IDENTIFICATION OF AN ATP-BINDING CASSETTE CYSTINE IMPORT SYSTEM OF *STREPTOCOCCUS MUTANS* AND ITS TRANSCRIPTIONAL REGULATION

By

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Jennifer Kim, Master of Science (2008)
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ABSTRACT

The polar amino acid ATP-binding cassette (ABC) transporter, CysBPA, was shown to mediate L-cystine uptake in *Streptococcus mutans*. Transcriptional analysis enabled identification of a tricistronic operon and the transcriptional start site for *cysBPA*. Phenotypic characterization of the Δ*cysBPA* mutant showed a 28% decrease in biofilm biomass and formation of sparse, fragile biofilms when compared to wild-type biofilm grown in a semi-defined minimal medium. While assessing cystine transport, the Δ*cysBPA* mutant was less efficient at L-cystine uptake and its ability to grow was severely impaired under L-cystine starvation conditions. A substrate competition assay showed that L-cystine uptake by CysBPA was strongly inhibited by DL-cystathionine and L-djenkolic acid and moderately inhibited by *S*-methyl-L-cysteine and L-cysteine. Through gene expression analysis, the cystine transporter was shown to be up-regulated under cystine starvation and in biofilm-grown cells under acidic conditions. In summary, the CysBPA transporter is involved in cystine transport, growth, and biofilm formation.

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CHAPTER 1: INTRODUCTION

1.1 Background and Cariogenicity of *Streptococcus mutans*

One of the most common bacterial infections in humans is dental caries, which leads to tooth decay (Kuramitsu, 1993; van Houte, 1994). Dental caries is the process of enamel, dentine, and cementum dissolution by acidic end-products due to bacterial fermentation of carbohydrates such as sucrose, glucose, or fructose (Selwitz *et al.*, 2007). When pH levels drop due to acid production and the 'critical pH' (approximately pH 5.5) is surpassed, the rate of demineralization exceeds remineralization of the tooth enamel, eventually resulting in decay. Critical pH, however, can vary among individuals depending on the calcium and phosphate concentrations in their saliva and plaque fluid (Dawes, 2003). Dental plaque or microbial biofilm formed on the tooth surface contributes to the occurrence of dental caries (Gibbons and van Houte, 1975). Plaque development can occur at stagnation sites and the properties of plaque, such as diffusion of carbohydrates and the retention of acids, contribute to the rampant occurrence of caries (Newman, 1986). The formation of caries is dependent on factors such as the concentration of carbon source, the amount and activity of the plaque microflora, the rate of salivary flow, and the specific tooth surface site (Hamilton, 2000).

In 1890, W. D. Miller postulated the parasitic germ theory of dental decay, which suggested that oral bacteria produced acid that solubilized calcium phosphate of enamel and caused caries. In 1924, J. K. Clarke isolated *Streptococcus mutans* from carious lesions, which later became the most investigated etiologic agent involved in dental caries.

In 1944, clinical studies using antimony electrodes for pH measurement showed that 'resting' plaque pH values decreased with increased caries activity, i.e. people with severe caries had low plaque pH values (Stephan, 1944). Then, in 1955, to further emphasize that caries was a bacteria-mediated disease, germ-free rats with a high sugar diet were infected with enterococci isolated from conventional rats with dental caries (Orland *et al.*, 1955). It was found that the infected germ-free rats also developed caries. Without fail, *S. mutans* can always be isolated from carious lesions or saliva from individuals with caries (Kohler *et al.*, 1981; Kristoffersson *et al.*, 1985).

In the late 1960s and early 1970s, Bratthall and co-workers showed that *S. mutans* was actually a group of organisms that could be distinguished on the basis of serology. Later, biochemical traits and phylogeny distinguished several species within the *S. mutans* group. Mutans streptococci (MS) members are found in plaque and possess the ability to ferment mannitol and sorbitol, produce extracellular glucans from sucrose, and induce cariogenicity in animal models (reviewed in Loesche, 1986). The MS members include *S. mutans*, *S. rattus*, *S. cricetus*, *S. sobrinus*, *S. ferus*, *S. macacae*, and *S. downei* (Coykendall, 1974, 1977; Facklam, 2002; Whiley and Beighton, 1998). Strains found in the MS group are usually α-hemolytic or non-hemolytic on blood agar (Whiley and Beighton, 1998). MS members are able to ferment a wide range of carbohydrates including *N*-acetylglucosamine, esculin, amygdalin, arbutin, cellobiose, galactose, inulin, lactose, maltose, mannitol, melibiose, raffinose, salicin, sorbitol, tagatose, and trehalose (Whiley and Beighton, 1998). In addition, MS group strains are identified by their resistance to bacitracin and a G+C molecular % content of 36-38% (Whiley and Beighton,

1998). Based on epidemiological studies, *S. mutans* was the predominant MS found in diverse populations and the first MS to colonize newly erupted teeth in infants (Berkowitz *et al.*, 1975).

1.2 Virulence Factors

1.2.1 Adherence and colonization by *S. mutans*

A biofilm is a complex aggregation of microorganisms enclosed in an extracellular matrix and attached to a surface. The steps involved in forming biofilms include: the initial attachment of the microbes to a surface, growth and aggregation of cells into microcolonies, maturation of the biofilm, and cell dispersion. Biofilms have interesting properties that pose clinical problems such as their tolerance to antimicrobial agents, environmental stresses, shear forces, protection from phagocytosis, host defences and predators, and changes in metabolic efficiency (reviewed in Marsh, 2000; O'Toole *et al.*, 2000). Dental plaque formation is a continuous, dynamic process involving several steps: 1) the tooth surface is covered by host and bacterial polymers that form a conditioning film (acquired enamel pellicle); 2) microbes are transported to the tooth surface via oral secretions; 3) the physico-chemical interactions between the acquired pellicle and the charged bacterial surface facilitate reversible adhesion; 4) the stereo-chemical interaction between bacterial adhesins and receptors in the acquired pellicle facilitate irreversible adhesion; 5) the co-aggregation of micro-organisms to the attached cells occurs; 6) bacterial cells divide and the biofilm develops and grows (reviewed in Marsh, 2000).

Immediately after eruption or mechanical scaling, the tooth surface is first covered by an acquired enamel pellicle consisting of proline rich proteins, glycoproteins, statherin, mucins, and α-amylase (reviewed in Lamont and Jenkinson, 2000). Early colonizing bacteria are predominantly streptococci but other early colonizers such as *Actinomyces naeslundii* and *Fusobacterium nucleatum* also express adhesins that facilitate attachment to the tooth surface. For example, statherin is recognized by fusobacteria and actinomyces but not streptococci, which bind to α-amylase (Kolenbrander *et al.*, 1993). One of the early colonizers, *Streptococcus sanguinis* is known to have a specific interaction with *S. mutans* through surface recognition (Liljemark and Schauer, 1977). Since streptococci and actinomyces are facultative anaerobes, their presence changes the environment and facilitates the acquisition of other oral bacteria such as veillonellae, propionibacteria, rothiae, capnocytophagae, and prevotellae (Kolenbrander *et al.*, 1993). Hence, the coaggregation of different oral bacteria contributes to the succession of plaque development.

S. mutans is capable of tooth colonization initially through sucrose-independent attachment to the acquired salivary pellicle. An *S. mutans* cell wall-anchored protein called antigen I/II (B, SpaP, P1, or Pac) is able to interact with salivary agglutinin glycoprotein facilitating non-sucrose-mediated adherence and aggregation of *S. mutans* (Douglas, 1994). Further colonization occurs through sucrose-dependent cellular accumulation. In the presence of sucrose, extracellular glucans are synthesized by secreted enzymes known as glucosyltransferases (GTFs). The glucans comprise the extracellular polysaccharide layer that contributes to dental plaque development by oral streptococci. It was demonstrated that three *gtf* genes (*gtfB*, *gtfC*, and *gtfD*) are present on the

chromosome of most strains of *S. mutans* (Kuramitsu, 1993). GtfB, GtfC, and GtfD synthesize water-insoluble glucans, partly water-soluble glucans, and water-soluble glucans, respectively. Streptococcal GTFs show distinct domains that facilitate their function including a signal peptide, a poorly conserved N-terminal region, a highly conserved catalytic domain, and a glucan-binding C-terminal domain (Honda *et al.*, 1990; Shiroza *et al.*, 1987; Ueda *et al.*, 1988). The glucan-binding domain mediates co-aggregation of *S. mutans*, surface adhesion, and overall cohesiveness of plaque. Gibbons and Fitzgerald (1969) showed that cell-bound GTFs facilitated agglutination of *S. mutans* in the presence of glucans, i.e. GTFs functioned as receptors for glucans. The expression and activity of GTF enzymes can be altered by environmental conditions such as pH, carbohydrate availability, and growth phase (Li and Burne, 2001; Wexler *et al.*, 1993).

The non-enzymatic glucan-binding proteins (GBPs) produced by *S. mutans* are also associated with its virulence. GBPs can occur as surface proteins or in secreted forms, which also become cell-associated when glucan is present (Banas *et al.*, 1990). *S. mutans* GBPs permit cohesive plaque formation, cellular adherence and accumulation (reviewed in Banas and Vickerman, 2003; Smith *et al.*, 1994). Various GBP knock-out studies concluded that GBPs were involved in cellular adherence, hence, contributing to the cariogenicity of *S. mutans* (Hazlett *et al.*, 1999).

1.2.2 Acidogenicity, aciduricity, and the acid tolerance response

S. mutans is classified as a heterofermentative bacterium, which metabolizes a wide range of carbohydrates to produce acidic end products (Hamada and Slade, 1980).

The way in which *S. mutans* metabolizes sugar is dependent on general environmental factors. Although the main fermented product is lactate, *S. mutans* is also able to produce other acids. Under aerobic conditions, *S. mutans* only produces lactate as pyruvate formate-lyase, an enzyme required in conversion of pyruvate to formate, acetate and ethanol, becomes inactivated (Iwami *et al.*, 2000). In contrast, in an anaerobic environment, the bacterium is able to produce lactate, formate, acetate, and ethanol (Iwami *et al.*, 2000). Lactate is the major end product when glucose is present in excess, while formate, acetate, and ethanol are formed under limited glucose growth conditions (Abbe *et al.*, 1982).

The ability to produce acidic end products is called acidogenicity and *S. mutans* is one of the most acidogenic species present in the oral flora. The fermentation of various sugars results in large quantities of lactate production. In turn, lactate complexes with calcium found in hydroxyapatite crystals of the tooth surface leading to its demineralization (Balakrishnan *et al.*, 2000). Compared with other streptococci, *S. mutans* produces acid at the fastest rate and over a wide pH range (pH 5-8) (de Soet *et al.*, 2000). *In vivo* plaque-pH telemetry studies showed that carbohydrate intake could lower pH from 7 to 4 in three minutes depending on the age of the biofilm and the concentration of carbohydrates (Jensen *et al.*, 1982).

Aciduricity is the ability to survive and grow at low pH. Since *S. mutans* generates large amounts of acid, it is imperative to have the physical and genetic components necessary to handle such harsh environments. When *S. mutans* grows at low pH, changes in cell physiology occur. During growth at pH 5.2, the proteins that showed an increase in synthesis include neutral endopeptidase, phosphoglucomutase, 60-kDa chaperonin, cell

division proteins, enolase, lactate dehydrogenase, fructose bisphosphate aldolase, acetoin reductase, superoxide dismutase, and lactoylglutathione lyase (Wilkins *et al.*, 2002). It is important to express certain proteins at higher levels because they are involved in protective mechanisms against shock and environmental stresses. In addition, proteins that showed a decrease in synthesis under acidic growth include protein translation elongation factors G, Tu, and Ts, DnaK, small-subunit ribosomal protein S1P, large-subunit ribosomal protein L12P, and components of both phosphoenolpyruvate:protein phosphotransferase and multiple sugar binding transport systems (Wilkins *et al.*, 2002). These proteins are found at low levels because the growth rate is reduced at low pH, thereby decreasing protein synthesis and altering sugar metabolism. As well, during glucose uptake and glycolysis, the pH optimum changes when cells are subjected to an acidic pH. The response to pH suggests that glucose transport mechanisms are also being regulated by the organism to quickly handle the new conditions (Hamilton and Buckley, 1991).

Under acidic conditions, *S. mutans* changes its physiology in order to adapt to the new environment. A pre-exposure to a non-lethal acidic pH (e.g. pH 5.5) can induce an adaptive response known as the acid tolerance response (ATR). Induction of the ATR by *S. mutans* enables better survival at very low pH. One feature of the ATR includes increased efficiency in the well-studied proton-translocating ATPase. The proton-translocating ATPase is necessary in order to maintain the pH gradient across the cell membrane (Belli and Marquis, 1991; Li *et al.*, 2001; Wilkins *et al.*, 2002). Other features of the ATR include decreased proton permeability (Bender *et al.*, 1986), increased synthesis of chaperonins (Jayaraman *et al.*, 1997), increased *ffh* (a 54kDa homologue of

the eukaryotic signal recognition particle) gene expression for targeting membrane-associated proteins (Gutierrez *et al.*, 1999), changes in membrane fatty acid composition (Quivey and Fozo, 2004), and up-regulation of DNA repair systems (Hanna *et al.*, 2001; Li *et al.*, 2001; Svensäter *et al.*, 2000).

1.3 *S. mutans* Growth and Amino Acid Requirements

For colonization of the tooth surface and dental plaque formation, microorganisms need to extract nutrients from their environment. The oral environment provides nutrients through host-derived factors, such as glycoproteins present in saliva and the host's diet. The production of bacterial exoglycosidases enable bacteria to break down the oligosaccharide side-chains found on glycoproteins into fermentable monosaccharides and amino sugars providing a readily available source of both carbon and nitrogen for bacterial growth (Smith and Beighton, 1986). The utilization of salivary glycoproteins is advantageous since the level of free sugars and free amino acids are low in saliva. Another main source of energy for oral bacteria comes from human dietary carbohydrates. However, availability fluctuates and bacteria need survival mechanisms when nutrients are limited. The carbohydrates are fermented by plaque bacteria into organic acids which, in turn, can be a source of nutrients to other oral bacteria.

Dental plaque can form with only the nutrients present in saliva (de Jong *et al.*, 1984; reviewed in Rudney, 2000). The availability of nutrients can impact the exopolysaccharide composition and community structure of biofilms at various stages of development. The nutrients and bacterial enzymes found in saliva can also affect the composition of surface conditioning films that facilitate bacterial adherence and help

propagate biofilm development (Bowden and Li, 1997). The type and concentration of certain nutrients can enable certain bacterial populations to be favored over others. For instance, the acquired enamel pellicle attracts early colonizers such as *S. sanguinis*, *Streptococcus mitis*, *Streptococcus oralis*, and *Actinomyces spp.* (Scheie, 1994). Once a biofilm becomes established, different bacterial communities will compete for nutrients and organisms need mechanisms of nutrient extraction and defence mechanisms to fend off other bacteria. The thickness of a biofilm also affects the diffusibility of nutrients to various areas of the biofilm.

In a study by Carlsson (1970), the nutritional requirements for *S. mutans* were investigated. A chemically-defined minimal medium was used to determine which components were absolutely required for *S. mutans* growth. Under aerobic cultivation, the amino acids that were proven to be essential were cysteine and glutamic acid. In addition, uracil was required for growth but the absence of adenine and guanine had no effect on growth yield. The vitamins required for growth include biotin, *p*-aminobenzoic acid, thiamine HCl, riboflavin, pyridoxine HCl, Ca-pantothenate and nicotinic acid. Folic acid was the only vitamin tested that was not necessary for growth. Finally, cysteine could be substituted with sodium thioglycolate. However, a later study by Terleckyj and Shockman (1975) showed that thioglycolate could not be substituted for cystine. In contrast, the requirements for growth under anaerobic conditions include the presence of glucose, cysteine, ammonium sulphate, salts, and all the required vitamins, except folic acid. Simple nutritional requirements may be advantageous for *S. mutans* especially in anaerobic environments with abundant ammonia and the lack of exogenous amino acids.

The presence of oxygen can affect the nutritional profile of *S. mutans*. For example, as little as 1.5% oxygen in the environment can alter the requirement for amino acids (Cowman *et al.*, 1974). Amino acid requirements varied depending on the *S. mutans* serological group. *S. mutans* serotype *c*, the predominant serotype strain found in the human oral cavity, had the simplest amino acid requirements among the different groups (Cowman *et al.*, 1974). Type *c* cultures only required aspartic acid or glutamic acid and cysteine for growth under aerobic conditions. Moreover, specific amino acids could cause an inhibitory effect on *S. mutans* growth. Depending on the serotype and oxygen levels, many *S. mutans* strains were inhibited by the presence of valine, isoleucine, leucine, lysine, or methionine (Cowman *et al.*, 1974).

Further studies showed that few amino acids were needed when *S. mutans* was grown anaerobically in the presence of carbonate or bicarbonate (Terleckyj and Shockman, 1975). Under aerobic and anaerobic conditions, cystine was the only amino acid that was required by all serotypes of *S. mutans* (Terleckyj and Shockman, 1975). The reduced form, cysteine, was also required for growth in lieu of cystine and no discernible differences were seen between either form of the amino acid (Terleckyj and Shockman, 1975). When grown aerobically in the absence of carbonate, it was observed that glutamate or glutamine was needed in addition to cystine (Terleckyj and Shockman, 1975). The amino acids that were not crucial for aerobic growth without carbonate include glycine, alanine, serine, threonine, methionine, proline, hydroxyproline, phenylalanine, and tryptophan (Terleckyj and Shockman, 1975). Also, the investigators could not reproduce the growth inhibition by leucine, valine, isoleucine, methionine, or lysine, as described previously by Cowman *et al.* (1974). Hence, controversy still remains over the

true nutritional requirements for *S. mutans* with these requirements likely varying between strains.

Despite studies showing the need for cysteine/cystine and glutamate/glutamine for growth, several laboratory strains of *S. mutans* are capable of growing anaerobically without any amino acid supply (St. Martin and Wittenberger, 1980). *S. mutans* was able to assimilate ammonia for the biosynthesis of amino acids through glutamate dehydrogenase, glutamine synthetase, or glutamate synthase activity (Cvitkovitch *et al.*, 1997; St. Martin and Wittenberger, 1980). Furthermore, *S. mutans* possesses a complete citrate pathway, which enables the anaerobic synthesis of glutamate and subsequently, the synthesis of other amino acids and proteins (Cvitkovitch *et al.*, 1997).

1.4 Sulfhydryls and the Importance of Cystine and Cysteine

Sulfur is important for all living organisms including bacteria. It is required for the synthesis of coenzymes and essential amino acids such as cysteine and methionine (Gottschalk, 1986). Sulfur is usually acquired as sulfate and then, reduced by the bacteria to sulfide. However, some bacteria are capable of utilizing reduced sulfur compounds. For example, hydrogen sulfide is required for growth of methanogenic bacteria. In other cases, bacteria such as thiobacilli or phototrophic bacteria use sulfide, elemental sulfur, or thiosulfate (Gottschalk, 1986). Thus, sulfur is necessary in sustaining life.

A sulfhydryl group is a chemical compound that contains the sulfur atom and hydrogen atom (-SH) as a functional group. In living cells, sulfhydryls can be found in proteins (due to the presence of cysteine residues) and as low-molecular-weight sulfhydryl

compounds in the cytoplasm. In all living organisms, protein sulfhydryl groups are crucial for proper enzyme activity and function of many proteins (Thomas, 1984). Furthermore, sulfhydryl groups provide reducing environments that protect against oxidation and other forms of chemical modifications (Thomas, 1984).

Bacteria can utilize both cysteine and cystine as sources of sulfur. Cysteine is one of the twenty naturally occurring amino acids and contains a sulfur atom (Fig. 1A). When two molecules of cysteine undergo oxidation, cystine is formed (Fig. 1B). Cystine possesses a disulfide bond, which in turn, may be required for proper protein folding or the stability and function of the protein (Ritz and Beckwith, 2001). The reduction and oxidation (redox) of disulfide bonds are mediated by thiol-redox enzymes, which facilitate the reversible process of turning a sulfhydryl into a disulfide. Disulfide bond reactions enable electron transfer to occur and allow enzymes to reactivate (Ritz and Beckwith, 2001). The redox state can also impart control over protein function. When cysteine residues undergo oxidation, the protein may be activated or inactivated, hence, cysteine and cystine are important to the organism's physiology.

In animals, cysteine can be synthesized only from methionine that is acquired from the host's diet. In contrast, bacteria are capable of synthesizing cysteine by acquiring inorganic sulfur from the environment. In enteric bacteria, *O*-acetylserine is formed from serine by serine transacetylase (CysE). Then, by incorporating sulfide or thiosulfate, cysteine can be formed by the catalytic action of *O*-acetylserine (thiol)-lyase-A (CysK) or *O*-acetylserine (thiol)-lyase-B (CysM) (Fig. 2). There are at least nine different groups of cysteine biosynthetic genes in enteric bacteria (Kredich, 1992). Among the nine groups, six or more operons are positively regulated, while *cysB* (transcriptional activator protein),

cysE, and *cysG* (sulfite reductase) are genes that are negatively autoregulated (Kredich, 1992). All the cysteine biosynthetic genes except *cysG* are considered to be a part of the cysteine regulon (Kredich, 1992). *Bacillus subtilis* can also convert inorganic sulfur in a pathway similar to enteric bacteria. *B. subtilis* possesses a *cysH* operon that encodes a 3'-phospho-adenosine-5'-phosphosulfate reductase, a sulfate permease (CysP) and enzymes that reduce sulfate to sulfite (Albanesi *et al.*, 2005). When sulfide is present, an *O*-acetylserine-(thiol)-lyase (cysteine synthetase) catalyzes the reaction for cysteine synthesis (Albanesi *et al.*, 2005) (Fig. 2). In *S. mutans*, putative cysteine biosynthesis genes are encoded in the genome, and the cysteine biosynthesis pathway is deduced from knowledge gained from other bacteria such as *Escherichia coli* and *B. subtilis*. Alternatively, the citrate pathway used by *S. mutans* for the anaerobic synthesis of glutamate and the assimilation of ammonia can lead to synthesis of other amino acids, including cysteine (Cvitkovitch *et al.*, 1997).

Cystine and cysteine can be used as a source of nutrition. Previous studies showed that various strains of *S. mutans* needed cystine or cysteine under all *in vitro* conditions tested (anaerobic, aerobic, and addition of carbonate) (Terleckyj and Shockman, 1975). However, cysteine can be toxic to many microorganisms and inhibition of *S. mutans* growth by cysteine was seen at concentrations higher that 1.67 mM while cystine was non-toxic up to 6 mM (Cowman *et al.*, 1983). Inhibition of *S. mutans* growth by cysteine could be overcome by the presence of valine, isoleucine, and leucine (Cowman *et al.*, 1983).

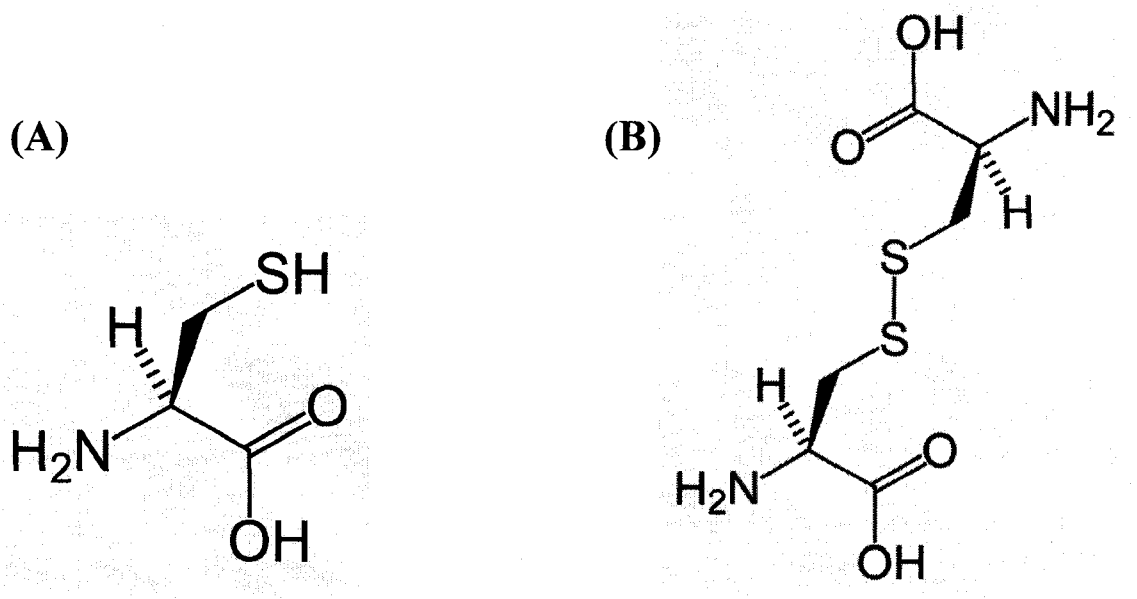


Figure 1. Chemical structures of cysteine (A) and cystine (B).

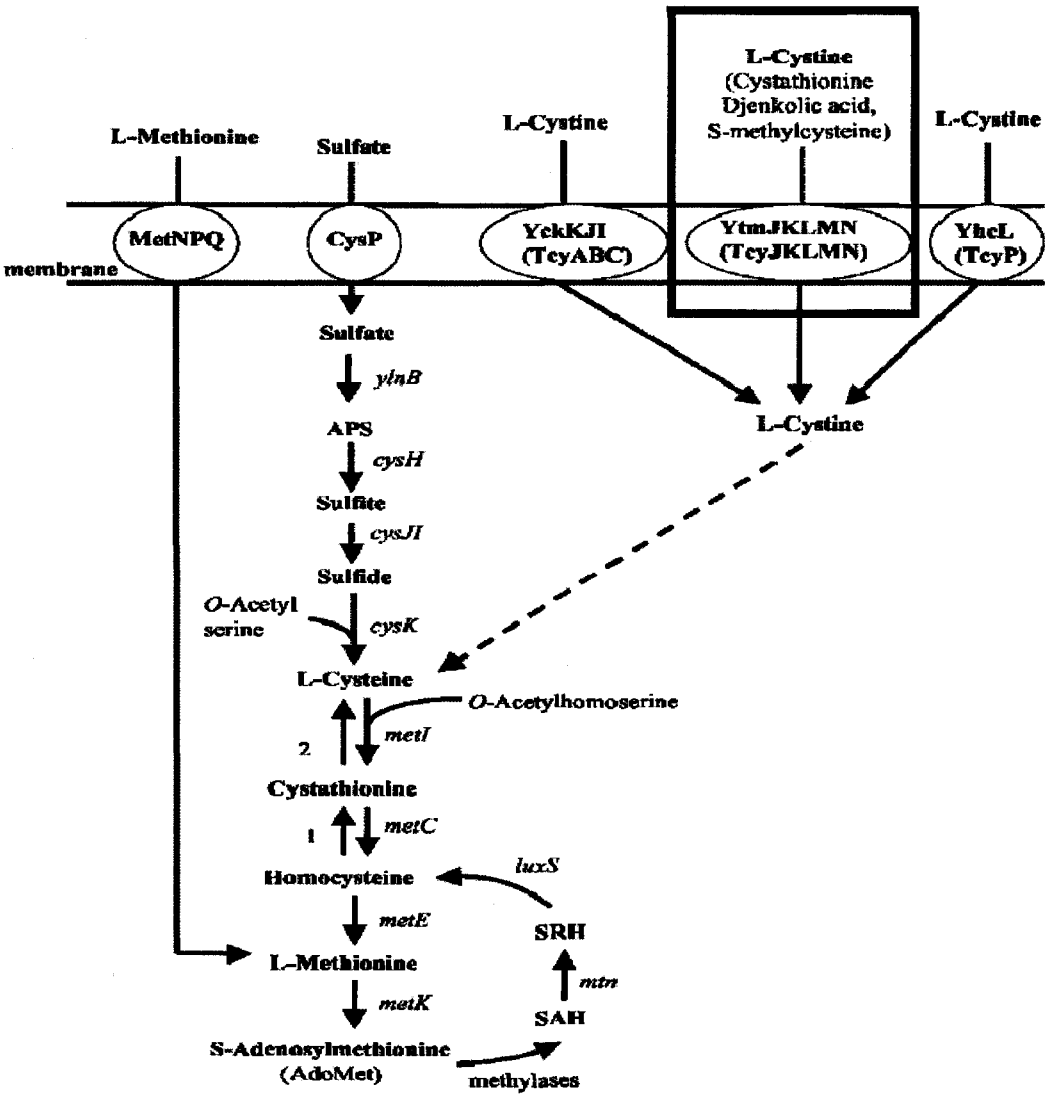


Figure 2. Transport and biosynthesis of cystine and sulfur-containing substrates in *B. subtilis.* The genes and corresponding enzyme are described as follows: *ylnB*, ATP sulfurylase; *cysH*, adenosine 5'-phosphosulfate reductase; *cysJI*, sulfite reductase; *cysK*, O-acetylserine sulfhydrylase; *metI*, cystathionine γ-synthase; *metC*, cystathionine β-lyase; *metE*, methionine synthase; *metK*, *S*-adenosylmethionine synthase; *mtnA*, *S*-adenosyl homocysteine/methylthioadenosine nucleosidase; *luxS*, *S*-ribosylhomocysteine hydrolase; *cysP*, sulfate permease; *metNPQ*, methionine permease; *tcyABC*, cystine ABC transporter; *tcyJKLMN*, cystine ABC transporter; *tcyP*, cystine symporter.

(Figure extracted from (Burguiere *et al.*, 2004)

1.5 ABC Transporters and Cystine Transport

The ATP-binding cassette (ABC) transporter superfamily contains both uptake and efflux transport systems (Higgins, 2001). According to the transport classification database (Saier Jr., 2000a) (www.tcdb.org), the ABC superfamily is currently divided into 22 subfamilies of prokaryotic importers, 24 subfamilies of prokaryotic exporters, and 10 subfamilies of eukaryotic proteins. These proteins bind ATP and use the energy to actively transport various molecules across cell membranes. The porters of the ABC superfamily consist of two integral membrane domains/proteins and two cytoplasmic domains/proteins. The uptake systems (but not the efflux systems) additionally possess extracytoplasmic solute-binding receptors. In Gram-negative bacteria, the solute-binding proteins are found in the periplasm while in Gram-positive bacteria, they are present either as a lipoprotein or as a cell surface-associated protein.

In microorganisms, ABC transporters are essential to antibiotic, antifungal resistance and the transport of various nutrients (Higgins, 2001). The bacterial ABC transporters involved in solute uptake require the presence of a ligand-binding protein. In Gram-negative bacteria, a periplasmic binding protein (PBP) interacts with the incoming substrate. In Gram-positive bacteria, which lack the outer membrane and periplasm, a substrate binding protein (SBP) is anchored to the transporter domains by lipoproteins or becomes cell surface-associated via electrostatic interactions. It is the PBP and SBP that determine specificity of the ABC transporter. The functions of the PBP and SBP include conferring high affinity and specificity for substrates and directionality of solute transport into the cell (Higgins, 2001). The substrate binding proteins are absolutely necessary

because they enable transmission of a transmembrane signal for ATP hydrolysis to occur (Davidson *et al.*, 1992; van der Heide and Poolman, 2002).

One family of the ABC superfamily is the polar amino acid uptake (PAAT) transporters. There are 15 types of PAAT transporters, in which the majority is very solute-specific or transports a narrow-range of substrates (Table 1). Typically, these transporters will only transport a single amino acid or a group of structurally related amino acids (Hosie and Poole, 2001). In stark contrast, there are some types of transporters that have a broad solute specificity. *Rhizobium leguminosarum* possesses a general amino acid permease (AapJQMP) which will transport a wide range of acidic, basic, and aliphatic L-amino acids (Walshaw and Poole, 1996). Narrow-range PAATs tend to have smaller integral membrane proteins than the broad-range PAATs (Hosie and Poole, 2001). The AapQM transmembrane protein of *R. leguminosarum* spans the membrane 8-9 times, while the narrow-range PAATs are predicted to have five transmembrane spanning segments (Walshaw *et al.*, 1997).

TABLE 1. Representative examples of bacterial polar amino acid ABC transporters*

Transport classification number	Species	ATP-binding protein	Transmembrane protein	Substrate-binding protein	Solute
3.A.1.3.1	*Salmonella typhimurium*	HisP	HisM, HisQ	HisJ ArgT	Histidine, Arginine, Lysine, Ornithine
3.A.1.3.2	*E. coli*	GlnQ	GlnP	GlnH	Glutamine
3.A.1.3.3	*E. coli*	ArtP	ArtQ, ArtM	ArtJ, ArtI	Arginine
3.A.1.3.4	*E. coli*	GltL	GltJ, GltK	GltI	Glutamate, Aspartate
3.A.1.3.5	*Agrobacterium tumefaciens*	OccP	OccQ, OccM	OccT	Octopine
3.A.1.3.6	*A. tumefaciens*	NocP	NocQ, NocM	NocT	Nopaline
3.A.1.3.7	*Rhodobacter capsulatus*	BztD	BztB, BztC	BztA	Glutamate, Glutamine, Aspartate, Asparagine
3.A.1.3.8	*Rhizobium leguminosarum*	AapP	AapQ, AapM	AapJ	L-amino acids
3.A.1.3.9	*Corynebacterium glutamicum*	GluA	GluC, GluD	GluB	Glutamate
3.A.1.3.10	*E. coli*	CysZ	CysY	CysX	Cystine, Diaminopimelate
3.A.1.3.11	*Pseudomonas aeruginosa*	AotP	AotQ, AotM	AotJ	Arginine, Ornithine
3.A.1.3.12	*Synechocystis* PCC6803	BgtA	BgtB	BgtB	Arginine, Lysine, Histidine, Glutamine
3.A.1.3.13	*B. subtilis*	TcyN	TcyL, TcyM	TcyJ, TcyK	L-cystine, L-cystathionine, L-djenkolate, *S*-methyl-L-cysteine
3.A.1.3.14	*B. subtilis*	TcyC	TcyB	TcyA	L-cystine
3.A.1.3.15	*B. subtilis*	YqiZ	YqiY	YqiX	Arginine

* According to Transport Classification Database (www.tcdb.org)

In the *S. mutans* UA159 genome, approximately 15% of the total ORFs are associated with transport systems (Ajdic *et al.*, 2002). Most of the *S. mutans* UA159 transporters are ATP-dependent and identified as either P-type (transports calcium and potassium), F-type (synthesizes ATP using a proton gradient), and ABC-type (transports solutes by ATP hydrolysis). The ABC-type is the most numerous in *S. mutans* UA159, as there are more than 60 different putative ABC transporters (Ajdic *et al.*, 2002). Some of the encoded ABC transporters include putative transporters for amino acids, branched-chain amino acids, aspartate, and glutamine (Table 2). However, phenotypic characterization of amino acid ABC transporters in *S. mutans* has been limited.

To date, there are three different L-cystine uptake systems in *B. subtilis* that have been identified. The symporter TcyP (YhcL) and two ABC transporters, TcyJKLMN (YtmJKLMN) and TcyABC (YckKJI) (Fig. 3) were identified using bioinformatic analysis and differential gene expression in response to sulfur availability or screening for selenocystine (a toxic analogue of L-cystine) resistance (Burguiere *et al.*, 2004). The TcyP symporter is a membrane protein belonging to the dicarboxylate amino acid:cation (Na^+ and/or H^+) symporter family (2.A.23) (Saier Jr. *et al.*, 2002), which transports dicarboxylic acids, semi-polar and neutral amino acids (alanine, serine, cysteine, and threonine), neutral and acidic amino acids, or dibasic amino acids (Saier Jr., 2000b). The ABC transporter TcyJKLMN belongs to the polar amino acid uptake transporter family (3.A.1.3) and it has high similarity to the L-cystine uptake system from *E. coli* (Coppee *et al.*, 2001). In *B. subtilis*, The TcyJKLMN transporter was found to transport other compounds including cystathionine, *S*-methyl-L-cysteine, djenkolic acid, and other sulfur sources (Albanesi *et al.*, 2005; Burguiere *et al.*, 2004). The TcyJKLMN transporter is

encoded within a large operon called the *ytmI* operon. The *ytmI* operon appeared to be the most sensitive to L-cystine starvation compared to the other transporters. Its expression was repressed more than 200-fold in the presence of sulfate or L-cystine. In addition, some genes from the operon were induced during disulfide stress by the thiol oxidant diamide, which caused the formation of non-native disulfide bonds (Burguiere *et al.*, 2004; Leichert *et al.*, 2003). Both the *ytmI* operon and the *tcyP* gene showed increased expression in the presence of methionine rather than in the presence of sulfate. In contrast, *tcyABC* mRNA levels remain unchanged at low concentration of sulfur (Burguiere *et al.*, 2004). The TcyABC transporter is also characterized as a polar amino acid ABC transporter (3.A.1.3) involved in L-cystine transport. According to L-cystine uptake experiments, TcyP and TcyJKLMN transporters had high affinity for L-cystine, while TcyABC had a lower affinity (Burguiere *et al.*, 2004).

In *B. subtilis*, YtlI is a positive regulator of the *ytmI* operon (Burguiere *et al.*, 2005). The *ytlI* gene is located upstream of the *ytmI* operon on the *B. subtilis* genome, however, they are divergently transcribed (Burguiere *et al.*, 2005). There was evidence for the existence of a cascade of regulation for the *ytmI* operon. Transcriptional regulation of *ytlI* occurred in response to sulfur availability independently of YtlI, suggesting the involvement of another regulator (Burguiere *et al.*, 2005). The other regulator involved in *ytmI* operon expression is YrzC, which acts as a negative regulator of the *ytmI* operon (Burguiere *et al.*, 2005). YrzC was identified while screening for mutations in *B. subtilis* with up-regulated *ribR* (a gene encoding a riboflavin kinase and situated within the *ytmI* operon) expression (Solovieva *et al.*, 2005). When *yrzC* mutants were complemented *in trans*, the transcription of the *ytmI* operon was stopped, thereby proving that YrzC acts as

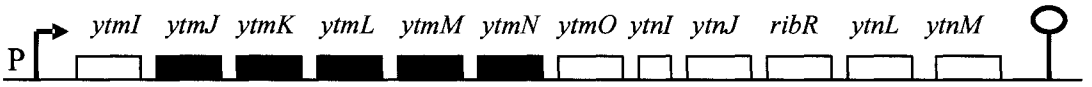
a negative regulator (Solovieva *et al.*, 2005). Further studies showed YrzC as a master regulator of sulfur metabolism in *B. subtilis* (Even *et al.*, 2006). When *B. subtilis* was grown in minimal medium with sulfate as the only sulfur source, YrzC acted as a repressor in gene expression for pathways involved in cysteine formation, such as OAS-thiol-lyase, L-cystine transporters, sulfonate assimilation, and methionine-to-cysteine conversion (Even *et al.*, 2006). The authors renamed YrzC to CymR for "cysteine metabolism repressor".

TABLE 2. *S. mutans* UA159 amino acid ABC transporter-encoding genes*

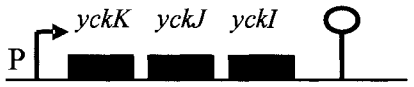
ATP-binding Domain	Membrane-spanning Domain	Solute-binding Domain	Function
SMU.241c	SMU.242c	SMU.242c	Glutamine?
SMU.461	SMU.460	SMU.459	Amino acid
SMU.568	SMU.567	-	Glutamine
SMU.805c	SMU.806c	SMU.806c	Amino acid
-	-	SMU.815	Aspartate
-	-	SMU.817	Amino acid
SMU.936	SMU.935 SMU.934	SMU.933	Glutamine/Truncation
SMU.1178c	SMU.1179c	SMU.1177c	Amino acid
-	SMU.1216c	SMU.1217c	Amino acid
SMU.1519	SMU.1521 SMU.1522	SMU.1520	Amino acid
SMU.1666 SMU.1665	SMU.1668 SMU.1667	SMU.1669	Branched chain amino acids

* According to Oral Pathogen Sequence Databases (www.oralgen.lanl.gov)

A) ABC transporter TcyJKLMN



B) ABC transporter TcyABC



C) Symporter TcyP

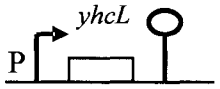


Figure 3. Genetic organization of different L-cystine transporters in *Bacillus subtilis*. The *ytmI*, *yckK*, and *yhcL* operons encode the TcyJKLMN, TcyABC, and TcyP cystine transporters, respectively. The putative promoters and transcriptional terminators are indicated. Red, blue, and green boxes correspond to solute-binding proteins, permeases, and ATP-binding proteins, respectively.

(Figure extracted from (Burguiere *et al.*, 2004)

1.6 Preliminary Data

Work previously done by Dr. Cvitkovitch identified environmentally regulated genes in *S. mutans* using transposon mutagenesis. One of the mutants with increased acid sensitivity, the AS36 mutant, had a 30% lower growth yield than the parent strain NG8 when grown in pH 5 medium. Sequence analysis of the region harboring the transposon revealed that Tn*917* was inserted near the 3' end of a gene (SMU.461) encoding a homolog of an ATP binding protein (Cvitkovitch *et al.*, 2000). The ATP-binding protein is a component of a putative polar amino acid ABC transporter. In this study, the functional role and specificity of the putative polar amino acid ABC transporter is investigated in *S. mutans* UA159.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Disruption of a putative L-cystine ABC transporter in *S. mutans* will lead to a mutant unable to effectively take up sulfur containing substrates and will likely express phenotypic alterations including changes in growth rates with sulfur containing compounds.

2.2 Objectives

1) Generation of mutants in *S. mutans* UA159 and determination of the phenotypic effects of the mutation.
2) Determination of substrate specificity for the putative polar amino acid ABC transporter.
3) Regulation of expression of the genes encoding the L-cystine transporter.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

All strains used in this study and their relevant characteristics are listed in Table 3. *S. mutans* UA159 wild-type strain was cultured on Todd-Hewitt (BD Biosciences) agar supplemented with 0.3% (wt/vol) yeast extract (THYE) or in THYE broth at 37°C in air with 5% CO_2 without agitation. Mutant strains were grown in THYE supplemented with erythromycin at 10 μg per ml. THYE medium was routinely used to grow the strains unless otherwise specified. Optical density (OD) was measured using an Ultrospec 3000 UV/Visible Spectrophotometer (Fisher Scientific).

TABLE 3. Bacterial strains used in the study

Strain or plasmid	Relevant characteristics[a]	Source or reference
S. mutans		
UA159	Wild-type strain; Em^S	J. Ferretti, U. of Oklahoma
SmCysB	UA159 Δ(*cysB*); Em^R	This study
SmCysP	UA159 Δ(*cysP*); Em^R	This study
SmCysA	UA159 Δ(*cysA*); Em^R	This study
SmCysBPA	UA159 Δ(*cysBPA*); Em^R	This study
SmCysR	UA159 Δ(*cysR*); Em^R	This study
Plasmid		
pALN122	*Streptococcus-E. coli* shuttle vector harboring erythromycin resistance cassette; Em^R	(Macrina *et al.*, 1983)

[a] Em: erythromycin

3.2 Construction of the Δ*cysB*, Δ*cysP*, Δ*cysA*, Δ*cysBPA*, and Δ*cysR* Mutants

The *S. mutans* Δ*cysB* (SmCysB), Δ*cysP* (SmCysP), Δ*cysA* (SmCysA), Δ*cysBPA* (SmCysBPA), and Δ*cysR* (SmCysR) mutants were constructed in strain UA159 by a PCR-based deletion strategy involving restriction-ligation and allelic replacement as described previously (Lau *et al.*, 2002). For each single insertion mutant, two sets of primer pairs, named P1-P2 and P3-P4 were used to amplify the 5' and 3' DNA flanking regions of the target region, respectively. The erythromycin resistance cassette was amplified from plasmid pALN122 using the primer pair Erm-F, Erm-R (Table 4). Mutant construction was confirmed by PCR and DNA sequencing using the primers used to generate the mutant.

TABLE 4. Primers used for construction of insertion mutants

Primer	Sequence (5' to 3')[a]	Amplicon (bp)
P1-cysB	GCTGATTTCAACTAAGGGACG	995
P2-cysB	GTAAGGTAAAAGCGACCAAGG	
P3-cysB	TCAGCAGTATTTAGCGGGTG	570
P4-cysB	GGTAAACCTGAGCAGTTGTCATC	
P1-cysP	CAACAGACTCAGATACAGCTCC	800
P2-cysP	CCGTTAGGTAAACTGGCAAC	
P3-cysP	AAGCTGTGGAAGGAGGTGTG	910
P4-cysP	ACGATAAAGAATCCAACCCG	
P1-cysA	CCGATCTTGGTTCAACTGATG	646
P2-cysA	CCGACAAGGGCTACAACTTC	
P3-cysA	ATTCTTGAGCAGGGAACGCC	847
P4-cysA	CGGAAAAAAGCACCATCAC	
P1-cysR	TGGACTGGGCAATCTCATCACC	663
P2-cysR	TGGTAACTGCTGGTTGTGTAATGTG	
P3-cysR	GAATCTCCTTTTTCTATCGCAG	728
P4-cysR	TCTGTCAGGCTTCCACTATTG	
Erm-F	GGCGCGCCCCGGGCCCAAAATTTGTTTGAT	876
Erm-R	GGCCGGCCAGTCGGCAGCGACTCATAGAAT	

[a]An *Asc*I restriction site has been added at the 5'-end of the P2 primers, while an *Fse*I restriction site has been added at the 5'-end of the P3 primers. Primers were designed and analyzed with MacVector 7.2 software.

3.3 Northern Blot

3.3.1 Northern blot experiments

S. mutans UA159 strain was grown to mid-log phase (OD_{600} ~0.4 – 0.5) in 25 ml of THYE broth. Cells were harvested by centrifugation (4,000 × *g*, 15 min, 4°C) and total RNA was extracted using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. Twenty microgram aliquots of each isolated RNA preparation were treated with 30 U of RQ1 RNase-free DNase (Promega) and repurified through RNeasy columns according to the manufacturer's protocol.

Five micrograms of each RNA sample and ladder (Invitrogen) were separated by electrophoresis on a 1% (wt/vol) agarose-formaldehyde gel and transferred to a nylon membrane as described by Sambrook and Russell (2001). RNA was stained with 0.03% (wt/vol) methylene blue solution after transfer from agarose-formaldehyde gel to nylon membrane (Herrin and Schmidt, 1988).

3.3.2 Generation of DIG-labeled probes

DNA probes were labeled with digoxigenin-dUTP with the PCR DIG Probe Synthesis Kit (Roche) as specified by the manufacturer. The *cysB*, *cysP*, and *cysA* probes were amplified by PCR from *S. mutans* UA159 genomic DNA using specific primers listed in Table 5.

DNA dot blotting was performed as follows to evaluate the probe labeling efficiency. Serial dilutions were made for each denatured probe and 1 µl of each dilution was spotted onto a nylon membrane. For comparison, known concentrations of a DIG-labeled control nucleic acid were spotted on the same membrane. The DNA was fixed to

the membrane by UV crosslinking. Chemiluminescent alkaline phosphatase substrate was used to visualize the DIG signal in the spots.

TABLE 5. DNA probes used in Northern blot experiments

Primer	Sequence (5' to 3')[a]	Amplicon (bp)
CysB-F	CAGGAAACAATCACTGTAGCAAC	364
CysB-R	GAATAGCAGCATAGTTAGAACCAGC	
CysP-F	CCTCAATCAAAAGATGGGGAC	419
CysP-R	CGATAAGACGACCAACTTGTTC	
CysA-F	TTCTGGTGCTGGGAAATCAAC	327
CysA-R	TGACCTCCTGAAAGATGGCG	

[a] Primers were designed and analyzed with MacVector 7.2 software.

3.3.3 DNA hybridization and detection

The membranes were prehybridized at 50°C in 10 ml of Dig Easy Hyb (Roche) solution for 3 h with constant agitation. DIG-labeled DNA probes (~25 ng/ml) were denatured by boiling for 5 min and rapidly cooling on ice and added to the membranes in 10 ml of fresh pre-warmed Dig Easy Hyb solution. The mixture was then incubated at 50°C overnight with constant agitation.

After hybridization, the membranes were washed twice with 2× SSC, 0.1% SDS buffer at room temperature for 5 min and twice with 0.1× SSC, 0.1% SDS buffer at 65°C for 15 min. The membranes were blocked with 1× Blocking Reagent (Roche) diluted with maleic acid buffer for 1 h. All procedures were carried out at room temperature with constant agitation. The membranes were transferred to 1× Blocking Reagent buffer containing alkaline phosphatase-conjugated anti-digoxigenin (Roche) diluted 1:10,000 for 30 min. The membranes were gently washed twice in 0.1× SSC, 0.1% SDS buffer for 15

min and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1M NaCl, pH 9.5) for 3 min. Transcripts were detected with the chemiluminescent substrate CDP-Star (Roche) and X-ray films (Kodak).

3.4 Rapid Amplification of 5' cDNA Ends (5' RACE-PCR)

The 5' RACE-PCR technique was used to define the transcriptional start site of *cysBPA* locus. Overnight cultures of *S. mutans* UA159 were diluted 1:50 in fresh THYE broth and incubated at 37°C until an OD_{600} of approximately 0.4 was reached. Total RNA was extracted using RNeasy Mini Kit (see section 3.3.1). DNA-free RNA (10 µg) was reverse transcribed using RACE outer primer (5'-CGATAACTGATAACGTCCTG-3') and Superscript II Reverse Transcriptase (Invitrogen) according to the supplier's instructions. RNaseH (USB) and RNase T1 (Roche) were then added and incubated at 37°C for 30 min and the cDNA was purified using the StrataPrep PCR Purification Kit (Stratagene) following the manufacturer's instructions. Tailing of purified cDNA using terminal deoxynucleotidyl transferase (Sigma) and dGTP/dTTP was done according to instructions. Tailed cDNAs were amplified by PCR using RACE universal primers (5'-GAATTCGAATTCCCCCCCCCCCC-3', 5'-GAATTCGAATTCAAAAAAAAAAAA-3') and RACE inner primer (5'-GCTGTATCTGAGTCTGTTGCTAC-3'). Amplicons were analyzed by agarose gel electrophoresis and sequenced using RACE inner primer.

3.5 Amino Acid Uptake and Substrate Specificity of Transporter

3.5.1 Culture conditions

Cells were grown in modified Berman's Broth (MBB) medium containing 2% (wt/vol) trypticase, 0.1% (wt/vol) yeast extract, 34 mM NaCl, 0.05% (wt/vol) sodium thioglycollate, 1 mM $MgSO_4$, 0.1 M $MnSO_4$, buffered to pH 7.3 with MOPS buffer and supplemented with 0.1% (wt/vol) glucose (Sato *et al.*, 1989). Overnight cultures were diluted 1:5 in MBB medium and grown until an OD_{600} of approximately 0.4 was reached. Cells were harvested by centrifugation (4,000 × *g*, 10 min, 4°C) and washed twice with cold buffer A (50 mM MOPS, 50 mM KPO_4, 10 mM $MgSO_4$). Cells were resuspended in buffer A to a final OD_{600} ~ 4.0 and kept on ice until used for transport assay.

3.5.2 Transport assay

The assay for uptake of cystine was performed essentially as described by Korithoski *et al.* (2005). The assay mixtures contained cell suspension (~ 1.5×10^9 CFU/ml), 1% (wt/vol) glucose, and 0.1 mg/ml chloramphenicol. Reaction mixtures were preincubated for 10 min at 37°C prior to the addition of substrates.

At time zero, L-[^{14}C]cystine and cold L-cystine were added at a concentration of 4 μM (2.6 mCi/mmol) and 200 μM, respectively, and the reaction mixtures were incubated at 37°C. Samples (100 μl) were removed at regular intervals and immediately filtered through 0.22-μm pore-size membranes (GV Durapore, Millipore). The filters were washed twice with 0.5 ml of buffer A and transferred to vials containing 5 ml of a scintillation fluid (ScintiSafe EconoF, Fisher Scientific) for determination of radioactivity. All transport experiments were carried out using three independent cultures and each time

point was sampled in duplicate. Final specific activity (rate of uptake) was calculated as: χ nmol substrate / mg cells / min. The specificity of cysBPA-mediated amino acid uptake was also examined using an amino acid competition assay. Uptake of L-[^{14}C]cystine (4 μM) was measured in the presence of 400 μM of the following cold L-amino acids: arginine, cysteine, glutamine, glutamate, leucine, and methionine. As a positive control and to determine total L-[^{14}C]cystine uptake, cells were incubated with radio-labeled cystine with no competing substrate. As a negative control, a reaction containing no cells was incubated with L-[^{14}C]cystine and no radioactivity was detected.

3.6 Growth Kinetics

3.6.1 Doubling time calculations

Strains were grown in THYE broth without antibiotic for 16 h to assay their growth kinetics. Overnight cultures were diluted 1:20 in triplicate into microtiter plates containing various media. A microbiology reader (Bioscreen C Labsystems; Helsinki, Finland), which provides constant temperature and linear shaking to prevent cell aggregation was used. Cultures were grown for 24 h with absorbance recorded every 20 min. The reader was equipped with Biolink software that allowed automatic recording and conversion of OD readings into growth curves. Wells without cells were used as negative controls. Doubling time (T_d) of the bacterial growth was calculated as measuring the slope of the logarithmic growth phase and using the following formula (Khalichi *et al.*, 2004):

$$T_d = (t_2 - t_1)\ln(2) / \ln(OD_2) - \ln(OD_1).$$

3.6.2 Bacterial growth in presence of various sulfur compounds

Strains were grown in a minimal medium (MM) containing: 56 mM glucose, 13.6 mM L-glutamic acid, 1.3 mM L-cysteine-HCl, 7 mM L-leucine, 19 mM NH_4Cl, 20 mM K_2HPO_4, 11 mM KH_2PO_4, 50 mM $NaHCO_3$, 4.9 mM $MgSO_4 \cdot 7\ H_2O$, 0.1 mM $MnCl_2 \cdot 4H_2O$, 72 μM $FeSO_4 \cdot 7\ H_2O$, 5.5 mM sodium pyruvate, 2.6 μM riboflavin, 1.4 μM thiamine-HCl, 0.4 μM biotin, 8 μM nicotinic acid, 0.7 μM ρ-aminobenzoic acid, 1 μM calcium pantothenate, and 5 μM pyridoxal-HCl, and buffered with 0.05 M Tris-maleate (pH 7.4) to a final pH of 7.1 (Fujiwara *et al.*, 1978). MM was supplemented with various sulfur compounds including L-cysteine, L-cystine, L-methionine, reduced L-glutathione, and $MgSO_4$ at various concentrations (0.1, 0.5, 1.0, 10, 50, or 100 mM).

To determine growth kinetics under cystine starvation, a modified MM which excluded L-cysteine-HCl was used and supplemented with various concentrations of L-cystine (0.1, 0.5, or 1 mM). To determine gene expression under cystine starvation, overnight THYE cultures were diluted 1:20 into modified MM and modified MM supplemented with 1 mM L-cystine, grown to mid-log phase, and harvested.

3.7 Quantitative Real-Time RT-PCR

3.7.1 Template preparation

For quantitative real-time RT-PCR experiments, cells were disrupted using the FastPrep FP 120 cell disrupter (BIO 101-Savant, Holbrook, NY) and total RNA was extracted using the TRIzol reagent (Invitrogen) as described previously by Hanna *et al.* (2001). A First Strand cDNA synthesis kit (MBI Fermentas) was used according to the manufacturer's specifications to generate single-stranded cDNA from 1 μg of DNA-free RNA samples. For each RNA sample, the cDNA synthesis reaction was also carried out without reverse transcriptase in order to identify and control for contamination by residual genomic DNA.

3.7.2 Efficiency of primer pairs

Serial dilutions (1.0, 2.5, 10, 50, 200, and 500 ng/μl) of cDNA were used to determine the efficiency of primers. Using cDNA templates, real-time RT-PCR reactions were carried out using the QuantiTect SYBR Green PCR Kit (QIAGEN) in an MX3000P system (Stratagene). Specific primers were designed and analyzed with MacVector 7.2 software for PCR products that were between 100-150 bp in length (Table 6). The PCR cycling conditions were as follows: 95°C for 15 min for the initial denaturation, followed by 40 cycles of a three-step profile consisting of 15 s of denaturation at 95°C, 15 s of annealing at 58°C, and 30 s of extension at 72°C. For each set of primers, cycle threshold (Ct) values were generated. Average Ct values were plotted against log concentration of cDNA to generate a standard curve. Efficiency (E) of primers was calculated using the formula (Pfaffl, 2001): $E = 10^{-1/slope}$.

TABLE 6. Primers used in real-time RT-PCR

Primer	Sequence (5' to 3')	Amplicon (bp)
CysB-RTF	CGTTACCCTAACCCAACGTC	144
CysB-RTB	TCACCACCAATCTTACCCTTG	
CysP-RTF	TGTTCAGGTTTACCGACGTG	101
CysP-RTB	CAAGAGAGGTTCCCTTGGTC	
CysA-RTF	AACCTTTTTGAGCGTCGGAC	125
CysA-RTB	TCAGAAAGTCCAACCTTGGC	
CysR-RTF	ACCGAGGAGAGATTGACTTTG	110
CysR-RTB	ACAAGCAGGAGAAGCCACTG	
16S-RTF	CTTACCAGGTCTTGACATCCCG	111
16S-RTB	ACCCAACATCTCACGACACGAG	

3.7.3 Relative quantification in real-time RT-PCR

A relative quantification based on the relative expression of a target gene versus a reference gene was used. Comparison of the expression of each target gene between its control and test conditions was determined according to the following formula (Pfaffl, 2001): Ratio = $(E_{target})^{\Delta Ct\ (control\text{-}test)}$ / $(E_{ref})^{\Delta Ct\ (control\text{-}test)}$. The *16S* rRNA gene was used as an internal reference as expression of this gene did not vary under the experimental assay conditions used ($P > 0.1$).

For each reaction, a master mix of the following components was prepared to the indicated end-concentration: 7.2 µl H_2O, 0.2 µl forward primer (0.5 µM), 0.2 µl reverse primer (0.5 µM), 10 µl QuantiTect SYBR Green PCR master mix (1×), and 2 µl cDNA (1 µg/ml) was added as PCR template.

3.8 Static Biofilm Assay

Biofilms were developed in 96-well and 12-well polystyrene microtiter plates. Biofilm growth was initiated by inoculating 10 µl of an overnight culture into 300 µl of semi-defined minimal (SDM) medium in the individual wells of a 96-well microtiter plate or by inoculating 100 µl into 3.0 ml of SDM medium in a 12-well plate. The SDM medium contained 57 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 34 mM NaCl, 5 mM glucose, 0.4% (wt/vol) casamino acids, 4 mM $MgSO_4 \cdot 7\ H_2O$ and was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 µM riboflavin, 0.25 µM thiamine HCl, 0.05 µM D-biotin) and amino acids (3.4 mM L-glutamic acid, 0.95 mM L-arginine HCl, 1.3 mM L-cysteine HCl, 0.1 mM L-tryptophan). The microtiter plates were incubated overnight at 37°C in air with 5% CO_2 without agitation. After the incubation, the planktonic cells were carefully removed and plates were air dried overnight at room temperature. The biofilms were stained with 0.01% (wt/vol) safranin for 10 min, rinsed with sterile distilled water, and air dried. Biofilm biomass was quantified by measuring the absorbance of stained biofilms at 490 nm with a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA). Biofilms formed in 12-well plates were not stained but used for gene expression analysis. Planktonic cells were carefully removed and biofilm cells left intact in the wells were rinsed once with sterile phosphate-buffered saline (PBS) to remove loosely bound cells. The biofilm cells were dislodged by gentle scraping using sterile pipette tips, collected by centrifugation (4,000 × *g*, 15 min, 4°C), and kept at -70°C until used for gene expression analysis (see section 3.7).

3.9 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on 16-h *S. mutans* biofilms. *S. mutans* was grown in SDM medium on sterile glass coverslips deposited in 24-well polystyrene microtiter plates. Preparation of biofilms for SEM was performed as described by Li *et al.* (2001) with minor modifications. After incubation, planktonic cells were removed and biofilm cells were washed once with 2.0 ml of sterile PBS. The samples were then dehydrated through a series of ethanol rinses (30, 50, 70, 95, and 100%), and critical-point dried with liquid CO_2. The coverslips were mounted and sputter coated with platinum. The samples were observed at various magnifications with a scanning electron microscope (model S-2500; Hitachi Instruments, San Jose, CA).

3.10 Statistical Analysis

Statistical significance was determined by a single factor analysis of variance (ANOVA) and/or one tail Student's *t*-test. A P value of <0.05 was considered significant.

CHAPTER 4: RESULTS

4.1 Identification of a polar amino acid ABC transporter in *S. mutans*

The *S. mutans cys* locus encodes a putative polar amino acid ABC transporter (Fig. 4). A BLASTP search was performed on the locus using the Transport Classification Database (www.tcdb.org). The gene SMU.459, hereafter referred to as *cysB*, encodes an amino acid ABC transporter binding protein of 273 aa. CysB is homologous (30% identity; 72/240) to the *B. subtilis* TcyJ (YtmJ) solute binding protein. The gene SMU.460, hereafter referred to as *cysP*, encodes an amino acid ABC transporter permease protein of 267 aa. CysP exhibits 34% identity (78/224) to the *B. subtilis* TcyM (YtmM) permease. Finally, SMU.461, hereafter referred to as *cysA*, encodes an amino acid ABC transporter ATP-binding protein of 247 aa. CysA is homologous (53% identity; 127/238) to the *B. subtilis* TcyN (YtmN) ATP-binding protein. The *B. subtilis tcyJ*, *tcyM*, and *tcyN* genes are part of the *ytmI* operon encoding an L-cystine ABC transporter. The *cys* locus is comprised of three genes transcribed in the same direction and collectively encodes a putative L-cystine ABC transporter, which will be named CysBPA.

According to the *S. mutans* UA159 genome sequence (Ajdic *et al.*, 2002), a probable RNA helicase (SMU.458) is encoded 5' proximal to the ABC transporter genes. Also, a putative transcription factor (SMU.462) is located directly downstream of the ABC transporter-encoding genes. In contrast, the *B. subtilis* ABC transporter TcyJKLMN is encoded in a large operon (*ytmI*) with genes encoding a hypothetical protein and a putative monooxygenase located directly upstream and downstream of the cystine

transporter-encoding genes, respectively. Moreover, the transcriptional activator of the *ytmI* operon, YtlI, is divergently transcribed from *ytmI*. Other genes of interest near the *cys* locus include SMU.466 and SMU.496, which code for a cysteine aminopeptidase C and a putative cysteine synthetase, respectively.

Lastly, a putative *cys* locus regulator was located in *S. mutans* UA159. The gene SMU.2060, hereafter referred to as *cysR*, encodes a LysR-type transcriptional regulator of 291 aa. CysR is homologous (24%, 65/263) to the *B. subtilis* YtlI regulator.

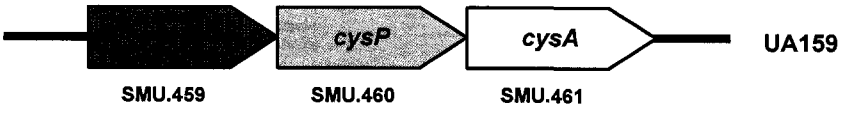


Figure 4. Map of the *S. mutans cys* locus. The *cysB*, *cysP*, *cysA* genes encode an amino acid (substrate)-binding protein, a permease, and an ATP-binding protein, respectively. Collectively, they encode for a putative polar amino acid ABC transporter.

4.2 Construction of *S. mutans* insertion mutants

The 5' and 3' regions of *cysB*, *cysP*, *cysA*, *cysBPA*, and *cysR* and an erythromycin resistance cassette were PCR amplified and restriction digested. The PCR products were ligated together and subsequently used to transform *S. mutans* UA159 cells. Colonies that grew in the presence of the antibiotic were further tested by PCR and the mutation confirmed by DNA sequencing. For PCR confirmation, primer pairs that were used were P1/Erm-R and P4/Erm-F to yield fragments A and B (Fig. 5).

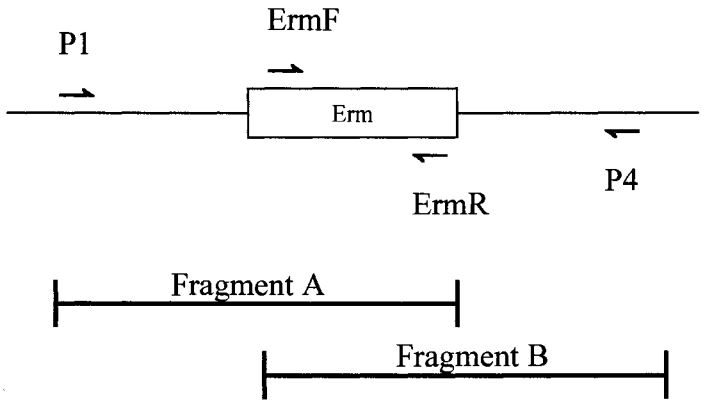


Figure 5. Confirmation of *S. mutans* insertion mutants by PCR using a combination of primer pairs. PCR products (Fragments A and B) were subjected to 1% agarose gel electrophoresis.

All insertion mutants for *cysB, cysP*, and *cysA* were successfully constructed. The PCR fragment length for each insertion mutant was identical to the theoretical band size (Table 7). An amplicon of ~ 880 bp corresponding to the erythromycin resistance cassette was obtained for all insertion mutants. DNA sequencing of fragments A and B for each mutant showed proper insertion of the erythromycin resistance cassette in the target gene (data not shown). Real-Time RT-PCR was also performed to ensure that in each insertion mutant, expression of the remaining two genes was still present. DNA gyrase-encoding gene *gyrA* (a common housekeeping gene) was used as a positive control and expression was seen in the wild-type strain and all mutants.

TABLE 7. PCR confirmation of *S. mutans* insertion mutants

Mutant	Fragment A (bp)	Fragment B (bp)
SmCysB (Δ*cysB*)	1871	1446
SmCysP (Δ*cysP*)	1676	1786
SmCysA (Δ*cysA*)	1522	1723
SmCysBPA (Δ*cysBPA*)	1855	1707

Theoretical fragment sizes determined by sequence analysis.

4.3 Transcriptional analysis of *cys* locus

A Northern blot experiment was performed to determine whether the three transporter-encoding genes formed an operon. Each probe allowed the detection of a single ~2.3 kb mRNA transcript in wild-type cells (Fig. 6). The size of this transcript was consistent with the co-transcription of *cysB*, *cysP*, and *cysA*. Background bands could be seen in the membrane that was probed using labeled *cysP*. The results clearly confirmed that the *cys* genes are co-transcribed and thus, form an operon.

Using the 5' RACE-PCR technique, the transcriptional start site of the *cysBPA* operon was located 8 nucleotides downstream from the last nucleotide of the inferred -10 box (Fig. 7).

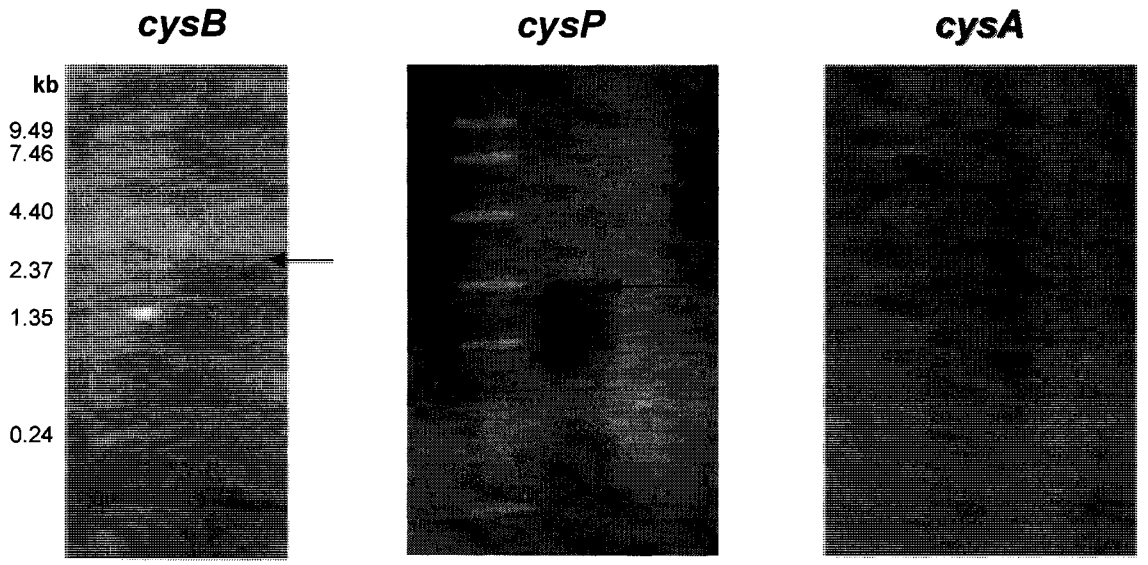


Figure 6. Northern blot analysis of the *cys* locus from *S. mutans* UA159. Arrows indicate the band size where each probe bound. Each membrane displays a 0.24-9.5 Kb RNA ladder (Invitrogen) for measuring transcript length.

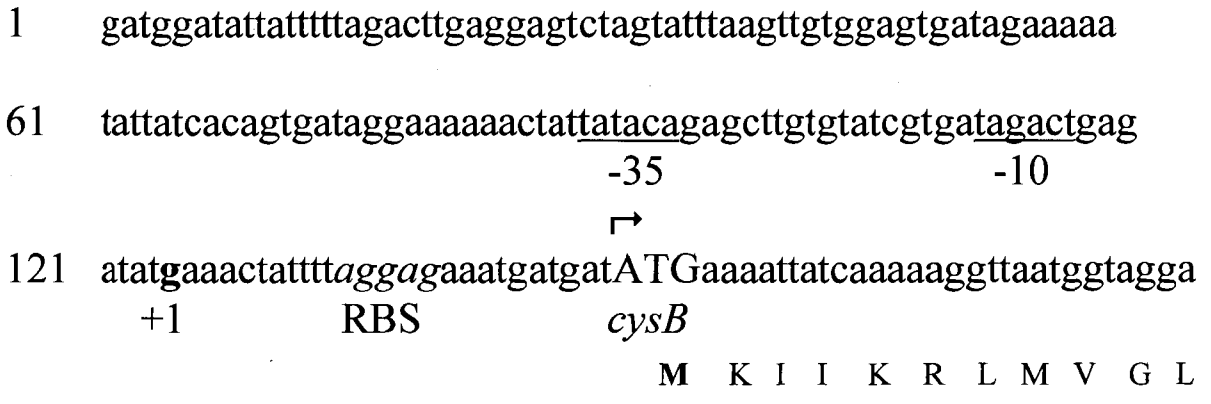


Figure 7. Partial nucleotide and deduced amino acid sequences of the *S. mutans* UA159 *cys* locus. Putative -35 and -10 promoter sites and a putative ribosome-binding site (RBS) are indicated. The transcriptional start site identified by 5' RACE-PCR is indicated below the sequence (+1).

4.4 Biofilm formation

To assess the role of the transporter in the expression of several *S. mutans* virulence properties, a number of phenotypic properties were analyzed including the ability to form biofilms. When *S. mutans* UA159 and its Δ*cys* transporter mutants were grown in SDM-glucose medium, a significant decrease in biomass was observed for all mutants relative to the wild-type UA159 strain (Fig. 8). All *cys* mutants showed a decrease in biomass compared to wild-type in sucrose-independent media. Indeed, SmCysB, SmCysP, SmCysA, and SmCysBPA mutants showed 39%, 40%, 30%, and 28% decreases in biomass, respectively, relative to that of the parent strain. When strains were grown in 4×-diluted THYE medium supplemented with 5 mM glucose or sucrose, no discernible difference in biomass was seen (data not shown). Also, no difference in biomass between the strains were seen when cells were grown in SDM medium supplemented with 5 mM sucrose (data not shown).

To examine the biofilm morphology by SEM, biofilms of *S. mutans* UA159, SmCysB, SmCysP, SmCysA, and SmCysBPA mutants were grown in SDM medium supplemented with 5 mM glucose, replicating the conditions for the biomass quantification experiment. Scanning electron microscopy confirmed the reduction of biomass. While *S. mutans* UA159 was able to form a dense, intricate biofilm, each of the *cys* transporter mutants formed extremely sparse biofilms (Fig. 9). At high magnifications, SmCysB, SmCysP, and SmCysBPA showed "bumpy" cell surfaces. The changes in cell surface morphology may indicate that the mutagenesis altered cell surface expression or it may be an artifact of the SEM preparation process. However, bumpy cell surfaces were

not seen in the wild-type biofilm under the same conditions. Hence, the *cys* genes appear to have a role in biofilm formation.

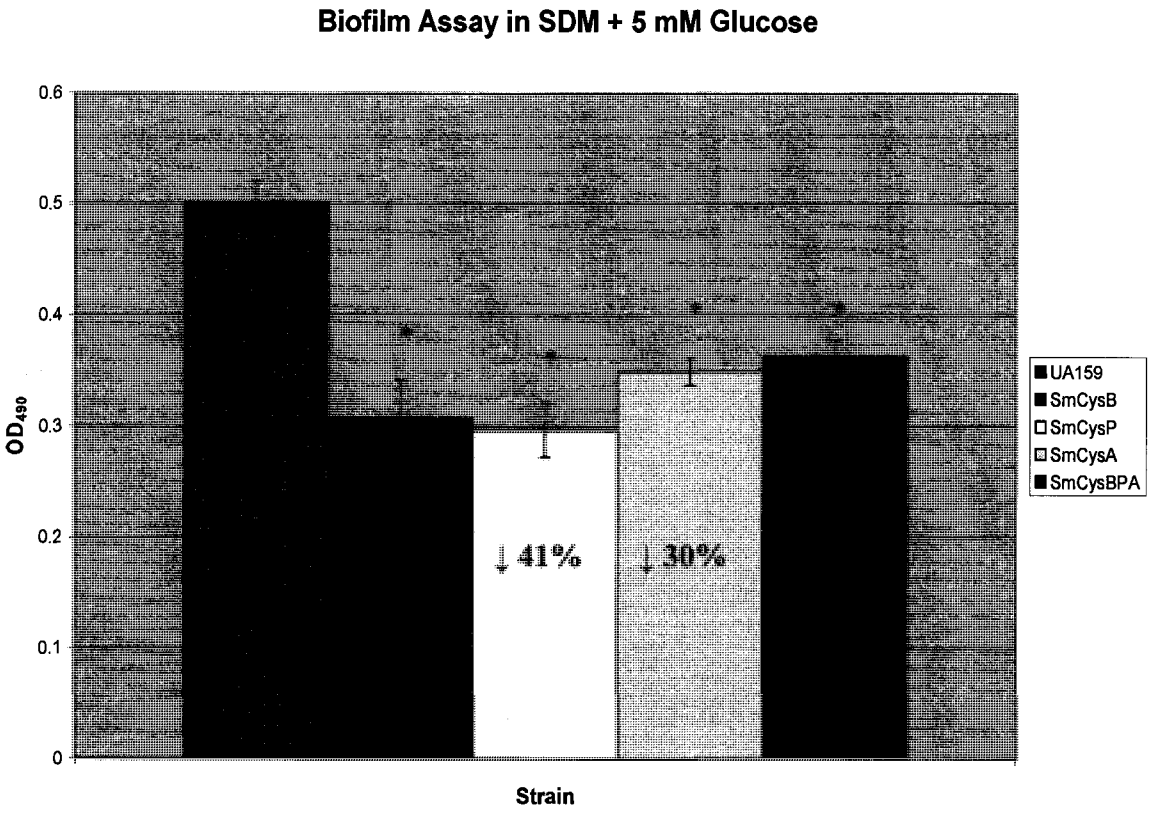


Figure 8. Biofilm biomass quantification of *S. mutans* UA159, SmCysB, SmCysP, SmCysA, and SmCysBPA mutants. * ≥ 20% is considered significant, ($P < 0.05$).

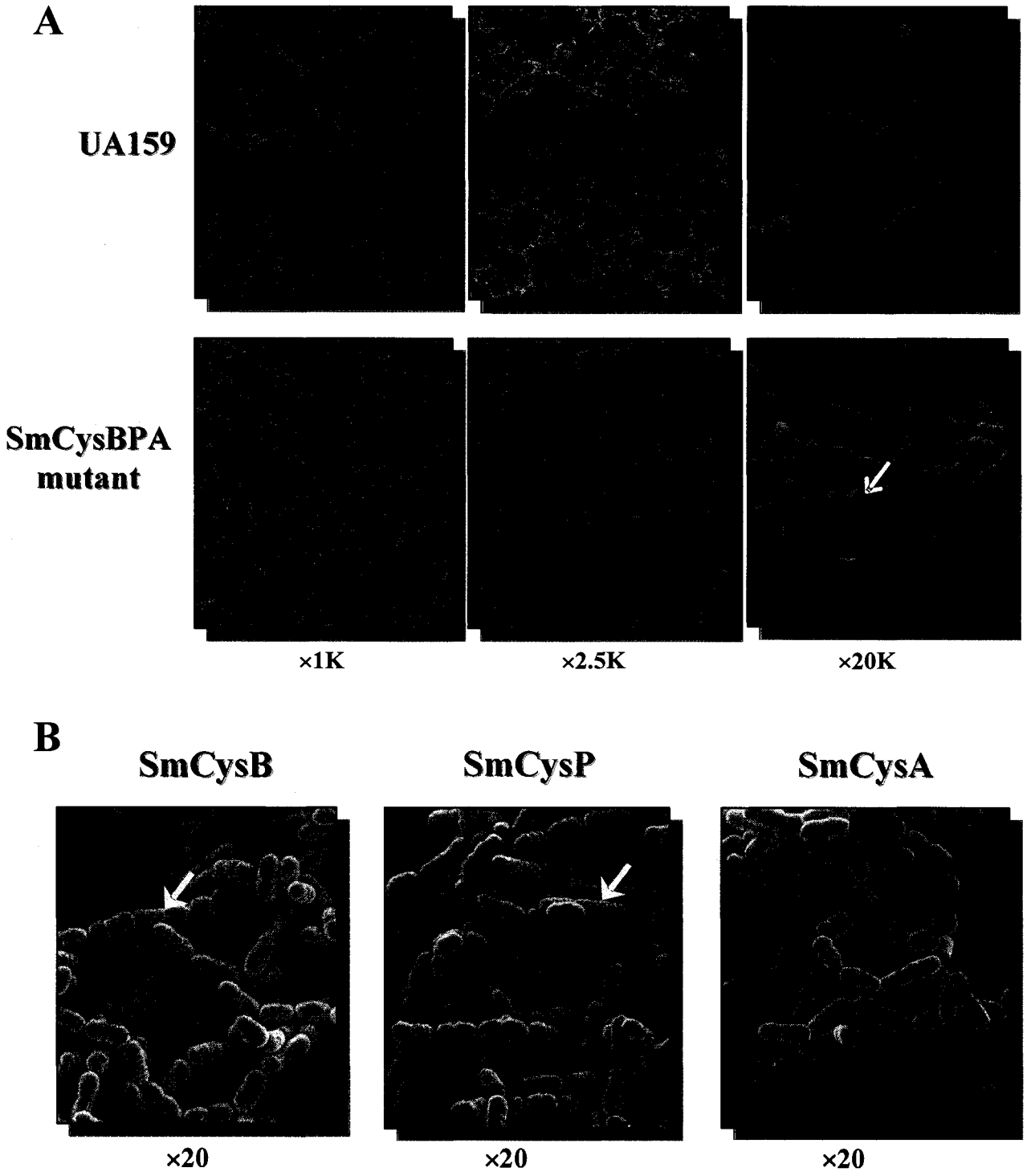


Figure 9. Scanning electron micrographs of *S. mutans* biofilms accumulated on the surface of glass discs. (A) *S. mutans* UA159 and SmCysBPA mutant biofilms. (B) SmCysB, SmCysP, and SmCysA mutant biofilms.

4.5 Acid challenge

4.5.1 Growth kinetics

Growth kinetics showed that all mutants grew similarly at pH 7.5 compared with UA159 wild-type strain (Table 8). However, the final growth yield of SmCysBPA after 16-h of growth in THYE (pH 7.5) was slightly reduced (Fig. 10). The phenotypic effect of the *cys* mutations on *S. mutans* acid resistance was determined by measurements of growth rates in THYE broth at pH 5.5. The results showed that growth at pH 5.5 was slower for all strains compared with growth at neutral pH. SmCysB and SmCysP mutants displayed a significantly slower doubling time compared with the wild-type strain at pH 5.5 (Table 8). Furthermore, the growth yield of SmCysBPA was slightly reduced compared to all strains grown at pH 5.5 (Fig. 11).

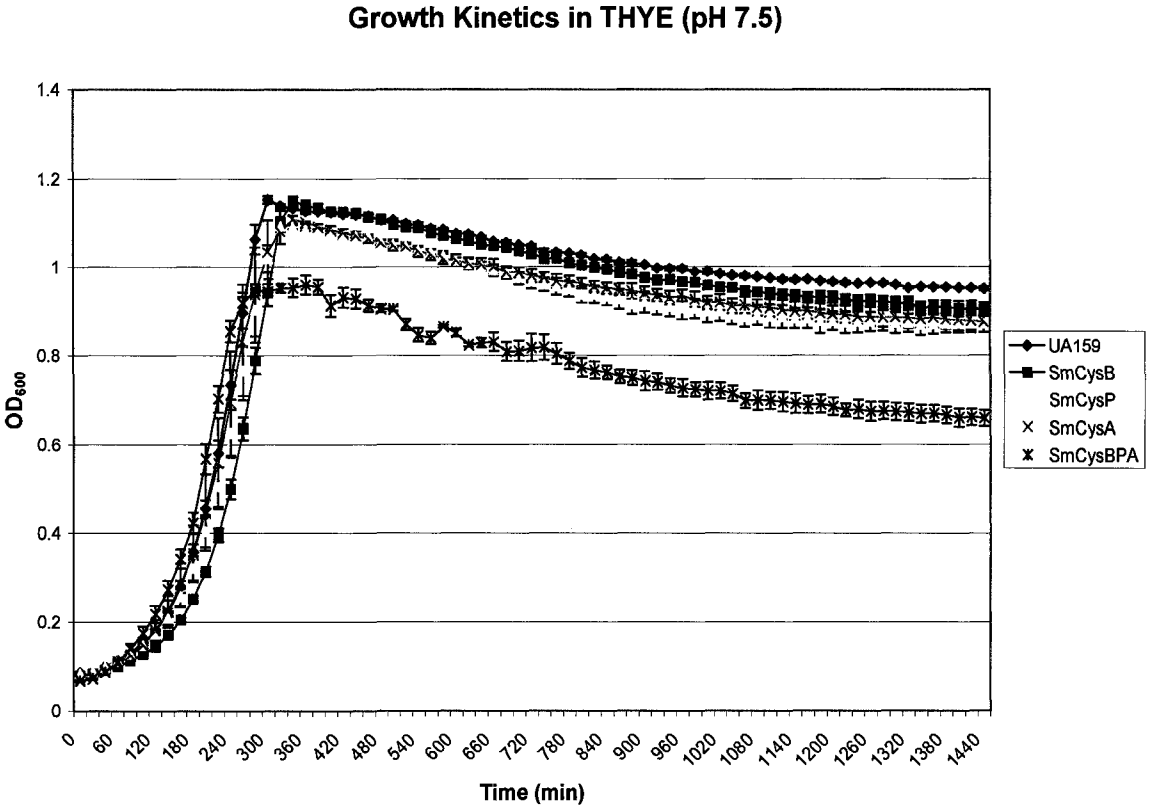


Figure 10. Growth curves of *S. mutans* UA159, SmCysB, SmCysP, SmCysA, and SmCysBPA in THYE (pH 7.5).

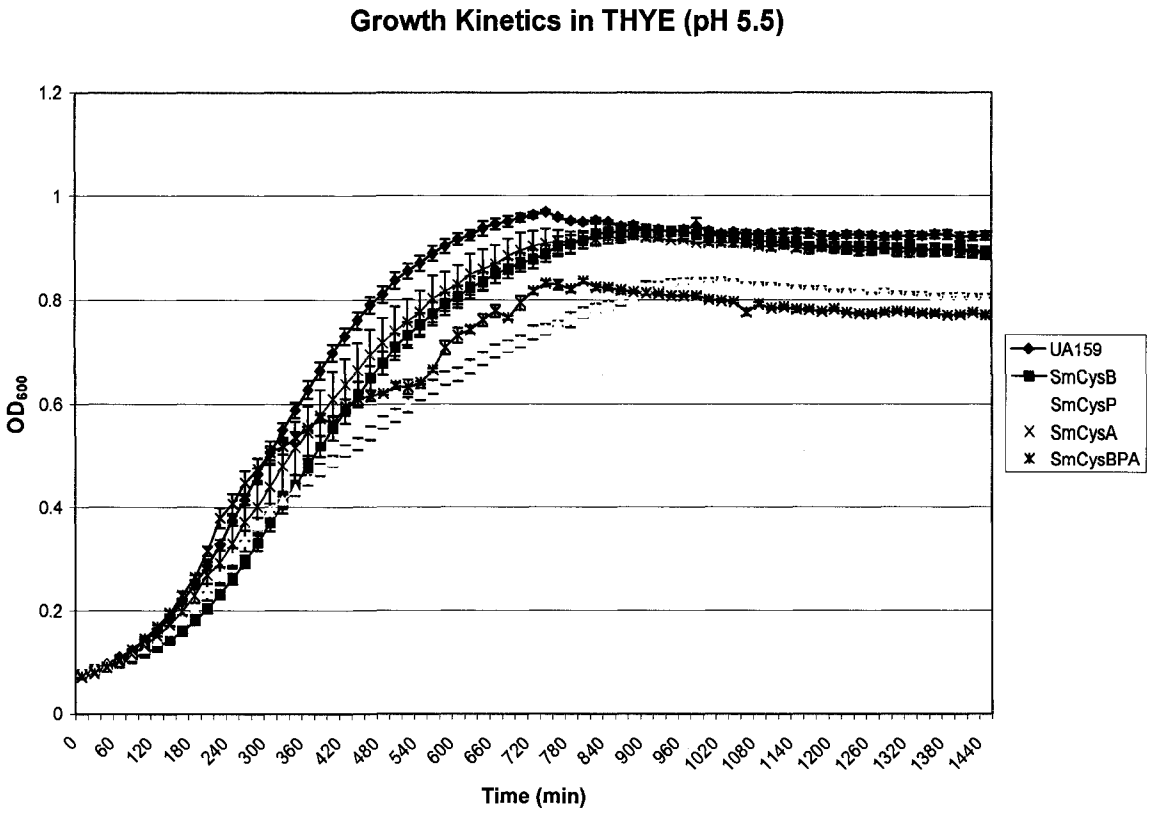


Figure 11. Growth curves of *S. mutans* UA159, SmCysB, SmCysP, SmCysA, and SmCysBPA in THYE (pH 5.5).

TABLE 8. Summary of doubling times (*T*d) for *S. mutans* UA159 and its mutants

Strain	THYE (pH 7.5) $T_d \pm SE$ (min)	THYE (pH 5.5) $T_d \pm SE$ (min)
UA159	57.3 ± 0.6	103.4 ± 1.6
SmCysB	59.6 ± 0.8	115.3 ± 4.5*
SmCysP	61.5 ± 0.5	112.5 ± 3.3†
SmCysA	59.6 ± 1.4	107.3 ± 7.5
SmCysBPA	61.7 ± 4.1	95.9 ± 0.9

Statistical significance compared to wild-type at pH 5.5 using Student's *t*-test: * ($P < 0.05$), † ($P < 0.01$).

4.5.2 Gene expression: biofilm vs. batch

Acid resistance in biofilm cells display phenotypic differences from cells grown in a batch culture (Welin-Neilands and Svensäter, 2007). Therefore, gene expression of the transporter during acid challenge was investigated in biofilm and batch cells. In batch cells, the expression of the *cys* transporter genes was down-regulated by ~2.4-fold indicating that there was significant change in *cys* locus gene expression in planktonic cells grown under acidic conditions relative to planktonic cells grown at pH 7.5 (Fig. 12). In contrast, biofilm cells showed a 4-fold average increase in *cys* locus gene expression when grown at an acidic pH compared to biofilm cells grown at neutral pH 7.5 (Fig. 12).

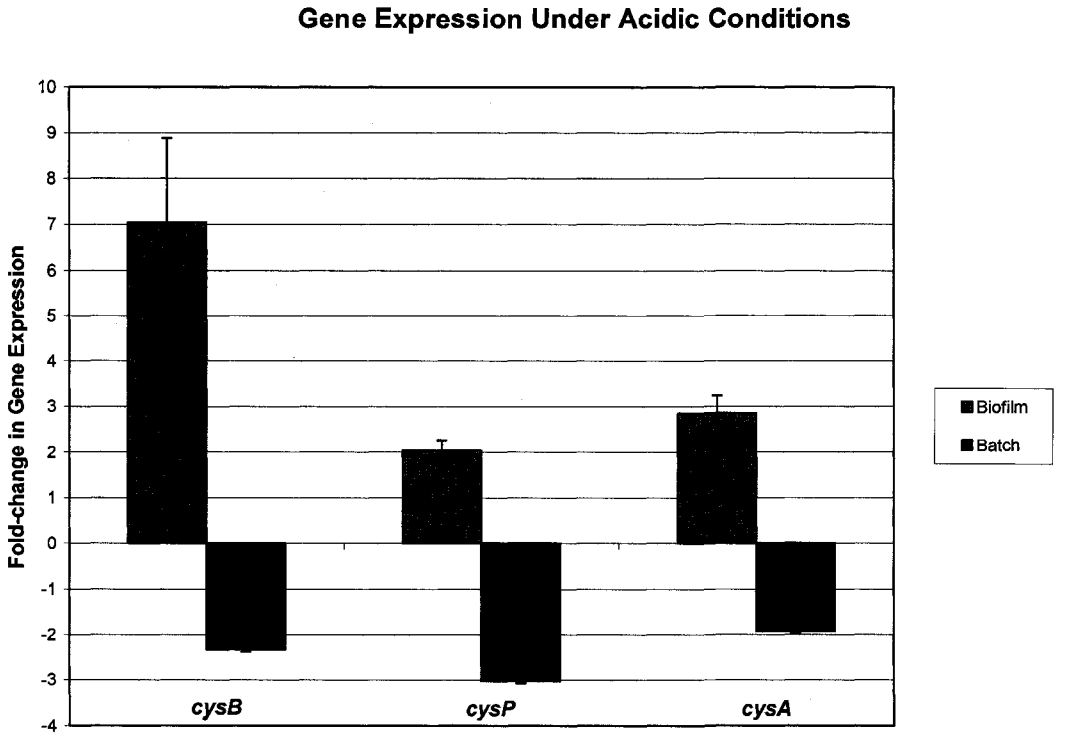


Figure 12. Differences in *cys* operon gene expression of *S. mutans* UA159 wild-type strain in biofilm or batch culture under acidic conditions. Results were normalized against the internal standard *16S* rRNA.

4.6 Transport assay

The *cys* operon is homologous to the *B. subtilis* TcyJKLMN cystine transporter. To confirm the role of the ABC transporter in cystine uptake, the abilities of *S. mutans* UA159 wild-type strain and its Δ*cysBPA* mutant to transport L-[^{14}C]cystine were tested. The rate of cystine uptake in wild-type cells was 1.06 ± 0.49 nmol/mg dry cell/min over a total time of 8 min. A significant decrease in L-cystine uptake was observed in the Δ*cysBPA* mutant compared to the wild-type strain (Fig. 13). The rate of cystine uptake in mutant cells was 0.48 ± 0.13 nmol/mg dry cell/minute over a total time of 8 min. Uptake after 10 min tapered off in both strains and therefore was not used to calculate the rate of transport. As L-cystine uptake detected in the mutant was significantly reduced (~55%) compared to the transport in wild-type strain, these results suggest that CysBPA is involved in L-cystine transport. Interestingly, despite a significant decrease in cystine transport by the mutant, transport of cystine was not completely abolished.

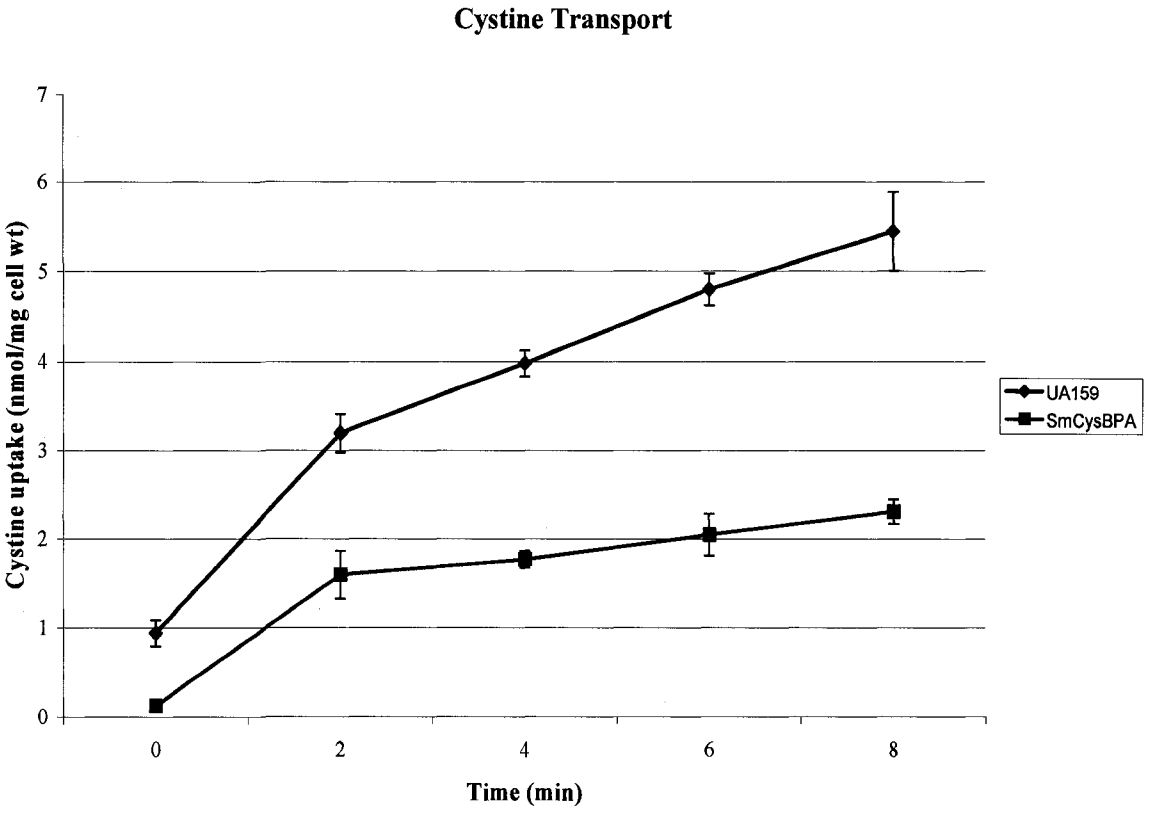


Figure 13. Time course for L-[^{14}C]cystine uptake in *S. mutans* UA159 wild-type strain and its SmCysBPA (*ΔcysBPA*) mutant. The cystine uptake analysis was performed in the presence of 4 μM L-[^{14}C]cystine and 200 μM of unlabeled L-cystine.

4.7 Competition assay

To determine the substrate specificity of the *S. mutans* CysBPA system, different amino acids and sulfur-containing compounds were tested for their ability to compete with transport of L-[^{14}C]cystine. The level of inhibition of labeled L-cystine uptake in the presence of a 100-fold excess of nonradioactive compounds was measured in the wild-type strain and its Δ*cysBPA* mutant. When unlabeled cystine was added in excess, a 94% decrease in L-cystine uptake was observed in the wild-type strain confirming the transporter specificity for cystine. In the wild-type strain, unlabeled cystathionine, djenkolic acid, *S*-methyl-L-cysteine, and cysteine competitively inhibited cystine uptake by 93%, 70%, 56%, and 51%, respectively (Table 9). Arginine, glutamine, glutamate, leucine, and methionine did not effectively inhibit cystine uptake. In addition, some sulfur compounds that did not inhibit cystine in wild-type cells include $MgSO_4$ and $FeSO_4$. In contrast, the SmCysBPA mutant showed very little L-cystine uptake and inhibition by unlabeled cystine, djenkolic acid, and *S*-methyl-L-cysteine was 19%, 9%, and 12%, respectively (Table 9). When the other competing substrates were tested in the mutant, more cystine was taken up relative to the control. Hence, cystine was still able to be transported into the cells despite the absence of the CysBPA system.

TABLE 9. Relative transport of CysBPA-dependent L-[^{14}C]cystine uptake in the presence of different competing substrates

Competitor[a]	Relative Transport (%)	
	***S. mutans* UA159**	**Δ*cysBPA***
L-Cystine	5.7 ± 1.1	81.2 ± 8.3
L-Arginine	84.3 ± 4.6	162.5 ± 40.9
L-Cysteine	49.1 ± 5.8	203.1 ± 56.7
L-Glutamine	91.8 ± 4.5	171.0 ± 64.4
L-Glutamate	90.0 ± 5.1	177.5 ± 14.9
L-Leucine	86.1 ± 2.9	144.3 ± 14.3
L-Methionine	90.3 ± 8.0	182.9 ± 61.3
DL-Cystathionine	7.3 ± 1.2	169.4 ± 2.0
L-Djenkolic acid	29.5 ± 4.8	91.5 ± 16.2
S-Methyl-L-cysteine	43.9 ± 8.0	87.8 ± 5.8
$FeSO_4$	90.1 ± 1.5	179.6 ± 21.7
$MgSO_4$	80.3 ± 4.0	149.1 ± 11.5

[a] The inhibition of radiolabeled L-cystine uptake was measured in the presence of a 100-fold excess of unlabeled compounds. The initial L-[^{14}C]cystine uptake rate was measured.

4.8 Growth kinetics of Δ*cysBPA*

4.8.1 Presence of sulfur compounds

Various sulfur sources such as cysteine, cystine, methionine, reduced L-glutathione, and $MgSO_4$ were used to supplement a minimal medium (MM) in order to determine the effect of these components on growth. A range of concentrations for each of the sulfur sources was tested. In all test conditions, the SmCysBPA mutant showed a noticeably slower growth rate than the wild-type strain except when cysteine was supplemented in the MM (Table 10). The growth rates of *S. mutans* UA159 remained relatively unchanged between 0 - 50 mM cysteine supplemented in the MM. The SmCysBPA mutant showed improved growth rates when the MM was supplemented with increasing amounts of cysteine. However, at 100 mM cysteine, both the wild-type strain and the SmCysBPA mutant grew at a very slow rate. Addition of up to 1 mM cystine showed no differences in growth rate for the wild-type strain or the SmCysBPA mutant strain. However, growth of the mutant was slightly improved with 1 mM cystine relative to the control. Higher concentrations of cystine were not used because it precipitated out of the MM at cystine concentrations of 10 mM or higher. Addition of reduced L-glutathione or methionine at concentrations up to 1 mM did not affect the growth rates of *S. mutans* UA159 wild-type strain. However, all concentrations of methionine showed slower growth in the SmCysBPA strain compared to the control. Finally, additional $MgSO_4$ did not affect wild-type growth but the doubling time of the SmCysBPA mutant was slower than the control.

TABLE 10. Doubling times and cellular growth yields of *S. mutans* UA159 and SmCysBPA (ΔcysBPA) mutant grown in MM with various supplemented sulfur sources at different concentrations.

Sulfur Compound Concentration (mM)	UA159 $T_d \pm$ SE (min)	Final OD_{600} T = 24 h	SmCysBPA $T_d \pm$ SE (min)	Final OD_{600} T = 24 h
Control	80.5 ± 1.1	1.6	136.4 ± 9.5*	1.4
Cysteine				
10	83.9 ± 2.0	1.5	86.6 ± 7.0†	1.4
50	95.5 ± 1.6†	1.4	90.0 ± 1.0†	1.4
100	212.3 ± 30.0†	0.8	211.8 ± 15.8†	0.8
Cystine				
0.1	83.3 ± 1.7	1.6	141.0 ± 1.6*	1.4
0.5	82.0 ± 0.3	1.6	131.3 ± 2.0*	1.5
1	81.0 ± 0.9	1.6	122.9 ± 0.9*, †	1.4
Methionine				
0.1	81.1 ± 1.4	1.7	157.4 ± 1.2*, †	1.4
0.5	80.2 ± 1.4	1.6	162.1 ± 0.8*, †	1.4
1	81.6 ± 0.7	1.6	158.3 ± 3.3*, †	1.4
50	121.0 ± 2.7†	1.4	260.2 ± 5.7*, †	1.1
L-glutathione reduced				
0.1	77.8 ± 0.2	1.7	145.1 ± 1.8*	1.5
0.5	75.7 ± 1.1	1.8	140.2 ± 1.4*	1.4
1	76.7 ± 0.2	1.7	131.1 ± 3.0*	1.5
50	101.5 ± 1.2†	1.4	146.7 ± 2.7*	1.2
$MgSO_4$				
10	84.8 ± 2.4	1.6	151.5 ± 2.0*, †	1.4

Statistical significance compared to wild-type using strain UA159 by using Student's *t*-test is indicated as follows: *, $P < 0.01$. Statistical significance compared to control (MM) by using Student's *t*-test is indicated as follows: †, $P < 0.01$.

4.8.2 Cystine starvation

To determine the effect of cystine starvation on *S. mutans* UA159 growth, the MM was modified by removing any similar compounds, hence, cysteine-HCl was not included in the MM. Growth of *S. mutans* UA159, SmCysBPA, and SmCysR mutants were affected when cells were starved for cystine relative to cultures provided with cystine (Table 11). In the presence of cystine, wild-type cells grew at a doubling time of 76.3 ± 1.5 min. However, cystine starvation caused the wild-type cells to grow at a slower doubling time of 93.3 ± 0.7 min with a decrease in cellular growth yield (Table 11, Fig. 14). When cystine was available, the SmCysBPA mutant grew very slowly at 118.2 ± 0.8 compared with the wild-type strain. However, the ability to grow under cystine starvation was abolished in the SmCysBPA mutant (Table 11, Fig. 14). Growth improved slightly in the SmCysBPA mutant when 0.1 mM cystine was supplemented to the modified MM.

A mutant with an inactivated *cysR* gene, a possible regulator to the *cys* locus, was also examined for growth kinetics and the effect of cystine starvation. The SmCysR (Δ*cysR*) regulator mutant doubling times increased from 117.2 ± 3.8 min when 1 mM cystine was provided relative to 261.0 ± 11.9 min under cystine starvation (Table 11, Fig. 14). A notable feature was the increase in lag growth phase of the SmCysR mutant in the presence or absence of cystine compared to the wild-type UA159 and SmCysBPA mutant strains.

To determine why the SmCysBPA transporter mutant was unable to grow in the absence of cystine, an examination of the different components of the ABC transporter was investigated. Growth curves for the individual transporter mutants (SmCysB,

SmCysP, SmCysA) were tested in modified MM without cystine (Fig. 15). As already observed, the *S. mutans* UA159 wild-type strain was able to grow without cystine, while the SmCysBPA mutant was unable to grow at all. However, SmCysB was able to grow without cystine with a doubling time of 131.3 ± 4.8 min. The SmCysA mutant was able to grow but at a slower rate than SmCysB with a doubling time of 214.8 ± 21.5 min. The SmCysP mutant was unable to grow at all under these conditions and growth curves were similar to the SmCysBPA mutant.

TABLE 11. Doubling times and cellular growth yields of *S. mutans* UA159, SmCysBPA, and SmCysR mutants under L-cystine starvation.

Cystine Concentration (mM)	UA159 T_d ± SE (min)	Final OD_{600} T = 24 h	SmCysBPA mutant T_d ± SE (min)	Final OD_{600} T = 24 h	SmCysR mutant T_d ± SE (min)	Final OD_{600} T = 24 h
1	76.3 ± 1.5	1.7	118.2 ± 0.8*	1.2	117.2 ± 3.8*	1.5
0.5	74.6 ± 0.2	1.7	197.4 ± 2.8†	1.0	119.7 ± 6.4	1.5
0.1	77.6 ± 1.0	1.7	Very slow growth†	0.8	118.7 ± 4.6	1.5
0	93.3 ± 0.7*	1.1	No growth†	0.3	261.0 ± 11.9¶	1.0

Statistical significance ($P < 0.01$) using Student's *t* test : *, statistical significance compared to UA159 wild-type cells grown with 1.0 mM L-cystine, †, statistical significance compared to Δ*cysBPA* mutant cells grown with 1.0 mM L-cystine, ¶, statistical significance compared to Δ*cysR* mutant cells grown with 1.0 mM L-cystine.

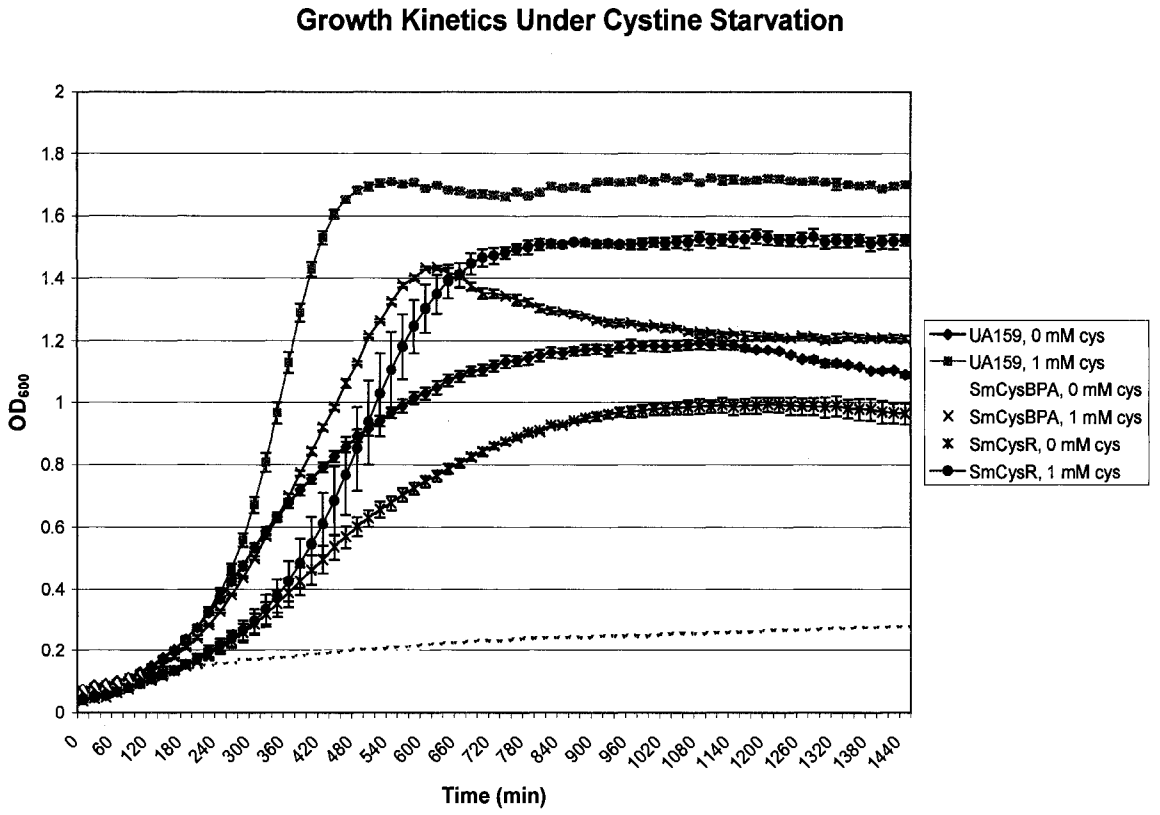


Figure 14. Growth of *S. mutans* UA159, SmCysBPA, and SmCysR strains in modified minimal medium in the presence or absence of L-cystine.

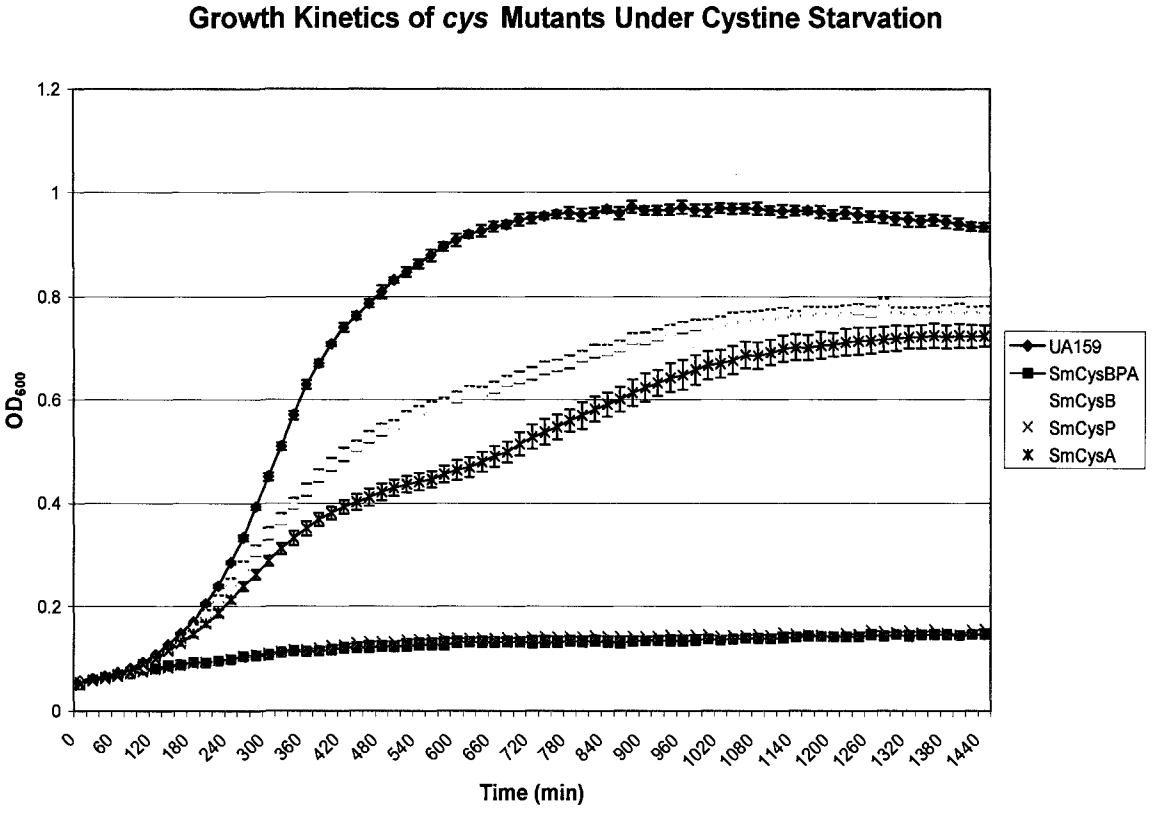


Figure 15. Growth of *S. mutans* UA159, SmCysBPA, SmCysB, SmCysP, and SmCysA strains in modified minimal medium in the absence of L-cystine.

4.9 Gene expression under cystine starvation

S. mutans UA159 was grown in modified MM or under cystine starvation until an OD_{600} of approximately 0.6 was reached. The gene expression of *cysB*, *cysP*, and *cysA* was analyzed by quantitative real-time RT-PCR to determine how the transporter gene expression is affected under a nutritional stress such as cystine starvation. Moreover, the gene expression of *cysR* was analyzed to determine its expression levels under the same conditions. Under cystine starvation, wild-type cells showed an up-regulation of the *cys* locus genes at least 2.4-fold (Fig. 16). The potential regulator of the *cys* locus, CysR, was also up-regulated ~2.6-fold under starvation conditions.

To determine the role of CysR on the expression of the CysBPA L-cystine transport system, a *cysR* insertion mutant (SmCysR) was constructed and tested under cystine starvation conditions. In the SmCysR mutant, expression of transporter genes was greatly increased (Figure 18). In *S. mutans* UA159 cells grown under starvation, gene expression levels for *cysB, cysP,* and *cysA* were 3.3-, 2.4-, and 2.8-fold up-regulated relative to cystine-fed cells. In contrast, in the absence of the regulator, *cysB, cysP,* and *cysA* gene expression levels were 10.8-, 13.1-, and 5.2-fold up-regulated under starvation relative to the cystine-fed state.

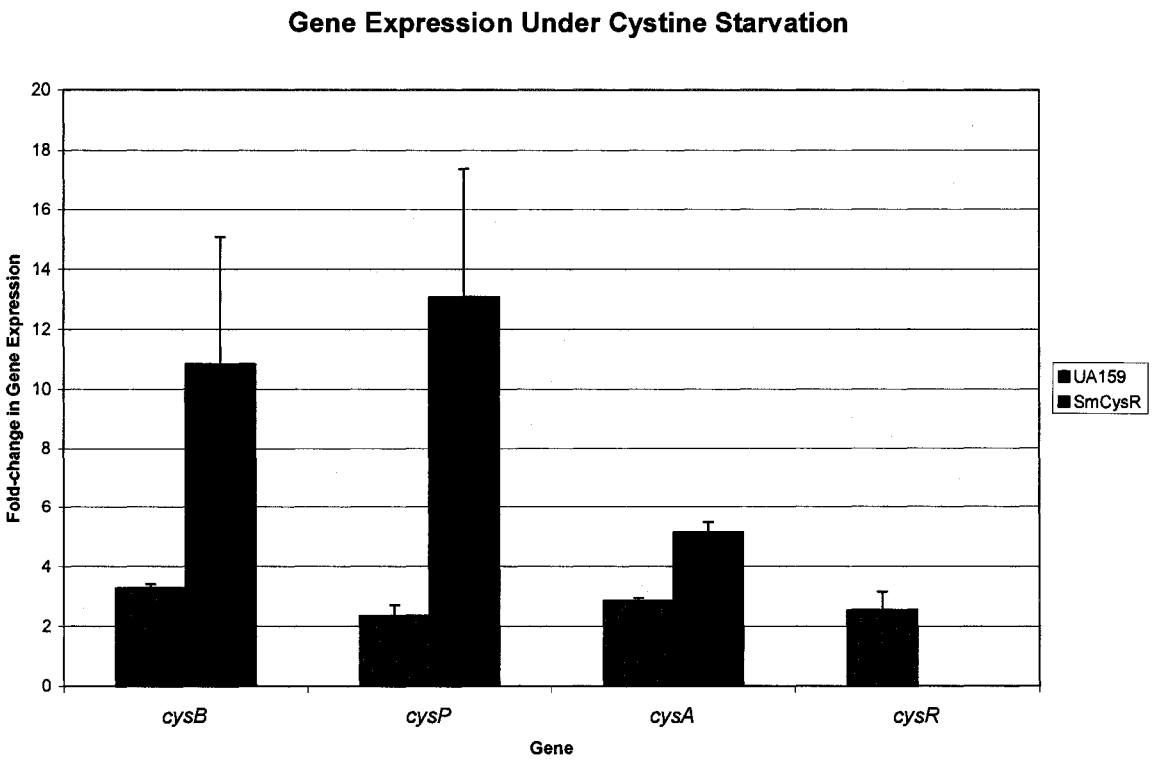


Figure 16. Fold difference in gene expression of the *cys* operon and its putative LysR-type transcriptional regulator in *S. mutans* UA159 wild-type strain and SmCysR mutant under cystine starvation. The fold-change in gene expression is expressed as cystine-starved cells relative to cystine-fed cells. Results were normalized against the internal standard *16S* rRNA.

CHAPTER 5: DISCUSSION

S. mutans is a biofilm-forming organism found in the oral cavity. It is found in dental plaque, which is a complex ecological system in which bacteria must overcome several conditions including nutritional starvation for survival. Further investigation is needed to determine the true nutritional requirements of *S. mutans* for growth and how the nutrients are acquired. In this study, an *S. mutans* amino acid transporter was characterized as a cystine transporter. The protein complex composed of 3 components: CysB (SMU.459), CysP (SMU.460), and CysA (SMU.461), is encoded by an operon that we proposed to rename *cysBPA*. It was demonstrated that the transporter was involved in cystine import, cystine starvation, growth, and biofilm formation. Furthermore, insight into the regulatory mechanism controlling the cystine transporter gene expression was gained.

A putative polar amino acid ABC transporter was previously identified in the *S. mutans* UA159 genome (Ajdic *et al.*, 2002). To gain insight into substrate specificities for the putative polar amino acid transporter, bioinformatic analysis was performed. Using the Transporter Classification Database, the best match to the target transporter sequence was a cystine uptake system in *B. subtilis*. The sequences for *cysB*, *cysP*, and *cysA* were relatively well conserved as determined by high scores on identity. The *S. mutans* UA159 genome encodes many ABC transporters, yet many remain uncharacterized. The characterization of a cystine transporter gives insight into its specificity in *S. mutans* and possibly in other bacteria.

In *B. subtilis*, three different cystine uptake systems are known (Burguiere *et al.*, 2004). The present study focused on an ABC transporter that was similar to the TcyJKLMN cystine uptake system found in *B. subtilis*. The *S. mutans* UA159 genome was also scanned for homologs to SMU.459 using tblastn to determine if there were other possible L-cystine uptake systems by searching for similar cystine-specific binding proteins. The results showed that another locus encoded a possible cystine transporter. The gene SMU.933, which encodes an amino acid ABC transporter binding protein, exhibited 29% identity (73/249) to the *B. subtilis* YtmJ protein. The genes found downstream of SMU.933 encode two probable transporter permease proteins (SMU.934 and SMU.935) and an ABC transporter ATP-binding protein (SMU.936). However, determining which system is preferred under various conditions by *S. mutans* has not been investigated, and it is beyond the scope of this study.

Potential cyst(e)ine metabolism genes were identified downstream of the *cysBPA* genes. A cysteine aminopeptidase C gene (SMU.466) was encoded near the *cys* locus. In lactococci species, the PepC aminopeptidase has broad substrate specificity and hydrolyzes napthylamide-substituted amino acids and di- and tri-peptides (Chapot-Chartier *et al.*, 1993). PepC is an enzyme that is 300-kDa in size and it is a hexamer of identical subunits with its peptidase activity inhibited by thiol-group-blocking compounds but not by serine or metalloproteinases inhibitors (Chapot-Chartier *et al.*, 1993). PepC from *Lactococcus lactis* subsp. *cremoris* AM2 was also found to have high homology to the mammalian bleomycin hydrolase (cysteine proteinase), which facilitates resistance to the anti-cancer drug bleomycin (Chapot-Chartier *et al.*, 1993). The presence of cysteine

aminopeptidases suggests *S. mutans* can access smaller peptides to free up amino acids such as cysteine.

In addition, a putative cysteine synthetase (SMU.496) was located downstream of the *S. mutans* cystine transporter *cysBPA* locus. During cysteine biosynthesis in *Escherichia coli* and *Salmonella typhimurium*, the final step involves the sulfhydration of *O*-acetyl-L-serine, which is facilitated by the cysteine synthetase enzyme (Hulanicka *et al.*, 1979). One study that used two-dimensional fluorescence difference gel electrophoretic analysis of *S. mutans* biofilms showed that cysteine synthetase A (*O*-acetylserine sulfhydrylase or CysK) was up-regulated in biofilms relative to planktonic grown cells (Rathsam *et al.*, 2005). Furthermore, *L. lactis* CysK had a role in formation of iron-sulfur clusters by regulating the mobilization of sulfur from cysteine (Fernández *et al.*, 2002). Hence, further study into the putative *S. mutans* cysteine synthetase (SMU.496) and its relation to the expression or regulation of the CysBPA transport system could be explored.

To confirm whether the *cysBPA* transporter genes were co-transcribed and formed an operon, Northern blot analysis was performed. It was determined that *S. mutans* cystine transporter genes were transcribed as a single transcript of mRNA containing *cysBPA*. In addition, 5'-RACE-PCR was conducted, and the start site of transcription was identified. Other characteristic sites were identified such as the ribosomal binding site and the -10 and -35 sites based on consensus sequences. In *B. subtilis*, the cystine transporter with the highest homology to *cysBPA* was found to be the TcyJKLMN transporter. Additionally, in *B. subtilis*, five genes comprise the cystine ABC transporter whereas *S. mutans* encodes the cystine transporter in only three genes. The TcyJKLMN cystine ABC transporter is part of a very large operon consisting of 12 genes called the *ytmI* operon. Transcriptional

analysis of the *ytmI* operon showed that the transcriptional start site was upstream of the *ytmI* gene, which encodes a protein of unknown function (Burguiere *et al.*, 2005). This differs from *S. mutans* where the transcriptional start site is located directly upstream of the transporter genes and the operon appears to consist of only the transporter-encoding genes. As identified in the *B. subtilis* genome sequence, TcyJ and TcyK encode the binding proteins of an amino acid ABC transporter, TcyL and TcyM encode for the permeases, while TcyN encodes the ATP-binding protein.

Transport assays using radio-labeled cystine and competing cold substrates were used to determine the substrate specificity of the *S. mutans* CysBPA transporter. In *S. mutans* wild-type, cystine uptake was not as fast as the rate observed in *B. subtilis*, however, this rate was calculated over the whole period of uptake (i.e. 8 min). In *S. mutans*, the highest rate of cystine uptake appeared to occur in the first 2 min. Then, transport of cystine continued linearly at a decreased rate. In *B. subtilis*, initial rates of L-cystine uptake were 1.9 nmol/min/mg of protein (Burguiere *et al.*, 2004). In wild-type *B. subtilis*, the rate of cystine uptake was linear for 6 minutes and started to plateau up to the 10 min mark. The Δ*tcyJKLMN* mutant in *B. subtilis* showed a decreased cystine transport rate of 1.4 nmol/min/mg of protein relative to the parent strain (Burguiere *et al.*, 2004). This decrease was not as pronounced as the decrease in rate of cystine uptake of the *S. mutans* SmCysBPA (Δ*cysBPA*) mutant relative to its parent strain. However, *B. subtilis* has three cystine transport systems, which explains why the rate of cystine uptake was not completely abolished as observed in *S. mutans*. Likewise, the SmCysBPA mutant was still able to transport cystine, thus, suggesting the presence of another cystine transport system in *S. mutans*.

To gain insight into the substrate specificity of CysBPA, various amino acids and sulfur-containing compounds were tested for their ability to competitively inhibit cystine uptake. In *S. mutans*, the competition assay showed that among all tested compounds, unlabeled cystine had the greatest inhibition at 94%. This confirmed the high affinity of the transporter for cystine. Furthermore, these results suggest that the CysBPA transport system is the primary transporter for cystine relative to any other potential cystine uptake systems in *S. mutans*. Cystathionine and djenkolic acid greatly inhibited cystine uptake by 93% and 70%, respectively. *S*-methyl-L-cysteine and cysteine also inhibited cystine uptake to a moderate degree at 56% and 51%, respectively, suggesting that the CysBPA transport system has broad specificity for substrates that are structurally similar to cystine. The inhibition of labeled L-cystine uptake in the presence of 10-fold excess of unlabeled competing substrates was tested in *B. subtilis* wild-type strain and a *B. subtilis* strain possessing only the TcyJKLMN transporter (Burguiere *et al.*, 2004). Substrates that inhibited L-cystine uptake in *B. subtilis* to a significant degree include L-cystine, L-djenkolic acid, DL-cystathionine, seleno-DL-cystine, and *S*-methyl-L-cysteine by 88%, 89%, 80%, 80%, and 73%, respectively (Burguiere *et al.*, 2004). Moderately inhibiting compounds in *B. subtilis* include L-histidine, L-methionine, DL-diaminopimelic acid, DL-lanthionine, L-glutamate, L-arginine, and DL-homocystine. Substrates that did not competitively inhibit cystine uptake in *B. subtilis* were L-cysteine and oxidized glutathione. In *B. subtilis*, cysteine had no inhibitory effect on cystine uptake, but cysteine was able to compete in *S. mutans* UA159. Hence, the CysBPA transport system in *S. mutans* has some affinity for cysteine. The competition assay was repeated in the *S. mutans* SmCysBPA mutant. Interestingly, some of the non-inhibiting compounds were

able to increase the uptake of cystine in the SmCysBPA mutant. It is possible that the absence of CysBPA enabled the function of another cystine uptake system in *S. mutans*, most likely with lower affinity for cystine.

Cystine is a good source of sulfur and as an amino acid for nutrition and growth. The effect of sulfur compounds on growth was tested by measuring growth kinetics of *S. mutans* wild-type and SmCysBPA mutant strains in a minimal medium supplemented with various sulfur sources. Additional sulfur compounds, such as cysteine, cystine, methionine, L-glutathione, and $MgSO_4$ at various concentrations did not affect growth in the wild-type UA159 strain except at concentrations of 50 mM or higher. In the minimal medium, the SmCysBPA mutant grew at a much slower rate than the wild-type strain. However, the SmCysBPA mutant showed improved growth with additional cysteine and cystine, while addition of methionine and $MgSO_4$ showed slower growth relative to the control. In the modified minimal medium, *S. mutans* UA159 wild-type growth was significantly slower when cystine or cysteine-like compounds were absent than when cystine was present. Nevertheless, *S. mutans* UA159 was still able to grow, therefore, cystine does not appear to be an essential requirement for growth. This suggested that the other amino acids present in the medium (i.e. glutamate and leucine) were sufficient to sustain growth. Also, the presence of inorganic sulfur and ammonium sources in the medium enabled the wild-type strain to utilize these compounds for growth. *S. mutans* possesses amino acid biosynthesis pathways and even though most amino acids are not freely available in the environment, some strains are able to synthesize intracellularly all the necessary amino acids (St. Martin and Wittenberger, 1980; Terleckyj and Shockman, 1975). The ability to synthesize its own amino acids is likely advantageous under thick

biofilm growth where conditions can become anaerobic and access to nutrients and any free amino acids is minimal. However, the SmCysBPA transporter mutant was able to grow at a significantly slower rate than the wild-type strain when cystine was available. When no cystine was provided, the transporter mutant was unable to grow past an OD_{600} = 0.3 and no exponential growth phase was detectable. The presence of the transporter was necessary for growth under these conditions suggesting that there was an essential component of the transporter that needed to be expressed in the wild-type strain to sustain viable growth. To determine the dispensability of the various subunit domains, individual *cys* mutants were tested under the same conditions, and it was found that the permease mutant, SmCysP, behaved exactly like the SmCysBPA mutant. When either the substrate-binding protein or the ATP-binding protein was absent, the mutants were still able to grow. It is possible that in the absence of the substrate-binding component, the other components of the ABC transporter can be utilized by other amino acid transporters in uptake of other substrates thereby enabling growth. Also, the absence of the ATP-binding protein did not completely impair growth and may be attributed to redundancy in the genome. It is also possible that genes encoding ATP-binding proteins elsewhere in the genome may be utilized to compensate or complement the ABC transporter since the protein is highly conserved between many transport systems. In contrast, the missing components among the *cys* mutants could possibly be substituted by ABC transporter components encoded elsewhere in the genome thereby partially re-establishing the cystine transporter. The poor growth exhibited by the SmCysP permease mutant may be attributed to a greater need for the expression of the permease. In *S. typhimurium*, the periplasmic histidine permease (ABC transporter) was disassembled and reassembled *in vitro* and it

was elucidated to have a possible regulatory or structural role for the integral membrane protein component of the transporter (Liu and Ferro-Luzzi Ames, 1998). Hence, the permease protein in the *S. mutans* cystine transporter could similarly play a larger role in cell physiology or morphology.

To gain insight into possible regulatory mechanism controlling *cysBPA* gene expression, the LysR-type regulator for cystine transport was inactivated in *S. mutans* UA159. When BLAST homology search was performed for a potential regulator in *S. mutans* UA159, a putative transcriptional regulator of the LysR-type family was encoded in the Open Reading Frame SMU.2060. The putative cystine transporter regulator (SMU.2060) gene had 24% similar identity (65/263) to the *B. subtilis* YtlI regulator. The SmCysR (ΔSMU.2060) regulator mutant was tested under the same cystine starvation conditions. When cystine was present, the SmCysR regulator mutant grew very slowly and when the mutant was starved for cystine, growth was markedly slow. Hence, the absence of the regulator did not completely abolish the growing ability of *S. mutans* in a modified minimal medium with the presence or absence of cystine. The LysR-type transcriptional regulators (LTTRs) are generally positive regulators in prokaryotes (Schell, 1993). In *E. coli*, LysR regulates genes involved in lysine biosynthesis, and it is co-induced by diaminopimelate, while CysB is a LysR-type transcriptional regulator of cysteine biosynthesis genes in *E. coli* and *S. typhimurium* (Schell, 1993). Most LTTRs, such as CysB, repress their own transcription, hence, they are negatively auto-regulated. In *B. subtilis*, YtlI is also a LysR-type regulator that is involved in the regulation of cysteine metabolism and the regulation of the cystine transport system, TcyJKLMN (Even *et al.*, 2006). In *B. subtilis*, YtlI and YrzC regulate the expression of the *ytmI* operon. The

YtlI regulator activates transcription of *ytmI* while YrzC, a negative regulator, controls *ytlI* expression (Even *et al.*, 2006). Therefore, it was deduced that *S. mutans* CysR could potentially be involved in the regulation of the CysBPA transport system.

To further understand the role of the *S. mutans* cystine transporter, expression of the *cysBPA* transporter genes was analyzed under cystine starvation conditions by quantitative real-time PCR. In *S. mutans* UA159, the cystine transporter was found to be significantly up-regulated under cystine starvation. An up-regulation of transporter expression suggests that cells are trying to scavenge for any available cystine or cystine analogues in the environment. The CysR regulator gene was also up-regulated under cystine starvation conditions suggesting it might be a positive regulator of *cysBPA*. In *B. subtilis*, the YtlI regulator is weakly expressed during growth with sulfate, cysteine, and thiosulfate, however, it is strongly expressed when glutathione, taurine and methionine are available (Coppee *et al.*, 2001). YtlI does not appear to be a global regulator of sulfur metabolism but it does actively regulate the TcyJKLMN cystine transporter. In our study, when *cysBPA* expression was tested in the SmCysR mutant under cystine starvation, the transporter genes were highly expressed. Although initially it was thought that CysR was a positive regulator, this result suggested that the regulator had a repressor role in *cysBPA* transporter gene expression. This differs from *B. subtilis*, which possesses a regulator that positively controls cystine transporter gene expression (Burguiere *et al.*, 2005). It is possible that the putative regulator (CysR) created an indirect effect on *cysBPA* expression in *S. mutans*. Furthermore, YtlI was found not to be a global regulator and a global regulator for sulfur or cyst(e)ine metabolism may be present in *S. mutans* but has yet to be identified.

For our study, individual *cys* insertion mutants were generated by PCR-ligation mutagenesis strategy for phenotypic characterization. The operon knock-out mutant was previously constructed in *S. mutans* UA159 strain by Lackovic (2003). The original mutagenesis of the transporter resulted in an acid-sensitive phenotype in the *S. mutans* NG8 strain. However, properties change from strain to strain, hence, it was important to assess whether the transporter knock-out in *S. mutans* UA159 also produced an acid-sensitive phenotype. The wild-type strain and all *cys* mutants were grown at pH 7.5 and pH 5.5. No difference in growth kinetics were seen between the strains at neutral pH, however, there were slight effects in growth kinetics at the acidic pH. Student's *t*-test showed that the difference between growth rates at pH 5.5 was statistically significant. However, a difference of 10 minutes in doubling time is generally not considered biologically significant. Hence, the mutants were not dramatically affected by the acidic environment and therefore, are not true acid-sensitive mutants.

Interestingly, gene expression analysis showed up-regulation of the cystine transporter in acidic biofilms relative to biofilms grown at neutral pH 7.5. It is possible that expression of the transporter is not connected to the acid tolerance response, but it may be a result of general stress experienced by the cells at low pH. Stress-responsive genes have been shown to be up-regulated during acid tolerance (Len *et al.*, 2004), and it may be possible that other regulators involved in stress could also control *cysBPA* expression. Alternatively, a regulator of the acid tolerance response could also regulate the expression of *cysBPA*. The cystine transporter appears to help facilitate growth under minimal conditions (see cystine starvation growth kinetics) and it is possible that the

cystine transporter is up-regulated during acid tolerance or nutrient limitation in a biofilm in order to aid in biofilm development and growth at low pH.

Further phenotypic characterization of the cystine transporter was investigated including the role of the transporter in biofilm formation. When biofilms were grown in a semi-defined minimal medium supplemented with glucose, all *cys* mutants showed a significant decrease in biofilm biomass relative to the biofilms formed by the parent UA159 strain. Since the cystine transporter appears to function in an important role in growth and nutrient acquisition, the removal of the transporter could affect growth during biofilm formation where nutrient availability can be quite variable. As seen in *E. coli*, cysteine can have an effect on biofilm formation. One study done in *E. coli* proposed that either intracellular or extracellular cysteine may be involved in biofilm formation (Sturgill *et al.*, 2004). The *E. coli* cysteine regulon is composed of genes involved in the biosynthesis of cysteine from serine and these genes are positively regulated by a CysB activator and the N-acetyl-L-serine molecule (NAS). NAS is produced through the enzyme action of CysE, a serine acetyltransferase. In *E. coli*, *cysE* null mutants formed biofilms faster and produced more biomass. However, addition of cysteine to the *cysE* mutant slowed down the rate of biofilm formation (Sturgill *et al.*, 2004). The authors proposed that cysteine acted as either an intracellular or extracellular signal to inhibit biofilm development (Sturgill *et al.*, 2004). In addition, it is known that cysteine can be toxic to *S. mutans* at concentrations of 1.67 mM or higher (Cowman *et al.*, 1983), which could inhibit growth. In this study, biofilms were grown in a semi-defined minimal medium, which may have signaled a growth stress or starvation pathway thereby requiring the presence of the cystine transporter. In the absence of the cystine transporter, biofilms were

unable to attain the same biomass as the wild-type strain. It is currently unknown whether cystine or cysteine could also function as a signal in *S. mutans* biofilm formation similarly to *E. coli* (Sturgill et al., 2004). Scanning electron microscopy showed that all the *S. mutans cys* mutants formed sparse, fragile-looking biofilms. In contrast, the wild-type biofilms that formed were dense and intricate. Again, this result reiterates the fact that the cystine transporter is necessary for optimal growth in biofilms. Cell surface structure of individual cells of the mutant strains showed the presence of a "bumpy" outer surface. It is possible that the absence of the cystine transporter altered the cell surface protein expression profile. Undoubtedly, the medium that was used to prepare biofilms for SEM could also affect the biofilm morphology. Regardless, a clear connection to biofilm formation is evident as *cys* mutants were unable to cluster together to form a dense biofilm biomass.

Cystine transporters are present in many bacteria. However, characterization and identification of these various cystine uptake systems have yet to be completed. *B. subtilis* has well-characterized cystine transport systems, which can help us further identify these systems in other species. In this study, a cystine transport system was characterized in *S. mutans* and possessed broad specificity for cystine analogues. The cystine transporter was highly expressed during cystine starvation conditions. Finally, the cystine transporter has a role in growth and biofilm formation. Continuing the work on describing regulatory mechanisms and the possibility of multiple interactions of different physiological pathways with regards to cystine transport may open doors to understanding *S. mutans* nutritional requirements and how amino acids are transported and used in other virulent, infectious bacteria.

CHAPTER 6: FUTURE DIRECTIONS

This study described the initial characterization of an amino acid transport system in *S. mutans* with specificity for cystine. The substrate specificity was identified and some of the conditions that induced its expression have been described. A putative regulator for the *cys* operon was identified, however, its function failed to be consistent with the characteristics of the LysR-type family of regulators in other Gram-positive bacteria. The true regulator for the *cys* operon has yet to be identified. This study characterized one cystine uptake system. However, BLAST searches showed that there may be a second cystine transport system in *S. mutans*. This hypothesized cystine transporter would also require transport assay and competition experiments to determine transporter specificity and whether it may be a lower affinity cystine transporter. It would be interesting to perform a double knock-out mutant for both transporters and determine the physiological effects, including the ability to transport cystine. It would also be important to determine if the true regulator for *cysBPA* also regulated the second cystine uptake system.

Cystine is a sulfur source and sulfur is important for all organisms. To determine the exact sulfur needs of *S. mutans* would require growing the bacteria in the presence of various sulfur sources and different combinations of sulfur sources. The minimal medium presented in this study is a good starting point for testing different combinations of nutrients for growth kinetics. It would also be interesting to see whether the presence of different sulfur sources affected the gene expression of the cystine transporter. Furthermore, radioactive S^{35} could be used to track the usage of sulfur within *S. mutans* and determine sulfur metabolism.

Nutritional stress, especially during growth, appears to affect the expression of the cystine transporter. If stress is related to cystine transport, then, understanding the regulatory mechanisms between stress and the cyst(e)ine metabolic pathway would be essential in further elucidating the relationship between stress and growth. This study has already shown that during cystine starvation or acid stress, the cystine transporter is up-regulated. It would be interesting to see whether other stresses, such as heat or oxidative stress, also up-regulate the cystine transporter.

Moreover, since the cystine transporter plays a role in growth, over-expressing the transporter under various conditions may affect its growth kinetics, biofilm biomass, and biofilm morphology. The cell surface may also be altered due to the absence of the transporter. Hence, it may be necessary to perform two-dimensional gel electrophoretic analysis to determine the changes in protein expression.

Finally, microarray experiments can also be performed to see which genes are up- or down-regulated during cystine starvation. It would be interesting to see whether cysteine-related genes are expressed in the same manner under varying conditions such as nutritional starvation or in the presence of different sulfur compounds.

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