusion of one PTS sugar by another PTS sugar (Ye and Saier, 1996). These various regulatory functions of P-(Ser)-HPr were characterized by employing site-directed mutagenesis of the Ser-46 residue. Site-directed mutagenesis studies have also identified the role of His-15 in P-(Ser)-HPr-mediated carbon catabolite repression of the *B. subtilis gnt* operon (Reizer *et al.*, 1996). Spontaneous mutagenesis has identified the importance of the Gly-67 HPr residue during interactions with EI or the EII complexes (Gauthier et *al.*, 1994). In the current study (Chapter 5), site-directed mutagenesis of the *S. mutans* HPr gene (*ptsH*) was the first step towards elucidating the role of specific HPr residues.

Site-directed, single-stranded mutagenesis and subsequent cloning steps produced the plasmid pHIK-2 carrying the

mutated *S. mutans ptsH* gene in which Gly-67 was replaced by an aspartate residue, the *ptsI* gene, a kanamycin resistance marker, and the 5' end of the *gapN* gene (Figure 5-1). Upon transformation of linearized plasmid carrying the mutated gene into *S. mutans*, selection of potential mutants was based on kanamycin resistance and Southern hybridization analyses confirmed integration of the kanamycin gene (Figure 5-2). Despite successful double-cross over to include the kanamycin gene, all the potential mutants readily fermented PTS sugars. We anticipated a PTS-negative phenotype upon successful integration of the mutated *ptsH* gene as previously established for a Gly67-Asp *S. salivarius* HPr mutant (Gauthier *et al.*, 1994).

We postulate that our inability to generate the *S. mutans* HPr mutant relates to the distance between the kanamycin gene and the mutated *ptsH* gene, as well as, the lack of a more discerning selection procedure, although the integration of the mutated HPr might have been a lethal event. In order to avoid introducing another PTS-related mutation, we decided to place the kanamycin gene between the *ptsI* and *gapN* genes. Although allelic exchange to integrate the kanamycin gene was verified, the results suggest that the more than 1700-bp distance between the kanamycin gene and the mutated *ptsH* gene may have influenced the rate of integration of the desired mutation. Cloning the mutated gene into a temperature-sensitive vector, such as pVE6004 (Maguin *et al.*, 1992) or

pGh9:ISS1 (Maguin *et al.*, 1996) may provide the necessary advantage for successful integration. In addition, a *S. mutans* mutant strain could be generated by integrating an antibiotic resistance gene within the *ptsH* gene and replacement by the desired mutation would be based on the loss of antibiotic resistance.

Regulation of sorbitol transport by *Streptococcus mutans*.

Sorbitol is transported into cells of *S. mutans* by an inducible sorbitol-PTS and intracellular sorbitol-6-phosphate is converted to fructose-6-P by an inducible sorbitol-6-P dehydrogenase (Brown and Wittenberger, 1973; Maryanski and Wittenberger, 1975). Sorbitol metabolism by *S. mutans* is subject to catabolite repression in the presence of glucose (Brown and Wittenberger, 1973; Dills and Seno, 1983), whereas *S. sanguis* has been shown to metabolize sorbitol and glucose concurrently (Hamilton and Svensater, 1991). Several studies have demonstrated the ability of *S. mutans* and *S. sanguis* to adapt to growth on sorbitol following exposure to sorbitol-containing gum or frequent mouth rinses with a sorbitol solution (Loesche *et al.*, 1984; Soderling *et al.*, 1989; Wennerholm *et al.*, 1994; Kalfas and Edwardsson, 1990).

In this study (Chapter 6), the sorbitol-defective mutant *S. mutans* BH96 was generated following transformation of the plasmid pα (Lunsford, 1995) into *S. mutans* LT11. The

sorbitol-negative phenotype was due to integration of Tn*4001* conferring kanamycin resistance to the mutant strain. *S. mutans* BH96 was unable to grow in the presence of sorbitol, however, growth on several other PTS sugars was not affected and the absence of kanamycin selection resulted in the reversion of the sorbitol-negative phenotype.

As well as establishing growth on various carbohydrate substrates, both *S. mutans* BH96 and wild-type *S. mutans* LT11 were grown in the presence of glucose, sorbitol and glucose+sorbitol (Figure 6-1 and 6-2). In the presence of glucose+sorbitol, *S. mutans* LT11 utilized glucose first (Figure 6-1) indicating that sorbitol metabolism is subject to catabolite repression by glucose as demonstrated by earlier research (Dills and Seno, 1983; Slee and Tanzer, 1983). In addition, a short lag period was observed during the transition to growth on sorbitol suggesting that the genes involved in sorbitol metabolism are expressed prior to glucose exhaustion. As predicted, *S. mutans* BH96 only grew in the presence of glucose as sorbitol was not consumed following growth on glucose (Figure 6-2).

Rescue of the transposon/genome junction involved transforming *S. mutans* BH96 with the plasmid pΩIS carrying a portion of the IS*256* sequences from Tn*4001* and integration of the plasmid occurred via Campbell-type integration (Figure 6-4). Genomic DNA from one of the successful transformants was

digested with *SstI*, as previous restriction mapping of *S. mutans* BH96 had indicated *SstI* sites within close proximity of the integrated transposon, and pΩIS carrying chromosomal flanking DNA was recovered and transformed into *E. coli* DH5α. Most of the transformants harbored a plasmid carrying approximately 1.0 kb of chromosomal DNA and one of these plasmids, designated pΩIS-SB, was selected for further study. To facilitate sequencing, a 1.1-kb *Hind*III fragment carrying mainly IS*256* sequence, was removed thereby generating the plasmid pΩ-SB.

The 948-bp chromosomal fragment from *S. mutans* BH96 carried on pΩ-SB was sequenced and the amino acid sequence was deduced for two partial ORFs using the DNA Strider program (Figure 6-7). These amino acid sequences were used in BLAST searches (Altschul *et al.*, 1990) of the GenBank Database (Figures 6-8 and 6-9). The first 474 bp of the sequence encodes 158 amino acids of the carboxy-terminus of a protein demonstrating homology with antiterminator regulatory proteins (Debarbouille *et al.*, 1990; Hassouni *et al.*, 1992) (Figure 6-8). A putative ribosome-binding site (RBS) is AAGA located at bases 629-632 and the start codon for the second ORF is ATG at bases 646-648. The second ORF encodes 101 amino acids of the N-terminus of a protein demonstrating homology to the EIIA component of mannitol EII complexes (Figure 6-9) (Akagawa *et al.*, 1995; Reiche *et al.*, 1988; van Weeghel *et al.*, 1991). In addition, two possible regions of

dyad symmetry were identified in the intergenic region of ORF1 and ORF2, bases 489-540 has a ΔG of -41.2 kJ and bases 544-622 has a ΔG of -43.7 kJ (Figure 6-7). Overall, the results suggest that Tn*4001* integrated in the BH96 chromosome within a gene coding for a transcriptional regulator, possibly an antiterminator, which may be a part of a operon involved in sorbitol transport and metabolism.

Despite the homology of ORF1 with the antiterminator proteins BglG/SacT family of antiterminators, as well as, the antiterminator domain of the regulatory protein LevR, specific characteristics would have to be identified in order to classify ORF1 as an antiterminator protein. These include the conserved Asp-His residues at position 100 and terminator structures 5' to ORF1 which may interact with the putative sorbitol antiterminator protein.

The plasmid carrying the partial open reading frames (ORFs), pΩ-SB, can be used to recover the remaining *S. mutans* sorbitol operon. Different methods could be employed including isolation of the region from a wild type genomic library, a marker rescue procedure, as well as, PCR-based methods. These approaches are described in more detail in the Discussion section of Chapter 6. Furthermore, in view of the interest in sorbitol metabolism by *S. sanguis* (Hamilton and Svensater, 1991; Svensater and Hamilton, 1991), the

information gained in this study may be applied to clone and identify the *S. sanguis* sorbitol-related genes.

In this study, both physiological and genetic techniques were employed to examine the PTS from *S. mutans* and the results have contributed to the fundamental knowledge of the PTS, including the role of P-(Ser)-HPr and a potential transcriptional regulatory protein involved in sorbitol metabolism. In general, *S. mutans* PTS research provides information regarding the role of the PTS in the life of *S. mutans* thereby providing insights into its mode of action in plaque and caries and, consequently, a better understanding of its pathogenesis. The short-term benefit of *S. mutans* PTS research is more comprehensive information regarding the physiology and pathogenesis of this organism. This information may be useful in the long-term if chemical or biological agents are to be developed to inhibit the growth and activity of *S. mutans* and other saccharolytic bacteria employing the PTS for sugar transport.

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Appendix

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

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Journal of Bacteriology 177:2251-2258

JOURNAL OF BACTERIOLOGY, May 1995, p. 2251–2258
0021-9193/95/$04.00+0
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Vol. 177, No. 9

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

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Received 29 December 1994/Accepted 25 February 1995

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_t of 125 μM for glucose and a V_{max} of 0.87 nmol 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 transport 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.**

Carbohydrate metabolism by *Streptococcus mutans* results in the formation of acid end products that can contribute to the demineralization of tooth enamel, leading to dental caries (11, 42). The phosphoenolpyruvate (PEP)-dependent 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, 17, 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) as two or more proteins, one of which is membrane bound (B and C) and one of which is soluble (IIA or EIII); (iii) with domains A and B fused into a single soluble protein and associated with two membrane components (C and D); or (iv) with domains IIA and IIB existing 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 available on the genetic arrangement of some, but not all, of the PTS and associated components responsible for the transport of sucrose (*scrA*) (35) and mannitol (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 report (21) has indicated that the gene for phospho-β-galactosidase (*lacG*), the enzyme that cleaves lactose phosphate 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 to 0.6 h^{-1} indicated two transport processes, a high-affinity system with K_t values of 6.8 to 8.0 μM, shown to be the PTS, and a lower-affinity system with K_t 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_t values of 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 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

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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 approximately 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 [φ80d Δ*lacZ* M15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *gyrA relA1* F$^-$ Δ(*lacZYA-argF*) *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* BM71 was utilized as a parent strain in the construction of *ptsI* mutant strain 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). The selective media, used to isolate PTS-defective streptococcal transformants, included (i) TYE containing 1.6% agar, 1% raffinose, and 8 μg of erythromycin ml^{-1} and (ii) blood agar (Oxoid blood base agar no. 2, 5% sheep blood, 0.0005% hemin) containing 8 μg of erythromycin ml^{-1}. Mutant DC10 was maintained on TYE-raffinose (0.3%) plates with 8 μg of 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 autotitration 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 primer labelling kit, and biotin-labelled DNA probes were prepared with 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 by alkaline lysis (1). *E. coli* was transformed by electroporation with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) 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 *Hin*cII-*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. Electrocompetent 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.); 125 μl of this culture was 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 at 600 nm of 0.25, when the cells were cooled on ice, centrifuged at 15,000 × 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, 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 Bio-Rad Gene Pulser. Immediately after pulsing, 1 ml of Todd-Hewitt broth was added to the cuvettes and the contents were transferred to a 1.5-ml microcentrifuge tube and incubated at 37°C for 90 min. The cells were centrifuged for 5 min at 15,000 × g, resuspended in 100 μl of Todd-Hewitt broth, 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 (SDS)-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 protein 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) by using purified carboxykinase from *E. coli*, kindly provided by H. Goldie, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The positive control contained 35 μg of protein from an *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 mol^{-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 addition of 10 μl of 188 mM Tris-HCl (pH 8.0), which contained 6% (wt/vol) SDS, 30% (vol/vol) glycerol, 6% (vol/vol) 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 Bio-Rad Mini Protean II apparatus. 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, recentrifuged at 27,000 × g for 20 min, and frozen at −70°C. The frozen cell pellet was used within 2 to 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 × g for 5 min. The supernatant was then centrifuged at 16,000 × g for 20 min to remove intact cells and cell debris and then subjected to centrifugation at 100,000 × g for 18 h 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 Tris-barbitone buffer containing (in 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 of 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 min at 10 V cm and 10°C on an LKB Bromma 2117 Multiphor apparatus. Electrophoresis in the second dimension, conducted for 19 h at 2 V/cm and 4°C, involved electrophoresis against a polyclonal rabbit antibody (8 mg per 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 Institutes of Health, Bethesda, Md.), and determining the number of pixels under each peak. These values were then compared to a standard curve (0 to 2,000 ng of HPr protein) to determine the number of nanograms of the various forms of HPr protein that existed in the

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(s)[a]	
	PTS	Non-PTS
BM71	Glucose, maltose, sucrose, mannitol, lactose, sucrose, fructose	Raffinose, melibiose
DC-10	Glucose	Raffinose, melibiose

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

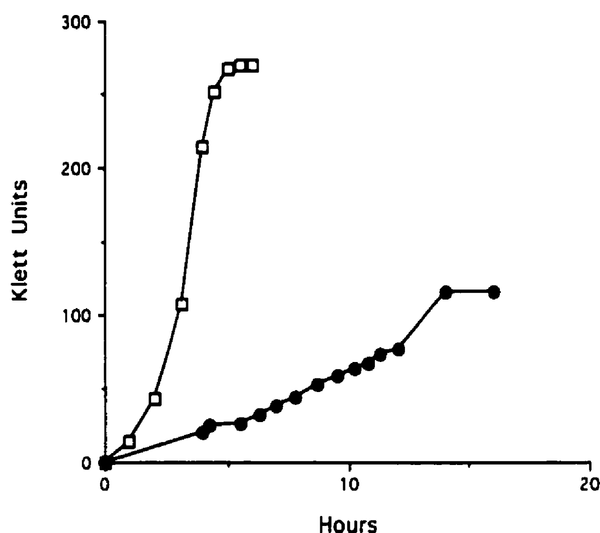

FIG. 1. Growth of wild-type *S. mutans* BM71 (□) and *ptsI* mutant DC10 (●) in TYE-0.3% glucose broth.

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 mol^{-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, filtered through 0.45-μm-pore-size 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 of Aquasol (NEN Research Products, Montreal, Quebec, Canada) in a liquid scintillation counter. Kinetics of glucose transport were determined essentially as previously described (19) with the concentrations of glucose ranging from 0.01 to 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 min and then filtered, washed, and counted. Samples were taken in quadruplicate, and the mean values were used to determine the relative activities.

Glucose phosphorylation. 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 addition of 2 mM [^{14}C]glucose (9 Ci mol^{-1}), and after 30 min of 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, Sainte 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, 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 nonfunctional. Growth curves of DC10 in TYE-0.3% glucose broth exhibited a doubling time of 180 min 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 56 ± 8 nmol 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 nmol of glucose mg of dry cells^{-1} min^{-1}) was only 1.2% of that exhibited by wild-type strain BM71 (115 ± 12 nmol mg of dry cells^{-1} min^{-1}).

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*. Figure 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 *Sst*I 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.

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 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 kDa, along with the predicted site of integration, we determined that the DC10

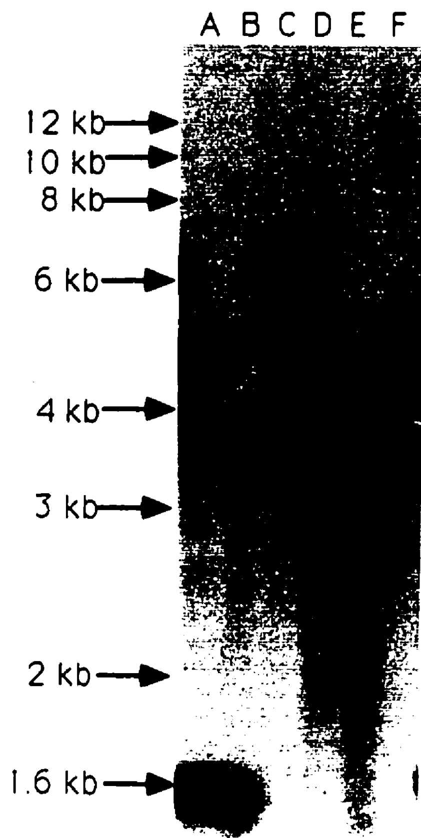

FIG. 2. Southern hybridization of DC10 and BM71 restriction-digested chromosomal DNAs probed with a radiolabelled 3.5-kb *Eco*RI-*Sst*I fragment containing the *ptsI* gene from *S. mutans*. Lanes: A, DC10 *Eco*RI; B, DC10 *Eco*RI-*Sst*I; C, DC10 *Sst*I; D, BM71 *Eco*RI; E, BM71 *Eco*RI-*Sst*I; F, BM71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

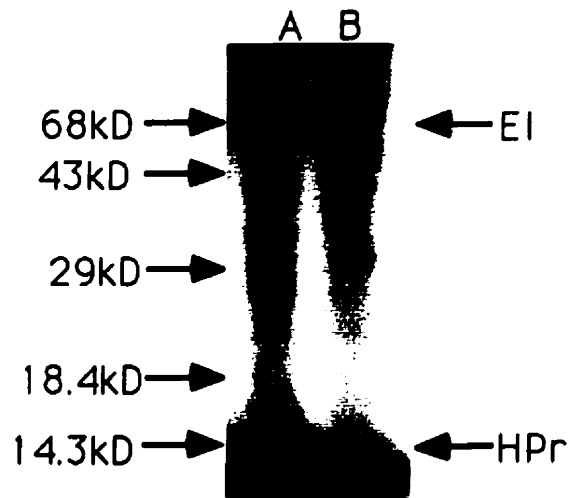

FIG. 4. Polyacrylamide gel electrophoresis of phosphorylated PTS proteins in reactions using ^{32}P-PEP with cytoplasmic extracts of *S. mutans* parent strain BM71 (lane A) and *ptsI* mutant DC10 (lane B). Each sample (35 μg) was incubated with ^{32}P-PEP for 5 min and then electrophoresed on an SDS–12% 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.

EI was missing approximately 150 of a total of 577 amino acid residues from the COOH terminus of the protein.

Further study of mutant DC10 involved examining the cytoplasmic proteins phosphorylated by ^{32}P-PEP. These experiments revealed that EI and HPr were phosphorylated in 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 has not been identified; however, it is probably not a PTS protein since it was observed in the mutant.

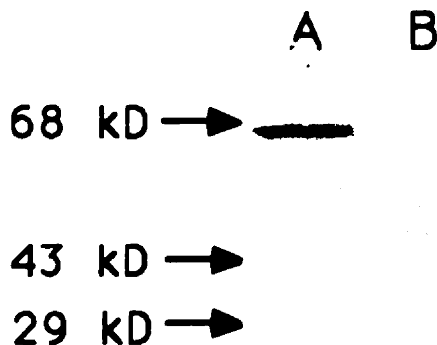

FIG. 3. Western blot of BM71 (lane A) and *ptsI* mutant DC10 (lane B) with anti-EI antibody. Each sample (total protein, 4 μg) was electrophoresed at 200 V for 60 min in a 12% polyacrylamide gel and blotted to polyvinylidene difluoride membranes (Immobilon P; Millipore) for 1 h at 100 V. Immunodetection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

Detection of the various forms of HPr. Since the possibility 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 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 samples of the same extract permits estimation of the cellular concentration of the four forms of HPr.

All four forms of HPr could be detected in wild-type *S. mutans* BM71 cell extracts; however, only nonphosphorylated HPr and P-(Ser)-HPr could be detected in mutant DC10 cell extracts (Fig. 5). Quantitative analysis of the peaks indicated that the total pool of HPr was not the same in both strains; 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 wild-type BM71, the major component was the doubly phosphorylated [P~(His)-P-(Ser)-HPr] fraction (63.0 μg mg^{-1}), while

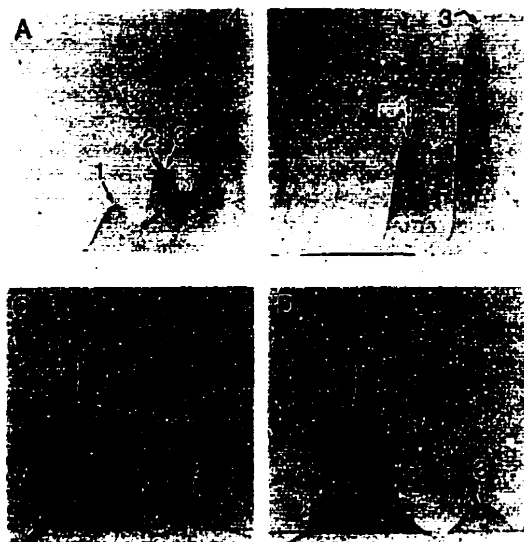

FIG. 5. Crossed immunoelectrophoresis of membrane-free extracts from *S. mutans* BM71 and DC10 cells grown in batch culture. Each sample contained 10 μg of cytoplasmic proteins and was probed with polyclonal anti-HPr rabbit antibodies directed against *S. mutans* DR0001. Panels: A, untreated extract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

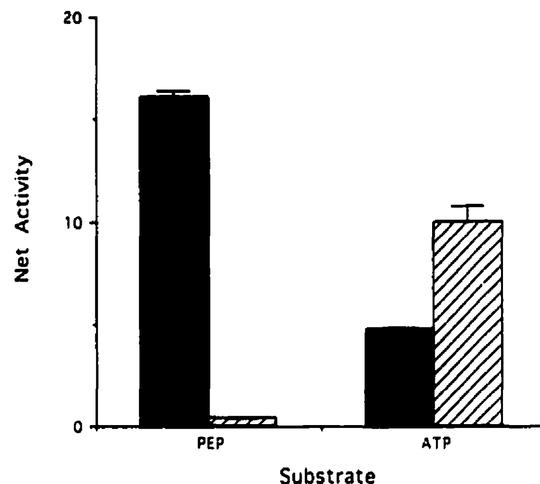

FIG. 6. Glucose phosphorylation by decryptified cells of *S. mutans* BM71 (■) and *ptsI* mutant DC10 (▨) incubated with PEP or ATP. Net activity is expressed as nanomoles of glucose phosphorylated milligram (dry weight) of cells^{-1} minute^{-1}.

P~(His)-HPr and free HPr were present at 19.5 and 12.1 μg mg^{-1}, respectively, and P~(Ser)-HPr was present at only 4.0 μg mg^{-1}. In mutant DC-10, 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 the 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 to 10 mM. Experiments with BM71 revealed apparent K_t values of 17 and 138 μM and V_{max} 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_t of 125 μM and a V_{max} 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 mutant DC10 but predominant in the parent strain. Substantial glucose-6-phosphate was formed in the presence of ATP in the mutant and to a lesser extent in the parent. The experiment was repeated with cells preincubated with 2-deoxyglucose to deplete the intracellular PEP pool, and the level of phosphorylation by PEP and ATP was similar to that seen with nondepleted 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 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), 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 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 fourfold 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; however, uptake studies with 6-deoxyglucose (6) questioned this model. This latter conclusion was

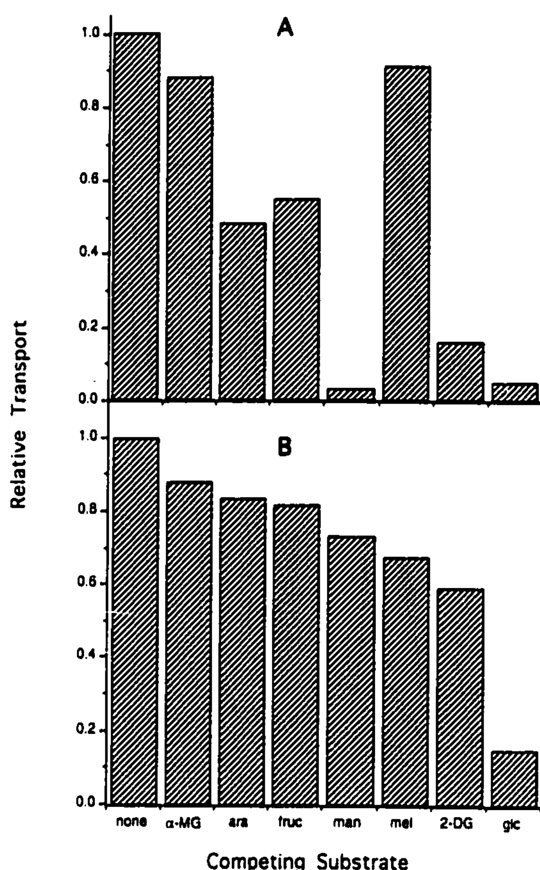

FIG. 7. Sugar competition for [^{14}C]glucose transport with intact cells of wild-type *S. mutans* BM71 (A) and *ptsI* mutant DC10 (B). Competing sugars were used at a concentration 100-fold in excess of that of the labelled glucose. Abbreviations: α-MG, α-methylglucoside; ara, arabinose; fruc, fructose;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

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_t 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 those 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_t 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_t 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 (36). 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.

ACKNOWLEDGMENTS

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

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