CHARACTERIZATION OF PknB, A PUTATIVE EUKARYOTIC-TYPE SERINE/THREONINE PROTEIN KINASE IN *STREPTOCOCCUS MUTANS*

By

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ABSTRACT

PknB is a putative transmembrane eukaryotic-type serine/threonine protein kinase (STPK) in the cariogenic bacterium *Streptococcus mutans* that affects biofilm formation, genetic competence and acid tolerance. PknB contains extracellular penicillin-binding and serine/threonine kinase associated (PASTA) domains predicted to bind the D-alanyl-D-alanine (D-ala-D-ala) dipeptide of unlinked peptidoglycan. D-ala-D-ala elicits responses dependent and independent of the presence of *pknB*. Biofilm-derived cells of a *pknB*-deficient mutant (PKNB) exhibited concentration-dependent growth enhancement with D-ala-D-ala, which was not a nutrient response as addition of L-alanine or D-alanine did not give the same results. A total of 77 genes were differentially expressed in PKNB, including 7 with putative functions in fatty acid biosynthesis. PKNB was more sensitive to cell wall- and membrane-targeting antibiotics compared to wild-type. Based on these results, PknB in *S. mutans* appears to play an important role in cell wall biosynthesis, response to membrane stress and/or regulation of cell membrane composition.

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List of Abbreviations

ABC; ATP-binding cassette

AgI/II; Antigen I/II

AHL; Acyl-homoserine lactone

AI-2; Autoinducer-2

AIP; Autoinducing peptide

ATR; Acid tolerance response

CHX; Chlorhexidine

CSP; Competence-stimulating peptide

D-ala; D-alanine

D-ala-D-ala; D-alanyl-D-alanine

DPD; 4,5-dihydroxy 2,3-pentanedione

FA; Fatty acid

Fab; Fatty acid biosynthesis

Fru-6-P; Fructose-6-phosphate

GBP; Glucan-binding protein

GBS; Group B Streptococcus

GlcN-6-P; Glucosamine-6-phosphate

GTF; Glucosyltransferase

HK; Histidine kinase

HMM; High molecular mass

IE; Infective endocarditis

L-ala; L-alanine

LMM; Low molecular mass

LTA; Lipoteichoic acid

m-Dpm; Meso-diaminopimelic acid

MIC; Minimum inhibitory concentration

MMG; Minimal medium with glucose

PASTA; Penicillin-binding protein and serine/threonine kinase associated

PBP; Penicillin-binding protein

PBS; Phosphate-buffered saline

PEN; Penicillin G

PEP; Phosphoenolpyruvate

PG; Peptidoglycan

PTS; Phosphotransferase system

RR; Response regulator

RT-PCR; Real-time PCR

SAH; S-adenosyl-L-homocysteine

SAM; S-adenosylmethionine

SDS; Sodium dodecyl sulfate

SEM; Scanning electron microscopy

SRCR; Scavenger receptor cysteine-rich

SRH; S-ribosylhomocysteine

SRP; Signal recognition particle

STPK; Serine/threonine protein kinase

STPP; Serine/threonine protein phosphatase

TCS; Two-component signal transduction system, two-component system

THYE; Todd-Hewitt yeast extract

TYEG; Tryptone yeast extract medium with 0.5% glucose

UDP; Undecaprenyl

UDP-GlcNAc; Undecaprenyl-N-acetylglucosamine

UDP-MurNAc; Undecaprenyl-N-acetylmuramic acid

VAN; Vancomycin

Chapter 1
INTRODUCTION

1.1 The Oral Environment

Dental plaque constitutes a biofilm in which diverse bacterial species are adhered to an oral surface and/or to other bacteria embedded in an exopolysaccharide matrix (Marsh 2004). The environment in the oral cavity is in constant flux, experiencing changes in pH, nutrient availability, oxygen tension, osmotic stress and temperature fluctuations (Lemos 2005; Biswas 2008). Despite these harsh conditions, it has been estimated that over 600 species of bacteria colonize the oral cavity (Paster 2001), with about 20% constituting streptococcal species (Kolenbrander 2000).

Oral bacteria exhibit tissue-specific tropisms and have been found to colonize the different surfaces of the oral cavity, such as the teeth, tongue and even other bacteria (Mager 2003). Studies focussed on examining which species preferentially colonize a particular biological surface in the mouth have found that *Actinomyces* species preferentially colonize hard tissues, such as the teeth, while *Prevotella melaninogenica* and *Veillonella parvula* colonize soft tissue surfaces in higher proportions (Mager 2003). Streptococcal species, such as *Streptococcus mitis, Streptococcus oralis,* and *Streptococcus salivarius* predominantly colonize the soft tissues and are found in higher concentrations in the saliva compared to other organisms (Papaioannou 2009). The tongue is predominantly colonized by Gram-negative organisms, such as *Capnocytophaga gingivalis* and *V. parvula* (Mager 2003). The fact that particular species tend to dominate in specific regions of the oral cavity suggests that bacteria express different receptors and adhesins that determine which surfaces they will ultimately colonize (Mager 2003).

The dental plaque biofilm originates with so-called "pioneer" organisms, or early colonizers, that adhere to host salivary components on the tooth enamel (Kolenbrander 2005; Yoshida 2006). Of these early colonizers, 60-80% are streptococcal species belonging to the viridians group, including *S. mitis, S. oralis* and *Streptococcus. sanguinis*, and form relatively simple biofilm communities (Kolenbrander 1993; Yoshida 2006). Following early colonization, the biofilm microflora becomes more complex as a result of co-aggregation between different

bacterial species. Adhesins on one species may be capable of recognizing a receptor on another cell type (Kolenbrander 1993), thus allowing for diversification of the biofilm community. These interactions have been extensively studied and various adhesins that promote co-aggregation have been identified. For example, the SsaB protein of *Streptococcus sanguis* is believed to mediate co-aggregation with *Actinomyces naeslundii* and *Streptococcus gordonii* (Kolenbrander 1993). In addition, co-aggregation with *A. naeslundii* and various streptococcal species, including *S. gordonii, S. oralis, S. mitis* and *S. sanguinis*, is believed to involve type 2 fimbriae on *A. naeslundii* and receptor polysaccharides on the streptococcal species (Yoshida 2006). Fusobacteria are able to co-aggregate with the early colonizers, as well as later colonizers, such as *Actinobacillus* and *Treponema* species, thereby serving as a bridge between early and late colonizers (Kolenbrander 1993). As a result of these interactions, the biofilm microflora becomes more diverse and complex interactions begin to develop. These interactions can either represent co-operation or competition between plaque bacteria.

The community lifestyle of dental plaque typically involves recycling of metabolic end products among various bacterial species. The metabolic end products of one organism can potentially be used by another organism or groups of organisms to facilitate the creation of food webs within the biofilm (Kuramitsu 2007). Studies have shown that lactic acid produced by *Streptococcus mutans* can be utilized as an energy source by *Veillonella* species and *Streptococcus oligofermentans* (Carlsson 2000; Kuramitsu 2007). In addition, a mutual relationship was observed between *S. oralis* and *A. naeslundii* when nutrient-limited saliva was provided as the sole energy source (Palmer 2001). When grown separately neither of these two species could survive. It was only when these two species were grown together that they were able to flourish. *S. mutans* requires p-aminobenzoic acid during anaerobic growth and studies have shown that *S. sanguinis* is able to provide *S. mutans* with this important vitamin (Carlsson 2000). Bacterial metabolism in dental plaque results in the establishment of pH, oxygen, nutrient and other gradients that are important for microbial growth. This heterogeneous environment allows for typically incompatible organisms to co-exist (Marsh 2005) and facilitates the creation of complex food webs among oral organisms.

Co-operative interactions among oral bacteria are common within the dental plaque community, but so too are antagonistic and competitive interactions. *S. mutans* produces

bacteriocins called mutacins that are small peptide antibiotics with bactericidal activity against closely related species (Qi 2001). Mutacin production is considered to be one of the factors that provides *S. mutans* with a competitive advantage over sensitive species, allowing for enhanced colonization (Kuramitsu 2007). Studies have demonstrated that mutacins produced by *S. mutans* can inhibit growth of *S. sanguinis*, while hydrogen peroxide produced by *S. sanguinis* can inhibit growth of *S. mutans* (Kreth 2008). In addition, there is also evidence that bacteriocins can act as analogues for signalling molecules, which was shown to be the case for *Streptococcus pyogenes* and *S. salivarius* (Upton 2001).

Wang and Kuramitsu (2005) found that when grown in mixed broth or biofilm cultures, strains of the oral streptococci *S. gordonii*, *S. sanguinis, S. mitis* and *S. oralis* were able to down-regulate mutacin production by *S. mutans*. When supernatant fluids from these oral streptococcal species were incubated with *S. mutans* competence-stimulating peptide (CSP), inactivation of CSP was observed, suggesting that interference with mutacin production in *S. mutans* by these oral streptococci may operate via the CSP-mediated quorum sensing system. It has also been shown that *S. gordonii* can attenuate genetic transformation by *S. mutans*, also via modulation of *S. mutans* quorum sensing (Kuramitsu 2006). The presence of both competition and co-operation among bacteria in the oral cavity highlights the vast complexity of interactions found within the dental plaque biofilm.

1.2 Diseases Associated with *S. mutans*

S. mutans is generally considered to be the primary etiological agent of human dental caries due to this organism's ability to produce and tolerate acid; however, in rare cases, *S. mutans* has also been shown to cause infective endocarditis (IE). The following sections summarize the role *S. mutans* plays in the development and progression of these diseases.

1.2.1 Dental Caries

Dental caries progression is a dynamic process involving dissolution of the tooth enamel, dentin and cementum by plaque bacteria, mainly via demineralization of the tooth enamel by acids produced during carbohydrate metabolism of acidogenic bacteria (Hamilton 2000). *S. mutans* is most often associated with human dental caries, as several studies have found higher

levels of *S. mutans* at carious lesions, thus ascribing this organism as the primary causative agent of this disease (Hamada 1980; Yamashita 1993; Liljemark 1996; Lemos 2005). Dental caries is the predominant cause of tooth loss in children and young adults, resulting in a high economic burden associated with this disease (Balakrishnan 2000; Islam 2007).

There are currently three major models to explain the etiology of dental caries: the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis (Aas 2008). The specific plaque hypothesis was first proposed by Clarke in 1924 (Clarke 1924; Balakrishnan 2000) and proposes that only a few species of bacteria, such as *S. mutans*, are involved in caries development (Aas 2008). This idea remains controversial because while many studies have reported that *S. mutans* is associated with dental caries (Hamada 1980; Liljemark 1996), there are other reports that indicate many non-mutans bacteria are also able to produce the amounts of acid necessary for caries formation (van Houte 1994; Kleinberg 2002; Aas 2008b).

The non-specific plaque hypothesis was first proposed by Miller in the late 1800s who believed that all bacteria in the mouth had the potential to be cariogenic (Balakrishnan 2000). This model is still gaining support among some investigators based on evidence that indicates *S. mutans* are not the only bacteria able to promote caries formation (Kleinberg 2002; Aas 2008). The relationship between *S. mutans* and caries is not absolute; high proportions of *S. mutans* may persist on the tooth surface without lesion development, and caries can develop in the absence of this species. In addition to *S. mutans*, lactobacilli, *Actinomyces* spp., *Veillonella* spp., and other non-mutans streptococci have also been found to play important roles in caries development (van Houte 1994; Kleinberg 2002; Aas 2008; Takahashi 2008)

The ecological plaque hypothesis suggests that plaque-mediated diseases, such as dental caries, result from imbalances in the resident microflora. Such imbalances arise from environmental factors that lead to enrichment for oral pathogens (Marsh 2003). This model incorporates aspects of the non-specific plaque hypothesis in that caries can be caused by any acidogenic organisms in the mouth, provided the local environmental conditions support the overall process of caries formation. Studies have shown that plaque pH is in constant flux, with pH falling due to acid production by acidogenic bacteria following intake of dietary

carbohydrate, and then rising due to alkali production by other plaque bacteria (Stephen 1944; Kleinberg 1961; Duquid 1985). It is believed that the balance between this acid-base formation contributes to the overall plaque pH and therefore governs which types of bacteria will predominate at the site, which in turn determines whether or not caries formation occurs (Kleinberg 2002; Takahashi 2008).

With respect to the role of *S. mutans* in the initiation and progression of dental caries, it has been found that this organism is able to metabolize a wide variety of carbohydrates, including sucrose, glucose, fructose and mannose (Ajdic 2002; Mitchell 2003). Fermentation of these carbohydrates via the glycolytic pathway results in the production of pyruvate, which is then converted into lactic acid (Mitchell 2003). When carbohydrate is limited, pyruvate is converted to formate, acetate and ethanol (Carlsson 1974). Lactic acid is the strongest of the acid end products produced by *S. mutans*; therefore, it is considered the most important acid involved in caries development (Balakrishnan 2000). Acids produced by *S. mutans* and other cariogenic plaque bacteria result in acidification of the local environment below the critical pH 5.5 that is required for remineralisation of enamel (Hamilton 2000; Mitchell 2003). With prolonged acid exposure, the demineralisation-remineralisation balance is shifted in favour of demineralisation of the tooth enamel and dental caries occurs (Kirkham 1994; Banas 2004).

1.2.2 Infective Endocarditis

IE is a life-threatening bacterial infection of the endothelial surface of the heart (Nakano 2007). The viridans group of streptococci, as well as *S. mutans* constitute the principle group of bacteria associated with this disease (Nagata 2006). Oral streptococci can often cause bacteraemia following various dental procedures, including oral surgery, flossing, or brushing, allowing these organisms to gain access and adhere to damaged heart valves, causing IE (Nagata 2006; Nakano 2007). It is estimated that about 20% of IE cases attributed to viridans streptococci are in fact caused by *S. mutans* (Banas 2004). Reports have identified a serotype-specific rhamnose-glucose polysaccharide (RGP) of *S. mutans* that acts as a putative adhesin for attachment to human monocytes and fibroblasts (Engels-Deutsch 2003) and human platelets (Chin 2004). Additionally, reports suggest that the *S. mutans* adhesin Antigen I/II may be involved in binding to the extracellular matrix components fibronectin, laminin and collagen

type 1, and the blood component fibrinogen, thus allowing for *S. mutans* to adhere to damaged heart tissue and cause disease (Beg 2002). Research continues in order to understand the pathology and causes of IE. In particular, to identify the various bacterial functions associated with infection and the role *S. mutans* plays in the pathogenesis of this disease.

1.3 Virulence Properties of *S. mutans*

In order for *S. mutans* to cause disease, it must first adhere to the tooth surface and form a biofilm. Once the biofilm is established, dietary carbohydrates can be utilized to produce acid and initiate caries progression. The major virulence properties associated with *S. mutans* are its adhesion to the tooth surface, biofilm formation, acid production (acidogenicity; discussed above), and acid tolerance (aciduricity) (Mitchell 2003; Banas 2004). The following sections will describe each of these virulence factors and how they relate to disease.

1.3.1 Adhesion

Adhesion of oral streptococci to dental surfaces constitutes the initial step of colonization and subsequent biofilm formation (Kelemen 2004). Salivary proteins and glycoproteins adsorb to oral surfaces, including enamel, dentin, epithelial cells, and even other bacteria (Douglas 1994). Interaction with these salivary constituents can not only facilitate adhesion, but also promote removal of the organisms via aggregation or direct killing, provide a source of nutrients, or allow for avoidance of the host's immune system by masking antigenic sites (Douglas 1994). Salivary agglutinin is a 500-kDa oligomeric protein complex consisting of the scavenger receptor cysteine-rich (SRCR) glycoprotein gp340, secretory IgA antibodies and an 80-kDa protein (Jakubovics 2005; Loimaranta 2005). The *S. mutans* adhesin Antigen I/II (AgI/II) binds to the gp340 component of salivary agglutinin to initiate attachment (Loimaranta 2005).

AgI/II (also called SpaP and P1 in *S. mutans*) represents a family of polypeptides expressed by many oral streptococci that demonstrate diverse binding specificities and affinities. But despite this diversity, the AgI/II family of proteins exhibits highly conserved domain structure (Kelemen 2004). The N-terminal domain comprises approximately 400 residues and contains an alanine-rich repeat A-region of about 320 residues. A central domain contains a variable V-region of roughly 360 residues, and the C-terminal domain of about 700 residues

includes a proline-rich P-region of approximately 180 residues (Kelemen 2004). AgI/II is a surface-expressed polypeptide. The C-terminal region contains an LPXTG motif that serves as a target for cleavage by sortase, resulting in linkage of AgI/II to the cell wall (Jakubovics 2005). The alanine-rich and proline-rich regions are believed to be responsible for interaction with salivary components (Hajishengallis 1994; Banas 2004).

Salivary gp340 exists in the fluid phase and attached to the tooth surface in the salivary pellicle (Jakubovics 2005). A study examining the binding capacity of AgI/II to these two different phases of gp340 (Loimaranta 2005) found that fluid-phase and surface-immobilized gp340 exhibit different binding capacities. This suggests that the phase of gp340 results in exposure or masking of different epitopes influencing how bacteria adhere to this protein. The fluid-phase of gp340 appears to promote aggregation of streptococci, including *S. mutans*, and is believed to aid in clearance of the bacteria, such as through swallowing. As a surface-immobilized substrate, gp340 is a receptor for adhesion following binding of this protein to the tooth surface as part of the salivary pellicle (Jakubovics 2005; Loimaranta 2005).

AgI/II has also been implicated in co-aggregation between different bacterial species in the oral cavity. A study by Jakubovics *et al.* (2005) demonstrated that the AgI/II of *S. gordonii*, SspB, was able to mediate co-aggregation with *A. naeslundii*, while other studies have shown that the SspA and SspB proteins were involved in binding to the periodontal pathogen *Porphyromonas gingivalis* (Demuth 2001; Lamont 2002; Jakubovics 2005). In addition, the presence of salivary agglutinin was shown to enhance co-aggregation of *S. mutans* with *S. sanguis* and *Actinomyces viscosus* (Lamont 1991).

AgI/II has been shown to elicit both antibody and T-cell proliferation responses (Ma 1991); therefore, AgI/II has been proposed as a possible vaccine target to control and prevent dental plaque formation and dental caries caused by *S. mutans* (Jakubovics 2005). Active immunization of primates and rodents with AgI/II resulted in protection from dental caries (Smith 2002; Jakubovics 2005) and application of synthetic AgI/II prevented *in vivo* recolonization of human teeth by *S. mutans* (Kelly 1999). While the development of an anti-caries vaccine based on the AgI/II polypeptide seems promising, it is important that the peptide

components confer protection and do not cross-react with non-pathogenic oral bacteria so as to avoid undesirable changes in the oral ecology (Hajishengallis 1994).

Sucrose-independent adhesion mediated by AgI/II appears to be important for initiating attachment of *S. mutans* to the tooth surface; however, sucrose-dependent adhesion mediated by attachment of glucan-binding proteins to synthesized glucan polymers is primarily responsible for establishing colonization and promoting biofilm formation (Banas 2004).

1.3.2 Biofilm Formation

Biofilms are comprised of aggregates of surface-adherent bacteria embedded in a polysaccharide matrix (Marsh 2004). It is believed that the biofilm mode of growth is the natural state adopted by most bacterial species (Jefferson 2004). Biofilms are typically comprised of diverse bacterial species that are spatially and functionally organized, which coincidentally, is the case in dental plaque (Marsh 2005). Several benefits have been proposed regarding the preference for a biofilm lifestyle. These benefits include defence against shear forces, stresses and antimicrobial agents, colonization of a favourable habitat, and a community lifestyle that promotes genetic transfer and sharing of metabolites (Jefferson 2004). Numerous studies have shown that biofilms are more resistant to shear forces, such as salivary flow, fluctuations in pH, oxidative stress, and antibiotics (Dashper 1990) than planktonic cells of the same species (Donlan 2002; Stoodley 2002; Yoshida 2002b; Welin J. 2004). It has been proposed that this could be due to altered diffusion of the antimicrobials and/or altered growth rates of biofilm cells (Donlan 2002).

There are 5 stages of biofilm development: initial attachment to a surface, which in *S. mutans* is governed by adhesins as described in the previous section; irreversible attachment as mediated by the production of extracellular polysaccharide (glucans in *S. mutans*); early development and maturation of biofilm architecture, and dispersal (Stoodley 2002). Biofilm maturation is associated with the production of a complex architecture, including microcolonies, channels and pores (Stoodley 2002). The water channels are found between microcolonies and serve to provide nutrients to the cells and remove wastes (Donlan 2002). Biofilm dispersal is believed to occur by shedding of daughter cells from actively growing cells, shearing of aggregates due to flow effects or detachment in response to nutrient depletion in the

environment. This would allow bacteria to search for new nutrient-rich environments to inhabit (Stoodley 2002; Sauer 2004). More recently, it has been shown that biofilm dispersal can be induced by short-chain fatty acid messengers produced by bacteria. Further studies are being carried out in order to determine the reason for this and identify the mechanisms involved in sensing and responding to these fatty acids (Davies 2009).

In addition to initial attachment via protein-surface interactions, *S. mutans* can utilize sucrose-dependent adhesion mediated by glucosyltransferase enzymes (Gtfs), which produce water-soluble and insoluble glucans from sucrose (Banas 2004). These enzymes are capable of splitting sucrose into its glucose and fructose components and add glucose to a growing glucan polymer (Banas 2004). *S. mutans* produces three Gtfs, GtfB, GtfC and GtfD. GtfB and GtfC are cell-associated and are responsible for synthesis of water-insoluble glucans linked predominantly by α-1,3-glycosidic bonds. GtfD is produced extracellularly and synthesizes water-soluble glucans linked by α-1,6-glycosidic bonds (Banas 2004; Tamesada 2004). Water-insoluble glucans comprise a major component of the plaque biofilm matrix and have been shown to facilitate not only adherence of *S. mutans* to the biofilm, but also co-adhesion between other oral streptococci (Tamesada 2004; Biswas 2007).

In order to adhere to these glucans, *S. mutans* produces four glucan-binding proteins (GBPs): GbpA, GbpB, GbpC and GbpD. Of these, GbpA, GbpB and GbpD can be both secreted and cell-associated, while GbpC is only found covalently attached to the cell wall (Stipp 2008). Various studies have been performed in order to examine the contribution of each of these GBPs to biofilm development. GbpA has been shown to be important for biofilm architecture in sucrose-dependent biofilms (Banas 2003). GbpB has been shown to be immunologically distinct from the other GBPs produced by *S. mutans* and is believed to be essential for viability with a potential role in cell-wall cycling and biosynthesis (Matsumoto-Nakano 2007; Fujita 2007b; Stipp 2008). Evidence suggests that GbpC is more important for early stages of biofilm formation and is involved in glucan-dependent aggregation of bacteria via binding to the water-soluble glucans produced by GtfD (Biswas 2007; Matsumoto-Nakano 2007). Finally, loss of GbpD has been shown to result in extremely fragile biofilms, suggesting that this GBP is important for providing biofilm scaffolding and promoting cohesiveness between glucan and bacteria in the biofilm (Banas 2003; Lynch 2007). Based on these studies, it is apparent that each

of the GBPs produced by *S. mutans* contributes to the overall biofilm architecture and is therefore important for biofilm formation and maturation.

Early stages of biofilm formation by *S. mutans* have been shown to involve the differential expression of 33 proteins, including those involved in carbohydrate metabolism, amino acid biosynthesis, protein folding and cell division (Welin J. 2004). Among the genes found to be required for biofilm formation are *gbpA*, encoding a glucan binding protein, *tarC* ,a regulator for glucan binding proteins, *comABCDE,* components of the quorum sensing system, *ccpA,* encoding for a carbon catabolite control protein, and *dgk,* encoding a stress response and mutacin regulator (Jefferson 2004). Biofilm formation is also associated with differential expression of various genes compared to planktonic growth. Genes that have been found to be differentially expressed in biofilm cells include the competence genes *comDE*, the heat shock protein DnaK, genes involved in carbohydrate metabolism, such as the gene encoding pyruvate kinase, and genes involved in cell division, such as *ftsZ* (Yoshida 2002b; Jefferson 2004; Welin J. 2004).

Studies have shown that the *S. mutans* quorum-sensing system is involved in regulating biofilm formation, as deletion of the *comC, comD* or *comE* genes results in biofilms with reduced biomass and/or altered architecture (Li 2001; Li 2002b). The Com system is also implicated in development of genetic competence (Suntharalingam 2005). Up-regulation of the *com* genes in *S. mutans* biofilm cells, as mentioned above, indicates competence is also increased in biofilms (Cvitkovitch 2003). It is believed that enhancing genetic competence facilitates horizontal gene transfer between bacterial species residing in the dental plaque biofilm, thus promoting genetic diversity and exchange of potentially beneficial genes (Cvitkovitch 2003; Suntharalingam 2005).

Biofilm formation has also been shown to be controlled by LuxS, a protein required for the synthesis of type 2 autoinducer AI-2. This molecule is believed to be involved in interspecies quorum sensing (Cvitkovitch 2003). Mutation of the *luxS* gene results in defective biofilm formation, suggesting that LuxS is required for proper biofilm development (Cvitkovitch 2003; Jefferson 2004; Yoshida 2005).

In addition to these quorum-sensing systems, biofilm formation in *S. mutans* has also been shown to be regulated by various two-component signal transduction systems (TCSs, discussed in a later section). Deletion of TCS-2 (CiaRH) resulted in biofilms of reduced biomass (Levesque 2007) and the VicRK system has also been shown to affect biofilm formation (Senadheera 2005). In addition, deletion of *hk11* or *rr11*, the components of the TCS-11 resulted in biofilms with a sponge-like structure, suggesting this TCS is important for controlling biofilm architecture (Li 2002; Levesque 2007).

The involvement of a number of systems in the control and regulation of biofilm development in *S. mutans* highlights the importance of this virulence property and the complexity of biofilm development. As development of a stable biofilm is important for the initiation of dental caries by *S. mutans,* studies examining the genetic involvement in biofilm formation are essential for understanding the process and allow for identification of potential targets to control cariogenesis.

1.3.3 Acid Tolerance

The dental plaque environment is continuously experiencing shifts in pH from above pH 7.0 to as low as pH 3.0 following ingestion of dietary carbohydrates (Stephen 1944; Kleinberg 1961). These conditions pose a considerable stress on organisms living within the dental plaque environment and for this reason some bacteria, including *S. mutans,* have evolved mechanisms to survive under acidic conditions. Various studies have shown that prior exposure of *S. mutans* to a sub-lethal pH of 5.5 results in enhanced survival at the extremely low killing pH of 3.0-3.5 (Svensater 1997; Hamilton 1998; Welin-Neilands 2007). This adaptive reaction has been called the acid tolerance response (ATR). It involves various alterations in physiology, including changes in gene and protein expression, increased glycolytic activity and increased activity of proton-translocating ATPases, that coincide with enhanced survival (Banas 2004; Welin-Neilands 2007).

The pH optimum of glycolytic enzymes in *S. mutans* is around pH 6.5 for cells growing in acidic conditions, while other oral bacteria have pH optima for glycolysis around pH 7.5 (Hamilton 1991). The ability of *S. mutans* to maintain a relatively alkaline intracellular pH allows glycolysis to be carried out at extracellular pH values as low as 4.0, thus providing a

competitive advantage for *S. mutans* over other less acid-tolerant oral bacteria (Hamilton 1991; Hamilton 1998). Growth of *S. mutans* below pH 5.5 is also accompanied by a decrease in glucose transport by the phosphoenolpyruvate (PEP) phosphotransferase system (PTS), which is one of the systems used to transport glucose into the cell (Hamilton 1991; Dashper 1992). This decreased activity has been attributed to repression of the membrane-bound enzyme II component of the glucose-PTS (Hamilton 1991). Although this apparent decrease in glucose transport would seem counterintuitive given *S. mutans* experiences increased glycolysis under low pH, there are suggestions that another glucose transport system operates at low pH and thus provides the cell with glucose (Dashper 1990; Hamilton 1991; Cvitkovitch 1995). Enhancement in the rate of glycolysis at low pH results in increased lactic acid production. This in turn results in further acidification of the environment allowing *S. mutans* to out-compete other oral bacteria that are unable to tolerate highly acidic conditions.

One of the main mechanisms involved in the ATR of *S. mutans* is the maintenance of intracellular pH (ΔpH). This process employs proton extrusion by end-product efflux and membrane-associated proton-translocating ATPases (also referred to as H^+/ATPases or F_1F_o-ATPases). These systems are responsible for maintaining transmembrane pH gradients with a more neutral intracellular pH and more acidic extracellular pH (Dashper 1992; Hamilton 1998). The lactic acid produced via glycolysis, as mentioned above, is exported from the cell in conjunction with protons, providing an energy-efficient way to remove glycolytic end products from the cell as well as help to maintain ΔpH (Dashper 1996).

Protons in the extracellular environment are able to diffuse through the cell membrane of *S. mutans* and acidify the cytoplasm (Banas 2004). In order to maintain a ΔpH so that cytoplasmic functions can continue at low environmental pH, these protons must be extruded and it is the F_1F_o-ATPases that are partially responsible for this. Activity of the F_1F_o-ATPases has been shown to increase under acidic conditions (Bender 1986; Hamilton 1991; Hamilton 1998), thus allowing for enhanced export of the increasing numbers of protons entering the cell during growth at low pH. Studies have indicated that more acid tolerant bacteria, such as *S. mutans*, have a pH optimum for F_1F_o-ATPases of approximately pH 6.0, while less acid tolerant bacteria exhibit pH optima closer to neutrality (Bender 1986; Hamilton 1991; Sturr 1992), which partly explains why these aciduric bacteria are able to survive under low pH conditions.

Another factor that contributes to the ATR of *S. mutans* is decreased permeability to environmental protons as a result of changes in cell membrane composition. When grown at pH 7.0, the cell membrane of *S. mutans* consists predominantly of short-chained saturated fatty acids, while growth at pH 5.0 is accompanied by a shift in membrane fatty acid composition with increased long-chain mono-unsaturated fatty acids (Quivey 2000; Fozo 2004c). Based on the results of Fozo and Quivey (2004b), it appears that this shift in membrane composition is not only responsible for reducing the permeability of *S. mutans* to protons, but also indirectly contributes to the increased activity of the membrane-bound F_1F_o-ATPases seen at low pH. In addition, it was reported that these mono-unsaturated fatty acids also contribute to increased glycolytic activity, possibly as a result of altered function of membrane-bound proteins.

The ATR of *S. mutans* has been shown to involve changes in protein synthesis, with a total of 199 acid-enhanced proteins, 25 of which appear within 30 minutes of acid shock (Hamilton 1998; Len 2004). These proteins include enzymes involved in metabolism, including glycolysis and branched-chain amino acid biosynthesis (Len 2004), the various subunits of F_1F_o-ATPases, general stress proteins such as heat shock proteins (Hamilton 1998), DNA repair enzymes (Len 2004), and chaperone proteins such as DnaK (Svensater 1997) and GroEL (Len 2004). These results indicate that pH acts as an important environmental stimulus to induce the production of proteins necessary for controlling acidification-associated cellular damage and consequently promoting cell survival (Banas 2004).

Studies examining the acid tolerance of *S. mutans* biofilms have shown that biofilm-derived cells are more resistant to acid than planktonic cells (Li 2001; Welin-Neilands 2007). This is believed to be due to cell density-dependent regulation of the ATR via the quorum-sensing system in *S. mutans* (Li 2001; Cvitkovitch 2003; Welin-Neilands 2007). The hypothesis is that exposure to low pH results in release of a signalling molecule (possibly the competence-stimulating peptide CSP) that subsequently induces the ATR in the population (Li 2001; Cvitkovitch 2003; Perry 2009).

The ATR is important for survival of *S. mutans* under acid stress. The extent of this response in *S. mutans* sets it apart from other less acid-tolerant oral bacteria. It provides *S.*

mutans with a competitive advantage, allowing it to dominate over other oral bacteria under the acidic conditions experienced in dental plaque and thus contributes to caries development.

1.4 Signal Transduction

Protein phosphorylation is an important mechanism by which extracellular signals, such as changes in the environment, are translated into cellular responses (Hussain 2006). In bacteria, the primary mechanism implicated in sensing and responding to changes in the environment involves two-component systems; however, research over the last decade has led to the identification of eukaryotic-type serine/threonine protein kinases in bacteria and archaea that represent a novel set of signalling systems in prokaryotes.

1.4.1 Two-Component Signal Transduction Systems

Two-component systems (TCS) are considered to be the primary means by which bacteria sense and respond to environmental stimuli, including oxidative stress, osmotic shock, nutrient starvation and pH fluctuations (Beier 2006; Levesque 2007; Biswas 2008). A typical TCS consists of a membrane-bound histidine kinase receptor (HK) and an intracellular response regulator protein (RR). The HK detects the environmental signal and undergoes ATP-dependent autophosphorylation at a conserved histidine residue in the intracellular kinase domain. The RR protein then catalyzes phosphoryl transfer from the HK to a conserved aspartate residue in its regulatory domain. Phosphorylation of the RR results in a conformational change that activates the RR and allows it to bind to promoter regions of target genes in order to activate and/or repress gene expression (Stock 2000).

Over 4000 TCSs have been identified in 145 bacterial genomes (Beier 2006). *S. mutans* possesses 14 TCS and one orphan RR, named GcrR (Ajdic 2002; Levesque 2007; Biswas 2008). Various studies have been carried out to identify the phenotypes regulated by these TCSs. The VicRK system (TCS-1) has been shown to be involved in regulating sucrose-dependent adhesion, biofilm formation, genetic competence development and response to oxidative stress (Senadheera 2005). Deletion of CiaH, the HK of TCS-2, was shown to result in biofilms of reduced biomass and cells growing in short chains, implicating this TCS in regulating biofilm formation, cell growth and/or cell division (Levesque 2007). TCS-3 was shown to be involved in

acid tolerance and may play a role in regulating expression of mutacin genes (Levesque 2007). TCS-11 is thought to play a role in cell segregation and biofilm architecture, as deletion of *hk11* or *rr11* results in sponge-like biofilms with cells growing in very long chains (Cvitkovitch 2003). This system is also believed to be involved in acid tolerance (Li 2002) and sensing and responding to cell envelope stress, as deletion of this system results in sensitivity to membrane-targeting antibiotics and chemicals (Suntharalingam 2009). For the most part, the signals detected by the HKs of the 14 TCSs in *S. mutans* remain largely undefined. The genes whose expression is altered via signalling through these systems have yet to be identified. Hence, further studies of these systems are necessary to increase our understanding of how *S. mutans* senses and responds to environmental changes. Perhaps the best-characterized TCS is the so-called quorum-sensing competence regulon that is present in many streptococcal species and has been found to control genetic competence development in *S. mutans*.

1.4.2 Quorum Sensing in Bacteria

Quorum sensing was first discovered by studying light production in the bioluminescent bacterium *Vibrio fischeri,* which was found to be controlled by an acyl-homoserine lactone autoinducer and a transcriptional regulator (LuxR) (Nealson 1970; Bassler 1999). Since that time, numerous studies have identified quorum sensing systems throughout all bacteria. Quorum sensing is a means by which bacteria communicate through the production and detection of small signalling molecules called autoinducers (AI) in order to sense population density and regulate gene expression (Syvitski 2007). These autoinducers are constitutively made and as the population grows so too does the extracellular concentration of AI. Once a threshold concentration is reached, the bacteria detect the signalling molecule and respond by altering gene expression (Bassler 2006).

Quorum sensing in Gram-negative bacteria involves detection of an acyl-homoserine lactone (AHL) autoinducer, which is produced by a LuxI-type autoinducer synthase (Bassler 2006). AHLs are able to diffuse across the cell membrane and when they reach a threshold concentration they become bound to a regulator protein (LuxR), which activates transcription of target genes (Bassler 1999). This quorum sensing system has been shown to be employed by over 30 species of Gram-negative bacteria and controls various functions, including swarming

motility and adhesion in *Serratia marcescens* (Parker 2009), virulence in *Pseudomonas aeruginosa* (Bassler 1999), and light production in *V. fischeri* (Engebrecht 1984).

Quorum sensing in Gram-positive bacteria utilizes short signal peptides, sometimes referred to as autoinducing peptides (AIP), that are exported by an ATP-binding cassette (ABC) export protein (Bassler 1999; Sturme 2002). The signalling peptide is then detected by the HK of a TCS. Signal transduction is achieved through a phosphorylation cascade that ultimately results in alteration of gene expression (Bassler 2006). Quorum sensing in Gram-positive bacteria has been shown to regulate a variety of processes, including competence development in *S. gordonii, Streptococcus pneumoniae,* and *S. mutans*, sporulation in *Bacillus subtilis*, and bacteriocin production in *Lactococcus lactis* (Cvitkovitch 2003).

1.4.3 Quorum Sensing in *Streptococcus mutans*

1.4.3.1 The LuxS System

The quorum sensing mechanisms described above constitute intraspecies communication involving the production and response to a unique autoinducing signal within one or closely related species. Studies of quorum sensing in *Vibrio harveyi* have discovered that this bacterium produces both a Gram-negative-type AHL and another autoinducer, named autoinducer-2 (AI-2), that is synthesized by the LuxS autoinducer synthase (Bassler 1999; Xavier 2003). The AI-2 molecule is a furanosyl borate diester that shares no similarity to other autoinducers identified to date (Xavier 2003). It is synthesized from S-adenosylmethionine (SAM), which serves as a methyl donor for RNA, DNA and proteins. Methyl donation converts SAM to a toxic intermediate S-adenosyl-L-homocysteine (SAH), which becomes hydrolyzed to S-ribosylhomocysteine (SRH) and adenine by a nucleosidase enzyme Pfs. LuxS catalyzes cleavage of SRH to homocysteine and 4,5-dihydroxy 2,3-pentanedione (DPD), which is believed to produce AI-2 spontaneously in solution (Xavier 2003; Sztajer 2008). The biosynthetic pathway of AI-2 up to the point of DPD production is the same for a number of species studied, including *E. coli, V. harveyi, S. aureus, Salmonella typhimurium* and *Enterococcus faecalis*; therefore, it is assumed that all bacteria containing LuxS produce the DPD precursor. However, subsequent steps in this pathway have not been characterized, so it is not known if there is a diverse array of AI-2 molecules produced by different species (Xavier 2003).

LuxS homologues have been found in a wide variety of both Gram-negative and Gram-positive bacteria, including *E. coli, B. subtilis, S. pneumoniae, S. pyogenes, Staphylococcus aureus*, and *S. mutans*, which suggests that AI-2 could serve as a universal signal that facilitates interspecies communication (Bassler 1999; Xavier 2003). Evidence for this comes from studies showing that culture supernatants from various *luxS*-containing bacteria, including *S. mutans*, are able to induce luminescence in *V. harveyi* (Merritt 2003; Wen 2004). It has also been shown that dual-species biofilm formation between *S. oralis* and *A. naeslundii* was abolished upon deletion of the *luxS* gene in *S. oralis*, while addition of synthetic AI-2 was able to restore dual-species biofilm formation (Rickard 2006). Moreover, biofilm formation by a *luxS* mutant of *S. mutans* could be restored to wild-type levels by adding cell-free supernatants of *S. gordonii, S. sobrinus* and *Streptococcus anginosus*, all of which contain a *luxS* gene. Together, these findings support a role for LuxS signalling in interspecies communication and suggest that this system is important for the development of mixed-species oral biofilms (Yoshida 2005).

Studies examining the effects of *luxS* mutation in *S. mutans* have found that LuxS is involved in biofilm formation, oxidative and acid stress, and bacteriocin production. Merritt et al. (2005) reported that a *luxS* mutant of *S. mutans* UA140 strain exhibited decreased production of mutacin I, a wide-spectrum bacteriocin. It was observed that this mutant showed decreased expression of *mutR*, a mutacin I regulator, and enhanced expression of *irvA*, a repressor of mutacin I production, providing a possible explanation for this observed phenotype. Findings of this study let to the implication that LuxS is responsible for repressing *irvA* so as to ensure production of mutacin I. A separate study using a different strain of *S. mutans*, UA159, which does not produce mutacin I, but does possess *irvA* and *mutR* homologues, showed decreased expression of *irvA*; however, the function of this repression in the absence of a mutacin I gene in UA159 remains unknown (Sztajer 2008).

A study by Wen and Burne (2004) demonstrated that a *luxS* mutant was more susceptible to oxidative and acid stresses, suggesting that LuxS plays a role in controlling expression of multiple stress response pathways. The *luxS* mutant also displayed down-regulation of acid-inducible DNA repair enzymes, which could explain why this mutant also displayed a deficiency in acid tolerance. This study also found that compared to wild-type, the *luxS* mutant produced weaker biofilms exhibiting a “hive-like” architecture with large gaps between microcolonies. A

similar architecture was seen in a study by Merritt *et al.* (2003); however, in this study, it was found that the mutant biofilms were actually stronger and more resistant to detergents than the wild-type. One hypothesis that was proposed to explain this observation was that the defect in interspecies communication by deletion of *luxS* would prevent the mutant strain from detecting other neighbouring species, thereby increasing biofilm production capacity. Another explanation for this comes from a study by Yoshida *et al.* (2005) that demonstrated increased activity of *gtfB* and *gtfC* in a *luxS* mutant. This would lead to increased extracellular glucan production, which is known to be important for proper biofilm formation in *S. mutans*.

The role of LuxS as a quorum sensing system has been challenged by some reports, including a study by Sztajer et al. (2008) that examined global gene expression in a *luxS* mutant of *S. mutans* UA159. This study reported expression of 30% of all genes were affected in this mutant, including genes involved in biofilm formation, acid tolerance, oxidative stress tolerance and bacteriocin production, which would explain the previously-reported phenotypes as mentioned above. However, the authors of this study suggest that LuxS serves as a central enzyme in a number of metabolic processes and the various phenotypes observed in *luxS* mutants are merely due to pleiotropic effects of *luxS* deletion. Further evidence supporting this claim stems from studying growth-phase-dependent gene expression in the *luxS* mutant. It has been previously shown that in *S. pneumoniae*, competence-stimulating peptide (CSP) induced early, late, and delayed phases of gene expression over a period of 22 minutes; however, no such temporal patterns of gene expression were seen for the *luxS* mutant, providing further evidence that LuxS does not serve as a quorum sensing system (Sztajer 2008). It is possible that AI-2 could be functioning to inform the recipient species about the density of other *luxS*-expressing bacteria in surrounding areas as opposed to its own cell density, as is the case with CSP-mediated signalling (Sztajer 2008). The role, if any, of LuxS in interspecies communication remains to be elucidated and further studies will be important in our understanding of this potential signalling system across the many bacterial species harbouring a *luxS* gene.

1.4.3.2 The Competence Stimulating Peptide Signalling System

Genetic transformation is the process by which bacteria take up exogenous DNA from the environment and integrate it into their own genomes. This enables the recipient to acquire

new genes that promote the development of antibiotic resistance and genetic variation (Li 2001b). In streptococci, competence develops in the early to mid-logarithmic phase of growth (Li 2001b).

In *S. mutans* a number of physiological processes are regulated by a peptide-based species-specific quorum sensing system, including biofilm formation (Li 2002b), acid tolerance (Li 2001), and genetic competence (Li 2001b). This quorum sensing system (Figure 1), herein referred to as the Com system, has been extensively studied as a competence-inducing system in *S. pneumoniae*, but is believed to operate in a similar manner in *S. mutans*. The Com system utilizes early gene products encoded by *comAB* and *comCDE* loci. C*omA* encodes an ABC transporter (ComA) and *comB* encodes an accessory protein (ComB). Together, these proteins are involved in the processing and export of the competence-stimulating peptide (CSP), which is encoded by *comC* (Petersen 2000). CSP is a 21-amino acid linear peptide that contains a leader peptide followed by a double-glycine motif. This leader sequence is cleaved during processing by ComAB to yield the mature signalling peptide (Li 2001b; Sturme 2002). ComD is the HK receptor that detects CSP and ComE is the RR responsible for activating expression of both *comAB* and *comCDE* operons, thus providing a positive regulatory loop. Late gene products are controlled by ComX, an alternate sigma factor that recognizes a *com*–box consensus sequence in the promoter region of operons involved in DNA uptake and recombination and initiates their transcription (Perry 2009). Inactivation of any of the *comCDE* genes of *S. mutans* results in a competence-deficient phenotype that can only be restored in the *comC* mutant through addition of exogenous synthetic CSP (Li 2001b), thereby providing strong evidence that this locus is involved in competence development in *S. mutans*.

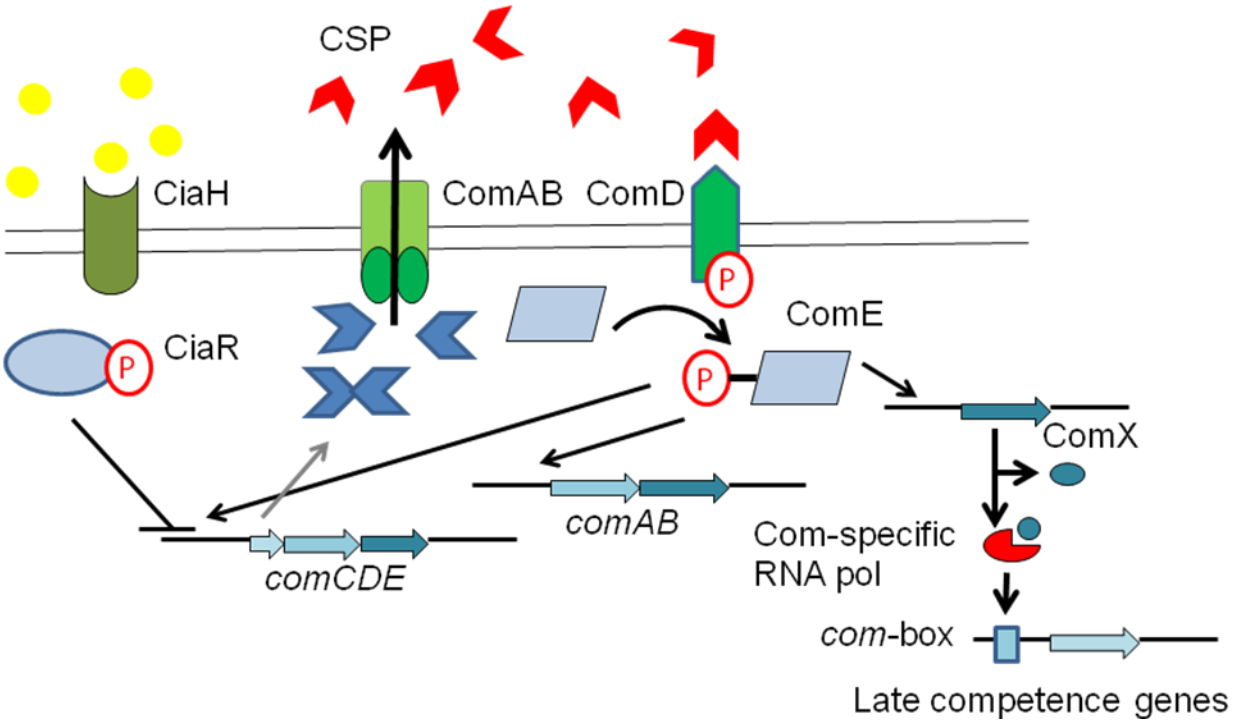

Figure 1. CSP-mediated quorum sensing system in *S. pneumoniae.* This system is thought to function in a similar manner in *S. mutans*. Figure modified from (Cvitkovitch 2003).

An *S. mutans comC* mutant unable to produce CSP produced biofilms with significantly altered architecture compared to the wild-type, which appeared to be due to the formation of very long chains of cells resulting in clumped biofilms with web-like microcolonies (Li 2002b). Mutants of *comD, comE,* or *comX* resulted in thinner biofilms with reduced biomass and in the case of the *comX* mutant, a competence-defective phenotype. While addition of synthetic CSP to the *comC* mutant could restore biofilm architecture, biofilm biomass was not restored in the *comD* or *comE* mutants. Addition of synthetic CSP to a *comCDE* mutant, which is unable to produce or respond to CSP, resulted in partial restoration of biofilm architecture. Taken together, these results not only establish a role for the Com system in biofilm formation of *S. mutans*, but also suggests the presence of another receptor that responds to CSP (Li 2002b) or alternative inputs via other TCS (Perry 2009). Currently, the identity of this second CSP receptor remains unknown.

1.4.4 Eukaryotic-type Serine/Threonine Protein Kinases

In 1991, a novel eukaryotic-type serine/threonine protein kinase (STPK), Pkn1, was identified in *Myxococcus xanthus* that was found to play a role in cellular development, as deletion of the *pkn1* gene resulted in premature differentiation and spore formation (Bakal 2000). This finding has led to the discovery of other STPKs, as well as serine/threonine protein phosphatases (STPPs) in many bacteria and archaea. To date, the majority of characterized and putative STPKs appear to be transmembrane proteins, suggesting that these proteins may function as receptors or sensors that directly interact with extracellular ligands and comprise novel signalling systems in prokaryotes, separate from traditional TCSs (Bakal 2000; Krupa 2005).

Numerous studies have sought to identify STPKs in a variety of bacteria and elucidate pathways that are under their control. Madec *et al.* (2002) identified PrkC and PrpC, an STPK and STPP, respectively, in *B. subtilis*. Deletion of either the *prkC* or *prpC* genes resulted in a significant reduction in sporulation efficiency, as well as reduced biofilm formation, suggesting that both of these proteins are involved in these processes. A recent study by Shah *et al.* (2008) demonstrated that PrkC was able to bind peptidoglycan (PG) fragments and initiate spore germination via phosphorylation of the essential ribosomal GTPase, EF-G. Germination could also be induced using PG from *E. coli*, which contains a meso-diaminopimelic acid (m-Dpm) as the third amino acid residue in the stem peptide. Germination could not be induced using PG from *S. aureus*, which contains an L-lysine residue at the third position. These findings led to the discovery that it is the third amino acid residue (m-Dpm vs. L-lys) in the stem peptide that confers specificity of PrkC for PG. While this study clearly demonstrates a role for PrkC in germination and detection of PG, the role of PrkC and PrpC in biofilm formation and spore development as reported by Madec *et al.* (2002) remains unclear.

Echenique *et al.* (2004) identified StkP in *S. pneumoniae* and demonstrated a role in virulence, as this protein was required for optimal lung infection and growth in blood following injection. StkP was also shown to positively regulate *comCDE* expression in an antagonistic role with CiaRH repression, thus implicating StkP in competence development. Saskova *et al.* (2007) demonstrated that an *stkP* mutant exhibited reduced transformation efficiency, providing further

support that StkP is involved in competence development in *S. pneumoniae.* The *stkP* mutant also exhibited a long lag growth phase and slower doubling time in early exponential growth phase, and was sensitive to a variety of stresses, including high temperature, oxidative stress, osmotic pressure and acid (Saskova 2007). Global transcriptome analysis revealed more than 4% of *S. pneumoniae* genes were differentially expressed in the mutant, including genes involved in oxidative stress response, cell wall biosynthesis, purine and pyrimidine metabolism, and genetic competence development (Saskova 2007). These findings suggest that the *stkP* mutation is broadly pleiotropic and StkP may function as a global regulator in *S. pneumoniae*.

Other roles for STPKs have also been discovered. For example, the STPK in *E. faecalis*, PrkC, is believed to be a general sensor for cell envelope stress, as the mutant was more sensitive to cell wall-targeting antibiotics (Kristich 2007). Stk1 in *Streptococcus agalactiae* has been implicated in growth, cell segregation and virulence, as the *stk1* mutant exhibited slow lag phase growth, long chain formation, and reduced virulence in rat infection models (Rajagopal 2003). SP-STK in *S. pyogenes* was shown to control virulence, growth and development, and modulation of the cell surface (Jin 2006). An *sp-stk* mutant also exhibited slow growth, as well as altered cell morphology and a loosely attached fibrillar outer layer structure (Jin 2006). PknA and PknB of *Corynebacterium glutamicum* were shown to play a major role in regulation of cell shape and PG synthesis (Fiuza 2008). Complete inactivation of *pknA* or *pknB* resulted in cell death, indicating these proteins are essential for viability in this organism. Partial depletion of either of these genes resulted in delayed growth and the production of elongated cells that were larger than wild-type, which was believed to be due to defects in PG synthesis (Fiuza 2008). As a final example, STK and STP (the phosphoprotein phosphatase) in *S. aureus* were shown to play a critical role in maintenance of cell wall architecture and therefore resistance to cell wall-targeting antibiotics (Beltramini 2009). Double mutants of both STK and STP displayed numerous cell division defects, including irregular shape and size, which also implicates these proteins in the regulation of cell division.

Many STPKs that are conserved among Gram-positive bacteria possess a C-terminal region similar to that of high molecular weight penicillin binding proteins, which contain penicillin-binding protein and serine/threonine kinase associated (PASTA) domains, and are believed to play a critical role in cell wall biosynthesis and cell division (Yeats 2002; Krupa

2005). These include PknB in *S. mutans* (Hussain 2006), PrkC in *B. subtilis* (Shah 2008), PrkC in *E. faecalis* (Kristich 2007), and StkP in *S. pneumoniae* (Echenique 2004). PASTA domains are small globular domains of approximately 70 amino acids (Yeats 2003) found at the C-terminus of penicillin-binding proteins (PBPs) and STPKs of Gram-positive bacteria (Pfam http://pfam.janelia.org/family?acc=PF03793; Yeats 2002). This domain binds to β-lactam antibiotics and is therefore believed to be responsible for detecting unlinked peptidoglycan of the cell wall, specifically the D-alanyl-D-alanine (D-ala-D-ala) dipeptide (Yeats 2002). PASTA-containing STPKs have been proposed to be the master regulators of peptidoglycan cross-linking, making them essential for growth and development in bacteria (Yeats 2003). Evidence for this stems from a variety of studies on PASTA-containing STPKs that have demonstrated a role in growth and/or development (Madec 2002; Rajagopal 2003; Kristich 2007; Beltramini 2009).

The kinase domain of several STPKs has been shown to undergo autophosphorylation via ATP hydrolysis (Obuchowski 2000; Rajagopal 2003; Novakova 2005; Jin 2006; Fiuza 2008). This added phosphate can then be transferred to various substrates and subsequently initiate a phosphorylation cascade that mediates a cellular response to the signal (Bakal 2000). Many studies have shown that STPPs are able to dephosphorylate their cognate STPKs (Rajagopal 2003; Novakova 2005; Jin 2006; Saskova 2007), and in some cases the STPP is responsible for dephosphorylation of STPK substrates (Gaidenko 2002; Rajagopal 2003). Reversible phosphorylation of STPKs and their substrates via the action of STPPs is believed to be an important mechanism governing the regulation of the numerous cellular responses thought to be under the control of STPKs. In a few instances, attempts to delete the STPP gene have been unsuccessful (Jin 2006), suggesting that this protein is essential for viability, although the precise mechanisms governing this have yet to be revealed.

Specific substrates for some STPKs have been identified. Stk1 in *S. agalactiae* was found to phosphorylate PpaC, a manganese-dependent inorganic pyrophosphatase responsible for regulating intracellular inorganic pyrophosphate (PPi) levels, which have global effects on cell metabolism, growth and division (Rajagopal 2003). SP-STK in *S. pyogenes* phosphorylates the histone-like protein Hlp (Jin 2006), which is a homolog of HlpA in *S. mutans* (Stinson 1998). Additionally, a study by Udo et al. (2000) demonstrated that Pkn2 in *M. xanthus* was also

responsible for the phosphorylation of the histone-like protein HU. Bacterial histone-like proteins participate in DNA replication and nucleoid organization, and also contribute to the regulation of gene expression (Jin 2006). *S. pneumoniae* StkP was found to phosphorylate phosphoglucosamine mutase (GlmM), which is responsible for catalyzing the initial step in the biosynthesis of UDP-N-acetylglucosamine, an important precursor for cell wall components such as PG and teichoic acids (Novakova 2005). Multiple STPKs of *Mycobacterium tuberculosis* are responsible for phosphorylation of EmbR, a transcriptional regulator that interacts with RNA polymerase and is essential for the regulation of an operon involved in cell wall biosynthesis (Sharma 2006). PknB of *M. tuberculosis* is responsible for phosphorylating the penicillin-binding protein PBPA, which is required for cell division and PG synthesis (Dasgupta 2006), as well as N-acetylglucosamine-1-phosphate uridyltransferase (GlmU) (Parikh 2009), an enzyme possessing a similar function to the previously mentioned GlmM in *S. pneumoniae*. Although some STPK substrates have been identified, the majority remain to be elucidated. The identification of kinase substrates is important for understanding the mechanisms by which STPKs regulate a seemingly wide range of cellular processes.

Recent studies have demonstrated that some STPKs are capable of phosphorylating the response regulators of TCS, suggesting that there is possible cross-talk between these signalling systems. In *S. pneumoniae,* StkP phosphorylates the DNA-binding domain of RitR, a response regulator that lacks a cognate HK, while the cognate phosphatase PhpP is capable of direct interaction with promoter-bound RitR (Ulijasz 2009). It is believed that interaction of RitR with PhpP may promote expression of *Piu* genes involved in haem transport, while StkP is involved in disrupting the RitR-PhpP complex and thus reducing *piu* expression (Ulijasz 2009). Studies of Group B Streptococcus (GBS) have demonstrated that Stk1 is able to phosphorylate the CovR response regulator, a homolog of VicR in *S. mutans* (Rajagopal 2006; Lin 2009). It was found that threonine phosphorylation of CovR by Stk1 resulted in decreased aspartate phosphorylation and DNA binding, while aspartate phosphorylation by the HK CovS reduces threonine phosphorylation. These results demonstrate that the sensor kinases CovS and Stk1 may respond to different extracellular signals that dictate whether CovR is phosphorylated on an aspartate residue by CovS or on a threonine by Stk1 (Rajagopal 2006). These different phosphorylation sites affect promoter DNA binding capacity of CovR, thus regulating gene expression (Rajagopal 2006; Lin 2009). Based on these studies, it is possible that STPKs and TCS can work together to

regulate gene expression in bacteria. Furthermore, this suggests that STPK signalling systems are not completely separate entities from traditional TCSs, as was previously believed.

STPKs have been extensively studied in eukaryotes, but comprise a relatively new field of research in prokaryotes. STPKs have been implicated in the regulation of a variety of cellular processes, including growth and development, biofilm formation, stress responses, virulence and genetic competence. Although STPKs and STPPs have been identified in several Gram-positive organisms, including *B. subtilis* (Madec 2002), *S. pneumoniae* (Echenique 2004; Saskova 2007), and *S. pyogenes* (Jin 2006), there is still little known about the signals they detect, their mechanism of action, and specific substrates.

1.4.4.1 PknB in *S. mutans*

A recent study by Hussain et al (2006) identified *pknB*, a gene encoding a transmembrane eukaryotic-type STPK, and its putative STPP, designated *pppL*, in *S. mutans*. A BLAST search revealed that PknB shared highest similarity to SP-STK of *S. pyogenes* (63% identity), Stk1 of *S. agalactiae* (59% identity) and StkP of *S. pneumoniae* (51% identity). The *pknB* gene overlaps and is co-transcribed with *pppL*, its putative cognate phosphatase. A consensus promoter sequence upstream of *pppL* and a transcriptional terminator downstream of *pknB* were also identified (Figure 2) (Hussain 2006). A topology analysis and protein domain search using the SMART database (http://smart.embl-heidelberg.de/) predicted that PknB is an N-in, C-out transmembrane protein with an intracellular kinase domain and three tandem PASTA domains exposed extracellularly, suggesting that PknB may detect unlinked peptidoglycan (Hussain 2006). The *pknB* gene is located directly upstream of the *hk11/rr11* locus (Figure 2), a TCS in *S. mutans* that has been shown to be involved in biofilm formation and acid tolerance. Since STPK genes are often adjacent to genes encoding their substrates, it has been proposed that these two systems could be involved in cross-talk; however, this has not been determined to date (Hussain et al, 2006).

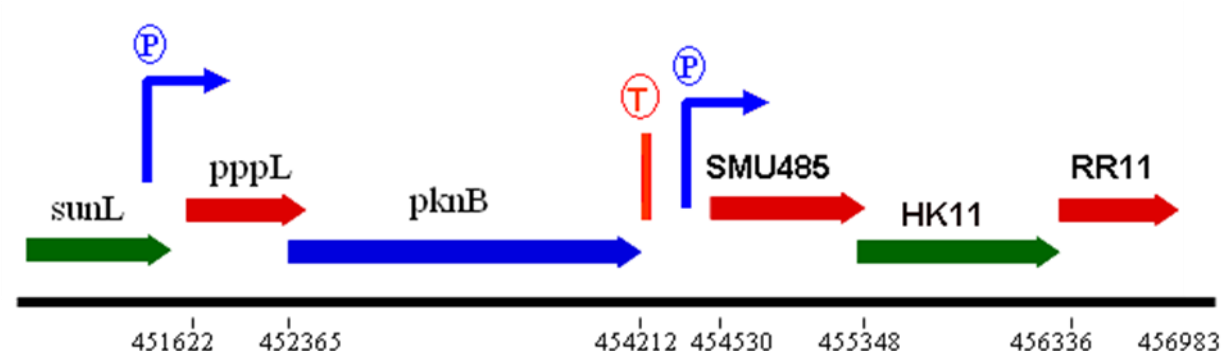

Figure 2. Gene map of the pknB locus in *Streptococcus mutans*. Putative promoter (P) and terminator (T) regions are shown. *pppL* encodes a putative phosphoprotein phosphatase and is co-transcribed with *pknB* (Hussain 2006). The operon is encoded on the plus strand and spans a region of approximately 2.6kb.

Deletion of the *pknB* gene resulted in biofilms of reduced biomass, with smaller microcolonies and larger gaps compared with *S. mutans* UA159 wild-type. The mutant was also sensitive to growth at a low pH of 5.5 and was deficient in genetic transformation, which could not be restored or improved by addition of exogenous CSP. The mutant also exhibited reduced growth in early lag phase compared to wild-type, and displayed a clumping phenotype when grown in liquid culture (Hussain 2006). The variety of phenotypes associated with the *pknB* mutant suggests a role for PknB in the regulation of these important processes in *S. mutans*.

Very little is known about the signalling mechanism via PknB, including the extracellular signal(s) being detected, possible phosphorylation substrates and downstream genes that are directly or indirectly affected by PknB. The aims of this study are to examine the response to the D-ala-D-ala dipeptide in the presence (UA159 wild-type) and absence (PKNB mutant) of *pknB* through DNA microarray analysis and to investigate the molecular basis for PknB-mediated phenotypes.

Chapter 2
HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The presence of PASTA domains in the extracellular region of PknB leads to the prediction that PknB may bind to β-lactam antibiotics, such as penicillin, and their peptidoglycan (PG) analogues, mainly the D-alanyl-D-alanine dipeptide of unlinked PG (Yeats 2002; Hussain 2006). If PknB detects unlinked PG via its PASTA domains, it could serve as a sensor for unlinked PG in the environment and differentially regulate genes involved in growth and cell wall biosynthesis (Yeats 2002).

A *pknB*-deficient mutant of *S. mutans* was shown to have defects in biofilm formation, growth at acidic pH and genetic competence (Hussain 2006), suggesting that PknB may be implicated in directly or indirectly regulating the expression of genes involved with these phenotypes. Since there is no putative signal, signalling pathway, or substrates associated with the PknB signalling system, we hypothesize the following:

An extracellular signal, possibly the D-alanyl-D-alanine dipeptide of unlinked peptidoglycan, is detected by the serine/threonine protein kinase, PknB in *S. mutans*, resulting in activation of a signalling pathway that leads to differential expression of genes involved in growth, biofilm formation, acid tolerance and genetic competence development.

2.2 Objectives

The objectives of the present study were as follows:

1. To examine the response to D-ala-ala in the presence and absence of *pknB*.
2. To investigate the molecular basis for phenotypes associated with PKNB

Chapter 3
METHODS

3.1 Bacterial Strains and Growth Conditions

S. mutans UA159 strain was used as the wild-type for this study. A *pknB*-deficient mutant derived from UA159, designated PKNB, was obtained from Elaine Allan at the Eastman Dental Research Institute in London, England and was previously described (Hussain 2006). All strains were grown in Todd-Hewitt yeast extract broth (THYE) containing 0.3% (wt/vol) yeast extract and incubated at 37°C with 5% CO_2. The PKNB strain was maintained in THYE supplemented with 1mg/ml kanamycin. Biofilms were grown in minimal medium (MMG) (50mM Trizma® maleate, 19mM NH_4Cl, 20mM K_2HPO_4, 11mM KH_2PO_4, 50mM $NaHCO_3$, 5.5mM sodium pyruvate, 0.1mM $MnCl_2{\cdot}4H_2O$, 72µM $FeSO_4{\cdot}7H_2O$, 2mM $MgSO_4{\cdot}8H_2O$) supplemented with filter-sterilized vitamins (2.7µM riboflavin, 1.5µM thiamine-HCl, 0.04µM biotin, 8µM nicotinic acid, 0.7µM p-aminobenzoic acid, 2µM calcium pantothenate, 5µM pyridoxal-HCl), amino acids (10mM L-glutamic acid, 3mM L-cysteine-HCl, 7mM L-leucine) and 1% (wt/vol) glucose. For measurement of growth kinetics in the presence of D-alanyl-D-alanine (D-ala-D-ala), strains were grown in tryptone yeast extract medium (10% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract and 17mM K_2HPO_4) supplemented with 0.5% glucose (TYEG).

3.2 DNA Microarrays

Four independent cultures of *S. mutans* UA159 and PKNB were grown overnight in THYE with antibiotic where required. Cultures were centrifuged at 4500rpm for 10min and resuspended in pre-warmed MMG medium then diluted 1:5 (8ml culture in 32ml MMG) and grown to mid-log phase (OD_{600}nm ≈ 0.4). Cultures were then centrifuged and washed three times in 10mM sodium-potassium buffer, with 10min centrifugation between washes. After the final wash, cells were resuspended in 20ml of buffer and incubated for 15min at 37°C with 5% CO_2. For the microarray experiment examining the response to D-ala-D-ala, cells were resuspended in 20ml of buffer and split into two 10ml cultures. 1mg/ml D-ala-D-ala was added to one culture, while 5mg/ml was added to the other. Samples were incubated for 15 min at 37°C 5% CO_2. Following incubation cultures were centrifuged and snap frozen in liquid nitrogen for subsequent RNA isolation and DNase treatment as described previously (Burne 1999).

Mutant and wild-type DNase-treated RNA was converted to cDNA and labelled with cyanine 3 (Cy3) fluorescent dye. RNA isolated from mid-log phase cultures of UA159 was prepared previously and used as reference samples. Reference cDNA was labelled with cyanine 5 (Cy5) fluorescent dye. Samples were labelled according to the Aminoallyl Labelling Procedure for Microarrays protocol (M007) (http://pfgrc.jcvi.org/index.php/microarray/protocols.html) provided by the J. Craig Venter Institute (JCVI) (formerly The Institute for Genomic Research, TIGR). Labelled cDNAs were then hybridized to *S. mutans* Spotted Array gene chips provided by JCVI, as outlined in their Hybridization of Labelled DNA Probes protocol (M008) (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). The following changes were made to the labelling and hybridization protocols: the ratio of aa-dUTPs:dTTP was changed to 2:1, the total amount of RNA used for cDNA synthesis was increased to 5µg, and slides were hybridized using the MAUI hybridization chamber (Bio Microsystems Inc) at 42°C. Slides were scanned using the GenePix 4000B scanner (Molecular Devices, Sunnyvale, California) at 535nm (Cy3) and 635nm (Cy5). Scanned images were then processed using TIGR's Spotfinder program with default settings. TIGR's MIDAS program was used to normalize output data and apply appropriate statistical and clustering algorithms to generate lists of affected transcripts. A cut-off of ≥2-fold up- or down-regulation was used to develop lists of differentially expressed genes in the presence and absence of D-ala-D-ala.

3.3 Quantitative Real-time PCR

Quantitative real-time PCR (RT-PCR) was carried out to confirm expression ratios of a total of 10 genes, 5 of which were obtained directly from microarray results in the absence of D-ala-D-ala. RNA was isolated from four independent cultures of UA159 and PKNB as outlined above, DNase treated (RQ1 DNase; Promega, Madison, WI), and converted to cDNA using the cDNA First Strand Synthesis kit (MBI Fermentas, Canada) according to the manufacturer's protocol. A control reaction for cDNA synthesis consisted of no-RT (reverse transcriptase) that subsequently used in RT-PCR with 16S rRNA primers to ensure the absence of contaminating genomic DNA. Table 1 summarizes the primers used for RT-PCR experiments. Primer efficiencies were determined for each set of primers prior to cDNA amplification. These were computed from cycle threshold (Ct) values obtained using 2µl of 50, 25, 5, 0.5 and 0.05µg/ml dilutions of UA159 genomic DNA as template. Temperatures resulting in efficiencies between

85 and 110% were used for further analysis. All primers were utilized at an annealing temperature of 55°C, with the exception of Smu1344 and Smu1745, which had an annealing temperature of 51°C. Amplification of cDNAs was performed in triplicate using the Stratagene Mx3000P^{TM} thermocycler (La Jolla, CA) with the following cycling conditions: initial denaturation at 95°C (15 min), then 40 cycles of denaturation at 94°C (15 s), primer annealing at 55°C or 51°C (30 s), and extension at 72°C (30 s). Expression was normalized to that of 16S rRNA, a house-keeping gene whose expression did not vary under said experimental conditions. The relative expression ratio for each gene was calculated according to the method by Pfaffl (2001).

Table 1: Real-time PCR primers used for microarray confirmation

Primer Name	Sequence	Primer Efficiency
16S Fwd	5'-CTT ACC AGG TCT TGA CAT CCC G-3'	83.8%
16S Rev	5'-ACC CAA CAT CTC ACG ACA CGA G-3'	
accA Fwd	5'-TGA GCC TCC TTC ACC AAT G-3'	112.4%
accA Rev	5'-GTC ACT TTT ATC AAC ACA GCA GG-3'	
accD Fwd	5'-GAA TGT CGC TTT ACA GCA GC-3'	81.5%
accD Rev	5'-TTG AGT ATG CCA GAG AAG AAC G-3'	
accC Fwd	5'-CCT TTT TAT CAA CCT TAC GGA TGC-3'	105.6%
accC Rev	5'-CAG ATG GGG AAG TTT TTA CTG CC-3'	
fabD Fwd	5'-AAA GAG CCG TAT GAA AGG GAC C-3'	75.8%
fabD Rev	5'-TGT TAT TGG TGG TGA AGT AGC AGC-3'	
fabH Fwd	5'-CAG CGT GTT CAC CTG ATG TC-3'	109.3%
fabH Rev	5'-CTG TTC TTT TTG GAG ATG GAG C-3'	
fabM Fwd	5'-GCA CGA GTA ATA CCA ATG G-3'	96.7%
fabM Rev	5'-TGC CAG TGA CAA AAC ACG C-3'	
dltA Fwd	5'-CGT CAA CTC TTC TCC GTC AAA ATA G-3'	89.7%
dltA Rev	5'-TTA CCC ATT GGT GTG TGG ACA TC-3'	
Smu1745 Fwd	5'-CCT ATC AAG TTG TGA ACG C-3'	96.6%
Smu1745 Rev	5'-ATG CCA ATG TGA CAC CGA G-3'	
Smu1335 Fwd	5'-TAA AAG CAG CCA TCA TAC-3'	89.3%
Smu1335 Rev	TGG TTC TAT CAC AAC AAT G-3'	
Smu1344 Fwd	5'-GAG AGA ATA CAT ACT CCC TTC ATC-3'	102.7%
Smu1344 Rev	5'-GCA GGA CAA AGT CTT GGT G-3'	

3.4 Effects of D-alanyl-D-alanine on Growth

3.4.1 Effects of D-alanyl-D-alanine on Biofilm-derived Cells

Overnight cultures of *S. mutans* UA159, PKNB, vicK-, HK11-, HK10-, and HK8- strains were prepared in THYE with antibiotic added as required (1mg/ml kanamycin for PKNB and

10μg/ml erythromycin for all other mutants). 30μl of each overnight culture was added to 3ml of pre-warmed MMG medium in 12-well plates. Each replicate consisted of 4 wells per plate. Biofilms were incubated for 24 hr at 37°C with 5% CO_2. The following day, 100-well honeycomb plates were prepared with 0.5, 1, 5 and 10mg/ml of D-ala-D-ala in a final volume of 350μl of TYEG. Controls consisted of THYE and TYEG without D-ala-D-ala. Biofilm cells were processed for growth analysis by removing planktonic cells and washing twice with pre-warmed MMG. After the final wash, biofilms were resuspended in 1ml MMG. The biofilm cells for each replicate were then pooled together and MMG was added to give a final volume of 5ml. Samples were vortexed briefly to mix and 10μl of biofilm cells were added to bioscreen plates. Growth was monitored by a Bioscreen C analyzer (Labsystems), which measured OD_{600} of the samples over 24 hr. Growth curves with varying concentrations of D-ala-D-ala were compared between UA159 wild-type and mutants. Final growth yields were averaged for each replicate and the Student's T-test employed to determine if yields were significantly different for UA159 and PKNB under various test conditions. The lag phase time was estimated from growth curves using a transformed logarithmic scale of OD_{600} vs. time. Lag phase ceased at the time the growth curve became linear; this represented the beginning of logarithmic phase growth. In order to assess if addition of D-ala-D-ala was enhancing PKNB growth due to nutrient addition, a similar experimental approach was employed using UA159 and PKNB 24 hr biofilm-derived cells with 0.5, 1, 5 and 10mg/ml of D-alanine or L-alanine.

To examine the effects of culture supernatants on growth of biofilm-derived cells, a similar bioscreen was performed using 24 hr biofilm cells of UA159 and PKNB. A 30ml overnight culture of UA159 in THYE was used to prepare the supernatants for experiments. First, 3ml of the overnight culture was used to prepare a 1:10 dilution in fresh THYE medium and the culture was grown to mid-logarithmic phase. Both the overnight and mid-log cultures were centrifuged for 10 min at 4500rpm and the supernatants were filter-sterilized using a 0.22μm filter. 1:10 dilutions of the filter-sterilized supernatants were prepared in fresh THYE medium (20ml final volume). Ten ml of the undiluted supernatants were transferred to a clean tube and boiled for 15 min. 10μl of UA159 and PKNB biofilm-derived cells were inoculated into 100-well honeycomb plates containing 350μl of the undiluted, diluted and boiled supernatants from UA159 overnight and mid-log phase cultures. A control consisted of THYE medium.

Growth curves were compared between UA159 and PKNB to examine effects of supernatants on growth. Final growth yields and lag phase times were calculated as described above.

3.4.2 Effects of D-alanyl-D-alanine on Planktonic Cells

Overnight cultures of *S. mutans* UA159 and PKNB were diluted 1:10 in fresh THYE medium and grown to mid-logarithmic phase (OD_{600} ≈0.4). 20µl of culture was used to inoculate 100-well honeycomb plates containing 350µl of TYEG medium with 0, 0.5, 1, 5 and 10mg/ml D-ala-D-ala. Controls consisted of TYEG and THYE without D-ala-D-ala. Final growth yields and lag phase times were calculated as described above.

3.5 Scanning Electron Microscopy

Biofilms of UA159 and PKNB were grown for 24 hours on glass discs in 12-well plates in MMG with 0, 0.5, 1, and 5mg/ml D-ala-D-ala. Biofilms were then washed with 10mM phosphate-buffered saline (PBS) and fixed in 2ml of 3.7% formaldehyde in PBS at room temperature for 24 hours. Samples were dehydrated by serial rinsing with 30, 50, 70, 95 and 100% ethanol and then dried at critical point with liquid CO_2. Discs were removed from plates, mounted on cylindrical stubs, sputter-coated with platinum and then examined with a model S-2500 scanning electron microscope (Hitachi Instruments, San Jose, California). Biofilms and cells were viewed at 200, 1000, 5000, 10 000, 50 000 and 80 000x magnification.

3.6 Quantitative Biofilm Biomass Assay

Overnight cultures of *S. mutans* UA159 and PKNB were prepared in THYE with antibiotic added as required. The following day, cultures were centrifuged and resuspended in MMG medium. A 96-well plate was prepared with MMG containing 0, 0.5, 1 and 5mg/ml D-ala-D-ala. Wells were inoculated with 10µl of culture in 300µl MMG (1:30 dilution) in triplicate with media-free wells left as controls. Biofilms were grown for 24hrs at 37°C with 5% CO_2. Following incubation, liquid media was aspirated from individual wells and plates air-dried upside-down for 24hrs. To each well, 200µl of 0.01% safranin was added and left for 10min at room temperature. Residual safranin was discarded and wells washed once with 200µl of distilled water to remove excess stain. Biofilm biomass was immediately quantified by measuring dye content at 490nm using the FL600 Microplate Fluorescence Reader (Bio-Tek Instruments Inc., VT). The absorbencies of media-free control wells were averaged and this

value was subtracted from all biofilm absorbance values in order to account for background staining of the plate material.

3.7 Acid Tolerance Response in the Presence of Exogenous Fatty Acids

Ten-ml overnight cultures of UA159 and PKNB in THYE medium with appropriate antibiotic were centrifuged for 10 minutes at 4500rpm. Supernatants were discarded and cells resuspended in 10ml TYEG medium (pH 7.5). Each culture was then used to prepare 1:10 dilutions in TYEG pH 7.5 with or without 10µg/ml cis-vaccenic acid ($C_{18:1}$) or 10µg/ml cis-eicosenoic acid ($C_{20:1}$). Samples were incubated at 37°C with 5% CO_2 until mid-log phase was reached (OD_{600} ≈0.4). Cultures were then divided into two equal amounts and centrifuged for 10 minutes at 4500rpm. Supernatant was discarded and half the samples were resuspended in TYEG pH 5.5 (adapted) with or without fatty acids. The other half were resuspended in TYEG pH 3.5 (unadapted) with or without fatty acids.

Unadapted cells were incubated at 37°C with 5% CO_2 and 1ml samples were taken every hour for 3 hours, beginning at time 0, and sonicated on ice for 10 seconds using theKontes Micro Ultrasonic Cell Disrupter set at power 6, tune 2. Serial dilutions were prepared up to 10^{-7} in PBS and 20µl of dilutions were plated in triplicate on THYE plates (+1mg/ml kanamycin for PKNB samples). Adapted cells were first incubated at 37°C with 5% CO_2 for 2 hr, and then centrifuged for 10 min at 4500rpm. Cultures were then resuspended in TYEG pH 3.5 and 1ml samples taken every hour for 3 hours as done for unadapted cells.

Colonies were counted after 48 hours of incubation at 37°C with 5% CO_2 and survival rate was calculated as follows:

$$\text{Viable count (cfu/ml)} = \frac{\text{average \# of colonies}}{\text{0.02ml x dilution factor}}$$

$$\text{Survival rate (\%)} = \frac{\text{viable count at any time point}}{\text{Viable count at time 0}} \times 100\%$$

Survival rates of UA159 and PKNB adapted and unadapted samples +/- fatty acids were compared to determine if addition of exogenous fatty acids improved the mutant's ability to survive in acidic conditions. Statistical analysis was conducted using the Student's T-test with a p value <0.05 considered to be significant.

3.8 Growth under Environmental Stresses

Overnight cultures of UA159 and PKNB were diluted 1:10 in fresh THYE medium and grown to mid-logarithmic phase (OD_{600}≈0.4). 20µl of each culture was then inoculated into wells of a 100-well honeycomb plate containing THYE medium with varying stressor concentrations. Stress conditions used were 0.4M and 0.2M sodium chloride (NaCl), 2% ethanol (EtOH), 0.0015% and 0.003% hydrogen peroxide (H_2O_2), 0.0015% and 0.001% sodium dodecyl sulphate (SDS), and 25mM paraquat. Growth was monitored by a Bioscreen C analyzer (Labsystems), measuring OD_{600} of the samples over 20 hours. Growth curves of UA159 and PKNB were compared to determine if PKNB was sensitive to any of the stresses used. Final growth yields and lag phase times were calculated as described above.

3.9 Antibiotic Minimum Inhibitory Concentration Assay

Overnight cultures of UA159 and PKNB grown in THYE media were diluted 1:20 in 10ml of fresh pre-warmed THYE and grown to mid-log phase (OD_{600} ≈ 0.4). Based on the OD_{600} of each culture, the volume of mid-log phase culture needed to be diluted in THYE to give 10^5 cells (OD_{600} of approximately 0.01) to be added to test antibiotic media was calculated using the following formula: (0.01/ OD_{600} culture) x 10ml.

Test antibiotics were prepared at the following starting concentrations: 10µg/ml vancomycin (VAN), 8µg/ml penicillin G (PEN), 16µg/ml chlorhexidine (CHX), and 2.5% sodium dodecyl sulfate (SDS). Serial 2-fold dilutions of each antibiotic were set up in 96-well microtitre plates. First, 100µl of normal THYE media was added to each well of the plate, and then 100µl of the starting concentration (high dose) of each antibiotic was added to the third column of the plate. Doubling dilutions were carried out across the plate, changing tips at each dilution step. Negative controls consisted of only sterile THYE media with no culture added and positive controls with bacterial cultures exposed to no antibiotic stress.

100µl of diluted mid-log phase cultures was used to inoculate the plates. Each culture was inoculated in duplicate on the plate with triplicates of each test condition. Plates were incubated at 37°C with 5% CO_2 for 24hrs. The absorbance at 490nm for each well was measured using the FL600 Microplate Fluorescence Reader (Bio-Tek Instruments Inc., VT). OD_{490} was also measured at 48hrs of growth.

Relative cell densities were calculated as (OD_{490} of culture in presence of each concentration of antibiotic) / (OD_{490} of culture in the absence of antibiotic) x 100. The minimum inhibitory concentration (MIC) was determined as the minimum antibiotic concentration needed to ensure that culture did not grow to over 10% of the relative cell density. MIC was also observed by eye as the lowest antibiotic concentration showing the absence of visible turbidity. PKNB was defined as susceptible to a particular antibiotic if it had an MIC at least 2-fold lower than that of the UA159 wild-type control.

Chapter 4
RESULTS

4.1 Transcriptome Analysis in the Presence of D-alanyl-D-alanine

The presence of PASTA domains in PknB suggests that this protein may act as a sensor for the D-ala-D-ala dipeptide of unlinked peptidoglycan. In order to examine the effects of this dipeptide on UA159 and PKNB mutant cells, microarray analysis was performed using 1mg/ml and 5mg/ml D-ala-D-ala. Addition of 1mg/ml D-ala-D-ala resulted in a total of 334 genes affected in UA159, 121 of which were up-regulated and 213 down-regulated. In the presence of 5mg/ml D-ala-D-ala, 266 transcripts were affected, with 99 up-regulated and 167 down-regulated (Table 2 and Appendix 1). When the affected transcripts were compared for UA159 at each D-ala-D-ala concentration, it was found that all of the genes affected at 5mg/ml D-ala-D-ala were affected to a similar extent in the presence of 1mg/ml. All of the affected transcripts represent genes that are up- or down-regulated in UA159 in response to D-ala-D-ala. Figure 3 displays the number of genes belonging to various functional groupings that were affected in UA159 in the presence of D-ala-D-ala. Many of the affected transcripts had putative roles in energy metabolism, fatty acid and lipid biosynthesis, translation, and solute transport and binding proteins. A large percentage of genes also belong to the unassigned category.

In the PKNB mutant, 126 genes were affected in the presence of 1mg/ml D-ala-D-ala, with 82 up-regulated and 44 down-regulated, while 287 transcripts were affected with 5mg/ml D-ala-D-ala, 178 of which were up-regulated and 109 down-regulated (Table 3 and Appendix 1). Comparison of PKNB gene expression in the presence of each D-ala-D-ala concentration revealed a total of 84 genes that were common to both test conditions, with the majority being specific to the presence of 5mg/ml D-ala-D-ala. Figure 4 displays the number of genes belonging to various functional groupings that were affected in PKNB in the presence of D-ala-D-ala. Many of the affected transcripts had putative roles in energy metabolism, translation, solute transport and binding proteins, and amino acid biosynthesis. Once again, a large percentage of genes belonged to the unassigned category. When affected transcripts were compared between UA159 and PKNB at each D-ala-D-ala concentration, a total of 49 genes were common to both strains at 1mg/ml and 84 genes at 5mg/ml D-ala-D-ala. Of these common genes, a total of 44 were similarly expressed in UA159 and PKNB. Of these genes, 5 were similarly expressed in

both strains at 1mg/ml, 23 were similar at 5mg/ml, and 16 were similar at both D-ala-D-ala concentrations (Table 2 and Appendix 1). A total of 46 genes displayed either opposing expression or 2-fold more up- or down-regulation in PKNB compared to UA159 at both D-ala-D-ala concentrations. Of these genes 7 were differentially regulated in PKNB at 1mg/ml, 27 at 5mg/ml, and 12 at both D-ala-D-ala concentrations. In addition, a total of 159 genes were found to be affected in PKNB, but were absent from UA159 at both D-ala-D-ala concentrations. Also, 174 genes were affected in UA159, but not in the PKNB mutant.

Table 2: Fold-expression ratios for some genes that are up- or down-regulated in UA159 and PKNB in response to D-ala-D-ala*

Gene ID	Putative Function	UA159		PKNB	
		‡1	5	‡1	5
Amino Acid Biosynthesis					
Smu1203	Putative branched-chain amino acid aminotransferase (IlvE)	2.00	2.10	--	--
Smu1269	Putative phosphoserine phosphatase (SerB)	--	--	2.86	3.28
Smu54	Putative amino acid racemase	-5.88	-6.45	--	--
Smu913	**Putative NADP-specific glutamate dehydrogenase**	**-3.30**	**-3.73**	**4.44**	**--**
Bacteriocins					
Smu150	**Non-lantibiotic mutacin IV, NlmA**	**-12.20**	**-9.26**	**-2.72**	**-3.92**
Smu151	Non-lantibiotic mutacin IV, NlmB	-17.86	-16.13	--	--
Smu1906	Bacteriocin-related protein	--	--	--	-5.59
Smu1914	Bacteriocin, BlpO-like protein	--	2.59	-4.13	-4.59
Smu299	Putative bacteriocin peptide precursor	--	--	-4.72	-14.09
Smu423	Possible bacteriocin	-10.31	-6.41	--	-3.13
Cell Envelope					
Smu1429	Putative UDP-N-acetylmuramyl tripeptide synthetase (MurC2)	--	--	3.88	2.76
Smu1691	Putative D-alanine-D-alanyl carrier protein ligase (DltA)	-1.94	-2.15	--	--
Smu1949	Putative membrane carboxypeptidase (Pbp2a)	2.50	2.67	--	--
Smu22	Putative secreted antigen GbpB/SagA putative peptidoglycan hydrolase (GpbB)	-2.52	-2.27	--	--
Smu456	Putative undecaprenyl-phosphate-UDP-MurNAc-pentapeptide transfersase (MraY)	-2.35	-2.15	--	--
Smu549	Putative undecaprenyl-PP-MurNAc-pentapeptide-UDP-GlcNAc GlcNAc transferase (MurG)	1.79	2.18	--	1.92

Smu755	Putative prolipoprotein diacylglycerol transferase	--	--	2.45	2.58
Smu889	Putative penicillin-binding protein, class C fmt-like protein (PbpX)	-3.07	-2.77	--	--
Cellular Processes					
Smu1324	Putative cell-division protein (FtsX)	--	--	--	-2.32
Smu1339	Putative bacitracin sythetase (BacD)	--	--	23.49	--
Smu15	Putative cell division protein (FtsH)	2.23	--	--	--
Smu188	Hsp33-like chaperonin (heat shock protein) (HslO)	-8.47	-8.77	--	--
Smu1948	**Putative preprotein translocase subunit (SecE)**	**-7.35**	**-7.19**	**--**	**-2.52**
Smu1954	Putative chaperonin (GroEL)	2.60	2.70	--	--
Smu2006	Putative preprotein translocase SecY protein	-3.04	-2.73	--	--
Smu629	Putative manganese-type superoxide dismutase, Fe/Mn-SOD (Sod)	1.72	1.41	2.78	--
Smu82	Molecular chaperone (DnaK)	3.93	3.25	--	--
Smu83	Heat shock protein (HSP-40) (DnaJ)	4.35	4.35	--	--
Central Intermediary Metabolism					
Smu1296	Putative glutathione S-transferase YghU	--	--	3.32	2.26
Smu1573	**Putative S-adenosylmethionine synthetase (MetK)**	**-2.11**	**-1.68**	**11.99**	**--**
Smu676	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GapN)	--	--	2.38	2.25
Smu943	**Putative hydroxymethylglutaryl-CoA synthase**	**-1.82**	**-2.13**	**2.35**	**2.41**
Energy Metabolism					
Smu1424	**Putative dihydrolipoamide dehydrogenase (PdhD)**	**5.66**	**7.16**	**35.72**	**41.30**
Smu1527	FoF1 proton-translocating ATPase, ε subunit (AtpC)	-3.41	-2.91	--	--
Smu1528	FoF1 proton-translocating ATPase, β subunit (AtpB)	-4.24	-4.48	--	--
Smu1530	FoF1 proton-translocating ATPase, α subunit (AtpD)	-3.52	-3.08	--	--
Smu1532	FoF1 membrane-bound proton translocating ATPase, b subunit (AtpF)	-3.72	-4.24	-2.52	-2.94
Smu1534	FoF1 membrane-bound proton translocating ATPase, c subunit (AtpH)	-3.97	-4.12	--	-2.23
Smu78	Fructan hydrolase exo-beta-D-fructosidase; fructanase (FruA)	--	--	--	2.25
Smu870	**Putative transcriptional regulator of sugar metabolism**	**-2.38**	**-2.55**	**--**	**2.54**
Smu871	Putative fructose-1-phosphate kinase (PfkB)	--	--	5.76	3.08

Smu942	**Putative hydroxymethylglutaryl-CoA reductase (MvaA)**	**-1.70**	**-1.79**	**2.15**	**2.45**
Fatty Acid and Lipid Biosynthesis					
Smu1335	Putative enoyl-(acyl-carrier-protein) reductase	-5.78	-4.18	--	--
Smu1344	Putative malonyl-CoA acyl-carrier-protein transacylase	-4.51	-2.87	--	--
Smu1734	**Putative acetyl-CoA carboxylase α subunit (AccA)**	**-1.84**	**--**	**--**	**2.88**
Smu1735	Putative acetyl-CoA carboxylase β subunit (AccD)	-2.12	-2.31	--	--
Smu1736	**Putative acetyl-CoA carboxylase biotin carboxylase subunit (AccC)**	**-1.71**	**--**	**--**	**2.47**
Smu1737	Putative 3-hydroxymyristoyl-(acp) dehydrogenase (FabZ)	-2.11	-2.13	--	--
Smu1738	Putative biotin carboxyl carrier protein of acetyl-CoA (BccP)	-2.41	-2.13	--	--
Smu1739	Putative 3-oxoacyl-(acp) synthase (FabF)	-3.13	-3.03	--	--
Smu1740	Putative 3-oxoacyl-(acp) reductase/ 3-ketoacyl-acp (FabG)	-2.00	-1.96	--	--
Smu1741	Putative malonyl-CoA (acp) transacylase (FabD)	-2.29	-2.59	--	--
Smu1744	Putative 3-oxoacyl-(acp) synthase III (FabH)	-2.75	-3.13	--	--
Smu962	Putative dehydrogenase	3.53	--	3.97	4.55
Purines, Pyrimidines, Nucleotides and Nucleosides					
Smu1221	Putative orotate phosphoribosyltransferase (PyrE)	10.92	6.28	--	--
Smu1222	Putative orotidine-5-decarboxylase (PyrF)	8.66	7.30	--	--
Smu1223	**Putative dihydroorotate dehydrogenase B (PyrDB)**	**4.50**	**3.92**	**-3.38**	**-3.60**
Smu1224	Putative dihydroorotate dehydrogenase, electron transfer subunit (PyrK)	--	--	--	-14.93
Smu50	Putative phosphoribosylaminoimidazole carboxylase catalytic subunit (PurE)	--	--	3.21	4.04
Smu51	Putative phosphoribosylaminoimidazole carboxylase ATPase subunit (PurK)	--	--	2.33	3.80
Smu856	**Putative pyrimidine operon regulatory protein (PyrR)**	**-6.67**	**-8.20**	**-21.28**	**-10.99**
Regulatory Functions					
Smu1008	Putative response regulator (RR8)	-8.13	-8.20	--	--
Smu1009	**Putative histidine kinase (HK8)**	**--**	**1.59**	**--**	**4.44**
Smu1128	Putative histidine kinase sensor (CiaH)	--	--	--	2.23
Smu1515	CovX (VicX)	2.59	2.47	--	--

Smu1516	Putative histidine kinase, VicK homolog	2.13	----	--	--
Smu1517	Putative response regulator, VicR homolog	2.04	--	-2.16	--
Smu1548	Putative histidine kinase	-2.19	-2.21	--	--
Smu1814	Putative histidine kinase, ScnK homolog	2.13	--	--	--
Smu1924	Response regulator GcrR for glucan-binding protein C	-2.12	-1.92	--	--
Smu1965	Putative histidine kinase	9.45	--	--	--
Smu1997	Putative ComX1, transcriptional regulator of competence-specific genes	--	--	--	4.52
Smu576	**Putative response regulator (LytR)**	**-2.92**	**--**	**--**	**2.40**
Smu577	Putative histidine kinase (LytS)	2.61	1.74	--	3.05
Smu660	Putative histidine kinase SpaK	-4.76	-4.81	--	--
Smu80	**Heat-inducible transcription repressor (HrcA)**	**--**	**2.13**	**-2.77**	**-4.35**
Smu928	Putative histidine kinase (RelS)	--	--	--	2.06
Replication and Repair					
Smu1967	Putative single-stranded DNA-binding protein (Ssb2)	-8.85	-6.25	--	--
Smu2085	Recombination protein (RecA)	1.72	2.00	--	--
Smu2091	DNA mismatch repair protein	--	--	--	2.46
Smu297	DNA polymerase I (PolI)	--	--	--	2.76
Smu60	DNA alkylation repair enzyme	-5.56	-5.62	--	--
Smu814	Putative MutT-like protein (MutT)	2.39	2.33	--	1.96
Transcription					
Smu124	Putative transcriptional regulator (MarR family)	2.94	3.06	--	--
Smu1361	Putative transcriptional regulator (TetR family)	6.85	6.75	--	--
Smu1745	Putative transcriptional regulator	-2.12	-1.96	--	--
Smu2001	DNA-directed RNA polymerase, α subunit (RpoA)	--	--	--	-2.06
Smu640	Putative transcriptional regulator (GntR family)	--	--	--	-11.77
Translation					
Smu359	Translation elongation factor G (EF-G)	-2.43	--	--	-3.30
Smu608	**Putative translation elongation and release factor (PrfC)**	**-12.20**	**-7.87**	**9.56**	**--**
Smu697	Putative translation initiation factor IF3 (InfC)	--	--	-4.69	-3.40
Smu714	Translation elongation factor EF-Tu (Tuf)	--	-1.91	--	-2.02
Transport and Binding Proteins					
Smu1561	Putative potassium uptake system protein (TrkB)	--	--	2.29	3.34
Smu1562	Putative potassium uptake protein TrkA	--	--	--	2.36

	(Trk)				
Smu1563	Putative cation-transporting P-type ATPase (PacL)	--	--	--	2.59
Smu1897	Putative ABC transporter, ATP-binding protein, CslA	--	--	-4.27	-3.43
Smu2116	Putative osmoprotectant amino acid ABC transporter ATP-binding (OpuCa)	2.87	2.63	--	--
Smu2117	Putative osmoprotectant ABC transporter permease protein (OpuCb)	2.78	--	--	3.02
Smu2118	Putative ABC transporter osmoprotectant-binding protein, glycine betaine/carnitine/ choline (OpuCc)	3.56	2.57	--	4.11
Smu2119	Putative osmoprotectant ABC transporter permease protein (OpuCd)	3.35	3.23	7.43	3.11
Unassigned Category					
Smu1059	Acid tolerance protein (SatC)	2.92	--	--	--
Smu1117	NADH oxidase (H_2O-forming) (NaoX)	-2.92	-4.31	--	--
Smu1617	GTP-binding protein Era homolog	--	--	2.19	2.55
Smu173	Putative ppGpp-regulated growth inhibitor	--	--	-5.15	-2.17
Smu1930	Putative cytoplasmic membrane protein LemA-like protein (LemA)	--	--	-1.93	-2.10
Smu2067	Putative stress response protein, possible glucosyltransferase involved in cell wall biogenesis (CsbB)	--	--	-4.12	-4.53
Smu412	Putative Hit-like protein involved in cell-cycle regulation	2.14	2.10	--	--
Smu646	**Putative phosphatase**	**-26.32**	**-24.39**	**--**	**1.95**

*Fold-expression ratios are compared to 0 D-ala-D-ala control condition, $p<0.001$. For a complete list of all affected genes in UA159 and PKNB see Appendix 1

‡mg/ml D-ala-D-ala

Genes in bold are potentially dependent on PknB for regulation in the presence of D-ala-D-ala

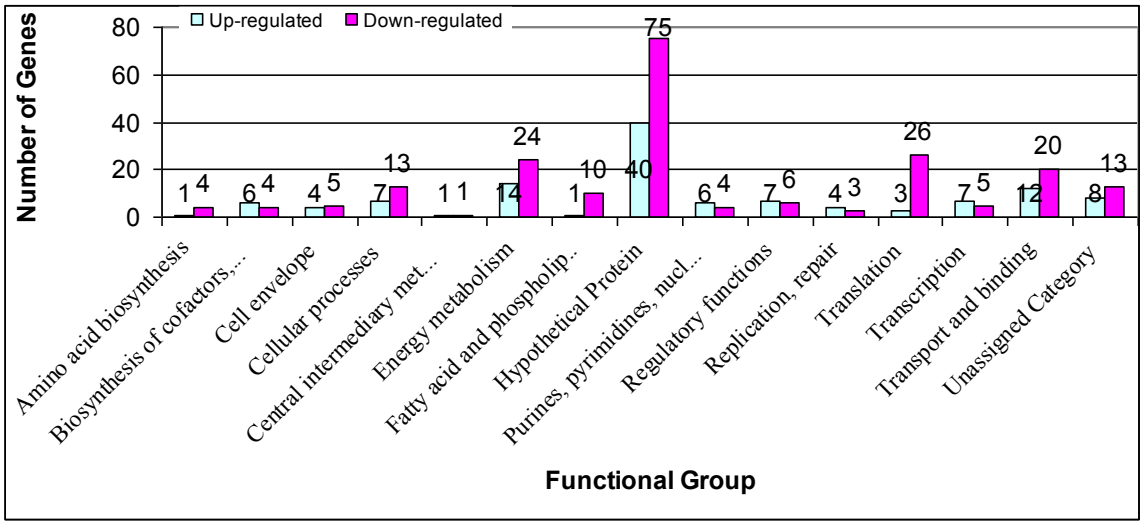

Figure 3. Functional groupings of differentially expressed genes in UA159 +1mg/ml D-ala-D-ala. Groupings for 5mg/ml not shown since all genes were common to those at 1mg/ml. The majority of affected transcripts have putative roles in energy metabolism, translation, solute transport and binding and cellular processes.

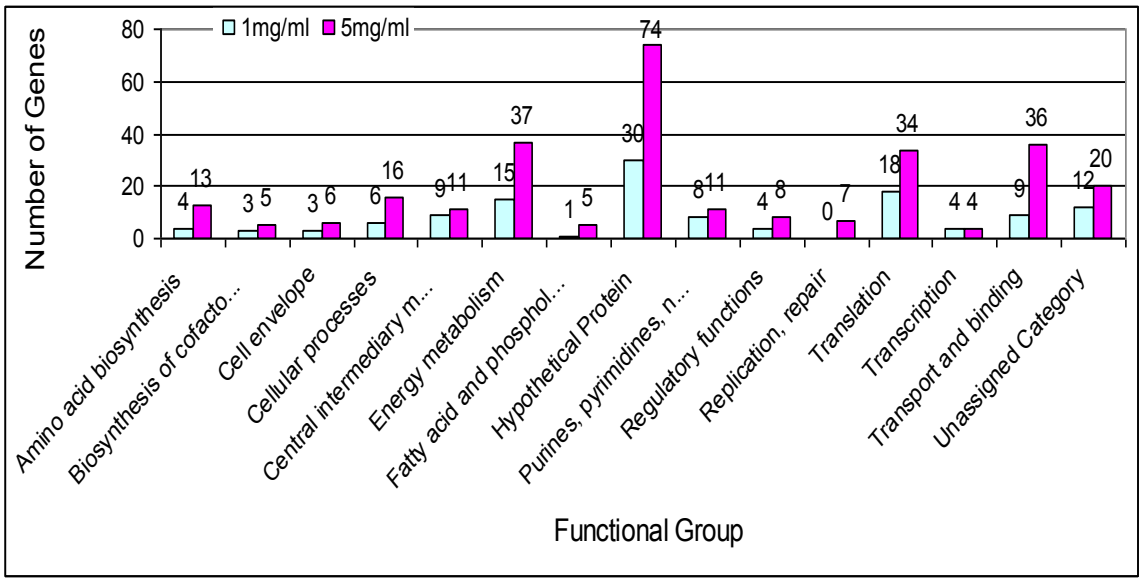

Figure 4. Functional groupings of differentially expressed genes in PKNB +1mg/ml and 5mg/ml D-ala-D-ala. A high percentage of affected transcripts have roles in energy metabolism, translation, transport and binding, and nucleotide synthesis. A greater number of genes were affected in each grouping at 5mg/ml D-ala-D-ala compared to 1mg/ml.

4.2 Effects of D-alanyl-D-alanine on Biofilm-derived Cells

Shah *et al.* (2008) demonstrated that PrkC in *B. subtilis* could respond to muropeptides in peptidoglycan in order to initiate germination of dormant spores. This led to the hypothesis that PknB in *S. mutans* could detect and respond to peptidoglycan fragments, specifically the D-ala-D-ala dipeptide, in order to promote growth of dormant cells derived from biofilms. In order to test this, 24 hr biofilm cells of UA159 and PKNB were exposed to varying concentrations of D-ala-D-ala and growth was monitored. Growth of PKNB biofilm cells was enhanced in the presence of D-ala-D-ala in a concentration-dependent manner, while growth of UA159 was not altered (Figure 5). The addition of D-ala-D-ala to PKNB resulted in final growth yields that were significantly higher than seen without the dipeptide. In TYEG without D-ala-D-ala, the final OD_{600} was approximately 0.90. At all D-ala-D-ala concentrations tested, final growth yields were approximately OD_{600} 1.1, a significant increase as determined by Student's T-test. While these final growth yields were higher compared to the TYEG control, they were still significantly lower than those of UA159, which were between OD_{600} 1.3 and 1.4 for all test conditions. The lag times were also much longer for PKNB compared to UA159; however, addition of D-ala-D-ala resulted in shorter lag times for PKNB in a concentration-dependent manner. A summary of final growth yields and estimated lag times for UA159 and PKNB at all D-ala-D-ala concentrations can be found in Table3.

Further analysis utilized a similar bioscreen experiment to measure growth rates of various mutants of histidine kinase receptors in other two-component signal transduction systems, including *vicK*-, HK11-, *lytS*- (HK10) and HK8-. All of these mutants grew similarly at all tested D-ala-D-ala concentrations (Figure 6) and had final growth yields similar to UA159.

Table3: Growth yields and estimated lag times for UA159 and PKNB with D-ala-D-ala

	UA159		PKNB	
	Final OD_{600}	**Lag (hr)**	**Final OD_{600}**	**Lag (hr)**
TYEG	1.31	2.1	0.90	8.60
0.5mg/ml	1.36	3.1	1.09*	8.75
1mg/ml	1.36	3.0	1.07*	8.40
5mg/ml	1.37	2.8	1.12*	7.19
10mg/ml	1.22	2.6	1.09*	6.64

* $p<0.01$ compared to TYEG

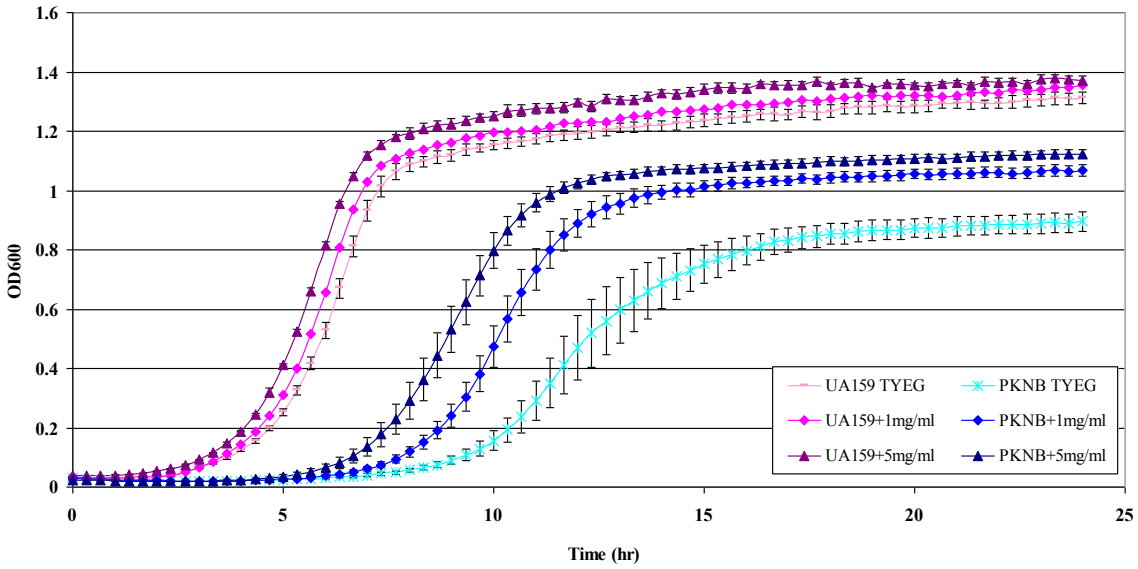

Figure 5. Growth of UA159 and PKNB biofilm-derived cells in the presence of D-ala-D-ala. Data represents the average of at least 3 replicates +/- standard error. Results for 0.5 and 10mg/ml not shown as growth curves are similar to 1 and 5mg/ml, respectively.

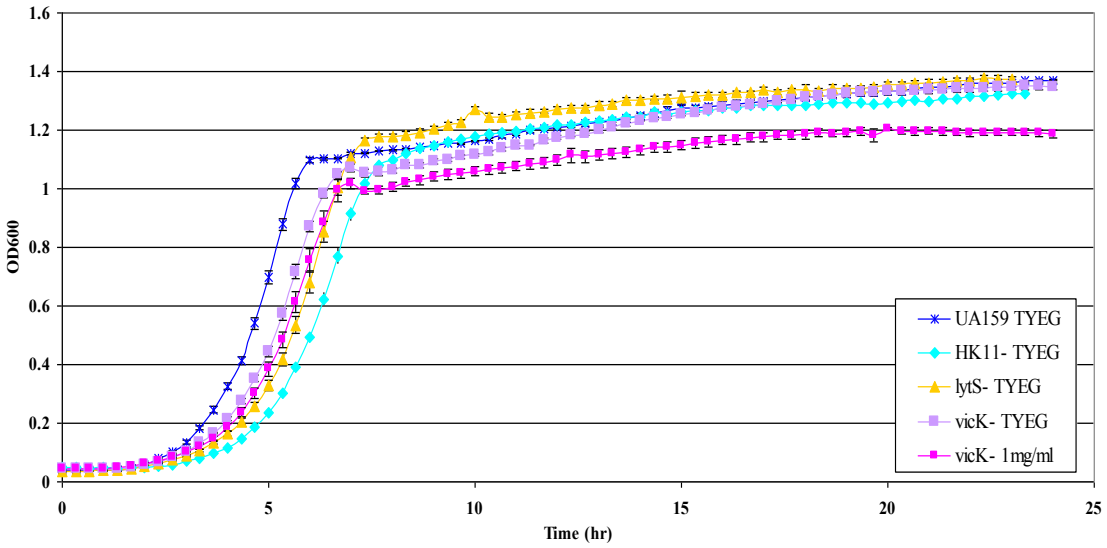

Figure 6. Effects of D-ala-D-ala on histidine kinase mutants. Strains deficient in HK11, VicK and LytS were exposed to varying D-ala-D-ala concentrations. Results for all concentrations yielded similar growth curves to TYEG control curves shown, except for vicK- where all other concentrations were similar to 1mg/ml curve shown. Data represents the average of 3 replicates +/- standard error.

In order to establish whether the phenotype seen for PKNB was due to merely supplying the mutant with a source of nutrients in the form of D-ala-D-ala, the same experiment was carried out using 0, 0.5, 1, 5 and 10mg/ml of D-alanine and L-alanine. As seen in Figure 7, L-alanine had no effect on growth of the PKNB mutant or UA159. D-alanine did not affect growth of UA159, but decreased growth of the mutant in a concentration-dependent manner (Figure 8). Final growth yields were significantly lower ($p < 0.05$) for PKNB compared to UA159 at all D-alanine concentrations (Table 4). In addition, estimated lag times were longer for PKNB in both D-alanine and L-alanine conditions.

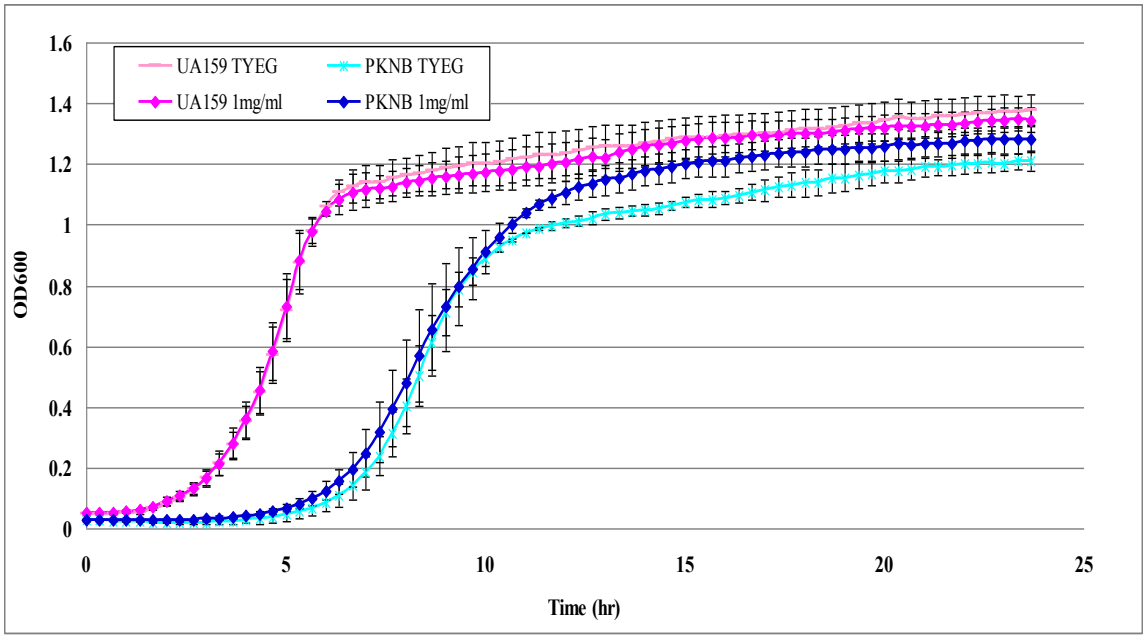

Figure 7. Growth of UA159 and PKNB biofilm-derived cells in the presence of L-alanine. Data represents the average of at least 3 replicates +/- standard error. Results for 0.5, 5 and 10mg/ml not shown as growth curves are similar to 1mg/ml.

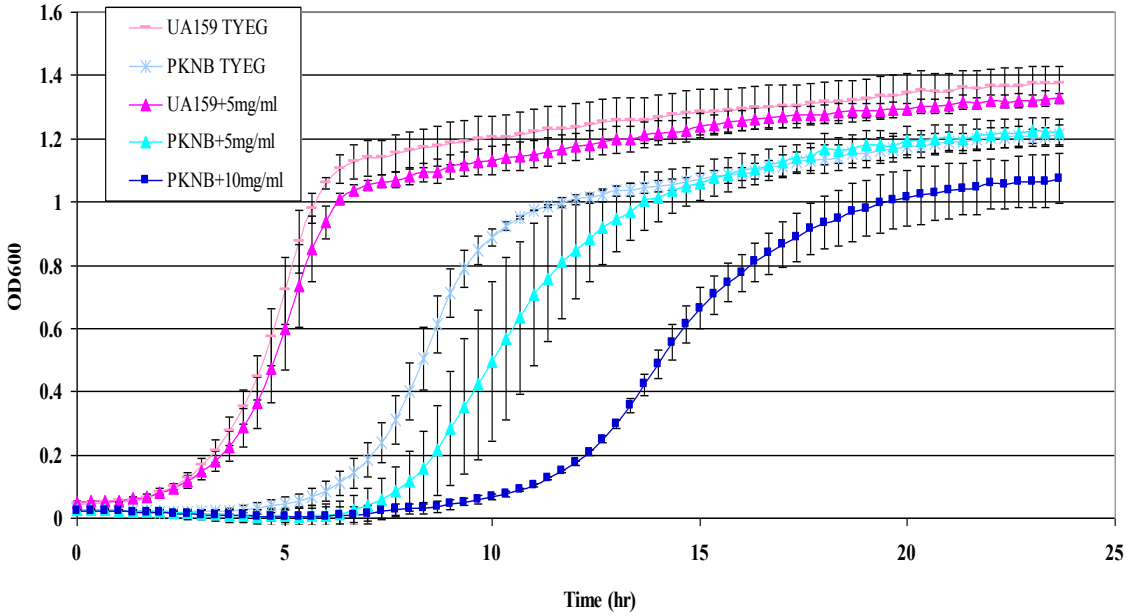

Figure 8. Growth of UA159 and PKNB biofilm-derived cells in the presence of D-alanine. Data represents the average of at least 3 replicates +/- standard error. Growth curves for UA159 in 0.5, 1 and 10mg/ml not shown as all results were similar to 5mg/ml. Results for PKNB +0.5mg/ml not shown as growth curves are similar to TYEG with no D-alanine. Growth curve of PKNB +1mg/ml is similar to that of 5mg/ml.

Table 4: Growth yields and estimated lag times for UA159 and PKNB with L-alanine and D-alanine

		UA159		PKNB	
		Final OD_{600}	**Lag (hr)**	**Final OD_{600}**	**Lag (hr)**
	TYEG	1.38	2.8	1.21	6.8
D-alanine	**0.5mg/ml**	1.38	2.8	1.24	6.8
	1mg/ml	1.34	2.9	1.24	8.1
	5mg/ml	1.33	2.9	1.22	8.5
	10mg/ml	1.30	2.9	1.07*	12.3
L-alanine	**0.5mg/ml**	1.37	3.1	1.28	6.9
	1mg/ml	1.35	3.2	1.28	7.1
	5mg/ml	1.38	3.2	1.27	6.9
	10mg/ml	1.34	3.2	1.23	6.7

* $p<0.05$ compared to TYEG

Next, growth of PKNB was assessed in UA159 overnight and mid-log phase filter-sterilized supernatants (Figure 9). Although there appeared to be no differences in growth between both overnight and mid-log phase diluted, undiluted and boiled supernatants, growth of PKNB was significantly increased in the presence of these supernatants compared to wild-type cells. Only results using overnight supernatants are shown, but results with mid-log phase culture supernatants were similar. PKNB lag phase time was reduced and final growth yield was slightly increased to a similar extent regardless of the type of supernatant used.

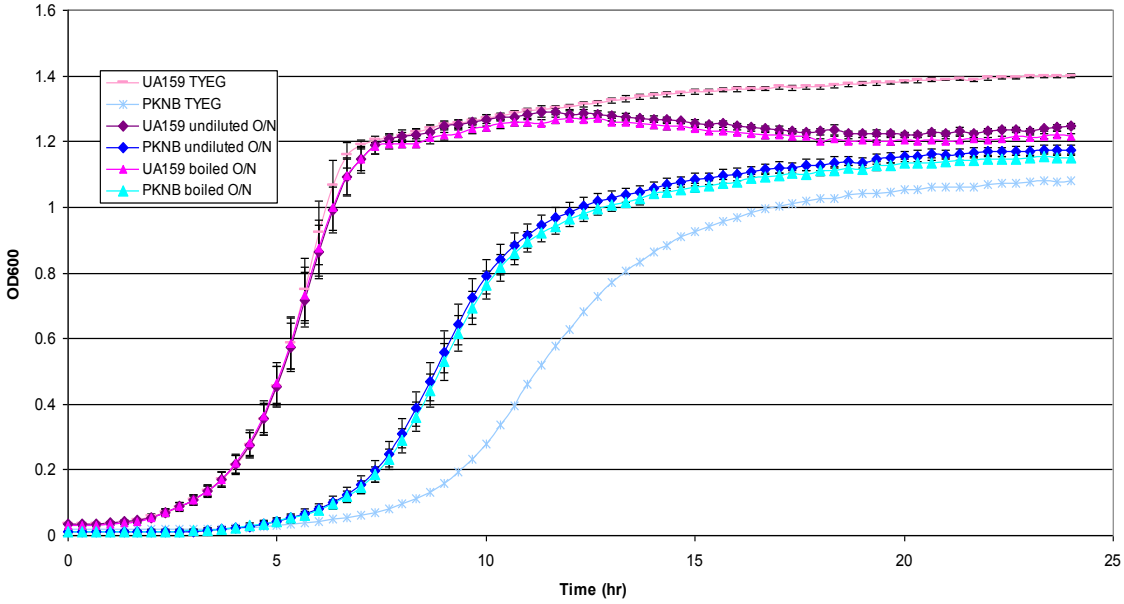

Figure 9. Growth of UA159 and PKNB biofilm-derived cells in the presence of cell-free UA159 overnight culture supernatant. Results for 1:10 diluted supernatant aren't shown as growth curves are similar to undiluted and boiled samples. Data represents the average of at least 3 replicates +/- standard error.

4.3 Effects of D-alanyl-D-alanine on Planktonic Cells

Since growth of biofilm-derived cells appeared to be enhanced by addition of D-ala-D-ala, we wished to examine the effects on planktonic cultures. Growth curves with varying concentrations of D-ala-D-ala were compared between UA159 wild-type and PKNB to examine effects of the D-ala-D-ala dipeptide on planktonic cultures and compare with effects on biofilm-derived cells. Addition of D-ala-D-ala to planktonic cells of UA159 resulted in a dose-dependent enhancement in growth (Figure 10). Specifically, two doses, 0.5 and 1mg/ml, showed similar reductions in lag phase time. Interestingly, 5 and 10mg/ml also showed similar results

accompanied by a further reduction in lag time compared to 0.5 and 1mg/ml. Final growth yields were similar at all D-ala-D-ala concentrations. Addition of D-ala-D-ala to PKNB planktonic cells (Figure 10) enhanced growth, but a concentration-dependent response was lacking, as 0.5, 5, and 10mg/ml all showed similar growth. Slightly less growth enhancement was seen at 1mg/ml compared to the other D-ala-D-ala concentrations. All D-ala-D-ala concentrations resulted in shorter lag times and significantly enhanced growth yields compared to the TYEG control ($p<0.05$). Growth yields and lag phase times for UA159 and PKNB are summarized in Table 5.

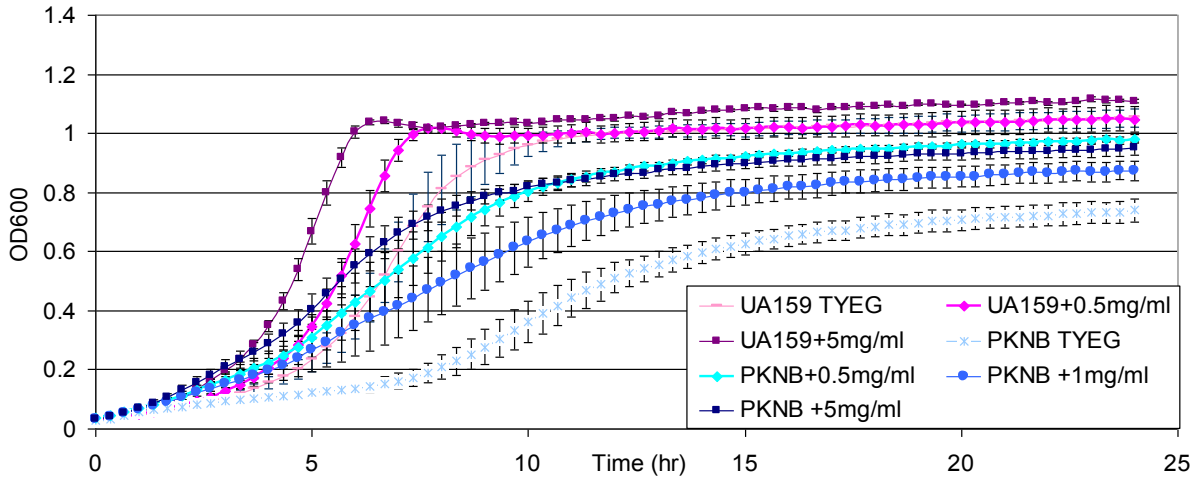

Figure 10. Effects of D-ala-D-ala on UA159 and PKNB planktonic cells. Growth curves of UA159 +1mg/ml and 10mg/ml D-ala-D-ala were similar to 0.5mg/ml and 5mg/ml, respectively. Data represents the average of at least 3 replicates +/- standard error.

Table 5: Growth yields and estimated lag times for UA159 and PKNB planktonic cells with D-ala-D-ala

	UA159		PKNB	
	Final OD_{600}	**Lag (hr)**	**Final OD_{600}**	**Lag (hr)**
TYEG	1.05	4.6	0.74	8.0
0.5mg/ml	1.05	3.2	0.98*	4.2
1mg/ml	1.05	3.7	0.87**	5.3
5mg/ml	1.11	2.3	0.95*	2.7
10mg/ml	1.03	2.3	0.90*	3.1

* $p<0.01$; ** $p<0.05$ compared to TYEG

4.4 Scanning Electron Microscopy

A study of PknB in *S. mutans* demonstrated that the PknB-deficient mutant produced biofilms of reduced density and biomass compared to UA159 (Hussain 2006). Growth kinetics

analyses showed enhanced growth of biofilm cells of PKNB in the presence of D-ala-D-ala. In order to determine whether this dipeptide has any effects on biofilm density, biofilms grown in the presence of varying D-ala-D-ala concentrations were observed by scanning electron microscopy (SEM). Micrographs of biofilms formed in the presence of 0, 0.5, 1 and 5mg/ml D-ala-D-ala are shown in Figure 11. From these images, it was difficult to conclude whether addition of D-ala-D-ala had any affect on biofilm density; therefore, further quantitative studies were required to determine whether D-ala-D-ala affects PKNB biofilm density.

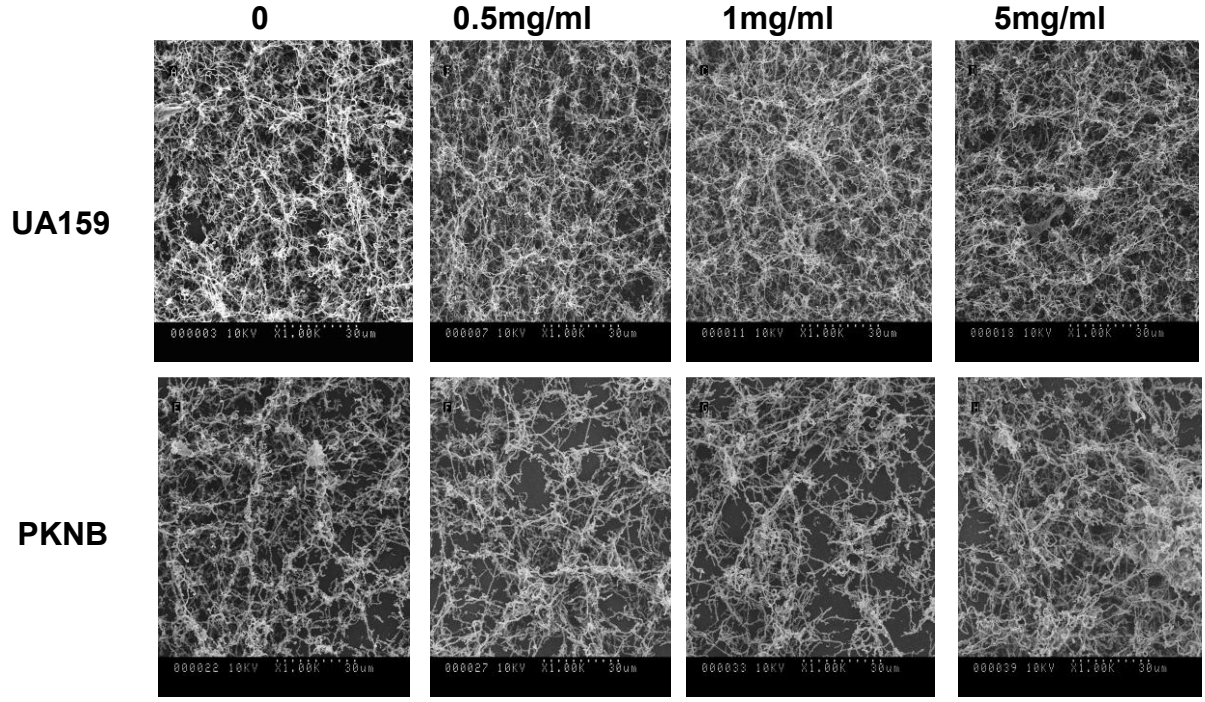

Figure 11. Scanning electron microscopy on UA159 and PKNB biofilms grown in the presence of D-alanyl-D-alanine. SEM micrographs at 1000x magnification for 0, 0.5, 1 and 5mg/ml D-ala-D-ala are shown. Based on these images it is difficult to determine whether or not D-ala-D-ala affected biofilm density of UA159 or PKNB.

To examine any visual defects in cell morphology, micrographs of mutant and wild-type cells were obtained at higher magnification (10 000x and 50 000x) (Figure 12). While UA159 cells grew and divided normally, PKNB cells appeared irregular in shape, with some cells in chains that appeared swollen or small in size (Figure 12, arrows). This suggests that the mutant may have impairments in cell division and/or growth. Also of note in these samples, there

appeared to be areas of the biofilm that consisted of what could potentially be excess extracellular material or lysed cells (Figure 12, triangles). This would suggest that PKNB produces excess extracellular polysaccharide or experiences increased cell lysis relative to the UA159 parent strain.

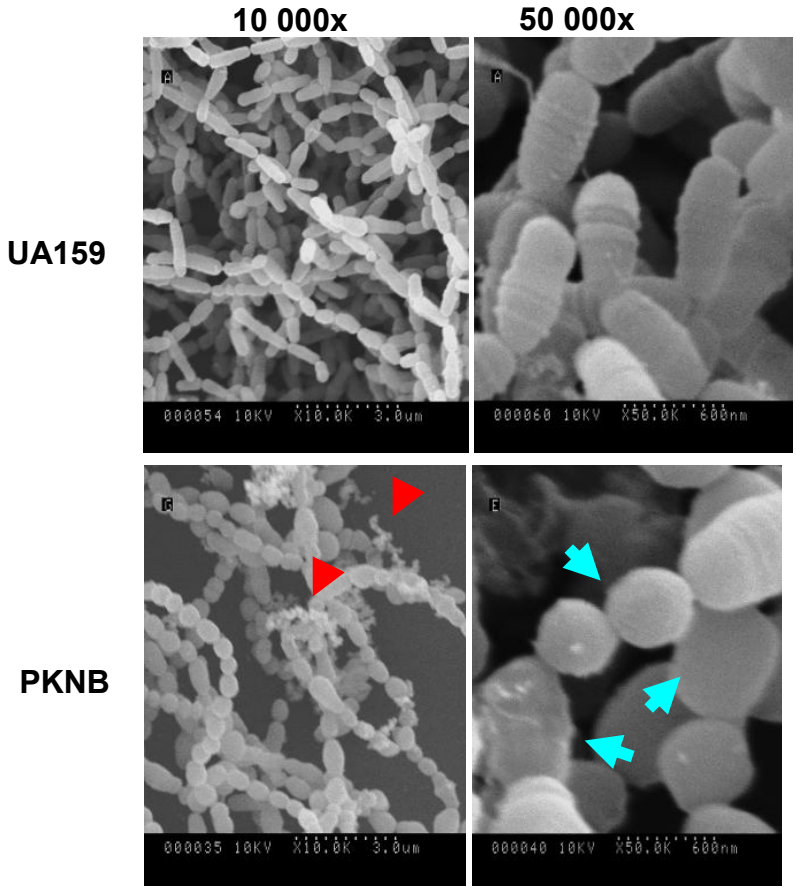

Figure 12. Cell morphology of UA159 and PKNB biofilm cells. Triangles indicate extracellular material while arrows highlight irregularly shaped cells.

4.5 Quantitative Biofilm Biomass Assay

Since SEM micrographs of UA159 and PKNB biofilms did not demonstrate clear differences in biofilm density, a quantitative biofilm biomass assay was performed using 24 hr biofilms of UA159 and PKNB grown in the presence of varying D-ala-D-ala concentrations. As illustrated in Figure 13, biofilms of PKNB consistently demonstrated reduced biomass compared to UA159, confirming the results of Hussain *et al.* (2006). In addition, there was no significant

change in biomass in the presence of 0.5, 1 and 5mg/ml of D-ala-D-ala, suggesting that this dipeptide does not affect biofilm density in PKNB. There was no significant change in biomass in UA159 biofilms with 0.5 and 1mg/ml D-ala-D-ala; however, biomass was increased at 5mg/ml ($p<0.05$), indicating that the wild-type strain had enhanced biofilm formation at high D-ala-D-ala concentrations.

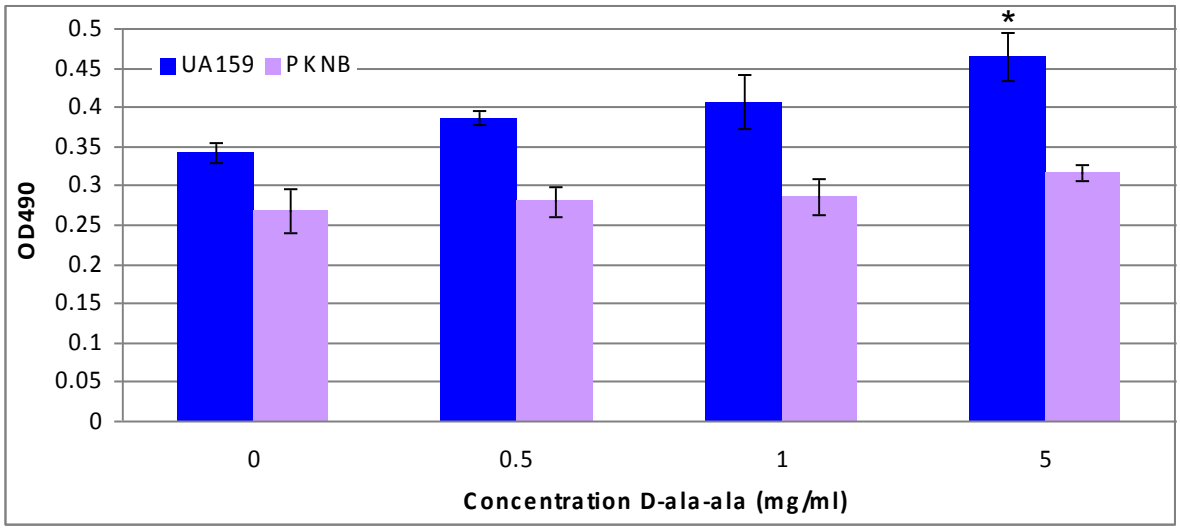

Figure 13. Quantitative biofilm biomass assay of UA159 and PKNB biofilms grown in the presence of D-ala-D-ala. Results shown represent the average of at least 2 independent experiments +/- standard error. (* $p<0.05$)

4.6 Global Transcriptome Analysis of PKNB

In order to identify genes that are potentially regulated by PknB, a DNA microarray was performed using the PKNB strain. A total of 77 genes were found to be differentially expressed greater than 2-fold in PKNB (p value <0.001) compared to UA159 wild-type. Of these genes, 63 were up-regulated, while 14 were down-regulated. Table 6 provides a complete list of the genes, organized by functional grouping, that were found to be differentially expressed in PKNB. Hypothetical proteins were omitted for clarity. Figure 14 shows the number of genes belonging to various functional groupings that were affected by *pknB* deletion. A high proportion of affected genes had putative functions in energy metabolism, transport and binding, and fatty acid biosynthesis. Genes of the fatty acid biosynthesis pathway were chosen for further investigation. Figure 15 displays quantitative real-time PCR (RT-PCR) data confirming results of the microarray.

Table 6: Differentially expressed genes in PKNB in the absence of D-ala-D-ala

Gene ID	Putative Function	Ratio*
Amino Acid Biosynthesis		
Smu.1173	putative O-acetylhomoserine sulfhydrylase (CysD)	3.14
Smu.54	putative amino acid racemase	-11.36
Bacteriocins		
Smu.150	Non-lantibiotic mutacin IV, NlmA	-9.62
Smu.151	Non-lantibiotic mutacin IV, NlmB	-18.87
Cell Envelope		
Smu.1691	putative D-alanine-D-alanyl carrier protein ligase (DltA)	-2.79
Smu.755	putative prolipoprotein diacylglycerol transferase	-2.22
Energy Metabolism		
Smu.1077	putative phosphoglucomutase (Pgm)	-2.75
Smu.137	malolactic enzyme (MleS)	-9.09
Smu.1877	putative PTS system, mannose-specific component IIAB (PtnA)	-6.71
Smu.1878	putative PTS system, mannose-specific component IIC (PtnC)	-5.43
Smu.2042	dextranase precursor (DexA)	-2.72
Smu.435	putative N-acetylglucosamine	-4.95
Smu.870	putative transcriptional regulator of sugar metabolism	-6.10
Fatty acid and phospholipid metabolism		
Smu.1016	putative acetyl-CoA carboxylase, biotin carboxyl carrier subunit (Bcc)	-2.19
Smu.1335	putative enoyl-(acyl-carrier protein) reductase	-41.67
Smu.1735	putative acetyl-CoA carboxylase beta subunit (AccD)	-3.94
Smu.1739	putative 3-oxoacyl-(acyl-carrier protein) synthase (FabF)	-3.02
Smu.1741	putative malonyl-CoA (acyl-carrier protein) transacylase (FabD)	-2.65
Smu.1744	putative 3-oxoacyl-(acyl-carrier protein) synthase III (FabH)	-2.87
Smu.962	putative dehydrogenase	2.93
Purines, pyrimidines, nucleotides and nucleosides		
Smu.1222	putative orotidine-5-decarboxylase (PyrF)	5.22
Smu.1223	putative dihydroorotate dehydrogenase B (PyrD)	4.72
Smu.32	phosphoribosylphrophosphate amidotransferase (PurF)	-250
Smu.34	putative phosphoribosylformylglycinamide cyclo-ligase (AIRS) phosphoribosyl aminoimidazole synthetase (PurM)	-34.48
Smu.356	purine operon repressor (PurR)	-2.37
Replication and Repair		
Smu.1967	putative single stranded DNA binding protein (Ssb2)	-10.42
Smu.561	putative hydrolase (MutT family)	-6.06
Smu.60	DNA alkylation repair enzyme	-7.81
Regulatory Functions		
Smu.91	Peptidyl-prolyl isomerase RopA (trigger factor)	-2.46
Smu.928	putative histidine kinase (RelS)	-2.17
Transcription and Translation		
Smu.1132	aminopeptidase N (PepN)	-2.03
Smu.124	putative transcriptional regulator (MarR family)	2.59
Smu.1626	50S ribosomal protein L1 (RplA)	-55.56
Smu.867	putative 16S rRNA processing protein (RimM)	-3.50

Smu.957	50S ribosomal protein L10 (RplJ)	-2.68
Transport and Binding Proteins		
Smu.1179	putative amino acid ABC transporter, permease protein	2.50
Smu.138	putative malate permease	-7.46
Smu.1668	putative branched chain amino acid ABC transporter (LivH)	-2.04
Smu.1993	putative ABC transporter, zinc permease protein (AdcB)	-5.05
Smu.998	putative ABC transporter, periplasmic ferrichrome-binding protein	-7.58
Unassigned Category		
Smu.1363	putative transposase	8.66
Smu.140	putative glutathione reductase	-7.58
Smu.201	putative transposon protein	-200
Smu.2050	putative methyltransferase (PrmA)	-2.06
Smu.2056	putative ATPase	-2.81
Smu.646	putative phosphatase	-28.57
Smu.728	putative oxidoreductase	-4.74

* Fold expression ratio calculated by dividing expression in PKNB with that of UA159; $p<0.001$

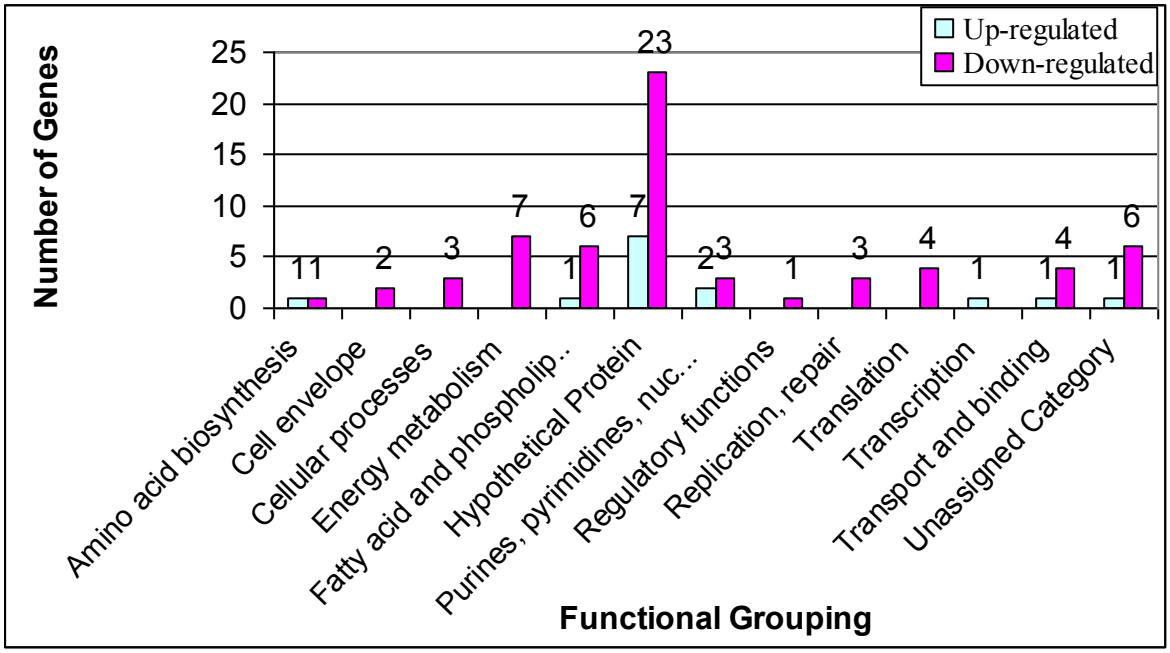

Figure 14. Functional grouping of differentially expressed genes in PKNB as determined by microarray analysis. A high percentage of affected transcripts had roles in energy metabolism, and fatty acid and lipid metabolism.

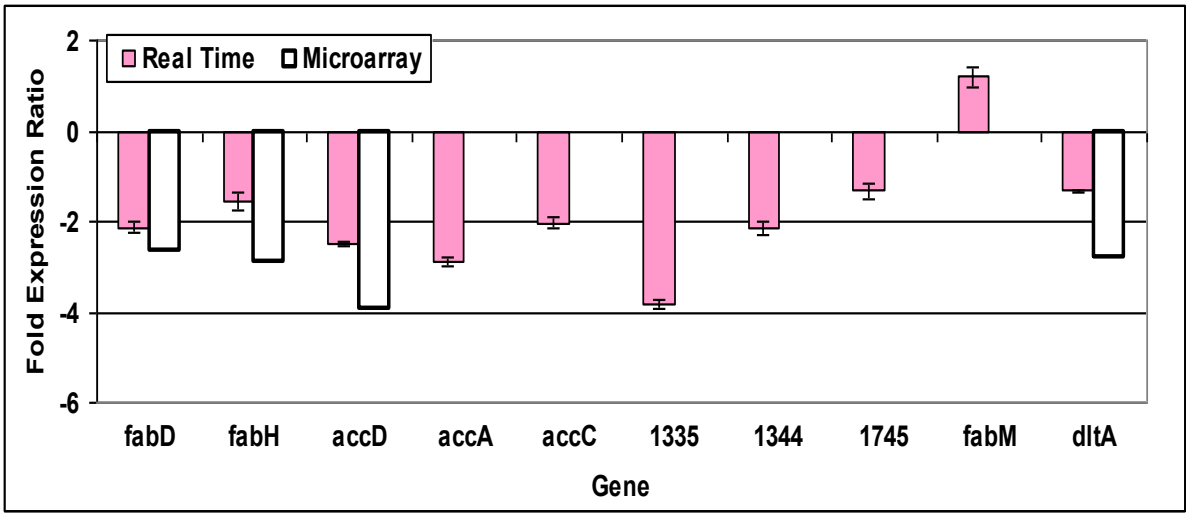

Figure 15. Confirmation of PKNB microarray results by quantitative real-time PCR. Smu1335 was down-regulated 41-fold according to microarray results (not shown here for clarity). Data represents results from four biological replicates +/- standard deviation.

4.7 Acid Tolerance Response with Exogenous Fatty Acids

A study on a *fabM* mutant of *S. mutans* demonstrated that this mutant was acid sensitive and addition of exogenous monounsaturated fatty acids was able to improve the mutant's acid survival (Fozo 2004b). The down-regulation of fatty acid biosynthesis genes in PKNB suggested that this mutant may exhibit an altered cell membrane composition. In addition, based on the results of Fozo *et al.* (2004b), it was hypothesized that the acid sensitive phenotype of PKNB could in part be due to alterations in fatty acid biosynthesis. In order to test this, an ATR assay was carried out on UA159 and PKNB in the presence and absence of the monounsaturated fatty acids cis-vaccenic and cis-eicosenoic acid.

Survival of UA159 and PKNB appeared unaffected by the presence of the two fatty acids used in this study. Survival of unadapted PKNB cells was significantly lower than that of UA159 at 1 hr ($p<0.05$), while at 2 and 3 hr survival was comparable between the two strains (Figure 16). Adapted PKNB cells showed significantly lower survival at 1 and 2 hr ($p<0.5$) compared to UA159, with comparable survival at 3 hr (Figure 17).

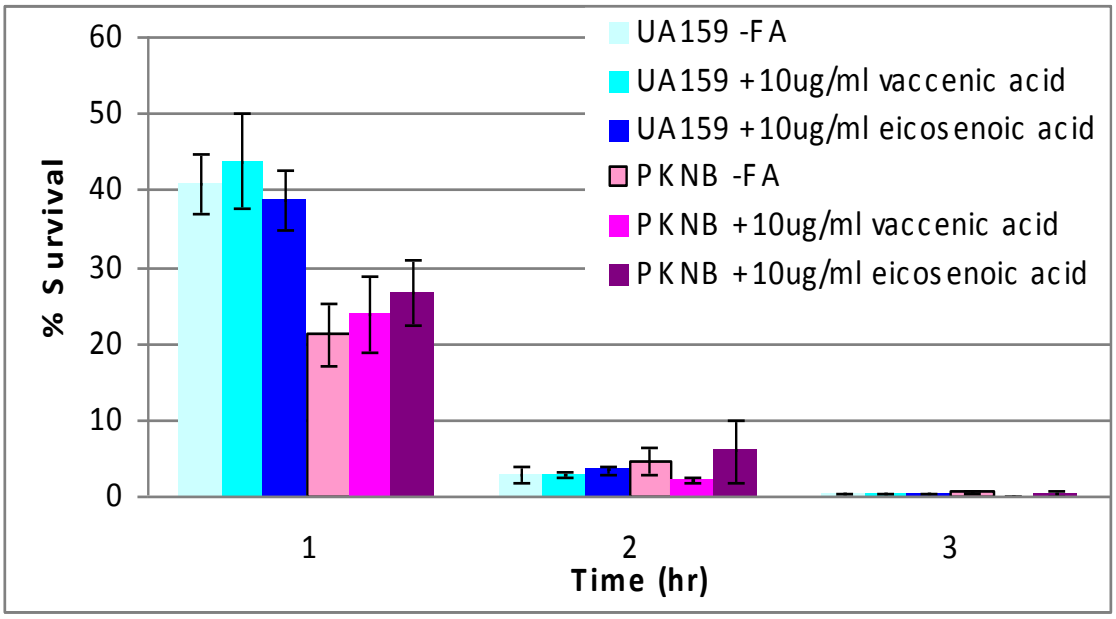

Figure 16. Acid tolerance response of unadapted cells in the presence and absence of exogenous fatty acids. There were no significant differences with addition of fatty acids compared to the no fatty acid controls. Results represent the average of at least 2 replicates +/- standard error. (-FA = no fatty acid added)

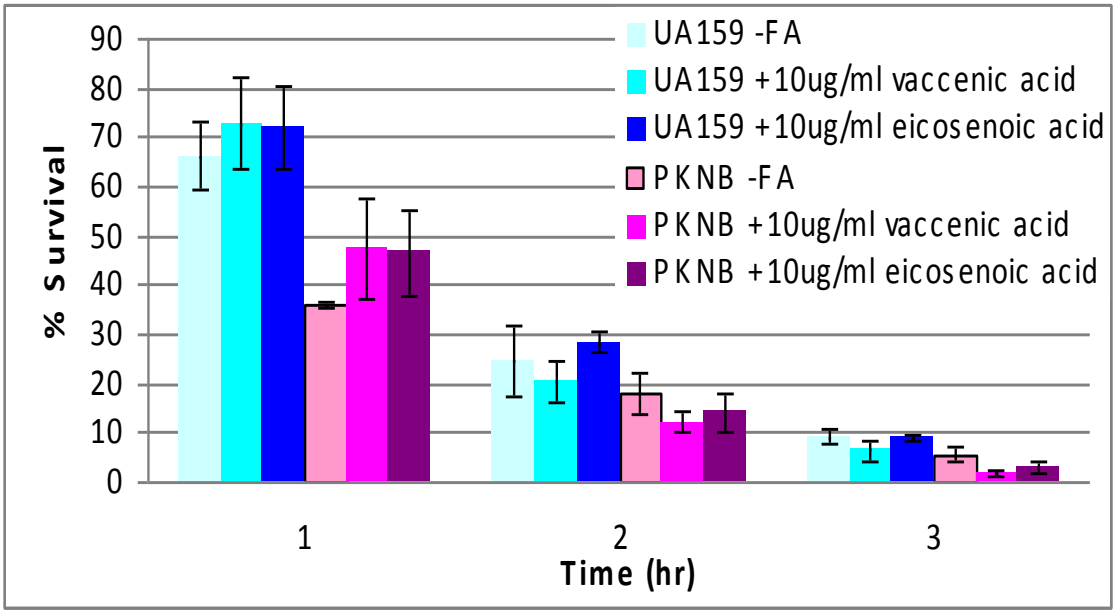

Figure 17. Acid tolerance response of adapted cells in the presence and absence of exogenous fatty acids. There were no significant differences in survival with added fatty acids compared to no fatty acid controls. Data represents the average of at least 2 replicates +/- standard error. (-FA = no fatty acid added)

4.8 Growth under Environmental Stresses

In order to assess the sensitivity of PKNB to environmental stresses, growth kinetics of UA159 wild-type strain and PKNB were monitored under various stress conditions. PKNB was susceptible H_2O_2 (Figure 18), as the mutant demonstrated increased lag phase growth compared to UA159 and the growth yield was markedly reduced. PKNB was also highly susceptible to SDS stress (Figure 19), since the mutant was unable to grow at either SDS concentrations used. Growth of PKNB appeared comparable to that of UA159 under NaCl, paraquat and EtOH stresses (Figure 20).

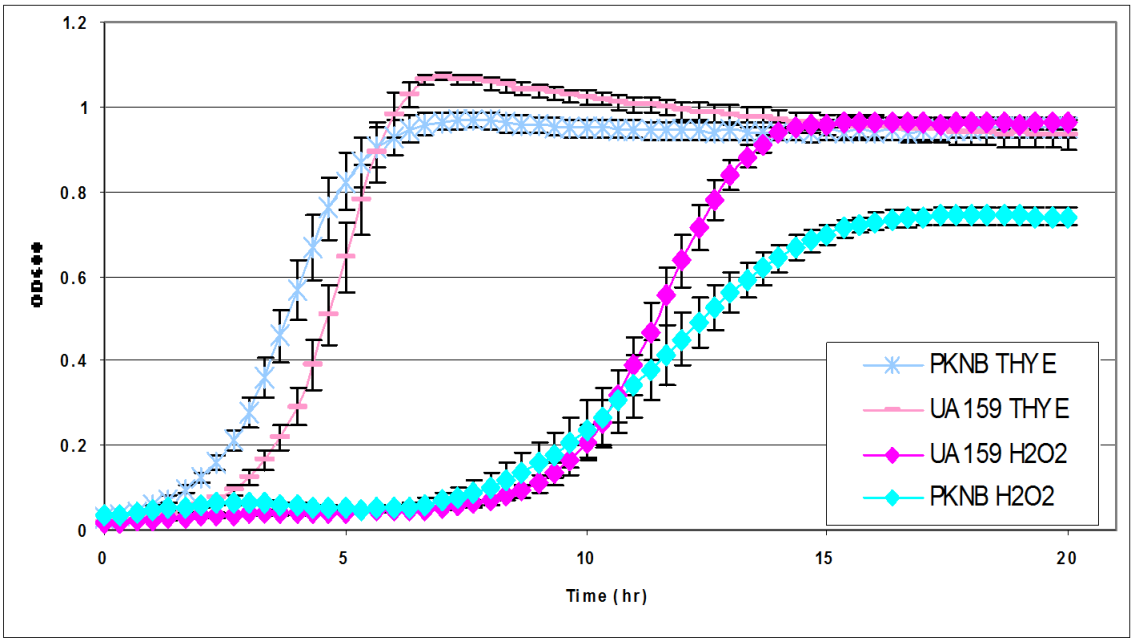

Figure 18. Growth of UA159 and PKNB in the presence of 0.003% hydrogen peroxide. Data represents the average of at least 5 replicates +/- standard error.

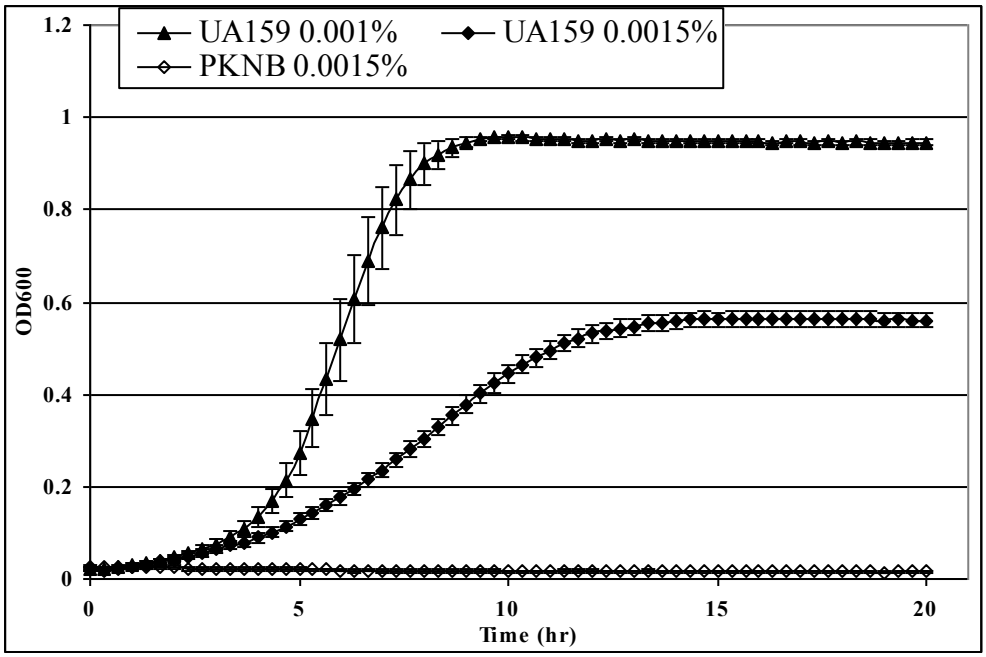

Figure 19. Growth of UA159 and PKNB in the presence of sodium dodecyl sulfate. Results for PKNB +0.001% SDS not shown as the growth curve is the same as that for 0.0015% shown. Data represents the average of at least 4 replicates +/- standard error.

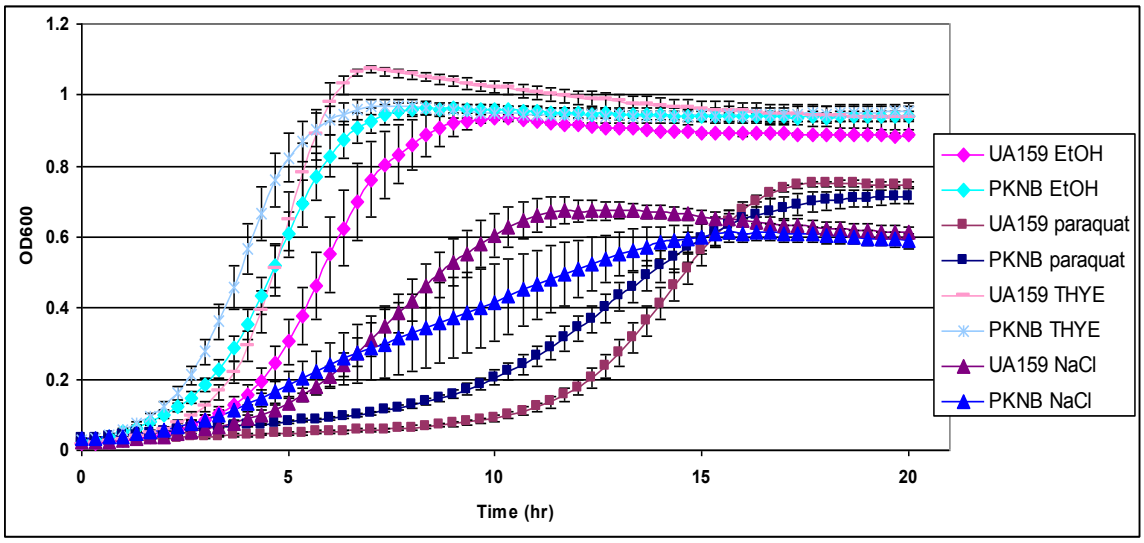

Figure 20. Growth of UA159 and PKNB under environmental stresses. Strains were grown with 2% ethanol (EtOH), 25mM paraquat and 0.4M sodium chloride (NaCl) in THYE medium. Data represents the average of at least 3 replicates +/- standard error.

4.9 Sensitivity to Cell Wall- and Cell Membrane-Targeting Antibiotics

PASTA domains are predicted to bind to β-lactam antibiotics, such as penicillin. Since PknB contains PASTA domains in the extracellular region, we tested susceptibility of PKNB to cell wall antibiotics VAN and PEN in antibiotic minimum inhibitory concentration (MIC) assays. The membrane-targeting agents SDS and chlorhexidine were also included in this analysis based on susceptibility of PKNB to SDS in growth kinetics analyses. The mutant was found to be 2-fold more sensitive to vancomycin, penicillin and chlorhexidine and 4-fold more sensitive to SDS compared to wild-type (Table 7).

Table 7: MIC Values of Cell Wall- and Membrane-Targeting Antibiotics for UA159 and PKNB Mutant

	UA159	**PKNB**
PEN (μg/ml)	0.125	0.0625*
VAN (μg/ml)	2.5	1.25*
% SDS	0.0195	0.0049**
CHX (μg/ml)	2	1*

PEN=penicillin G; VAN=vancomycin; SDS=sodium dodecyl sulfate; CHX=chlorhexidine
*2-fold reduction in MIC; **4-fold reduction in MIC

Chapter 5
DISCUSSION

The presence of exogenous D-ala-D-ala appeared to have extremely diverse effects on gene expression in both UA159 and PKNB (Table 2 and Appendix 1). Affected pathways included amino acid biosynthesis, cell wall biogenesis, DNA replication and repair, central metabolism, and solute transport and binding proteins. A high percentage of affected transcripts also belonged to the unassigned category; therefore, further studies are warranted to characterize the function of these gene products in order to further understand mechanistically how *S. mutans* responds to D-ala-D-ala.

Addition of D-ala-D-ala to UA159 cultures resulted in differential expression of 334 genes, 273 of which were similarly expressed at both 1mg/ml and 5mg/ml D-ala-D-ala. This suggests the absence of concentration-dependent effects on gene expression in the wild-type strain. Addition of D-ala-D-ala to the PKNB mutant resulted in differential expression of 126 genes at 1mg/ml and 287 genes at 5mg/ml. A total of 84 genes were common to both D-ala-D-ala concentrations and they demonstrated similar expression, once again suggesting the absence of concentration-dependent effects on gene expression in the mutant. There were also a number of genes that were found to be differentially regulated in UA159, but not in PKNB at both D-ala-D-ala concentrations, and vice versa (Table 2 and Appendix 1). These affected transcripts represent genes that may or may not be dependent on PknB for regulation in the presence of D-ala-D-ala, but further experiments on expression analysis of these genes in UA159 and PKNB in the presence of D-ala-D-ala would be required to confirm this.

When examining genes that were common to both UA159 and PKNB at each D-ala-D-ala concentration, it was important to differentiate those that displayed similar up- or down-regulation in PKNB compared to UA159 at each D-ala-D-ala concentration and those that displayed opposing or enhanced up- or down-regulation in PKNB. A total of 44 genes were similarly expressed in UA159 and PKNB at both D-ala-D-ala concentrations. These affected transcripts represent genes that are controlled independently of the presence of PknB in response to D-ala-D-ala. This is because a similar response to D-ala-D-ala is observed in both the presence (UA159) and absence (PKNB) of PknB. These results suggest that there is another receptor

independent of PknB that is able to detect D-ala-D-ala and elicit a response that causes the up- or down-regulation of a number of genes. There were also a number of transcripts whose expression in PKNB was opposite to that seen in UA159 (i.e. up-regulated in PKNB, but down-regulated in UA159 and vice versa) or highly enhanced or repressed in PKNB (more than 2-fold up- or down-regulation in PKNB compared to UA159). These affected transcripts represent genes that are dependent on PknB for regulation in the presence of D-ala-D-ala (Table 2 and Appendix 1). These results suggest that PknB is also able to detect D-ala-D-ala and affect transcription of various target genes. It is important to note, however, that PknB is a membrane-bound protein and therefore is likely not involved in directly binding to target promoters in DNA in order to promote or inhibit transcription. PknB is probably responsible for phosphorylating another protein(s) that is responsible for affecting gene transcription; however, the identity of this other protein(s) is currently unknown. Interestingly, the response regulator VicR represented one of the affected transcripts that was dependent on PknB for regulation in response to D-ala-D-ala. There is evidence that TCS homologous to the *S. mutans* VicRK system are involved in detecting and responding to cell wall peptidoglycan (Dubrac 2008). This suggests that perhaps PknB is responsible for phosphorylating VicR in order to mediate the response to D-ala-D-ala; however, there is currently no evidence of this.

Addition of D-ala-D-ala to UA159 led to the up-regulation of a number of stress response genes, including genes encoding for the heat shock proteins and chaperones DnaJ, DnaK and GroEL. The role of chaperone proteins is to assist in the folding of newly synthesized or denatured proteins and they are often up-regulated when the cell is under stress (Lemos 2001). In *E. coli*, DnaK functions by binding to hydrophobic regions of a peptide as it is synthesized. This binding is initiated by DnaJ, which binds to the extended substrate and delivers it to DnaK-ATP, triggering ATPase activity that strengthens binding and allows for folding of the protein (Masters 2009). Once the protein has folded correctly, GrpE stimulates release of ADP by DnaK, which facilitates release of the substrate (Masters 2009).

In *S. mutans*, DnaK and GroEL are part of a general stress response and expression of these genes is controlled by the HrcA repressor (Lemos 2001). The *dnaK* operon in *S. mutans* consists of the *hrcA* repressor, *grpE*, *dnaK* and *dnaJ* (Lemos 2001). It has been shown that *groEL* and *dnaK* expression are increased during acid shock and it is believed that DnaK is

important for either the biogenesis or stabilization of the proton-extruding F_1F_o-ATPases at low pH (Lemos 2001; Lemos 2007). In addition, complexes of GroEL and DnaK are thought to regulate signal transduction pathways by controlling the stability and activities of response regulators and histidine kinases (Lemos 2007). Depletion of *dnaK* or *groE* in *S. mutans* has been shown to result in slower growth, the formation of longer chains of cells, clumping in liquid culture, reduced viability, and increased susceptibility to stresses, such as hydrogen peroxide and temperature (Lemos 2001; Lemos 2007).

GroEL expression was up-regulated approximately 2.7-fold in UA159 in the presence of D-ala-D-ala, while *dnaJ* and *dnaK* were up-regulated between 3.3- to 4.4-fold. This suggests that D-ala-D-ala is initiating a stress response in wild-type cells. It is likely that the D-ala-D-ala serves as a signal for the presence of unlinked peptidoglycan (Yeats 2002), which would indicate a weakness in the cell wall. Alternatively, the cells could be detecting products of lysis from neighbouring cells. Perhaps the detection of exogenously added D-ala-D-ala leads to a stress response in which the cells sense that the cell wall is significantly weakened and chaperone proteins, such as DnaJK and GroEL, are up-regulated in order to ensure proper protein folding.

One of the genes that was found to be dependent on PknB for regulation in the presence of D-ala-D-ala was *hrcA*. This gene encodes for the HrcA regulator and was found to be up-regulated approximately 2-fold in UA159, but down-regulated 2.8- to 4.4-fold in PKNB. HrcA has been shown to negatively regulate the *groE* and *dnaK* operons in *S. mutans* (Lemos 2001); therefore, the apparent up-regulation of *dnaJK* and *groEL* in UA159 is surprising. The down-regulation of *hrcA* in PKNB would suggest that *dnaJK* and *groEL* would be up-regulated, although this was not observed in the PKNB microarray. Further studies are warranted to confirm these microarray results in order to determine if these chaperone proteins are in fact up-regulated in UA159 and to examine their expression in PKNB.

A number of genes involved in cell division were also affected by addition of D-ala-D-ala, including *divIVA, ftsH* and *ftsY*. FtsY is a receptor for the signal recognition particle (SRP), which is a highly conserved protein-ribonucleotide complex involved in co-translational targeting of a subset of proteins to the cell membrane (Hasona 2005). The SRP delivers incompletely translated proteins containing a signal peptide sequence to FtsY for subsequent

completion of translation and translocation via the SecYEG translocon (Hasona 2005). It is believed that disruption of the SRP pathway may indirectly affect cell division because many essential division proteins are located in the membrane (de Leeuw 1999). Expression of *ftsY* is down-regulated approximately 3.7-fold in UA159. If D-ala-D-ala serves as a signal to the cell that the cell wall is damaged, it is possible that targeting of proteins to the membrane via FtsY and the SRP pathway is decreased. This would allow damage to be repaired before integral membrane proteins are inserted into a weakened cell wall. This hypothesis is also supported by the fact that genes encoding components of the SecYEG translocon, *secE* and *secY*, were also down-regulated 3- to 7-fold in UA159 in the presence of D-ala-D-ala.

FtsH is a membrane-anchored ATP- and Zn^{2+}-dependent metalloprotease that is present in most bacteria (Schumann 1999). In *E. coli*, it is required for the degradation of certain unstable proteins, including the heat shock sigma factor σ^{32}, the SecY subunit of the translocon complex, and the α subunit of the F_0-ATPase (Schumann 1999). In addition to its protease activity, FtsH has also been shown to possess chaperone activity (Akiyama 1998). A study of FtsH in *B. subtilis* has shown that deletion of the *ftsH* gene results in a pleiotropic phenotype that includes filamentous growth (Wehrl 2000). It was found that FtsH localizes to the mid-cell during septation; however, this only occurred in approximately 15% of dividing cells (Wehrl 2000). FtsH was also shown to degrade the cell division initiation protein FtsZ *in vitro*; therefore, it has been proposed that FtsH functions as a proteolytic regulator for cell division via degradation of FtsZ (Anilkumar 2001). Because FtsH is a stress-responsive protease and mid-cell localization occurs only in a fraction of dividing cells, it is likely that proteolytic regulation of FtsZ by FtsH is restricted to specific growth conditions/stresses (Wehrl 2000; Anilkumar 2001).

The role of FtsH as a stress-responsive protease and potential chaperone would explain its up-regulation in UA159 in the presence of 1mg/ml D-ala-D-ala. If detection of excess D-ala-D-ala serves as a stress signal, then FtsH may be up-regulated in order to assist in protein folding, as well as prevent cell division from occurring by degrading FtsZ; however, further studies are required in order to determine the role (if any) of FtsH in the D-ala-D-ala response and cell division modulation in *S. mutans*.

DivIVA is a cell division protein that has been extensively studied in Gram-negative bacteria and the Gram-positive organism *B. subtilis*. In Gram-negative species, the selection of cell division sites is controlled by MinCDE proteins; however, Gram-positive bacteria lack the MinE protein and possess the unrelated DivIVA protein instead (Ramirez-Arcos 2005). In *B. subtilis*, DivIVA, along with MinCD, defines the mid-cell and is involved in cell division and chromosome segregation (Ramirez-Arcos 2005). In contrast to rod-shaped bacteria, cocci, such as *E. faecalis, S. pneumoniae* and *S. aureus*, lack Min proteins, but all possess DivIVA homologues. It has been shown that DivIVA localizes to sites where peptidoglycan synthesis occurs and it interacts with many components involved with early and late stages of cell septation during division (Fadda 2003). DivIVA has also been shown to interact with proteins involved in cell wall rearrangement, such as LytB and PcsB (Vicente 2007). It has been proposed that DivIVA in Gram-positive bacteria may be primarily involved in acting as a cytoskeletal stress-bearing mechanism related to cell wall growth (Vicente 2007).

Deletion of *divIVA* in *S. pneumoniae* has been shown to result in severe growth inhibition, accompanied by defects in cell shape, nucleoid segregation and division (Fadda 2003). DivIVA is essential in *E. faecalis* and is involved in cell division, viability and chromosome segregation (Ramirez-Arcos 2005). Surprisingly, DivIVA in *S. aureus* is not involved in cell division or chromosome segregation (Pinho 2004). Based on the results of these studies, it is believed that the mechanisms by which DivIVA functions varies across different species, likely due to differences in species-specific protein interactions (Ramirez-Arcos 2005).

The *divIVA* gene was found to be down-regulated 2.3-fold in UA159 in the presence of 1mg/ml D-ala-D-ala. Due to its involvement in cell division, it is possible that the response to D-ala-D-ala results in down-regulation of cell division genes, although growth kinetics analysis showed enhanced growth in the presence of D-ala-D-ala. As stated previously, this could be because the detection of unlinked peptidoglycan via D-ala-D-ala signals the cell that the cell wall is damaged and/or weakened. Since cell wall synthesis is coupled to division, continuation of cell division may occur improperly if there is cell wall damage. It would be worthwhile to examine expression of *divIVA* and other cell division proteins in PKNB, as differential expression of these genes could provide insight into how PknB is involved in D-ala-D-ala signalling and/or modulation of cell division.

Synthesis of cell wall peptidoglycan involves a number of intracellular and extracellular processing steps. The assembly of the N-acetylglucosamine and N-acetylmuramic acid disaccharide peptide monomers occurs in the cytoplasm or inner leaflet of the cell membrane (Fiuza 2008b). GlmS is an amidotransferase that catalyzes the first committed step of amino sugar metabolism (Barreteau 2008). Glucosamine-6-phosphate (GlcN-6-P) is a central component of cell wall synthesis and glycolysis that is processed to undecaprenyl-N-acetylglucosamine (UDP-GlcNAc) for cell wall synthesis, or isomerized to fructose-6-phosphate (Fru-6-P) for glycolysis (Komatsuzawa 2004). A study in *S. aureus* demonstrated that GlmS is the key enzyme leading from glucose to cell wall by catalyzing the conversion of Fru-6-P to GlcN-6-P for use in PG synthesis (Komatsuzawa 2004). Inhibitors of GlmS activity impede cell wall synthesis and a *glmS*-deficient mutant of *S. aureus* was found to be unable to grow in media containing glucose as a sole carbon source because this mutant could not utilize glucose for cell wall synthesis (Komatsuzawa 2004). The up-regulation of *glmS* by 4.8- to 5.6-fold in UA159 suggests an increase in cell wall synthesis in response to D-ala-D-ala, thereby supporting results of growth kinetics experiments in which planktonic cultures of UA159 exhibited enhanced growth in the presence of D-ala-D-ala. This is plausible if the cell is sensing cell wall damage via D-ala-D-ala detection, thereby causing the cells to increase peptidoglycan synthesis to replace supposedly damaged cell wall material.

Synthesis of the UDP-N-acetylmuramic acid (UDP-MurNAc) moiety of PG is synthesized by the cytoplasmic enzymes MurA and MurB, with MurA forming UDP-GlcNAc-*enol*-pyruvate (Sylvester 2001; Barreteau 2008). MurB then reduces this product to D-lactate and UDP-MurNAc (Sylvester 2001; El Zoeiby 2003). The MurB enzyme has been found to be essential for PG synthesis in bacteria (Pucci 1992; Rowland 1995). MurB was highly up-regulated (24-fold) in PKNB with 1mg/ml D-ala-D-ala, suggesting that MurB enzymatic activity is highly active in the mutant. *MurB* was not detected in UA159 in the presence of D-ala-D-ala; therefore, it is not possible to determine if this gene is dependent or independent of PknB for regulation in the presence of D-ala-D-ala. It is possible that PG synthesis is also enhanced in the mutant in response to D-ala-D-ala, which appears to be the case for UA159, but gene expression analysis will be required to confirm whether this gene exhibits the extremely high up-regulation observed in this microarray study and to determine any differential expression in the wild-type.

The Mur ligases (MurC, D, E and F) catalyze the step-wise formation of the peptide side chain of PG (Fiuza 2008b). MurC is responsible for adding the first residue (usually L-alanine) to UDP-MurNAc (Barreteau 2008; Fiuza 2008b). MurD, E and F add subsequent amino acid residues, namely D-glutamic acid (MurD), *meso*-diaminopimelic acid or L-lysine (MurE) and the D-ala-D-ala dipeptide (MurF) (Barreteau 2008). The up-regulation of MurC2 in PKNB (2.8- to 3.9-fold) is once again indicative of enhanced PG synthesis in response to D-ala-D-ala detection, but the absence of this gene from UA159 results once again requires further study to determine dependence on PknB. Interestingly, MurC of *C. glutamicum* has been shown to be negatively regulated by phosphorylation by the STPK PknA (Fiuza 2008b). Also, MurD of *M. tuberculosis* was shown to be phosphorylated by PknA of this bacterium (Thakur 2008). These results suggest that perhaps PknB of *S. mutans* is able to phosphorylate one or more of the Mur ligases in order to regulate their activity; however, further studies would be required to confirm this.

The UDP-MurNAc-peptide precursor is then transferred from soluble UDP onto the undecaprenyl phosphate carrier by the MraY translocase, forming lipid I (Bouhss 2008; Fiuza 2008b). MurG then catalyzes the second membrane-associated step of PG synthesis by transferring the GlcNAc moiety from UDP-GlcNAc to lipid I, forming lipid II (Bouhss 2008). MurG interacts with MraY, and this complex is believed to participate in cell elongation (Bouhss 2008). *MraY* has been shown to be essential for viability and over-expression in *E. coli* has been associated with enhanced transferase activity (Bouhss 2008). *MurG* was similarly up-regulated in UA159 and PKNB, suggesting that this gene is not dependent on PknB for regulation in response to D-ala-D-ala and is likely under the control of another receptor that responds to this dipeptide. However, these results do support the hypothesis that PG synthesis is enhanced by the presence of exogenous D-ala-D-ala. Surprisingly, however, *mraY* is down-regulated approximately 2-fold in UA159 with D-ala-D-ala. This would suggest that formation of the lipid I intermediate is reduced in UA159, but the up-regulation of a number of other PG synthesis genes, including those involved in steps following MraY activity suggests that PG synthesis remains enhanced. Further studies, including gene expression analysis, will be required to confirm the results of this microarray and determine the effects of D-ala-D-ala on PG synthesis in UA159 and PKNB.

Following translocation of lipid II to the outer leaflet of the cell membrane, the PG glycan chains are polymerized and cross-linked by the penicillin-binding proteins (PBPs)

(Sauvage 2008). Two types of PBPs exist in bacteria; high molecular mass (HMM) PBPs, which possess transglycosylation (polymerization) and/or transpeptidase (cross-linking) activities; and low molecular mass (LMM) PBPs that possess carboxypeptidase activity, which removes the terminal D-alanine residue from the peptide chain (Laible 1992; Ghosh 2008; Sauvage 2008). Pbp2a and Pbp1b are both HMM PBPs, with Pbp2a possessing only transpeptidase activity (Sauvage 2008). Pbp2a has been shown to be required for normal outgrowth of *B. subtilis* spores (Murray 1998), while the presence of at least one PBP with dual transglycosylation and transpeptidation activities (e.g. Pbp1b) is necessary for growth in most bacteria (Sauvage 2008). *Pbp1b* and *pbp2a* are both up-regulated (2.5- to 2.7-fold) in UA159 in the presence of D-ala-D-ala, suggesting once again that PG synthesis and cross-linking is up-regulated in response to excess D-ala-D-ala, perhaps in attempts to cross-link the supposedly damaged PG. Once again, further studies are warranted to examine the effects of D-ala-D-ala on PG synthesis in UA159 and PKNB.

It is evident from these results that the effects of D-ala-D-ala on both UA159 and PKNB are extremely diverse. Numerous metabolic pathways are affected, including glycolysis, amino acid and cell wall biosynthesis, cell division, nucleotide biosynthesis, and stress responses. It is clear that further studies are necessary to understand the mechanisms involved in the response to D-ala-D-ala, not only to understand how *S. mutans* responds to this dipeptide, but also to determine what role, PknB plays in the detection and response to D-ala-D-ala.

A recent study by Shah *et al.* (2008) demonstrated that the STPK, PrkC, in *B. subtilis* was able to respond to muropeptides in peptidoglycan in order to initiate germination of dormant spores. *S. mutans* PknB shares 39% identity to PrkC of *B. subtilis*, as determined by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi), suggesting that perhaps PknB is also able to respond to peptidoglycan fragments. Although *S. mutans* does not form spores, the hypothesis was that PknB could detect and respond to peptidoglycan fragments, specifically the D-ala-D-ala dipeptide, in order to promote growth of dormant cells derived from biofilms. In order to test for this, a bioscreen was carried out using 24 hr biofilm-derived cells of UA159 and PKNB and growing them in a planktonic mode of growth with 0, 0.5, 1, 5 and 10mg/ml D-ala-D-ala. It was observed that growth of PKNB was enhanced in the presence of D-ala-D-ala in a concentration-dependent manner (Figure 5), while growth of UA159 was not altered.

In order to establish whether the phenotype seen for PKNB was due to merely supplying the mutant with a source of nutrients in the form of D-ala-D-ala, the same experiment was carried out using 0, 0.5, 1, 5 and 10mg/ml of D-alanine and L-alanine. L-alanine had no effect on growth of the PKNB mutant or UA159. D-alanine did not affect growth of UA159, but decreased growth of the mutant in a concentration-dependent manner. The fact that addition of D-alanine or L-alanine did not produce growth curves similar to those obtained from the addition of D-ala-D-ala indicates that the results of the initial bioscreen represent a specific response to D-ala-D-ala and were not due to supplying the cells with a source of added nutrients. Taken together with the results from addition of D-ala-D-ala, it appears that there is another receptor independent of PknB that is able to detect D-ala-D-ala.

A recent review by Dubrac *et al.* (2008) discusses the role of the WalKR signal transduction pathway in regulating cell wall metabolism. Based on a study by Dubrac (2007), it was discovered that this system appeared to function as a master regulator for cell wall metabolism. The homologous system in *S. mutans* is the VicKR TCS. It was predicted that WalK, the histidine kinase receptor, is responsible for detection of D-ala-D-ala (Dubrac 2008), suggesting that the WalK homolog in *S. mutans,* VicK, may also detect this dipeptide. Due to implications that VicK is associated with D-ala-D-ala detection, a VicK-deficient mutant was also used in this bioscreen experiment. Growth of the mutant was not affected by the addition of D-ala-D-ala (Figure 6), suggesting that VicK is not responsible for D-ala-D-ala detection, at least under the conditions used.

A similar growth rate experiment was carried out using mutants of histidine kinase receptors in other two-component systems, including HK11-, lytS- and HK8-. HK11 was chosen because it is located downstream of the p*knB* gene, suggesting possible cross-talk between these systems. HK8- and lytS-deficient mutants were chosen because these genes were found to be up-regulated in UA159 and PKNB microarrays with 5mg/ml D-ala-D-ala. All of the mutants grew similarly to wild-type at all D-ala-D-ala concentrations tested.

The D-ala-D-ala dipeptide appeared to enhance the growth of dormant biofilm-derived cells of PKNB; however, there were no observable effects on growth of UA159 or any of the other mutants used. These results do not eliminate the possibility that PknB is able to detect D-

ala-D-ala since addition of other compounds, specifically L-alanine and D-alanine, does not produce the same effects in the mutant. These results suggest that there is another receptor other than PknB that is able to respond to D-ala-D-ala. It is possible that there is another system with lower affinity for D-ala-D-ala detection and PknB is the higher affinity system; however, it is currently unknown what other system could be responsible for detection of D-ala-D-ala in *S. mutans*. Evidence suggests that the WalKR system is involved in detection of D-ala-D-ala (Dubrac 2007; Dubrac 2008), suggesting that the homologous system in *S. mutans*, VicRK, could be the system involved in the dose-dependent response observed in PKNB; however, the WalK sensor of *S. aureus* used in these studies contains an extensive extracellular domain that VicK in *S. mutans* lacks (Dubrac 2007; Dubrac 2008). This indicates that VicK is likely not able to detect D-ala-D-ala directly, as this dipeptide is found outside the cell. It is possible that VicK and PknB function together, whereby PknB acts as the sensor for D-ala-D-ala and co-ordinates with VicK in order to mediate a response to D-ala-D-ala. This hypothesis is supported by Saskova *et al.* (2007) who reported that several genes belonging to the WalR regulon in *S. pneumoniae* were also controlled by StkP, an STPK homologous to that of PknB in *S. mutans*, thus suggesting that StkP could be influencing WalKR activity. It is therefore possible that a similar situation exists for the VicRK system and PknB in *S. mutans*, although we have no evidence of this.

The addition of cell-free supernatants prepared from UA159 overnight and mid-logarithmic phase cultures also resulted in enhanced growth of the PKNB mutant; however, no dose-dependent response was observed and there was no difference in the response when either overnight or mid-log phase supernatants were used. The purpose of boiling a portion of the supernatants was to denature many proteinaceous components that could have elicited an enhanced growth response. The fact that the boiled supernatants gave the same result as samples that were not boiled suggests that the factor responsible for enhanced growth of PKNB is not a heat-denaturable peptide. The identity of the specific factor(s) responsible for this enhanced growth is currently unknown. It is well known, however, that during cell division of Gram-positive organisms a large amount of cell wall material is lost to the environment (Mauck 1971; Doyle 1988). It is believed that perhaps the enhanced growth response seen in PKNB is due to detection of this extracellular peptidoglycan material that acts as a signal to increase growth of the cells. Further experiments are needed to confirm if this is in fact the case.

When planktonic cultures of UA159 and PKNB were exposed to varying concentrations of D-ala-D-ala, a dose-dependent enhancement in growth was observed for UA159 (Figure 10), with 0.5 and 1mg/ml showing similar curves and 5 and 10mg/ml showing similar results with greater enhancement than 0.5 and 1mg/ml. In the case of PKNB, growth rates appeared similar for all concentrations used. While it appeared that the growth rate at 1mg/ml was less affected than the other D-ala-D-ala concentrations, there was large variation resulting in trends overlapping with the 0.5mg/ml curve, concealing potentially biologically relevant differences.

The fact that these experiments showed enhanced growth of UA159 in the presence of D-ala-D-ala and a lack of a dose-dependent growth increase of PKNB cells indicates that there are likely differences between cellular responses to D-ala-D-ala in planktonic versus biofilm-derived cells. During cell division by planktonic or biofilm cells, peptidoglycan is shed into the extracellular environment (Mauck 1971; Doyle 1988). It is possible that during planktonic growth the UA159 cells are sensing increased amounts of cell wall material in the form of added D-ala-D-ala. This could serve as a signal for cells to recognize an environment favourable for growth. This in turn could help promote biofilm formation, as more cells are available to adhere to a surface in order to initiate biofilm development. The lack of response in biofilm-derived cells is unclear. It is possible that these cells are less responsive to the D-ala-D-ala signal, as a stable biofilm has already been formed; however, in the case of PKNB, which produces less dense biofilms as confirmed by a safranin stain assay, the D-ala-D-ala signal could aid in promoting planktonic growth of the cells. The results of this experiment are preliminary and further studies are required to determine if planktonic cells do in fact exhibit altered responses to D-ala-D-ala compared to biofilm-derived cells.

A study of PknB in *S. mutans* demonstrated that the PknB-deficient mutant produced biofilms of reduced density and biomass compared to UA159 (Hussain 2006). In the study presented here, SEM was utilized to examine biofilm structure and cell morphology. In addition, because growth kinetics analyses showed enhanced growth of biofilm cells of PKNB in the presence of D-ala-D-ala, biofilms grown in the presence of D-ala-D-ala were also examined.

From the SEM images at higher magnifications (Figure 12), it appeared that UA159 cells were growing and dividing normally, whereas PKNB cells were irregular in shape, with some

cells in chains appearing swollen or small and round. This suggests that the mutant had impairments in cell division and/or growth. Studies of PknB homologs in other organisms have also implicated a role for these STPKs in cell division. For example, an SP-STK-deficient mutant of *S. pyogenes*, which shares 65% identity to PknB in *S. mutans*, demonstrated altered cell morphology and incomplete septation (Jin 2006). A PknB-depleted strain of *C. glutamicum* (31% identity to *S. mutans* PknB) exhibited an elongated phenotype due to inhibition of the final stages of cell division (Fiuza 2008). Also, studies of *M. tuberculosis* demonstrated that PknB phosphorylates the penicillin-binding protein PBPA, which is involved in cell division, in order to regulate cell wall synthesis at the division septum. Based on these findings and the results of SEM experiments, it is possible that PknB in *S. mutans* is also involved in cell wall maintenance and cell division in a similar manner to these other organisms.

There were also areas of the biofilm that consisted of what could potentially be excess extracellular material or lysed cells, which would suggest that PKNB produces excess extracellular polysaccharide or undergoes increased cell lysis. Autolysins are enzymes with peptidoglycan hydrolase activity, whose expression is believed to be controlled by the LytRS system in *S. mutans* (Chatfield 2005). *LytR* and *lytS* were found to be 2.4- and 3.1-fold up-regulated in PKNB in the presence of 5mg/ml D-ala-D-ala compared to the absence of the dipeptide, thus suggesting increased peptidoglycan turnover in these cells. Although *lytRS* were not found to be differentially regulated in PKNB in the absence of D-ala-D-ala, it is possible that given less strict statistical cut-offs, they may have appeared on the gene list. Quantitative real-time PCR and autolysis assays would be warranted to examine expression of these genes in PKNB and determine if in fact this mutant experiences elevated autolysis activity compared to UA159. Brunskill and Bayles (1996) studied a *lytS*-deficient mutant of *S. aureus* and found that this mutant formed aggregates when grown in liquid culture. PKNB also exhibits a clumping phenotype when grown in liquid culture, suggesting that differential expression of autolysin genes in the mutant could be responsible for this clumping phenotype. An alternate explanation for these areas seen on SEM micrographs could be that PKNB exhibits enhanced expression of genes involved with extracellular polysaccharide formation, such as the glucosyltransferases (*gtfBCD*) and glucan-binding protein (*gbpB*). This was found to be the case for a VicK-deficient mutant, which also exhibited clumping when grown in liquid culture (Senadheera 2005); however, in the experiments described here there was no sucrose added to the growth medium,

so any effects on polysaccharide formation would be dependent on glucose. It would be interesting to perform additional experiments, including RT-PCR and carbohydrate staining to determine if PKNB produces excess exopolymer material.

Micrographs of PKNB and UA159 biofilms were difficult to interpret in terms of relative biofilm density; therefore, a quantitative biofilm biomass experiment was warranted to determine whether D-ala-D-ala affected biofilm biomass. It was observed that 24 hr biofilms of PKNB grown in the presence of 0, 0.5, 1 and 5mg/ml D-ala-D-ala consistently produced biofilms of reduced biomass compared to UA159 (Figure 13), thus confirming previous observations (Hussain 2006). There was also no significant difference in biomass upon addition of varying D-ala-D-ala concentrations, indicating that this dipeptide does not affect biofilm biomass in the mutant. These observations are in contrast to results of growth rate analyses in which PKNB biofilm-derived cells exhibited enhanced growth in the presence of D-ala-D-ala. This suggests that the state in which the cells are grown (biofilm vs. planktonic) could be important for the type of response that is elicited in the presence of D-ala-D-ala. Biomass of UA159 biofilms was similar at 0, 0.5 and 1mg/ml D-ala-D-ala, while at 5mg/ml, biomass appeared to be significantly increased ($p<0.05$). It is possible that at higher concentrations, D-ala-D-ala promotes increased biomass of UA159 biofilms; however, the mechanisms behind this are currently unknown and further experiments are required to establish whether or not D-ala-D-ala affects UA159 biofilm biomass.

In order to assess the sensitivity of PKNB to environmental stresses, growth kinetics of UA159 wild-type strain and PKNB under various conditions were examined. Mid-log phase cells were exposed to varying concentrations of sodium chloride (NaCl), ethanol (EtOH), hydrogen peroxide (H_2O_2), sodium dodecyl sulphate (SDS), and paraquat. PKNB was found to be significantly more susceptible to SDS and H_2O_2 compared to the wild-type, while growth of PKNB appeared comparable to that of UA159 under NaCl, paraquat and EtOH stresses.

PASTA domains are predicted to bind to β-lactam antibiotics, such as penicillin. PKNB was found to be 2-fold more sensitive to VAN, PEN and CHX and 4-fold more sensitive to SDS compared to UA159. Penicillin G and vancomycin are antibiotics that target steps in cell wall biosynthesis. Greater sensitivity of the PKNB mutant to these agents suggests that PknB is

involved in generation of the cell wall and/or sensing cell wall stress. A number of STPKs have been linked to antibiotic susceptibility and hence, cell wall biosynthesis. A recent report of Stk in *S. aureus* revealed that a mutant lacking both STK and its cognate phosphatase, STP, demonstrated increased sensitivity to cell wall-acting antibiotics and exhibited defects in cell division (Beltramini 2009). PrkC of *E. faecalis* was also shown to play a role in antibiotic resistance and it was suggested that this STPK acts as a receptor that monitors cell wall integrity (Kristich 2007). Given that a PknB-deficient mutant of *S. mutans* also exhibits sensitivity to cell wall-targeting antibiotics, it is possible that PknB is also involved in detecting cell wall integrity and mediates the necessary responses when cell wall integrity is compromised.

Both SDS and CHX target the cell membrane. Since the PKNB mutant is highly sensitive to these antimicrobials, it is also possible that PknB plays a role in sensing and responding to cell membrane stress and/or regulation of cell membrane composition. A study by Molle *et al.* (2006) reported that an STPK in *M. tuberculosis* is involved in regulation of fatty acid synthesis. This is the first report of an STPK directly regulating components of the type II fatty acid synthase machinery. These findings, along with the down-regulation of fatty acid biosynthesis genes in the PKNB microarray, suggests that perhaps PknB in *S. mutans* is also involved in direct regulation of fatty acid synthesis, leading to modulation of cell membrane composition. Deletion of *pknB* would therefore result in alterations in fatty acid synthesis and would explain the increased sensitivity of PKNB to the membrane-targeting agents SDS and CHX used in the experiments reported herein. It was recently reported that TCS-11 (renamed LiaFSR), located directly downstream of the *pknB* locus, is involved in sensing cell envelope stress (Suntharalingam 2009). *Lia* mutants were found to be susceptible not only to various cell wall-targeting agents, but also to the membrane-targeting detergents SDS and CHX. Given the close proximity of these systems in the *S. mutans* genome, it is possible that PknB is involved in cross-talk with the LiaFSR system in order to mediate a response to membrane stress. Based on these findings, it appears that cell wall and cell membrane integrity are closely linked. It is possible that the sensitivity of PKNB to SDS and CHX is due to effects of PknB deletion on cell wall components and susceptibility to membrane-acting agents arises from modulations in cell wall structure as opposed to direct modulation of membrane composition. Further studies are warranted to determine the mechanisms behind PKNB's susceptibility to cell wall and membrane stresses.

Studies on STPKs in other bacteria have demonstrated mixed results in terms of sensitivity to osmotic stress (NaCl). A study of StkP in *S. pneumoniae* revealed that this STPK mutant is susceptible to osmotic stress (Saskova 2007); however, a study of a PrkC mutant of *B. subtilis* showed this mutant was not sensitive to NaCl (Madec 2002). It appears that the *S. mutans* PKNB mutant is similar to the *B. subtilis* mutant with respect to this phenotype. PknB also doesn't seem to be involved with response to ethanol stress and intracellular oxidative stress (paraquat). Based on these preliminary growth rate analyses, PknB appears to play a role in responding to extracellular oxidative stress (H_2O_2), as well as cell membrane stress, since the mutant was highly susceptible to the presence of SDS in the growth medium.

A study of the PknB homolog in Group B streptococci (GBS), Stk1, demonstrated that an Stk1-deficient mutant was attenuated for *de novo* purine biosynthesis and exhibited decreased levels of G nucleotides (GMP, GDP, and GTP) (Rajagopal 2005). It was shown that Stk1 was responsible for phosphorylating and thus inhibiting PurA activity. PurA (adenylosuccinate synthetase) is an enzyme involved in synthesis of AMP. The model proposed by the authors is that Stk1 phosphorylates PurA when A nucleotide pools or ATP concentrations increase within the cell, thus suppressing further AMP and ATP synthesis in order to control intracellular nucleotide pools. In the absence of Stk1, PurA activity is not regulated and therefore leads to continuous synthesis of A nucleotides, ultimately leading to decreased G nucleotide synthesis (Rajagopal 2005). This work presents a novel role for serine/threonine kinases in regulation of purine biosynthesis in GBS, and potentially other bacteria as well. A high number of genes involved in purine and pyrimidine biosynthesis were affected in PKNB, including *purM* and *purF*, which are involved in *de novo* purine biosynthesis (Rajagopal 2005), as well as *pyrD* and *pyrF*, which are involved in pyrimidine synthesis (Jensen 1979). In light of the results presented by Rajagopal *et al.* (2005), it is possible that PknB in *S. mutans* is also involved in regulation of the purine and/or pyrimidine biosynthesis pathways in this organism.

A total of 6 genes involved in fatty acid biosynthesis were down-regulated 2- to 4-fold in PKNB relative to UA159. Of these affected transcripts, 4 are found within the fatty acid biosynthesis operon in *S. mutans* (Figure 21). Fatty acids (FAs) are one of the major components of the bacterial cell membrane, as they are used for the synthesis of phospholipids and lipopolysaccharides (Lu 2004). FAs are produced by a group of enzymes called the type II fatty

acid synthase system (Rock 2002). The FA biosynthetic pathway in *Escherichia coli* has been extensively studied and serves as a model for Type II systems in other bacteria; however there is considerable diversity within prokaryotes (Marrakchi 2002). An overview of the fatty acid biosynthesis pathway in *S. pneumoniae* is shown in Figure 22.

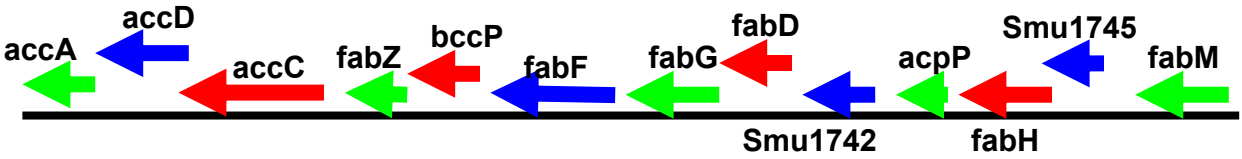

Figure 21. Fatty acid biosynthesis operon in *S. mutans*. *accA* (Smu1734), acetyl-CoA carboxylase subunit alpha; *accD* (Smu1735), acetyl-CoA carboxylase subunit beta; *accC* (Smu1736), acetyl-CoA carboxylase biotin carboxylase subunit; *fabZ* (Smu1737), (3R)-hydroxymyristoyl-ACP dehydratase; *bccP* (Smu1738), acetyl-CoA carboxylase biotin carboxyl carrier protein subunit; *fabF* (Smu1739), 3-oxoacyl-(acyl carrier protein) synthase II; *fabG* (Smu1740), 3-ketoacyl-(acyl-carrier-protein) reductase; *fabD* (Smu1741), acyl-carrier-protein S-malonyltransferase; Smu1742, putative trans-2-enoyl-ACP reductase; *acpP* (Smu1743), acyl-carrier protein; *fabH* (Smu1744), 3-oxoacyl-(acyl carrier protein) synthase III; Smu1745, putative transcriptional regulator; *fabM* (Smu1746), *trans*-2, *cis*-3-decenoyl-ACP isomerase.

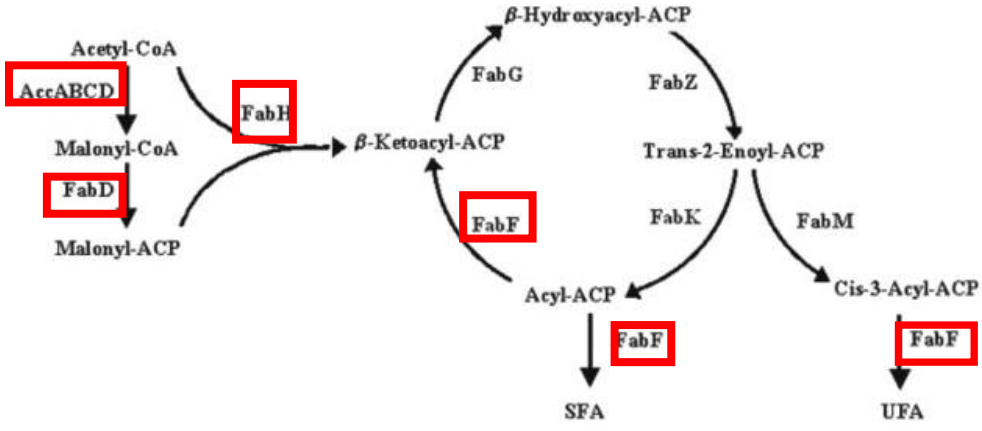

Figure 22. The fatty acid biosynthesis pathway in *Streptococcus pneumoniae*. The first step is carboxylation of acetyl-CoA by the heterotetrameric enzyme complex acetyl-CoA carboxylase (Acc), which is encoded by four individual genes (*accABCD*). The product,

malonyl-CoA, is then transferred to the acyl carrier protein (ACP) by FabD, the malonyl-CoA:ACP transacylase. The β-ketoacyl-ACP synthase III (FabH) catalyzes condensation of acetyl-CoA with malonyl-ACP to initiate cycles of fatty acid elongation that are carried out by the elongation condensing enzyme, FabF. (DiRusso 1999; Marrakchi 2002; Rock 2002) Enzymes outlined in boxes indicate the corresponding genes that were down-regulated in PKNB, with the exception of AccA, AccC and AccB (not identified in *S. mutans*). Figure from (Mohedano 2005)

Fatty acid composition dictates fluidity of the cell membrane and has been linked to a variety of processes, including thermal regulation (de Mendoza 1983), cell division and motility (DiRusso 1999), survival at acidic pH and sugar transport (Quivey 2000; Fozo 2004b; Fozo 2004c). Acid adaptation of *S. mutans* involves many mechanisms, including a shift in membrane fatty acid composition to include a higher proportion of long-chained, monounsaturated fatty acids (Fozo 2004c). These changes in FA composition cause the membrane to become less permeable to protons, thus allowing for survival in low pH environments (Quivey 2000). These results suggest that the acid sensitive phenotype exhibited by PKNB could be in part due to differential expression of the *fab* genes and genes encoding for the Acc complex, resulting in an altered cell membrane composition that prevents the bacteria from being able to survive under acidic conditions.

Down-regulation of the *accD* gene, as well as the *fab* genes (*fabDFH*) in PKNB suggested that this mutant exhibits altered expression of genes responsible for FA biosynthesis, and therefore possesses an altered cell membrane composition. Quantitative real-time PCR (RT-PCR) was performed in order to confirm down-regulation of *accD, fabDH, dltA* and Smu.1335 in PKNB (Figure 15). Expression of *accA, accC* and Smu.1344 were also examined as these genes belong to the same operon as *accD* and Smu.1335, respectively. *fabM* expression was examined as it belongs to the *fab* operon and has been linked with acid sensitivity ((Fozo 2004b). In addition, expression of Smu.1745 was examined as this gene is located directly upstream of the *fab* locus and is predicted to be a transcriptional regulator for these genes (Fozo 2004b).

Recent studies have identified a gene encoding a *trans*-2, *cis*-3-decenoyl-ACP isomerase (*fabM*), which is believed to be responsible for the production of monounsaturated fatty acids

(Fozo 2004b; Altabe 2007). Studies of a *fabM*-deficient mutant of *S. mutans* have demonstrated that monounsaturated fatty acids are required for survival in acidic pH and this mutant exhibits altered membrane permeability, F_1F_o-ATPase activity, (Fozo 2004b), and reduced virulence (Fozo 2007).

The *fabM* gene did not appear in the microarray experiments and RT-PCR demonstrated only a 1.2-fold up-regulation in PKNB relative to UA159, which could suggest that mono-unsaturated fatty acid synthesis is not affected in the mutant; however, the down-regulation of the other *fab* genes responsible for synthesizing the precursor components used by *fabM* might still result in altered fatty acid membrane composition and in turn acid sensitivity. In order to determine whether the addition of exogenous monounsaturated fatty acids could alleviate PKNB's acid sensitive phenotype, as was shown to be the case for the *fabM* mutant (Fozo 2004b), an acid tolerance response (ATR) assay was carried out in the presence and absence of two monounsaturated fatty acids. Cis-vaccenic and cis-eicosenoic acid were chosen for this experiment, as it was shown that these are the two major fatty acids present in the *S. mutans* cell membrane during acid adaptation (Quivey 2000; Fozo 2004c). This experiment did not demonstrate any differences in survival of PKNB in the presence and absence of exogenously added fatty acids, suggesting that alterations in monounsaturated fatty acid synthesis are not responsible for the acid sensitive phenotype of PKNB, at least under the conditions tested. The fact that many of the *fab* genes were affected in the mutant, as well as the fact that PKNB is highly sensitive to the cell membrane targeting antibiotics SDS and chlorhexidine, still potentiates the involvement of these genes in differential fatty acid biosynthesis in PKNB. A study of *M. tuberculosis* revealed that an STPK of this organism could phosphorylate enzymes of the type II fatty acid synthase system involved in mycolic acid biosynthesis, including FabD (Molle 2006). This report provided evidence that an STPK can control fatty acid synthesis, thus opening new doors to the possibility that this could also be the case for other bacterial STPKs, including PknB of *S. mutans*.

Alternatively, it is possible that PKNB's acid sensitivity is due to the down-regulation of the *dlt* genes, as these have been associated with acid sensitivity (Boyd 2000). The *dltA* gene was down-regulated approximately 3-fold in PKNB as determined by microarray analysis, while real-time PCR confirmation yielded a down-regulation of only 1.3-fold. *dltA* is the first gene of the

dlt operon (Figure 23), which is involved in synthesis of D-alanyl-lipoteichoic acid (LTA) (Figure 24) (Boyd 2000). This gene encodes Dcl, a D-alanine-D-alanyl carrier protein ligase, which catalyzes activation and ligation of D-alanine to the D-alanine carrier protein (Dcp) in an initial step to incorporation of D-alanine into membrane-associated LTA (Heaton 1994; Boyd 2000). Disruption of the *dltC* gene in *S. mutans* has been shown to result in acid sensitivity, possibly due to alterations in cell permeability to protons (Boyd 2000). Only *dltA* expression was examined by RT-PCR; however, it is possible that expression of the other *dlt* genes, namely *dltBCD*, are also affected in the mutant. Therefore, further studies are warranted to determine if differential expression of the *dlt* operon could be responsible for PKNB's acid sensitivity.

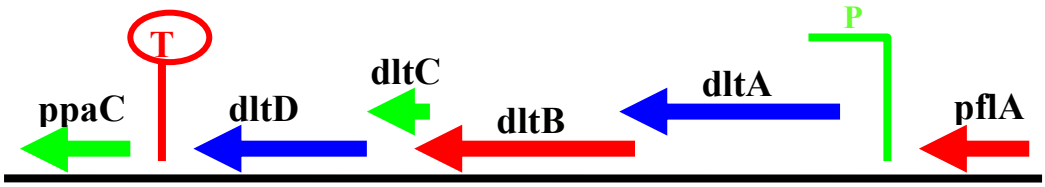

Figure 23. The *Dlt* operon in *S. mutans* encodes proteins involved in LTA synthesis. Putative promoter and terminator regions are shown. *dltA* encodes the D-alanine-D-alanyl carrier protein ligase (Dcl); *dltB* is a hypothetical protein; *dltC* encodes the carrier protein (Dcp); and *dltD* encodes a putative membrane protein (Heaton 1994).

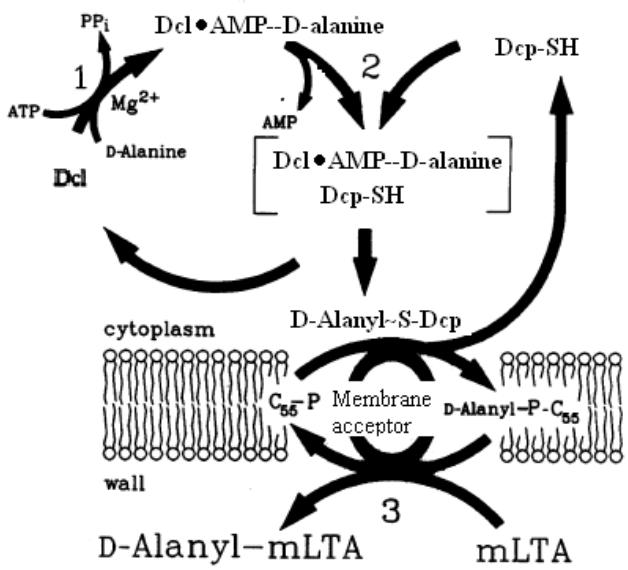

Figure 24. The lipoteichoic acid (LTA) biosynthesis pathway in *Lactobacillus casei.* Figure modified from (Heaton 1994).

From the results of these experiments, it appears that PknB in *S. mutans* is involved in the regulation of a wide variety of pathways, from cell wall biosynthesis and nucleotide metabolism, to fatty acid biosynthesis and cell growth and division. Identifying the signal that PknB is detecting, whether that is the D-ala-D-ala dipeptide or some other environmental signal, will be important for understanding how this STPK responds to extracellular stimuli. Further, the identification of kinase substrates for PknB will be critical in understanding its role in the regulation of these pathways. Together, these results may provide a novel target (PknB) for control of *S. mutans* growth.

Chapter 6
FUTURE DIRECTIONS

Studies of STPKs in prokaryotes represent a relatively new field of research; therefore, much remains to be discovered regarding the roles these proteins play in various regulatory and metabolic pathways in bacteria. While specific roles and substrates are being identified for a variety of bacterial STPKs, only one report has been published on PknB in *S. mutans* (Hussain 2006); therefore, many questions remain unanswered regarding the specific functions and substrates of this STPK. The presence of PASTA domains in PknB and a variety of other STPKs from Gram-positive organisms has led to the prediction that these STPKs detect the D-ala-D-ala moiety of unlinked peptidoglycan (PG) (Yeats 2002; Krupa 2005). Currently, the only report of a bacterial STPK responding to PG fragments is that of Shah *et al.* (2008) who examined PrkC of *B. subtilis*. It is important to note, however, that while these investigators demonstrated a response of PrkC to PG, it was not the D-ala-D-ala dipeptide that was determined to be the signal. It therefore remains unknown whether D-ala-D-ala serves as a signal for PASTA domain-containing STPKs.

Based on microarray analysis, it is evident that the presence of D-ala-D-ala affects a wide range of pathways in *S. mutans* UA159 and the PKNB mutant. It appears that D-ala-D-ala elicits a stress response, as demonstrated by the up-regulation of a variety of heat shock proteins, accompanied by down-regulation in cell division genes and up-regulation of cell wall synthesis genes. It would be beneficial to further examine the effects of D-ala-D-ala on UA159 in order to understand the various signalling pathways and response mechanisms involved with exposure to D-ala-D-ala.

The different growth curves obtained from biofilm-derived and planktonic cultures of UA159 and PKNB to varying concentrations of D-ala-D-ala suggest that the response to D-ala-D-ala depends on the growth state of the cells; however, the reasons for this are unknown. It would be interesting to perform transcriptome analysis of biofilm-derived cells exposed to D-ala-D-ala in order to compare affected transcripts with those of the current microarray studies using planktonic cells. This would allow for the identification of any genes that are specific to the response of biofilm cells to D-ala-D-ala and may provide information regarding the enhanced growth response of PKNB biofilm-derived cells in the presence of this dipeptide.

Due to evidence suggesting that the WalRK system in bacteria is able to regulate cell wall synthesis, likely via detection of D-ala-D-ala (Dubrac 2007; Dubrac 2008), it will be extremely interesting to determine if VicK or LiaS and PknB in *S. mutans* are able to interact in order to co-ordinate a response to D-ala-D-ala. This could be achieved through the use of *in vitro* phosphorylation assays; however, the identification of a specific substrate of PknB may be required in order to perform these experiments.

Another question that remains to be answered is whether PknB is able to differentially regulate purine and pyrimidine biosynthesis, as well as fatty acid synthesis, as suggested by the microarray results. Rajagopal *et al.* (2005) were able to determine that Stk1 of *S. agalactiae* was responsible for modulating purine biosynthesis by growing an *stk1*-deficient mutant in media lacking purine compounds. Performing similar experiments with PKNB may reveal a role for PknB in *S. mutans* in controlling nucleotide synthesis. In addition, the down-regulation of a number of fatty acid synthesis genes suggests that the cell membrane of PKNB exhibits differences in fatty acid composition compared to the wild-type. Analyzing the fatty acid profiles of membranes from UA159 and PKNB will be able to determine if this is in fact the case.

Based on preliminary microarray results, it was believed that alterations in fatty acid biosynthesis in PKNB were responsible for this mutant's sensitivity to acid; however, ATR assays in the presence and absence of exogenously added monounsaturated fatty acids did not reveal any differences in survival. These results suggest that there are other mechanisms responsible for PKNB's acid sensitive phenotype, such as the down-regulation of *dlt* genes, although further studies will be required to determine this.

In addition to acid sensitivity, the mechanisms involved in other PKNB-related phenotypes, such as decreased biofilm density, clumping, and competence deficiency, remain unexplained. The presence of potential areas of lysed cells or excess exopolymer in SEM micrographs could explain the clumping phenotype; however, further experiments, such as autolysis assays and carbohydrate stains will be necessary to determine if increased autolysis and/or the production of excess exopolymer by PKNB is responsible for the clumping phenotype. A study by Saskova *et al.* (2007) demonstrated that in an *stkP*-deficient mutant of *S. pneumoniae,* late competence genes are only weakly expressed both in the presence and absence

of CSP, suggesting that the reduced transformation efficiency of the *stkP* mutant may be due to a weak induction of DNA uptake and processing genes. It would therefore be interesting to examine gene expression of various early and late competence genes in *S. mutans* to determine if differential expression of certain competence genes in PKNB could be responsible for this mutant's deficit in transformation efficiency.

Perhaps one of the most important questions that remains to be answered is what are the substrates being phosphorylated by PknB? The identification of kinase substrates will be instrumental in understanding which pathways are regulated by PknB and will aid in providing answers for many of the questions postulated above. It is evident that further studies on PknB are required, as much remains to be understood about the role of PknB in *S. mutans*.

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Appendices

Appendix 1: Complete list of affected transcripts in UA159 and PKNB with 1mg/ml and 5mg/ml D-ala-D-ala

Gene ID	Putative Function	UA159*		PKNB*	
		‡1	5	‡1	5
Amino Acid Biosynthesis					
Smu1023	Putative pyruvate carboxylase/oxaloacetate decarboxylase, α subunit (PycB)	--	--	--	2.68
Smu1203	Putative branched-chain amino acid aminotransferase (IlvE)	2.00	2.10	--	--
Smu1261	**Putative phosphoribosyl-ATP pyrophosphohydrolasae**	**-1.73**	**--**	**--**	**4.00**
Smu1265	Putative phosphoribosyl formimino-5-amino-imidazole carboxamide ribonucleotide isomerase (HisA)	--	--	--	2.64
Smu1269	Putative phosphoserine phosphatase (SerB)	--	--	2.86	3.28
Smu1270	Putative histidinol dehydrogenase (HisD)	--	--	--	2.20
Smu1271	Putative ATP phosphoribosyltransferase (HisG)	--	--	--	2.06
Smu1675	Putative cystathionine γ-synthase possible bifunctional enzyme (MetB)	--	--	--	3.47
Smu1721	putative diaminopimelate decarboxylase	2.08	1.71	--	--
Smu1748	Putative aspartokinase (Akh)	--	--	2.15	2.67
Smu496	Putative cysteine synthetase A, O-acetylserine lyase (CysK)	--	--	3.71	--
Smu54	Putative amino acid racemase	-5.88	-6.45	--	--
Smu665	putative acetylglutamate kinase (ArgB)	-7.19	-7.30	--	--
Smu680	Putative γ-carboxymuconolactone decarboxylase subunit	--	--	--	2.69
Smu779	Putative 3-dehydroquinate synthase (AroB)	--	--	--	2.29
Smu780	Putative chorismate synthase (AroC)	--	--	--	2.28
Smu781	Putative prephenate dehydrogenase	--	--	--	2.43
Smu786	Putative prephenate dehydratase (PheA)	--	--	--	2.51
Smu913	**Putative NADP-specific glutamate dehydrogenase**	**-3.30**	**-3.73**	**4.44**	**--**
Bacteriocins					
Smu150	**Non-lantibiotic mutacin IV, NlmA**	**-12.20**	**-9.26**	**-2.72**	**-3.92**
Smu151	Non-lantibiotic mutacin IV, NlmB	-17.86	-16.13	--	--
Smu1906	Bacteriocin-related protein	--	--	--	-5.59
Smu1914	**Bacteriocin, BlpO-like protein**	**--**	**2.59**	**-4.13**	**-4.59**
Smu299	Putative bacteriocin peptide precursor	--	--	-4.72	-14.09
Smu423	**Possible bacteriocin**	**-10.31**	**-6.41**	**--**	**-3.13**
Biosynthesis of Cofactors, Prosthetic Groups and Carriers					
Smu1073	putative formyl tetrahydrofolate synthetase (FthS)	3.15	4.29	4.47	4.41
Smu1074	Putative flavoprotein involved in panthothenate	--	--	--	3.07

	metabolism				
Smu1075	putative DNA/pantothenate metabolism flavoprotein (Afp)	4.19	4.03	--	--
Smu1084	putative protoporphyrinogen oxidase (HemK)	3.76	3.25	--	--
Smu1430	**putative cobyric acid synthase (CobQ)**	**-2.28**	**-2.05**	**3.01**	**--**
Smu1799	putative nicotinate mononucleotide adenylyltransferase (NadD)	2.21	--	--	--
Smu267	putative glutamate-cysteine ligase	2.83	2.25	--	2.51
Smu582	putative farnesyl diphosphate synthase	2.06	1.79	--	--
Smu669	putative glutaredoxin	-3.40	--	--	--
Smu842	Putative thiamine biosynthesis protein (ThiI)	--	--	2.44	3.32
Smu85	putative phosphomethylpyrimidine kinase (ThiD)	-15.39	-17.86	--	--
Smu917	putative 6-pyruvoyl tetrahydropterin synthase	-4.83	-5.35	--	--
Smu969	Dihydropteroate synthase (FolP)	--	--	--	2.41
Cell Envelope					
Smu1004	Glycosyltransferase-I (GtfB)	--	--	--	-3.65
Smu1105	putative phosphoglycerate mutase-like protein	-2.97	-3.65	--	--
Smu1302	Putative surface adhesion protein (AdcA)	--	--	--	3.58
Smu1429	Putative UDP-N-acetylmuramyl tripeptide synthetase (MurC2)	--	--	3.88	2.76
Smu1437	putative UDP-N-acetylglucosamine 2-epimerase (EpsC)	2.37	--	--	--
Smu1461	putative glucose-1-phosphate thymidyltransferase (RmlA)	2.01	--	--	--
Smu1588	Putative hexosyltransferase	--	--	--	2.65
Smu1691	Putative D-alanine-D-alanyl carrier protein ligase (DltA)	-1.94	-2.15	--	--
Smu1949	Putative membrane carboxypeptidase (Pbp2a)	2.50	2.67	--	--
Smu1991	putative membrane carboxypeptidase, penicillin-binding protein 1b (Pbp1b)	2.74	--	--	--
Smu22	Putative secreted antigen GbpB/SagA putative peptidoglycan hydrolase (GpbB)	-2.52	-2.27	--	--
Smu456	Putative undecaprenyl-phosphate-UDP-MurNAc-pentapeptide transfersase (MraY)	-2.35	-2.15	--	--
Smu549	Putative undecaprenyl-PP-MurNAc-pentapeptide-UDP-GlcNAc GlcNAc transferase (MurG)	1.79	2.18	--	1.92
Smu609	putative 40K cell wall protein precursor	-7.09	-6.37	--	--
Smu716	Putative peptidoglycan branched peptide synthesis protein alanine (MurN)	--	--	--	2.25
Smu755	Putative prolipoprotein diacylglycerol transferase	--	--	2.45	2.58
Smu889	Putative penicillin-binding protein, class C fmt-like protein (PbpX)	-3.07	-2.77	--	--
Smu972	Putative UDP-N-acetylenolpyruvoylglucosamine reductase (MurB)	--	--	23.93	--
Cellular Processes					
Smu1003	putative glucose-inhibited division protein (Gid)	2.98	--	--	--

Smu1050	Putative phosphoribosylpyrophosphate synthetase, PRPP synthetase (KrpS)	--	--	--	2.20
Smu1286	Putative permease multidrug efflux protein	--	--	--	2.24
Smu1324	Putative cell-division protein (FtsX)	--	--	--	-2.32
Smu1339	Putative bacitracin sythetase (BacD)	--	--	23.49	--
Smu1451	**putative alpha-acetolactate decarboxylase (AldB)**	**-2.70**	**-2.49**	**--**	**2.41**
Smu15	Putative cell division protein (FtsH)	2.23	--	--	--
Smu1609	Putative membrane protein involved in protein secretion (SecG)	--	--	--	-4.67
Smu1614	Putative formamidopyrimidine-DNA glycosylase (Fpg)	1.77	--	--	3.80
Smu1651	putative arsenate reductase	-2.05	-1.91	--	--
Smu1850	Putative aminopeptidase P (PepP)	--	--	--	2.77
Smu188	Hsp33-like chaperonin (heat shock protein) (HslO)	-8.47	-8.77	--	--
Smu1948	**Putative preprotein translocase subunit (SecE)**	**-7.35**	**-7.19**	**--**	**-2.52**
Smu1954	Putative chaperonin (GroEL)	2.60	2.70	--	--
Smu2006	Putative preprotein translocase (SecY)	-3.04	-2.73	--	--
Smu2141	glucose inhibited division protein homolog (GidA)	-52.63	-50	--	--
Smu557	putative cell division protein (DivIVA)	-2.33	--	--	--
Smu589	putative DNA-binding protein	-2.72	-2.37	--	--
Smu629	Putative manganese-type superoxide dismutase, Fe/Mn-SOD (Sod)	1.72	1.41	2.78	--
Smu71	putative cation efflux pump	-3.36	-2.90	--	-1.53
Smu744	putative cell division protein (FtsY); signal recognition particle	-3.68	--	--	--
Smu764	Alkyl hydroperoxide reductase (AhpC)	--	--	--	2.42
Smu82	Molecular chaperone (DnaK)	3.93	3.25	--	--
Smu83	Heat shock protein (HSP-40) (DnaJ)	4.35	4.35	--	--
Smu853	putative lipoprotein signal peptidase (LspA)	5.83	5.16	--	--
Smu924	thiol peroxidase (Tpx)	2.16	2.42	--	--
Smu940	Putative hemolysin III	--	--	--	2.17
Central Intermediary Metabolism					
Smu1187	glucosamine-fructose-6-phosphate aminotransferase (GlmS)	5.59	4.83	--	--
Smu1296	Putative glutathione S-transferase YghU	--	--	3.32	2.26
Smu1299	Putative acetate kinase	--	--	24.91	2.58
Smu132	Putative hippurate amidohydrolase	--	--	4.33	5.31
Smu1573	**Putative S-adenosylmethionine synthetase (MetK)**	**-2.11**	**-1.68**	**11.99**	**--**
Smu1674	Putative aminotransferase probable beta-cystathionase (MetC)	--	--	3.38	4.16
Smu2127	Putative succinate semialdehyde dehydrogenase	--	--	3.86	3.12
Smu318	putative hippurate hydrolase	-1.96	-2.12	--	--
Smu360	Extracellular glyceraldehyde-3-phosphate dehydrogenase (GapC)	--	--	--	-3.53

Smu542	**putative glucose kinase (Glk)**	**--**	**2.14**	**2.14**	**4.24**
Smu676	NADP-dependent glyceraldehyde-3- phosphate dehydrogenase (GapN)	--	--	2.38	2.25
Smu938	Putative phosphomevalonate kinase	--	--	--	3.35
Smu939	**Putative dehydrogenase (FMN-dependent family)**	**-1.60**	**--**	**--**	**2.07**
Smu943	**Putative hydroxymethylglutaryl-CoA synthase**	**-1.82**	**-2.13**	**2.35**	**2.41**
Energy Metabolism					
Smu1077	**putative phosphoglucomutase (Pgm)**	**1.70**	**1.41**	**3.67**	**4.97**
Smu1102	6-phospho-β-glucosidase (Arb)	--	--	--	2.34
Smu1185	PTS system, mannitol-specific enzyme IIBC component (MtlA1)	-17.24	--	--	--
Smu1191	6-phosphofructokinase (PfkA)	-1.98	-2.00	--	-2.73
Smu128	putative acetoin dehydrogenase, E1 component (AdhB)	2.38	2.18	2.45	3.12
Smu129	putative dihydrolipoamide acetyltransferase (AdhC)	2.78	2.57	2.35	3.87
Smu1247	putative enolase (Eno)	-1.89	-2.27	--	--
Smu130	Putative dihydrolipoamide dehydrogenase (AdhD)	--	--	--	2.34
Smu1420	putative oxidoreductase	5.89	4.11	--	--
Smu1421	Putative dihydrolipoamide acetyltransferase, E2	--	--	--	7.57
Smu1422	**Putative pyruvate dehydrogenase E1 component β subunit (PdhB)**	**-5.21**	**--**	**--**	**11.84**
Smu1423	**putative pyruvate dehydrogenase, TPP-dependent E1 component (PdhA)**	**6.79**	**4.57**	**10.89**	**21.22**
Smu1424	**Putative dihydrolipoamide dehydrogenase (PdhD)**	**5.66**	**7.16**	**35.72**	**41.30**
Smu1452	alpha-acetolactate synthase (AlsS)	--	-2.24	--	--
Smu148	Putative alcohol-acetaldehyde dehydrogenase (AdhE)	--	--	--	-3.97
Smu1492	PTS system, lactose-specific enzyme IIA EIIA-LAC) (LacF)	--	-4.90	--	--
Smu1527	FoF1 proton-translocating ATPase, ε subunit (AtpC)	-3.41	-2.91	--	--
Smu1528	FoF1 proton-translocating ATPase, β subunit (AtpB)	-4.24	-4.48	--	--
Smu1530	FoF1 proton-translocating ATPase, α subunit (AtpD)	-3.52	-3.08	--	--
Smu1531	FoF1 membrane-bound proton translocating ATPase, δ subunit (AtpE)	--	--	--	-2.56
Smu1532	FoF1 membrane-bound proton translocating ATPase, b subunit (AtpF)	-3.72	-4.24	-2.52	-2.94
Smu1533	FoF1 membrane-bound proton translocating ATPase, a subunit (AtpG)	-2.99	-3.31	--	--
Smu1534	FoF1 membrane-bound proton translocating ATPase, c subunit (AtpH)	-3.97	-4.12	--	-2.23

Smu1535	Glycogen phosphorylase (PhsG)	--	--	--	3.29
Smu1536	Putative starch (bacterial glycogen) synthase (GlgA)	--	--	--	2.67
Smu1537	putative glycogen biosynthesis protein (GlgD)	2.11	2.02	--	--
Smu1539	Putative 1,4-α-glucan branching enzyme (GlgB)	--	--	--	4.20
Smu1590	intracellular alpha-amylase (AmyA)	2.70	--	--	--
Smu1602	Putative NAD(P)H-flavin oxidoreductase	--	--	2.72	--
Smu1692	pyruvate-formate lyase activating enzyme (PflA)	2.24	2.73	--	--
Smu1840	Putative fructokinase (ScrK)	--	--	--	2.02
Smu1841	Putative PTS system, sucrose-specific IIABC component (ScrA)	--	--	--	3.50
Smu1867	putative alcohol dehydrogenase	2.51	2.37	4.43	2.01
Smu1877	putative PTS system, mannose-specific component IIAB (PtnA)	-3.29	-3.77	--	--
Smu1878	putative PTS system, mannose-specific component IIC (PtnC)	-3.65	-5.38	--	--
Smu1958	putative PTS system, mannose-specific IIC component	-5.68	-5.24	--	--
Smu1960	putative PTS system, mannose-specific IIB component	-2.48	--	--	--
Smu1978	**putative acetate kinase (AckA)**	**-2.32**	**-1.60**	**2.39**	**--**
Smu2037	putative trehalose-6-phosphate hydrolase (TreA)	2.44	1.98	--	--
Smu2038	Putative PTS system, trehalose-specific IIABC component (PttB)	--	--	--	5.08
Smu2042	Dextranase precursor (DexA)	--	--	--	3.59
Smu231	Acetolactate synthase, large subunit (AHAS) (IlvB)	--	--	--	-2.81
Smu268	adenylosuccinate synthetase (PurA)	-2.30	-2.74	--	--
Smu270	putative PTS system, membrane component (UlaA)	2.16	2.76	2.88	--
Smu271	putative PTS system, enzyme IIB component (PtxB)	2.21	2.49	--	--
Smu307	glucose-6-phosphate isomerase (Pgi)	-2.59	--	--	--
Smu309	Regulator of sorbitol operon	--	--	--	5.87
Smu323	putative glycerol-3-phosphate dehydrogenase (GpsA)	4.03	3.96	--	--
Smu435	putative N-acetylglucosamine-6-phosphate deacetylase	-7.30	-5.88	--	--
Smu361	Phosphoglycerate kinase (Pgk)	--	--	--	-2.03
Smu463	putative thioredoxin reductase (NADPH) (TrxB)	-2.31	--	--	--
Smu465	NAD(+) synthetase (nitrogen-regulatory enzyme) (NadE)	--	--	--	2.58
Smu490	Putative pyruvate formate-lyase activating enzyme (PflC)	--	--	--	3.57
Smu563	Putative ornithine carbamoyltransferase	--	--	--	-13.89
Smu596	Phosphoglyceromutase (PmgY)	-2.42	-2.29	--	--

Smu675	Phosphoenolpyruvate:sugar phosphotransferase system enzyme I	--	--	2.19	2.21
Smu715	triosephosphate isomerase (TpiA)	-2.40	-1.47	--	--
Smu78	Fructan hydrolase exo-beta-D-fructosidase; fructanase (FruA)	--	--	--	2.25
Smu870	**Putative transcriptional regulator of sugar metabolism**	**-2.38**	**-2.55**	**--**	**2.54**
Smu871	Putative fructose-1-phosphate kinase (PfkB)	--	--	5.76	3.08
Smu872	putative PTS system, fructose-specific enzyme IIABC component	-3.16	-2.48	--	--
Smu886	galactokinase (GalK)	-2.37	--	--	--
Smu887	galactose-1-P-uridyl transferase (GalT)	-2.13	--	--	--
Smu888	UDP-galactose 4- epimerase (GalE)	-3.75	-3.98	-4.05	-5.62
Smu942	**Putative hydroxymethylglutaryl-CoA reductase (MvaA)**	**-1.70**	**-1.79**	**2.15**	**2.45**
Smu952	Putative methyltransferase (MmuM)	--	--	--	3.09
Smu981	Putative BglB fragment (BglB1)	--	--	--	-3.88
Smu99	Fructose-1,6- bisphosphate aldolase (FbaA)	-1.93	-1.89	-2.53	-4.67
Fatty Acid and Lipid Biosynthesis					
Smu131	Putative lipoate-protein ligase (LplA)	--	--	--	5.13
Smu1335	Putative enoyl-(acyl-carrier-protein) reductase	-5.78	-4.18	--	--
Smu1344	Putative malonyl-CoA acyl-carrier-protein transacylase	-4.51	-2.87	--	--
Smu1443	Putative tributyrin esterase	--	--	--	2.72
Smu1734	**Putative acetyl-CoA carboxylase α subunit (AccA)**	**-1.84**	**--**	**--**	**2.88**
Smu1735	Putative acetyl-CoA carboxylase β subunit (AccD)	-2.12	-2.31	--	--
Smu1736	**Putative acetyl-CoA carboxylase biotin carboxylase subunit (AccC)**	**-1.71**	**--**	**--**	**2.47**
Smu1737	Putative 3-hydroxymyristoyl-(acp) dehydrogenase (FabZ)	-2.11	-2.13	--	--
Smu1738	Putative biotin carboxyl carrier protein of acetyl-CoA (BccP)	-2.41	-2.13	--	--
Smu1739	Putative 3-oxoacyl-(acp) synthase (FabF)	-3.13	-3.03	--	--
Smu1740	Putative 3-oxoacyl-(acp) reductase/ 3-ketoacyl-acp (FabG)	-2.00	-1.96	--	--
Smu1741	Putative malonyl-CoA (acp) transacylase (FabD)	-2.29	-2.59	--	--
Smu1744	Putative 3-oxoacyl-(acp) synthase III (FabH)	-2.75	-3.13	--	--
Smu2151	putative phosphotidylglycerophosphate synthase (PgsA)	-3.08	--	--	--
Smu962	Putative dehydrogenase	3.53	--	3.97	4.55
Purines, Pyrimidines, Nucleotides and Nucleosides					
Smu1221	Putative orotate phosphoribosyltransferase (PyrE)	10.92	6.28	--	--
Smu1222	Putative orotidine-5-decarboxylase (PyrF)	8.66	7.30	--	--
Smu1223	**Putative dihydroorotate dehydrogenase B (PyrDB)**	**4.50**	**3.92**	**-3.38**	**-3.60**

Smu1224	Putative dihydroorotate dehydrogenase, electron transfer subunit (PyrK)	--	--	--	-14.93
Smu1227	Putative purine nucleoside phosphorylase (DeoD)	--	--	--	2.36
Smu14	Putative hypoxanthine-guanine phosphoribosyl-transferase (HprT)	--	--	--	-2.58
Smu1467	putative adenine phosphoribosyltransferase (Apt)	-2.22	-2.15	--	--
Smu1625	Putative uridylate kinase (PyrH)	--	--	--	-2.56
Smu2071	Putative anaerobic ribonucleotide reductase activating protein	--	--	-2.72	--
Smu29	putative phosphoribosylaminoimidazole-succinocarboxamide synthase SAICAR synthetase	--	-4.10	--	--
Smu32	phosphoribosylpyrophosphate amidotransferase (PurF)	-52.63	-62.5	--	--
Smu322	Glucose-1-phosphate uridylyltransferase	--	--	4.83	2.21
Smu325	putative dUTPase (Dut)	-2.24	-2.10	--	--
Smu34	putative phosphoribosylformylglycinamide cyclo-ligase (AIRS) phosphoribosyl aminoimidazole synthetase (PurM)	--	-9.26	--	--
Smu35	putative phosphoribosylglycinamide formyltransferase (GART) (PurN)	--	-50.00	--	--
Smu48	putative phosphoribosylamine-glycine ligase phosphoribosyl glycinamide synthetase (GARS) (PurD)	2.17	1.83	--	--
Smu50	Putative phosphoribosylaminoimidazole carboxylase catalytic subunit (PurE)	--	--	3.21	4.04
Smu51	Putative phosphoribosylaminoimidazole carboxylase ATPase subunit (PurK)	--	--	2.33	3.80
Smu59	adenylosuccinate lyase (PurB)	2.83	2.58	5.34	2.72
Smu667	Putative ribonucleotide reductase, small subunit (NrdF)	--	--	3.12	2.19
Smu856	**Putative pyrimidine operon regulatory protein (PyrR)**	**-6.67**	**-8.20**	**-21.28**	**-10.99**
Smu860	carbamoylphosphate synthetase, large subunit (CarB)	102.1	51.36	--	--
Regulatory Functions					
Smu1008	Putative response regulator (RR8)	-8.13	-8.20	--	--
Smu1009	**Putative histidine kinase (HK8)**	**--**	**1.59**	**--**	**4.44**
Smu1128	Putative histidine kinase sensor (CiaH)	--	--	--	2.23
Smu1184	Putative transcriptional regulator, antiterminator	--	--	--	2.64
Smu1515	CovX (VicX)	2.59	2.47	--	--
Smu1516	Putative histidine kinase, VicK homolog	2.13	--	--	--
Smu1517	**Putative response regulator, VicR homolog**	**2.04**	**--**	**-2.16**	**--**
Smu1548	Putative histidine kinase	-2.19	-2.21	--	--
Smu1578	Putative biotin operon repressor (BirA)	--	--	2.91	
Smu1814	Putative histidine kinase, ScnK homolog	2.13	--	--	--
Smu1924	Response regulator GcrR for glucan-binding	-2.12	-1.92	--	--

	protein C				
Smu1965	Putative histidine kinase	9.45	--	--	--
Smu1997	Putative ComX1, transcriptional regulator of competence-specific genes	--	--	--	4.52
Smu424	Negative transcriptional regulator (CopY)	--	--	-5.92	--
Smu427	Putative copper chaperone (CopZ)	--	--	-5.32	--
Smu486	putative histidine kinase	2.59	--	--	--
Smu576	**Putative response regulator (LytR)**	**-2.92**	**--**	**--**	**2.40**
Smu577	Putative histidine kinase (LytS)	2.61	1.74	--	3.05
Smu660	Putative histidine kinase SpaK	-4.76	-4.81	--	--
Smu80	**Heat-inducible transcription repressor (HrcA)**	**--**	**2.13**	**-2.77**	**-4.35**
Smu928	Putative histidine kinase (RelS)	--	--	--	2.06
Smu953	**putative transcriptional regulator/ aminotransferase**	**-2.98**	**-3.79**	**--**	**2.81**
Replication and Repair				--	--
Smu1204	Topoisomerase IV, subunit A (ParC)	--	--	--	2.11
Smu1210	Putative DNA topoisomerase IV, subunit B (ParE)	--	--	--	2.07
Smu1499	Putative exonuclease (RexA)	--	--	--	2.78
Smu1649	putative exodeoxyribonuclease III (ExoA)	2.49	1.93	--	--
Smu1859	Putative single-stranded DNA-binding protein (Ssb)	--	--	--	-2.36
Smu1870	Putative DNA mismatch repair protein (MutS2)	--	--	--	2.13
Smu1967	Putative single-stranded DNA-binding protein (Ssb2)	-8.85	-6.25	--	--
Smu2085	Recombination protein (RecA)	1.72	2.00	--	--
Smu2091	DNA mismatch repair protein	--	--	--	2.46
Smu2156	putative RecF protein, ATPase involved in DNA (RecF)	4.56	--	--	--
Smu297	DNA polymerase I (PolI)	--	--	--	2.76
Smu598	putative recombination protein (RecM)	-2.47	--	--	--
Smu60	DNA alkylation repair enzyme	-5.56	-5.62	--	--
Smu814	Putative MutT-like protein (MutT)	2.39	2.33	--	1.96
Smu821	putative DNA primase (DnaG)	4.41	3.63	--	--
Transcription					
Smu1168	putative transcriptional regulator	-4.78	--	--	--
Smu1193	Putative transcriptional regulator	--	--	--	-2.64
Smu124	Putative transcriptional regulator (MarR family)	2.94	3.06	--	--
Smu1361	Putative transcriptional regulator (TetR family)	6.85	6.75	--	--
Smu1409	putative transcriptional regulator	12.31	11.44	--	--
Smu1419	putative transcriptional regulator	3.41	--	--	--
Smu1514	Putative ribonuclease III (Rnc)	--	--	9.15	--
Smu161	putative transcriptional regulator	-6.58	-7.58	--	--
Smu1647	putative transcriptional regulator	-166.7	-142.9	--	--
Smu1707	Putative rRNA methylase	--	--	311.6	--
Smu1745	Putative transcriptional regulator	-2.12	-1.96	--	--
Smu1926	**putative transcriptional regulator (PsaR)**	**4.75**	**4.59**	**--**	**2.36**

Smu2001	DNA-directed RNA polymerase, α subunit (RpoA)	--	--	--	-2.06
Smu2084	Transcriptional regulator Spx	1.64	2.68	2.72	2.23
Smu349	Dimethyladenosine transferase	--	1.79	2.33	--
Smu38	putative transcriptional regulator	-4.67	--	--	--
Smu611	putative ATP-dependent RNA helicase, DEAD-box family	-1.76	-4.08	--	--
Smu640	Putative transcriptional regulator (GntR family)	--	--	--	-11.77
Smu822	DNA-dependent RNA polymerase sigma subunit major sigma (RpoD)	2.48	--	--	--
Smu852	Putative transcriptional regulator CpsY-like	--	--	340.2	--
Smu921	putative transcriptional regulator	3.96	3.71	--	--
Translation					
Smu1044	Putative pseudouridylate synthase	--	--	--	3.25
Smu1051	Putative iron-sulfur cofactor synthesis protein NifS family	--	--	--	2.77
Smu1127	Putative 30S ribosomal protein S20 (RpsT)	-2.50	--	--	-2.38
Smu1132	Aminopeptidase N (PepN)	--	--	--	2.64
Smu1218	putative amidase (NylA)	--	--	-2.87	--
Smu1239	Putative dipeptidase (PepV)	--	--	--	2.42
Smu1272	Putative histidyl-tRNA synthetase (HisZ)	--	--	2.32	2.88
Smu1288	50S ribosomal protein L19 (RplS)	--	--	--	-7.19
Smu1311	putative asparaginyl-tRNA synthetase (AsnC)	-2.70	-2.83	--	--
Smu1477	putative tRNA isopentenylpyrophosphate transferase (MiaA)	-2.92	-3.14	--	--
Smu1512	putative phenylalanyl-tRNA synthetase (alpha subunit) (PheS)	-29.41	-33.33	--	--
Smu154	30S ribosomal protein S15 (RpsO)	-5.65	-2.46	--	--
Smu1592	Putative dipeptidase (PepQ)	--	--	--	-2.23
Smu1606	putative SsrA-binding protein homolog (SmpB)	2.12	1.83	--	--
Smu1626	50S ribosomal protein L1 (RplA)	--	--	59.5	46.75
Smu1627	50S ribosomal L11 protein (RplK)	-2.25	-2.48	--	--
Smu169	50S ribosomal protein L13 (RplM)	--	--	--	-2.83
Smu170	30S ribosomal protein S9 (RpsI)	--	--	--	-2.43
Smu1711	putative pseudouridylate synthase B, large subunit (RluB)	1.81	1.97	--	2.36
Smu1779	putative RNA methyltransferase	2.75	2.77	--	--
Smu1819	**Putative glutamyl-tRNA (Gln) amidotransferase subunit B (GatB)**	**-1.41**	**--**	**--**	**2.11**
Smu1847	Putative translation elongation factor P (Efp)	--	--	--	-2.17
Smu1858	30S ribosomal protein S18 (RpsR)	--	--	--	-2.07
Smu1860	30S ribosomal protein S6 (RpsF)	--	--	-2.17	-3.58
Smu1886	putative seryl-tRNA synthetase (Sys)	3.13	2.57	--	--
Smu1970	Putative phenylalanyl-tRNA synthetase, β subunit	--	--	2.43	--
Smu2000	50S ribosomal protein L17 (RplQ)	-2.59	-1.97	--	--
Smu2002	30S ribosomal protein S11 (Rs11)	-2.32	-1.96	-2.25	--
Smu2007	50S ribosomal protein L15 (RplO)	-2.24	--	--	--

Smu2010	**50S ribosomal protein L18 (RplR)**	**-5.38**	**-6.33**	**--**	**-2.17**
Smu2011	50S ribosomal protein L6 (BL10) (RplF)	-2.15	-2.35	--	--
Smu2014	30S ribosomal protein S14 (RpsN)	-2.05	--	--	--
Smu2015	50S ribosomal protein L5 (RplE)	-3.00	-4.70	--	--
Smu2016	50S ribosomal protein L24 (RplX)	-1.95	-2.09	--	--
Smu2017	50S ribosomal protein L14 (RplN)	--	-3.82	--	--
Smu2020	50S ribosomal protein L16 (RplP)	-3.14	-4.41	--	--
Smu2024	50S ribosomal protein L4	-1.96	-2.46	--	-2.48
Smu2025	50S ribosomal protein L3 (RplC)	-2.36	-2.53	--	--
Smu2026	30S ribosomal protein S10	-2.71	-3.14	-3.40	-3.17
Smu2032	30S ribosomal protein S2 (RpsB)	-2.36	-2.51	--	-2.17
Smu2098	Putative arginyl-tRNA synthase (ArgS)	--	--	--	2.43
Smu2135	30S ribosomal protein S4 (RpsD)	--	--	-2.70	-5.18
Smu340	**50S ribosomal protein L34 (RpmH)**	**-1.81**	**--**	**-4.00**	**-4.39**
Smu357	30S ribosomal protein S12 (RpsL)	-1.35	-1.63	-2.30	-2.53
Smu358	30S ribosomal protein S7	--	--	-2.23	-2.36
Smu359	Translation elongation factor G (EF-G)	-2.43	--	--	-3.30
Smu421	translation initiation factor 2 (InfB)	1.81	2.94	--	--
Smu608	**Putative translation elongation and release factor (PrfC)**	**-12.20**	**-7.87**	**9.56**	**--**
Smu697	Putative translation initiation factor IF3 (InfC)	--	--	-4.69	-3.40
Smu698	50S ribosomal protein L35 (RpmI)	-2.06	-1.71	-3.73	--
Smu699	50S ribosomal protein L20 (rplT)	-2.21	--	-2.62	-2.84
Smu714	Translation elongation factor EF-Tu (Tuf)	--	-1.91	--	-2.02
Smu818	**30S ribosomal protein S21 (RpsU)**	**-5.29**	**--**	**--**	**-7.69**
Smu84	**putative tRNA pseudoruridine synthase A (TruA)**	**-14.71**	**-20.83**	**-10.10**	**-7.41**
Smu846	50S ribosomal protein L21 (RplU)	--	--	-2.83	-3.62
Smu849	50S ribosomal protein L27 (RpmA)	--	--	-2.11	-2.39
Smu865	30S ribosomal protein S16 (RpsP)	-2.19	--	--	-2.76
Smu957	**50S ribosomal protein L10 (RplJ)**	**-5.29**	**-5.26**	**--**	**-1.98**
Smu960	**50S ribosomal protein L7/L12 (RplL)**	**-4.05**	**-3.09**	**--**	**-1.83**
Transport and Binding Proteins					
Smu1013	putative Mg2+/citrate transporter	-4.08	-3.72	--	--
Smu1041	Putative ABC transporter, ATP-binding protein	--	--	--	-2.35
Smu1063	putative ABC transporter, proline/glycine betaine (OpuAa)	3.49	2.72	--	2.06
Smu1067	Putative ABC transporter, permease protein	--	--	--	2.48
Smu1068	Putative ABC transporter, ATP-binding protein	--	--	--	2.15
Smu1118	putative ABC sugar transporter, permease protein	-2.33	-2.45	--	--
Smu1134	Putative phosphate ABC transporter, ATP-binding	--	--	--	2.26
Smu1163	Putative ABC transporter, ATP-binding protein	--	--	--	2.38
Smu1179	Putative amino acid ABC transporter, permease protein	--	--	--	-2.60
Smu1194	putative ABC transporter, ATP-binding protein	-3.02	-3.01	-3.55	-2.33
Smu1217	Putative ABC transporter, amino acid binding	--	--	--	-2.31

Smu1348	putative ABC transporter, ATP-binding protein	-28.57	-23.81	--	--
Smu1412	Putative ABC transporter, membrane protein subunit	--	--	--	3.10
Smu1446	putative ABC transporter, permease protein	-2.24	-2.72	--	-2.53
Smu1521	putative amino acid ABC transporter permease protein	2.48	1.86	--	2.79
Smu1561	Putative potassium uptake system protein (TrkB)	--	--	2.29	3.34
Smu1562	Putative potassium uptake protein TrkA (Trk)	--	--	--	2.36
Smu1563	Putative cation-transporting P-type ATPase (PacL)	--	--	--	2.59
Smu1568	putative maltose/maltodextrin ABC transporter, sugar-binding protein (MalX)	3.29	3.47	--	--
Smu1665	**Putative branched chain amino acid ABC transporter (LivF)**	**-1.74**	**--**	**--**	**2.95**
Smu1666	**putative branched chain amino acid ABC transporter (LivG)**	**-1.87**	**-2.36**	**--**	**2.95**
Smu1667	putative branched chain amino acid ABC transporter (LivM)	-2.24	-2.55	--	--
Smu1668	putative branched chain amino acid ABC transporter (LivH)	-2.13	-2.65	--	--
Smu1708	putative potassium uptake system protein (TrkA)	-2.10	-1.88	--	--
Smu1852	putative magnesium/cobalt transport protein	-83.33	--	--	--
Smu1881	Putative ABC transporter, ATP-binding protein	--	--	--	-4.10
Smu1897	Putative ABC transporter, ATP-binding protein, CslA	--	--	-4.27	-3.43
Smu1927	putative ABC transporter, ATP-binding protein	-30.30	-27.03	--	--
Smu1938	Putative ABC transporter, permease protein	--	--	-2.60	--
Smu1966	putative periplasmic sugar-binding protein	-41.67	--	--	--
Smu1993	**putative ABC transporter, zinc permease protein (AdcB)**	**-2.65**	**-2.86**	**--**	**5.68**
Smu1994	Putative ABC transporter, ATP-binding protein (AdcC)	--	--	--	2.14
Smu2116	Putative osmoprotectant amino acid ABC transporter ATP-binding (OpuCa)	2.87	2.63	--	--
Smu2117	Putative osmoprotectant ABC transporter permease protein (OpuCb)	2.78	--	--	3.02
Smu2118	Putative ABC transporter osmoprotectant-binding protein, glycine betaine/carnitine/ choline (OpuCc)	3.56	2.57	--	4.11
Smu2119	Putative osmoprotectant ABC transporter permease protein (OpuCd)	3.35	3.23	7.43	3.11
Smu2159	putative ABC transporter, ATP-binding protein	3.09	3.43	--	--
Smu248	Putative ABC transporter, membrane protein	--	--	--	2.62
Smu286	putative ABC transporter, ATP-binding protein NlmT	2.67	--	--	--
Smu396	**putative glycerol uptake facilitator protein (GlpF)**	**-3.13**	**-3.22**	**--**	**-8.93**
Smu408	possible xanthine-uracil permease	-1.84	-2.32	--	--

Smu432	Putative ABC transporter, integral membrane protein	--	--	--	2.53
Smu461	putative amino acid ABC transporter, ATP-binding protein	-11.11	--	--	--
Smu525	putative ABC transporter, ATP-binding protein	3.78	3.44	--	--
Smu602	**putative sodium-dependent transporter**	**-9.43**	**-9.90**	**--**	**-4.55**
Smu652	Putative ABC transporter, ATP-binding protein possible nitrate	--	--	4.91	11.01
Smu653	Putative ABC transporter, permease protein	--	--	--	5.14
Smu772	putative glucan-binding protein D BglB-like protein (GbpD)	-3.22	--	--	--
Smu806	Putative glutamine ABC transporter	--	--	--	4.33
Smu817	putative amino acid transporter, amino acid-binding protein	2.10	2.33	--	--
Smu819	Putative large conductance mechanosensitive channel (MscL)	--	--	-3.06	-4.83
Smu857	Putative uracil permease	--	--	-15.38	-4.57
Smu879	multiple sugar-binding ABC transporter, permease protein (MsmF)	-5.75	-5.88	--	--
Smu880	multiple sugar-binding ABC transporter, permease protein (MsmG)	-4.41	-4.76	--	--
Smu882	multiple sugar-binding ABC transporter, ATP-binding protein, (MsmK)	-3.58	-3.11	--	--
Smu909	putative malate permease	3.29	2.82	--	--
Smu923	Putative ABC transporter, ATP-binding protein	--	--	--	3.65
Smu934	Putative amino acid ABC transporter, permease	--	--	3.17	4.19
Smu936	Putative amino acid ABC transporter, ATP-binding	--	--	--	3.46
Smu998	putative ABC transporter, periplasmic ferrichrome-binding protein	-3.94	--	--	--
Unassigned Category					
Smu06	putative GTP-binding protein	--	2.14	--	--
Smu1022	2'-(5''-triphosphoribosyl)-3'-dephospho-CoA:apo-citrate lyase (CitX)	--	--	--	2.51
Smu1059	Acid tolerance protein (SatC)	2.92	--	--	--
Smu1080	Hypothetical protein possibly transposon-related	--	3.04	--	--
Smu1117	NADH oxidase (H_2O-forming) (NaoX)	-2.92	-4.31	--	--
Smu1323	possible hydrolase	-2.08	-2.01	--	--
Smu1327	conserved hypothetical protein possible 4Fe-4S ferredoxin	4.99	4.93	--	--
Smu1331	putative transposase	4.29	3.94	--	--
Smu1345	Putative peptide synthetase similar to MycA	--	--	--	3.58
Smu1363	putative transposase	5.37	4.82	--	--
Smu1392	Putative acetyltransferase	--	--	--	2.12
Smu1432	**putative endoglucanase precursor**	**--**	**2.23**	**--**	**--**
Smu1449	putative fibronectin/fibrinogen-binding protein	-200	--	--	--

Smu1473	Putative oxidoreductase	--	--	--	3.13
Smu149	Putative transposase	--	--	-3.25	--
Smu1593	**possible CDP-diglyceride synthetase**	**-2.09**	**--**	**3.00**	**--**
Smu1617	GTP-binding protein (Era)	--	--	2.19	2.55
Smu1620	Putative phosphate starvation-induced protein (PhoH)	--	--	2.11	1.69
Smu1700	conserved hypothetical protein possible LrgB family protein	-5.78	-6.67	--	--
Smu1727	putative inner membrane protein	2.03	1.94	--	2.29
Smu173	Putative ppGpp-regulated growth inhibitor	--	--	-5.15	-2.17
Smu1747	putative phosphatase	-2.94	-3.06	--	--
Smu1807	Putative integral membrane protein	--	--	9.98	--
Smu1816	putative maturase-related protein	4.07	3.48	--	--
Smu1817	putative maturase-related protein	-13.51	-13.16	--	--
Smu187	possible dehydrogenase	1.83	1.61	--	2.60
Smu1930	Putative cytoplasmic membrane protein LemA-like protein (LemA)	--	--	-1.93	-2.10
Smu1988	putative DNA binding protein	-9.35	-5.68	--	--
Smu201	putative transposon protein	-25.00	-22.73	--	--
Smu2056	Putative ATPase	--	--	2.86	2.43
Smu2067	Putative stress response protein, possible glucosyl-transferase involved in cell wall biogenesis (CsbB)	--	--	-4.12	-4.53
Smu222	Hypothetical protein, possible integrase fragment	--	--	15.41	--
Smu320	possible 5-formyltetrahydrofolate cyclo-ligase	-2.16	-2.46	--	--
Smu328	**Possible carbonic anhydrase**	**-1.64**	**-1.73**	**--**	**2.25**
Smu355	putative CMP-binding factor	1.93	1.64	--	2.59
Smu412	Putative Hit-like protein involved in cell-cycle regulation	2.14	2.10	--	--
Smu623	putative deacetylase	-7.58	--	--	--
Smu641	Putative oxidoreductase	--	--	--	2.12
Smu646	**Putative phosphatase**	**-26.32**	**-24.39**	**--**	**1.95**
Smu647	putative methyltransferase	2.05	2.09	--	--
Smu720	Conserved hypothetical protein possible Na+/solute symporter	--	--	--	-2.25
Smu728	**Putative oxidoreductase**	**-2.67**	**--**	**--**	**3.60**
Smu747	conserved hypothetical protein putative permease	1.74	--	2.99	4.86
Smu801	**Putative GTP-binding protein**	**1.68**	**--**	**--**	**-2.05**
Smu862	Conserved hypothetical protein putative permease	--	--	111.8	
Smu88	Conserved hypothetical protein possible mechanosensitive ion channel	--	--	--	2.52

*Ratios obtained by dividing expression value at 1mg/ml or 5mg/ml by that of 0 D-ala-D-ala control condition for UA159 and PKNB; $p<0.001$

‡mg/ml D-ala-D-ala

Hypothetical proteins have been omitted for clarity. **Genes highlighted in bold are likely dependent on the presence of PknB for regulation in the presence of D-ala-D-ala**