Genetic and Phenotypic Analysis of Genes Involved in Bacitracin Resistance in *Streptococcus mutans*

by

Naif A. Jalal

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Genetic and Phenotypic Analysis of Genes Involved in Bacitracin Resistance in *Streptococcus mutans*" by Naif A. Jalal in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor: _____

Co- Supervisor: _____

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DEDICATION

It is my pleasure to dedicate my whole work and degree to my parents, my beloved wife, my beloved son, my sister, and my brother for their understanding and patience throughout the time and being there when help is needed.

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ABSTRACT

Streptococcus mutans is considered to be the primary causative agent of dental caries in humans. In the oral cavity, *S. mutans* frequently exposed to antimicrobial compounds secreted from competing species and from the host. To survive in the oral cavity, *S. mutans* must be able to sense and respond to these antimicrobial compounds. For example, *S. mutans* is well known to resist bacitracin; however, only a few studies have explored the molecular mechanisms of resistance of *S. mutans* to bacitracin or other related antibiotics. By screening a high-density transposon mutant library constructed previously in our laboratory, we identified a transposon insertion mutant in SMU.244 gene that was sensitive to bacitracin. The objective of this study was to characterize potential roles of SMU.244 in response and resistance to bacitracin and other cell wall-acting antibiotics. We also extended our investigation into another genetic locus SMU.862-864 that encoded an ABC transporter (exporter) and might be involved in bacitracin response in *S. mutans*. Our results confirmed that SMU.244 encoded a homolog of BacA or UppP protein that is involved in the cell wall biosynthesis. Deletion of SMU.244 resulted in SmΔbacA mutant that was 32-fold more sensitive to bacitracin and 2-fold more sensitive to penicillin G, vancomycin, and nisin than the parent strain UA159. This defect in antibiotic resistance was completely restored in the complement strain (Sm-pCpbacA). SmΔbacA also showed a slower growth rate (T_d = 1:22 h^{-1}) than the parent (T_d = 1:05 h^{-1}) and its growth nearly ceased in the presence of 0.48 U/ml of bacitracin. In addition, SmΔbacA mutant formed a biofilm with reduced biomass, especially in the presence of bacitracin (0.48 U/ml), suggesting that it may play a role in biofilm formation. The work using qRT-PCR revealed that the *bacA* gene might not be directly regulated by the BceABRS system and its expression appeared to be independent from induction by bacitracin. Taken together, we conclude that SMU.244 encodes a BacA homolog that plays important roles in resistance to cell wall-acting antibiotics in *S. mutans*. Our work also showed that SMU.862-864 might not be involved in resistance to cell wall-acting antibiotics. With the methods used in this study, there was no sufficient evidence to support that this ABC transporter played direct roles in biofilm formation, acid resistance and stress responses in *S. mutans*.

LIST OF ABBREVIATIONS USED

ABC	ATP-binding cassette
BacA	Bacitracin resistant protein A
BrpA	Biofilm regulatory protein A
CSP	Competence-stimulating peptide
C_t	Threshold cycle
CTAB	Cetyltrimethylammonium bromide
Dgk	Diacylglycerol kinases
EDTA	Ethylenediaminetetraacetic acid
Farnesyl-PP	Farnesyl pyrophosphate
GlcNAc	*N*-acetylglucosamine
IPP	Isopentenyl pyrophosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
MIC	Minimum inhibitory concentration
MurNAc	N-acetylmuramic acid
PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
RBS	Ribosome-binding site
RGP	Rhamnose-glucose polysaccharide
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth with 20 mM glucose
TAE	Tris-acetate and EDTA
TCSTSs	Two-component signal transduction systems
TE	Tris-EDTA
THYE	Todd-hewitt supplemented with 0.3% yeast extract
TSS	Transcription start site
TTE	Triton X-tris-EDTA
UK	Undecaprenol kinase
Up	Undecaprenyl-phosphate

Upp	Undecaprenyl pyrophosphate
UppP	Undecaprenyl pyrophosphate phosphatase
UppS	*cis*-prenyl pyrophosphate synthase
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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Chapter 1. Introduction

1.1 *Streptococcus mutans*

Streptococcus mutans is a Gram-positive, facultative anaerobic coccus that belongs to the phylum Firmicutes and the group of lactic acid-producing bacteria (Ajdic *et al.,* 2002; Clarke, 1924; Jordan *et al.,* 2008). *S. mutans* was first isolated from carious lesions by Clarke (1924). When first isolated, this bacterium looked more oval in its morphology than round by Gram-staining and appeared to be a mutant-form of a Gram-positive coccus. This bacterium was designated as *S. mutans* (Clarke, 1924). However, it was not until 1960's that the association between *S. mutans* and dental caries was confirmed by a number of studies from epidemiology, microbiology, and germ-free animal models (Banas, 2004; Hamada and Slade, 1980; Loesche, 1986). In the oral cavity, *S. mutans* depends on a "biofilm lifestyle" for survival and persistence in dental plaque, a multispecies biofilm community, which may consist of more than 700 species of microorganisms (Hamada and Slade, 1980; Kuramitsu *et al.,* 2007).

1.2 The Oral Environment

The oral environment is known to support the growth of a wide range of microorganisms, including *S. mutans*. In the oral cavity, many factors are known to influence the growth of oral microorganisms, such as temperature, pH, salivary flow, and the availability of nutrients (Marcotte and Lavoie, 1998). The oral cavity is a moist environment with a relatively constant temperature (34~36°C). The pH in the oral cavity is maintained near neutrality (about pH 7.0) by the flow of saliva. Saliva contains several buffering systems, including bicarbonate and phosphates (Marcotte and Lavoie, 1998; Schenkels *et al.,* 1995). These buffering systems or molecules can neutralize acids produced by oral bacteria from carbohydrate metabolism (Marcotte and Lavoie, 1998).

In addition to its buffering activity, saliva is also considered as an important source of nutrients for the oral microflora, since it provides carbohydrates, glycoproteins, proteins, amino acids, water, and minerals including sodium, potassium, calcium, and chloride (Schenkels *et al.,* 1995). The dietary components such as carbohydrates and proteins are also considered as an important source of nutrients to the oral microorganisms (Marcotte and Lavoie, 1998).

In the oral cavity, microorganisms including *S. mutans* usually colonize and reside in a densely packaged microbial mass on teeth, known as dental plaque, which is a multispecies biofilm community organized in a complex matrix composed of salivary compounds and microbial extracellular products (Hamada and Slade, 1980; Kuramitsu *et al.,* 2007). In dental plaque, oral microorganisms often experience a wide range of environmental stresses, such as acidic stress, osmotic stress, and oxidative stress, which largely result from frequent consumption of diet and metabolic activities of microorganisms in the oral cavity (Lemos and Burne, 2008). In addition, the antimicrobial compounds produced by the host or by the competing species of bacteria also act as a life-threatening stress to the oral microorganisms (Jordan *et al.,* 2008; Lemos and Burne, 2008).

Following the consumption of carbohydrate-rich diet, pH in dental plaque may drop to as low as 4.0 (Matsui and Cvitkovitch, 2010; Welin-Neilands and Svensater, 2007). This occurs due to the production of lactic acid through fermentation of sugars by acidogenic bacteria such as *Lactobacillus* and *S. mutans* (Matsui and Cvitkovitch, 2010). This low pH environment may lead to an alteration in the plaque microbiota and a selection of aciduric bacteria. The selection of aciduric bacteria in turn leads to a sustained acidic environment in dental plaque, playing a major role in the development of dental caries. Thus, the abilities to survive extreme and rapid changes in pH are considered as an important virulence factor of cariogenic bacteria (Matsui and Cvitkovitch, 2010).

Once pH in dental plaque drops to 5.4 or lower, this causes demineralization of the tooth enamel (Banas, 2004). The demineralization may increase the concentration of

calcium salts so that increasing the ionic strength of the dental plaque fluid (~150 mM) (Gao *et al.,* 2001). In addition, accumulation of solutes from diet, such as NaCl, in the extracellular matrix might also increase osmolality in the local environment. Thus, microorganisms in the dental plaque may face a challenge of high osmotic stress (Lemos *et al.,* 2005).

In the dental plaque environment, oral microorganisms are also frequently exposed to oxidative agents, such as peroxide-containing oral hygiene products (Lemos *et al.,* 2005; Marquis, 1995). In addition, a number of oral bacteria are also known to produce H_2O_2, which is a powerful oxidative molecule, resulting in oxidative stress to the microorganism in dental plaque (Kreth *et al.,* 2008; Lemos *et al.,* 2005). In this regard, a number of studies have shown that hydrogen peroxide produced by either *Streptococcus sanguinis* or *Streptococcus gordonii*, under aerobic condition, are able to inhibit the growth of *S. mutans* (Kreth *et al.,* 2005; Kreth *et al.,* 2008). This is because *S. mutans* do not posses catalase enzyme, which is responsible for the detoxification of H_2O_2 to water and oxygen. Thus, in dental plaque H_2O_2 can act as antimicrobial compound to inhibit the growth of susceptible oral bacteria (Lemos *et al.,* 2005).

1.2.1 Antibiotic Stress in the Oral Environment

It is well known that all lineages of bacteria, including those in dental biofilms, produce bacteriocins that are potent peptide antibiotics responsible for "chemical warfare" in a microbial community (Jordan *et al.,* 2008; Kuramitsu *et al.,* 2007). It is also known that human hosts produce many defense peptides, such as α-defensin, β-defensin, LL-37 and others, which bind to bacterial cell envelope and kill the bacteria. In the oral cavity, β-defensin is expressed in gingiva, tongue, salivary gland and mucosa. In addition to β-defensin, saliva also contains many other antimicrobial compounds such as α-defensin, and LL-37 (Jenssen *et al.,* 2006; Wiesner and Vilcinskas, 2010). The antimicrobial action of these peptides usually occurs via pore formation on the cell membrane of bacteria. This makes the susceptible bacteria more permeable to small

molecules, resulting in decreased bacterial viability (Dale and Fredericks, 2005; Jordan *et al.,* 2008).

In addition, secretory immunoglobulin A (SIgA) is a major specific defense factor of saliva, which is produced by salivary glands (Marcotte and Lavoie, 1998). SIgA is known to protect the oral cavity from microbial adherence to epithelial and tooth surfaces, by neutralizing enzyme activities such as glucosyltransferases of streptococci, and by acting in synergy with other antibacterial factors in the saliva such as lysozyme (Marcotte and Lavoie, 1998).

The presence of antibiotics or host defense peptides in the environment is a common threat to microbial life. The sensitive detection and subsequent induction of appropriate resistance mechanisms is therefore a prerequisite for microbial survival. For example, bacitracin is a cyclic polypeptide antibiotic that is produced by certain species of the *Bacillus,* such as *Bacillus subtilis* and *Bacillus licheniformis* (Froyshov, 1974; Johnson *et al.,* 1945). This antibiotic can target essential components of the cell wall and suppresses the biosynthesis of peptidoglycan in Gram-positive bacteria (Jordan *et al.,* 2008). However, these organisms have developed effective mechanisms to cope with bacitracin and protect themselves from the killing by their own-produced antibiotics (Mascher *et al.,* 2006b; Ohki *et al.,* 2003b).

1.3 The Cell Envelope in Gram-Positive Bacteria

The cell envelope in Gram-positive bacteria refers to two functional layers: a cytoplasmic membrane that encloses the cellular contents and a thick cell wall that primarily consists of peptidoglycan and surrounds the cytoplasmic membrane (Jordan *et al.,* 2008; Navarre and Schneewind, 1999). Therefore, the cell envelope is an essential structure that gives bacterial cell its shape and counteracts the high osmotic pressure. It also provides an important sensory interface, mediating both information flow and transport of solutes from and out of a bacterial cell (Jordan *et al.,* 2008). The bacterial cell envelope is the first line of defense against threats represented by antimicrobial

compounds such as bacitracin from the surrounding environment. Thus, the monitoring and maintenance of cell envelope integrity, and an effective response to threats is crucial for bacterial survival especially for those bacteria that live in a complex and dynamic environment (Jordan *et al.,* 2008; Ouyang *et al.,* 2010). The cell wall of Gram-positive bacteria consist mainly of a mesh-like sacculus (peptidoglycan) surrounds the cytoplasmic membrane and is composed of glycan chains cross-linked by short peptides (Typas *et al.,* 2011). The cell wall play several important roles in bacteria such as maintaining the cell integrity, defined cell shape, involved in cell growth and cell division, and serves as a scaffold for anchoring other cell wall components such as teichoic acids (Typas *et al.,* 2011; Vollmer *et al.,* 2008).

In Gram-positive bacteria such as *S. mutans,* the synthesis of the cell wall can be divided into three steps. The first step occurs in the cytoplasm, while the second and third steps occur in the cell membrane and in the cell wall, respectively (Jordan *et al.,* 2008). The process of cell wall biosynthesis is initiated by generating uridine diphosphate-N-acetylmuramic acid (UDP-MurNAc) from uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate, followed by the addition of pentapeptide to UDP-MurNAc. The UDP-MurNAc-pentapeptide molecule is then phosphodiester linked to the lipid carrier molecule undecaprenyl-phosphate (Up) to generate lipid I precursor. UDP-GlcNAc is then linked to lipid I to generate the disaccharide-pentapeptide molecule or lipid II precursor, which may undergo further species-specific modification by the addition of amino acids to the lysine (Navarre and Schneewind, 1999).

The lipid II precursor is translocated across the cytoplasmic membrane and serves as a substrate for the newly synthesized peptidoglycan. Cell wall assembly is then catalyzed by glycosyltransferases, which catalyze the polymerization of the glycan chains, and transpeptidases or penicillin binding proteins (PBPs), which catalyze the transpeptidation (cross-linking) of wall peptides (Navarre and Schneewind, 1999; Typas *et al.,* 2011). Following the polymerization of the disaccharide-pentapeptide molecule, the lipid carrier will be released as an undecaprenyl pyrophosphate (Upp), which needs to be recycled to Up before it can be reused (Figure 1.1) (Navarre and Schneewind, 1999).

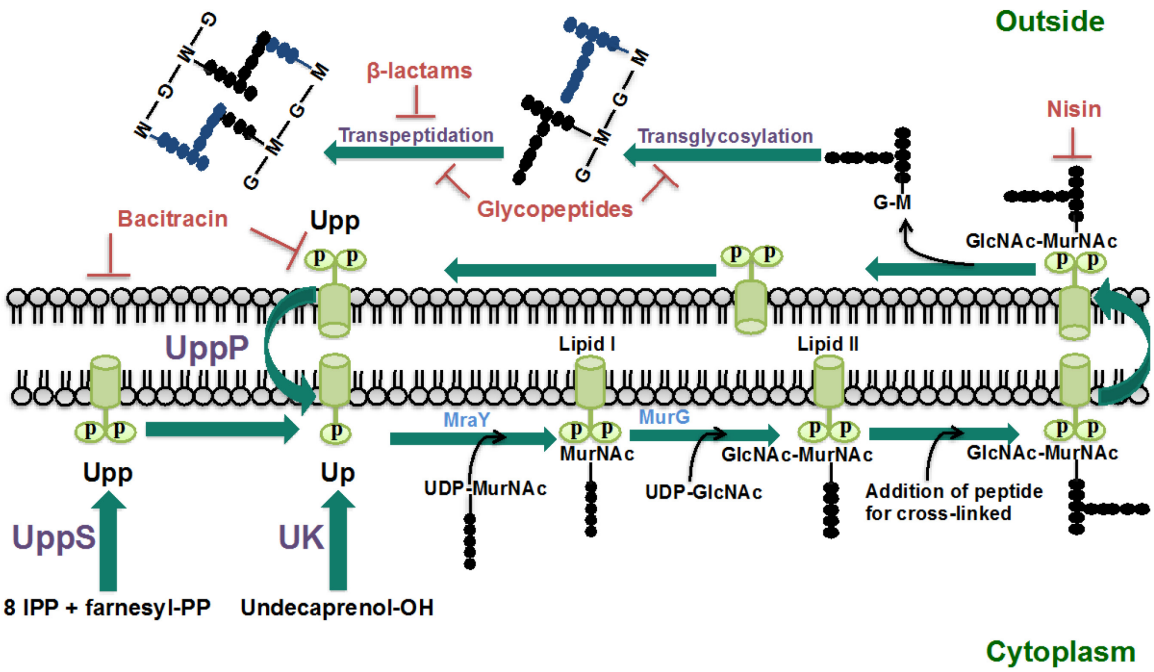

Figure 1. 1. A schematic diagram describing the biosynthesis of undecaprenyl phosphate (Up) and its role in peptidoglycan biosynthesis. The Up-precursor is synthesized via the enzymatic activity of UppS using eight molecules of IPP (isopentenyl pyrophosphate) and farnesyl-PP (farnesyl pyrophosphate) as substrates. The product is then dephosphorylated into Up by the activity of UppP enzyme. Up also arises by the phosphorylation of undecaprenol by undecaprenol kinase **(**UK) enzyme or by the recycling of Upp after it releases the peptidoglycan precursor. The MraY enzyme (UDP-MurNAc-pentapeptide phosphotransferase) catalyzes the transfer of MurNAc-pentapeptide motifs onto the Up lipid carrier, generating lipid I. MurG enzyme (UDP-GlcNAc-undecaprenoyl-pyrophosphoryl-MurNAc-pentapeptide transferase) catalyze the transfers of the GlcNAc motifs onto the lipid I complex, generating lipid II intermediates. The lipid II is then translocated to the outer side of the cell membrane where the newly disaccharide pentapeptide unit of peptidoglycan is released. The lipid carrier is released as Upp and dephosphorylated to Up before being reused. Modified from Typas *et al.* (2011).

The lipid carrier undecaprenyl phosphate (Up) is a key component in the cell wall synthesis of the bacteria (Coker and Palittapongarnpim, 2011; Navarre and Schneewind, 1999). It is required for the transportation of hydrophilic cell wall precursors such as peptidoglycan or teichoic acid across the hydrophobic cell membrane. Undecaprenyl phosphate arises from the dephosphorylation of the undecaprenyl pyrophosphate (Upp), which originates from either *de novo* synthesis by *cis*-prenyl pyrophosphate synthase (UppS) or release after the transfer of the peptidoglycan precursor. The dephosphorylation of Upp is catalyzed by undecaprenyl pyrophosphate phosphatase (UppP). However, it is still unknown whether this reaction occurs immediately after the release of peptidoglycan precursor at the outer side of the cell membrane or after the translocation of the free-Upp to the inner side of the cell membrane (Coker and Palittapongarnpim, 2011; Tatar *et al.,* 2007). An alternative pathway to produce Up is also reported in Gram-positive bacteria. In this pathway, Up could result from the phosphorylation of undecaprenol (U-OH) via the activity of an enzyme termed undecaprenol kinase (UK or diacylglycerol kinases, DgkA) (Figure 1.1) (Coker and Palittapongarnpim, 2011; Jerga *et al.,* 2007). In addition, Upp has been shown to act as a phosphate donor in the phosphorylation of lipid A via an enzyme termed LpxT in *E. coli*. This reaction resulted in the generation of Up (Touze *et al.,* 2008).

Bacitracin is known to require a divalent metal ion, such as zinc, for proper biological activity (Stone and Strominger, 1971). Bacitracin inhibits the growth of sensitive organisms by binding tightly to undecaprenyl pyrophosphate (Upp), thereby preventing the synthesis and recycling of the lipid carrier (Jordan *et al.,* 2008). This in turn leads to a reduction in the number of available lipid carrier undecaprenyl phosphate molecules and subsequently inhibits the dynamic process of cell wall synthesis (Figure 1.1) (Jordan *et al.,* 2008; Stone and Strominger, 1971). The enzyme responsible for the conversion of Upp (target site of bacitracin) to Up, UppP, is strongly linked to bacitracin resistance. A previous study has shown that overexpression of the *bcrC* gene that encoded UppP enzyme in *B. subtilis* resulted in increased resistance to bacitracin compared to the wild type (Bernard *et al.,* 2005). A similar result was obtained in Gram-negative bacteria *E. coli* when the *uppP* gene that encoded UppP enzyme was

overexpressed (Cain *et al.,* 1993; El Ghachi *et al.,* 2004). In addition to *uppP* another three genes termed *ybjG*, *yeiU*, and *pgpB* in *E. coli* were found to have UppP activity. Overexpression of each of these genes resulted in an increased level of UppP activity and bacitracin resistance phenotype (El Ghachi *et al.,* 2005; Tatar *et al.,* 2007).

In *S. pneumoniae*, deletion of *bacA* gene resulted in a mutant that was more sensitive to bacitracin and showed a significant attenuation in the virulence in a respiratory tract mouse model of infection (Chalker *et al.,* 2000). Similar results were also obtained when the *bacA* gene was deleted from *S. aureus*, which resulted in an 8-fold increase in sensitivity to bacitracin and attenuation in the virulence in a pyelonephritis mouse model of infection (Chalker *et al.,* 2000). Furthermore, deletion of *bacA* gene in *Streptococcus gordonii* resulted in defect in biofilm formation with a significant reduction in biomass accumulation compared to the parent strain (Loo *et al.,* 2000). Although *bacA* is known to be important in bacitracin resistance and virulence in these streptococci, it remains to be unclear whether *bacA* or its gene product UppP plays the same role in *S. mutans*. Lis and Kuramitsu have shown that the *dgk* gene in *S. mutans* encodes a protein that shares homology with *E. coli* diacylglycerol kinase (Lis and Kuramitsu, 2003). This gene product mainly recognizes a lipid substrate, most likely undecaprenol, and may act as undecaprenol kinase. This finding was supported by a significantly higher sensitivity of the Δ*dgk* mutant to bacitracin compared to the parent strain, supporting the role of this gene product as undecaprenol kinase (Lis and Kuramitsu, 2003). In addition to bacitracin, several others cell wall-acting antibiotics are known to inhibit the process of cell wall biosynthesis either by sequestering the substrate of a given step or by inhibiting the enzymatic activity of these reactions. For example, nisin is known inhibit the growth of their susceptible bacteria by sequester 'hijack' lipid II complex and use it as a docking molecule to form pores (Jordan *et al.,* 2008). While, glycopeptide antibiotics (e.g. vancomycin) block glycan polymerization and cross-linking by binding tightly to the D-alanyl-D-alanine dipeptide terminus of lipid II and nascent peptidoglycan. In addition, β-lactams (e.g. penicillin G) inhibit peptidoglycan synthesis by covalent binding to the active site of transpeptidases enzyme by mimicking the D-alanyl-D-alanine terminus of the pentapeptide side chain (Typas *et al.,* 2011).

1.4 Antibiotic-Induced Cell Envelope Stress in *S. mutans*

It is well known that the cell envelope of bacteria is the target site for numerous antibiotics or cell envelope-disturbing conditions (Navarre and Schneewind, 1999; Vollmer *et al.,* 2008). A stress caused by such antibiotics, agents or conditions that disturb or destroy the integrity of the cell envelope of bacteria is called cell envelope stress response (CESR). Therefore, the maintenance of the cell envelope integrity or homeostasis is crucial for bacterial survival in the presence of cell envelope disturbing agents such as antibiotics (Jordan *et al.,* 2008).

Over the past few years, studies showed that *S. mutans* is able to sense and respond to the presence of antibiotics in the surrounding environment. This response is often characterized by altered expression of many genes involved in multiple signal transduction systems, the cell wall biosynthesis and the virulence of *S. mutans* (Mazda *et al.,* 2012; Ouyang *et al.,* 2010; Suntharalingam *et al.,* 2009). Bacitracin is a good example of the ability of *S. mutans* to sense, respond and adapt to antibiotics in the surrounding environment (Jordan *et al.,* 2008; Ouyang *et al.,* 2010). Although, *S. mutans* does not produce bacitracin and bacitracin producers, such as *B. licheniformis* and *B. subtilis* are not common members in dental plaque. These mechanisms utilized by *S. mutans* to resist bacitracin may also be used by this pathogen to resist other antimicrobial molecules produced from competing bacterial species in dental plaque or from the host, such as human α-defensin and β-defensin. Nevertheless, only few studies have explored the mechanisms of bacitracin resistance in *S. mutans* (Ouyang *et al.,* 2010; Suntharalingam *et al.,* 2009).

1.4.1 Two-Component Signal Transduction Systems (TCSTS)

The cell envelope stress response in Gram-positive bacteria involve multiple signaling transduction systems, which are required for sensing and responding to the presence of cell wall-acting antibiotics or to the damages caused by these antibiotics (Jordan *et al.,* 2008). A major mechanism of signal transduction, widespread in bacteria, is the so-called two-component signal transduction systems (TCSTSs) (Hoch, 2000). These TCSTSs normally consist of a membrane-associated sensor protein that senses specific stimuli outside the cell, and cytoplasmic response regulator protein that enables the cells to respond to the stimuli. These systems provide the major means by which bacteria communicate with each other and the outside world for optimal physiology, stress response, and adaptation (Stock *et al.,* 2000).

There are fourteen TCSTSs that have been identified in the *S. mutans* genome (Ajdic *et al.,* 2002; Biswas *et al.,* 2008). A number of studies have shown that these TCSTSs are able to sense a wide range of environmental stimuli such as pH, oxygen, calcium (He *et al.,* 2008), antibiotics (e.g. bacitracin) (Ouyang *et al.,* 2010), and signal peptides (e.g. competence-stimulating peptide or CSP) (Allan *et al.,* 2007; Li *et al.,* 2001b). In response to these stimuli, TCSTSs regulate the expression of a number of genes encoding stress responsive proteins and virulence factors in *S. mutans*, such as biofilm formation, genetic competence, and stress tolerance including antibiotic stress (Li *et al.,* 2002b; Ouyang *et al.,* 2010; Qi *et al.,* 2004). Among the fourteen TCSTSs in *S. mutans*, at least three systems have been shown to play roles in antibiotic-induced cell envelope stress response. These include the BceABRS (Ouyang *et al.,* 2010), LiaFSR (Suntharalingam *et al.,* 2009), and CiaXRH (Mazda *et al.,* 2012).

1.4.1.1 The BceABRS System

The detoxification module that consists of a TCSTS and ATP-binding cassette (ABC) transporter is known to play most important role in bacitracin resistance (Jordan *et al.,* 2008). In *S. mutans*, an example of this detoxification module is the four-component system BceABRS that has been shown to play a role in the cell envelope stress response (Ouyang *et al.,* 2010). This system consists of a TCSTS BceRS and an ABC transporter BceAB. A deletion of any gene of this system resulted in mutants with 100-fold increase in sensitivity to bacitracin compared to the parent strain UA159 (Ouyang *et al.,* 2010; Tsuda *et al.,* 2002). In addition, BceRS positively regulates the expression of the *bceABRS* operon in response to bacitracin. This system also regulates the expression of three additional genetic loci SMU.862, SMU.302, and SMU.1856c in response to bacitracin. However, the roles of these genes in bacitracin stress response in *S. mutans* are still unclear. The up-regulation of the *bceABRS* operon and the other three genetic loci via the BceRS requires the presence of an intact ABC transporter BceAB, which suggest that the BceAB transporter may act as a co-sensor, together with the BceRS (Ouyang *et al.,* 2010) (Figure 1.2). In addition, the up-regulation of *bceAB* genes was abolished when the aspartate residue (the predicted phosphorylation site) at position 54 of the BceR (response regulator) was mutated (Kitagawa *et al.,* 2011).

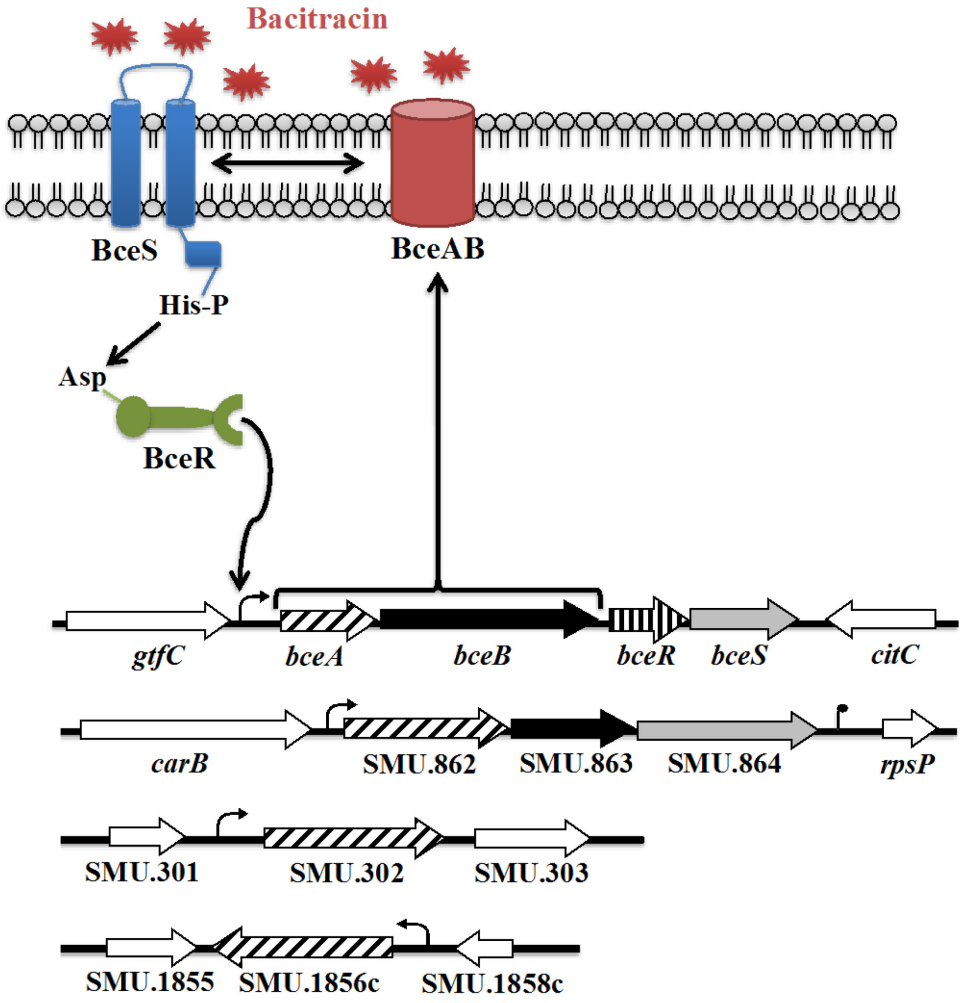

Figure 1. 2. Schematic diagram describing the BceABRS four component system in *S. mutans*. The double head arrow indicates that both BceS and BceAB are required for bacitracin sensing via this system. The target genetic loci that are regulated by the BceABRS system are listed and the promoter of each genetic locus is indicated. Modified from Jordan (2008).

It is known for years that the BceRS in *B. subtilis* is involved in bacitracin stress response (Mascher *et al.,* 2003b; Ohki *et al.,* 2003a). This TCSTS also regulates the expression of an ABC transporter termed BceAB in response to bacitracin. This system appears to be the most sensitive bacitracin resistance determinant in *B. subtilis*. The BceRS is also known to affect the activity of another ABC transporter termed YvcRS in response to bacitracin (Jordan *et al.,* 2008; Rietkotter *et al.,* 2008). A similar observation was also seen in *Staphylococcus aureus*, in which the BceRS was found to play an important role in responding to the cell envelope stress. In *S. aureus*, this TCSTS is found to regulate the expression of two ABC transporters termed BceAB and VraDE in response to bacitracin. Deletion of *bceAB* or *vraDE* genes resulted in mutants that were 2-4 fold more sensitive to bacitracin compared to the parent strain, respectively (Matsuo *et al.,* 2011; Yoshida *et al.,* 2011). These results suggest that the other ABC transporters, such as those encoded by SMU.862-864, regulated by the BceABRS system in *S. mutans* may also paly a role in antibiotics resistance similar to that found in *S. aureus*.

1.4.1.2 The LiaFSR System

In addition to BceABRS system in *S. mutans*, another TCSTS termed LiaFSR has also been shown to play an important role in responding to antibiotic-induced cell envelope stress (Suntharalingam *et al.,* 2009). This system was first characterized in *S. mutans* by Li and colleagues and was initially named Hk11-Rr11 (Li *et al.,* 2002a). Later, Suntharalingam and colleagues showed that a deletion of either of genes encoding LiaSR system resulted in mutants that are more susceptible to lipid II interfering antibiotics and cell membrane disrupting agents. They also showed that the LiaSR system induces the transcription of several genes involved in membrane protein synthesis and peptidoglycan synthesis, such as the *rgpG* gene, under bacitracin stress condition. In contrast, in the absence of bacitracin LiaF appears to repress the LiaSR system (Suntharalingam *et al.,* 2009).

Similarly, the LiaFSR system in *B. subtilis* was found to play a role in cell envelope stress response. Unlike BceABRS system that directly senses and response to bacitracin, the LiaRS TCSTS appears to sense damage in the cell envelope of *B. subtilis* (Rietkotter *et al.,* 2008). The LiaRS system responds to a large variety of cell wall-acting antibiotics, especially those interfering with lipid II cycling, detergents and organic solvents (Mascher *et al.,* 2004). The promoter of the *lia* operon in *B. subtilis* can be induced when the cells enter the stationary phase without any external stimulus, further suggesting that LiaRS in *B. subtilis* does not directly sense antibiotics (Jordan *et al.,* 2007). However, it is unknown whether the LiaRS system in *S. mutans* directly senses antibiotics or the cell envelope damage. The LiaF was found to act as a negative regulator to control expression of *liaRS* genes in *B. subtilis*, as a deletion of *liaF* gene resulted in constitutive expression of *liaRS* (Jordan *et al.,* 2006). In addition to the *B. subtilis*, the liaFSR system in *S. pneumoniae* also plays a similar role in responding to the cell envelope stress (Eldholm *et al.,* 2010).

1.4.1.3 The CiaXRH System

Previous studies showed that CiaXRH system played a regulatory role in mutacin production, genetic competence, biofilm formation and acid tolerance in *S. mutans* (Liu and Burne, 2009; Qi *et al.,* 2004). Recently, Mazda and colleagues have shown that CiaXRH system also plays a role in the resistance of *S. mutans* to antimicrobial peptides in the biofilm. In this regard, they have found that CiaXRH system of *S. mutans* grown in biofilm regulates the expression of *dltC*, which is involved in teichoic acid biosynthesis and is important for the resistance of *S. mutans* to antimicrobial peptide such as human α- and β-defensin (Mazda *et al.,* 2012). Similarly, the CiaRH in *S. pneumoniae* was found to prevent lysis during stress induced by cell wall-acting antibiotics (Mascher *et al.,* 2006a). Inactivation of *ciaRH* in *S. pneumoniae* resulted in a mutans that are sensitive to cell wall-acting antibiotics including bacitracin (Mascher *et al.,* 2006a). The CiaRH TCSTS in *S. pneumoniae* is also found to regulate the expression of *dltA-D* operon similar to that found in *S. mutans* (Mascher *et al.,* 2003a).

1.4.2 Other Components Involved in Bacitracin Resistance in *S. mutans*

A recent study shows that a surface membrane-associated protein, termed biofilm regulatory protein A (BrpA), may play a role in the cell envelope stress response in *S. mutans* (Bitoun *et al.,* 2012). Deletion of *brpA* gene results in a down-regulation of a number of genes involved in peptidoglycan synthesis, such as the *ddlA* (D-alanine-D-alanine ligase) gene (Bitoun *et al.,* 2012). In addition, Δ*brpA* mutant is more susceptible to antimicrobial agents, especially lipid II interfering antibiotics such as bacitracin with a reduction in MIC of 2-fold compared to wild type (Bitoun *et al.,* 2012). The *brpA* gene is also up regulated in response to a sub-inhibitory concentration of different cell wall-acting antibiotics, such as bacitracin. The *ΔbrpA* mutant showed an attenuation of the virulence of *S. mutans* in a *Galleria mellonella* (wax worm) model (Bitoun *et al.,* 2012). The Δ*brpA* mutant was also reported to form longer chains and exhibited a defect in biofilm formation with reduced biomass. This mutant was also more susceptible to acid killing (pH 2.8 for 45 min) and killing by 0.2% hydrogen peroxide compared to the parent strain (Wen and Burne, 2002; Wen *et al.,* 2006).

Tsuda and colleagues have identified another genetic locus, *rgpA-F*, which encodes enzymes involved in the biosynthesis of cell wall-associated rhamnose-glucose polysaccharide (RGP) in *S. mutans* (Tsuda *et al.,* 2002; Yamashita *et al.,* 1998). Deletion of any gene of *rgpA-F* genes results in mutants that are five-fold more sensitive to bacitracin than the wild type of *S. mutans* (Tsuda *et al.,* 2002). In addition to this genetic locus, another gene termed *rgpG* is found to be involved in the synthesis of rhamnose-glucose polysaccharide in *S. mutans* (Yamashita *et al.,* 1999). However, the role of this gene product in bacitracin resistance in *S. mutans* is still unclear.

1.5 Association Between Antibiotic Resistance and Virulence

The relationship between antibiotic resistance and bacterial virulence factors was reported previously. For example, antibiotics can act as signaling molecules to induce the expression of numerous genes involved in stress response and virulence factors (Linares *et al.,* 2006). This respond is mediated by TCSTSs or some extracytoplasmic function σ factors (ECF σ), and is usually aimed to maintain the integrity of the microorganism under stress condition (Jordan *et al.,* 2008). Some of the virulence-associated traits, such as biofilm formation, are also known to increase the ability of bacteria to resist antibiotics (Martinez and Baquero, 2002).

S. mutans has many virulence factors that enable it to initiate dental caries. These include the early attachment to and aggregation with other bacteria in the biofilm, acid production, acid tolerance, and genetic competence (Banas, 2004; Loesche, 1986). The formation of biofilms by *S. mutans* involves a multistep mechanism that starts with the deposition of an acquired enamel pellicle (AEP) on the tooth surface, which then gets coated with the salivary component such as salivary agglutinin glycoprotein (SAG) and bacterial component such as glucosyltransferase (Gtf-IS) (Islam *et al.,* 2007; Mitchell, 2003). After that the earlier attachment of *S. mutans* to the SAG via the sucrose-independent attachment occurs followed by the proliferation and aggregation of *S. mutans* and other dental pathogens in this site (Islam *et al.,* 2007).

In *S. mutans*, the two-component signal transduction systems (TCSTSs) are known to be involved in the regulation of biofilm formation. For example, ComDE (Li *et al.,* 2002b), LiaRS (HK/RR11) (Li *et al.,* 2002a), VicRK (Senadheera *et al.,* 2005), and CiaRH (Qi *et al.,* 2004), an orphan response regulator CovR (Biswas and Biswas, 2006; Idone *et al.,* 2003), and a quorum sensing system LuxS (Merritt *et al.,* 2003) are involved in regulation of biofilm in response to different stimuli (Senadheera and Cvitkovitch, 2008).

The formation of biofilms by *S. mutans* has been liked to the virulence and cariogenicity of this pathogen. Previous studies showed that *S. mutans* cells that grow in biofilm showed significantly higher resistance to acid killing (pH 3.5) (Li *et al.,* 2001a; Welin-Neilands and Svensater, 2007). In biofilm, *S. mutans* could be transformed at a frequency of 10 to 600 fold higher than the planktonic counterparts (Li *et al.,* 2001b). Most of all, the biofilm life style of *S. mutans* has been shown to increase the ability of this pathogen to resist antimicrobial agents (e.g. chlorhexidine) (Marsh, 2004; Wilson, 1996).

In *S. mutans*, LiaFSR system, which is important in responding to the cell envelope stress, is known to play an important role in biofilm formation. Li and colleagues showed that deletion of LiaSR (Hk11-Rr11) resulted in defects in biofilm formation with reduced biomass and sponge-like architecture (Li *et al.,* 2002a). In addition, simultaneous inactivation of ComCDE and LiaSR resulted in an attenuated *S. mutans* virulence and cariogenicity in a rat model of infection (Li *et al.,* 2008). LiaSR in *S. mutans* also appears to induce the expression of *rgpG* gene, which encodes an N-acetylglucosamine-1-phosphate transferase, in the presence of bacitracin (Suntharalingam *et al.,* 2009; Yamashita *et al.,* 1999). This enzyme, together with the proteins encoded by *rgpA-F*, plays important roles in the biosynthesis of *S. mutans* cell wall rhamnose-glucose polysaccharide (Tsuda *et al.,* 2002; Yamashita *et al.,* 1999). This polysaccharide is found to play a role in the pathogenesis of *S. mutans* by inducing the secretion of several inflammatory mediators such as tumor necrosis factor alpha (TNF-α) from human monocytes, which is a key mediator of the early host immune response in terms of induction of inflammation (Soell *et al.,* 1995). Antibiotics also found to act as signaling molecules to induce biofilm formation in many Gram-positive organisms. For instance, sub-inhibitory concentration of ampicillin and cefalotin were found to induce biofilm formation of *Streptococcus intermedius* and *S. aureus*, respectively (Kaplan, 2011; Subrt *et al.,* 2011). However, it is unknown if phenomenon also occur in *S. mutans*.

S. mutans is considered to be one of the aciduric bacteria in the oral cavity. Its ability to produce and tolerate acidic environment is considered to be important virulence factors for this pathogen to cause dental caries. *S. mutans* maintains its intracellular pH homeostasis mainly through the prevention of proton influx and the increase of proton efflux (Matsui and Cvitkovitch, 2010). *S. mutans* can prevent proton influx through the alteration of the fatty acid composition of its membrane by increasing in the level of monounsaturated and longer chain fatty acids (Quivey *et al.,* 2000). Another mechanism for *S. mutans* to maintain its intracellular pH homeostasis is through efflux pumps, either through end-product efflux (lactic acid) or proton efflux (by F_1F_0-ATPase proton pumps) (Kuhnert *et al.,* 2004; Matsui and Cvitkovitch, 2010).

Biofilm lifestyle of *S. mutans* also contributes to the ability of this pathogen to adapt to the acidic environment. Recent study by Welin-Neilands and Svensater showed that biofilm cells have an increased ability to tolerate acidic environment compared to planktonic cells. In addition, they also showed that the adhesion of *S. mutans* to a surface is an important step in the development of this tolerance, and that different strains of *S. mutans* have different degrees of acid tolerance (Welin-Neilands and Svensater, 2007). Furthermore, *S. mutans* biofilm cells appear to be more resistant to acid killing at pH 3.5 for 2 hours compared to planktonic cells. The pre-exposure of biofilm cells to pH 5.5 for 6 hours greatly enhance the survival of the cells to a subsequent exposure to killing pH 3.5 compared to planktonic cells (McNeill and Hamilton, 2003). Additionally, Li and colleagues have shown that cell density and biofilm growth mode modulate the ability of *S. mutans* to adapt to low pH environment; in their study pre-adapted biofilm cells from a low cell density or light biofilm exhibited decreased resistance to the killing pH 3 than cells extracted from a dense biofilm (Li *et al.,* 2001a).

Genetic competence is considered to be an important virulence factor for *S. mutans*, which allows this pathogen to acquire new genes that may encode for antibiotic resistance or virulence factors (Li *et al.,* 2008; Perry and Kuramitsu, 1981). Li and colleagues identified the peptide system (ComCDE) that controls the genetic competence in *S. mutans*. In this system, they showed that *comC* is encoding a competence-stimulating peptide (CSP), and *comDE* are encoding a histidine kinase and a response regulator respectively that sense and respond to the presents of the CSP in the surrounding environment (Li *et al.,* 2001b). Additionally, Petersen and Scheie identified an ABC transporter termed ComAB, which is thought to be responsible for the secretion of the CSP in *S. mutans* similar to that found in *S. gordonii*. Defect in this transporter resulted in a mutant deficient in transformability (Petersen and Scheie, 2000).

In *S. mutans*, ComCDE system also known to regulates production of bacteriocins and bacteriocin self-immunity proteins (Matsumoto-Nakano and Kuramitsu, 2006; van der Ploeg, 2005). It has been shown that ComCDE system is required for expression of the *nlmAB* genes, which encode two-peptide non-lantibiotic bacteriocin, and *bip* gene, which encodes a bacteriocin immunity protein (van der Ploeg, 2005). Deletion of any gene of *cmoCDE* results in mutants that are unable to produce bacteriocins (van der Ploeg, 2005). In addition, inactivation of *bip* gene affects the sensitivity of *S. mutans* to a variety of antimicrobial agents. This gene is also up regulated in response to sub-inhibitory concentrations of different antibiotics (Matsumoto-Nakano and Kuramitsu, 2006).

Antibiotics-induced stress in turn is also known to affect the genetic competence of Gram-positive bacteria (Charpentier *et al.,* 2012). For instance, a sub-inhibitory concentration of antibiotics such as aminoglycoside (e.g. kanamycin and streptomycin), fluoroquinolone (e.g. norfloxacin, levofloxacin, and moxifloxacin), and mitomycin C can induce transformation in *S. pneumoniae* (Prudhomme *et al.,* 2006). This induction in transformation required an intact peptide system ComCDE, which controls the genetic competence, and appropriate stress response (Charpentier *et al.,* 2012).

Chapter 2. The Rationale and Objectives of this Study

Dental plaque is a microbial biofilm community that consists of diverse species of microorganisms. Many organisms in dental plaque produce a number of antimicrobial molecules known as bacteriocins to fight against related species and to compete for nutrients and colonization sites. The production of various bacteriocins or other toxic compounds by microorganisms may result in "chemical warfare" in the dental biofilms, leading to various life-threatening challenges to the organisms. The inability of organisms, such as *S. mutans*, to cope with such challenges would result in rapid elimination from the community. The oral mucosa and saliva produce and secrete a number of antimicrobial compounds, including salivary antimicrobial peptide, histatin, β-defensin, and cathelicidin, which may also suppress or even kill many bacteria such as *S. mutans* in dental biofilms. To survive and initiate infections, *S. mutans* must be able to sense, respond and adapt to these stresses by inducing an appropriate resistance mechanisms.

S. mutans is known to resist bacitracin, a cyclic polypeptide antibiotic that inhibits bacterial cell wall biosynthesis by preventing the recycling of the lipid carrier(Jordan *et al.,* 2008). Previous studies have reported several mechanisms in which *S. mutans* copes with and resists bacitracin, such as BceABRS four-component system that allows this organism to sense, respond to and resist bacitracin or probably other antibiotics (Ouyang *et al.,* 2010). These mechanisms are not only important for *S. mutans* to cope with life-threatening insults including antibiotics, but also important for the virulence and pathogenic potential of this organism in dental caries.

By screening a high-density transposon mutant library, which was previously constructed in *S. mutans* UA159, we identified several transposon insertion mutants that were sensitive to bacitracin. One of these mutants was confirmed to have a transposon insertion in SMU.244 locus. This gene encodes a protein that is highly homologous to BacA in *S. pneumoniae* or UppP in *E. coli*, which play an important role in the cell wall biosynthesis in these organisms. Also, the *bacA* knockout mutant in *S. pneumoniae* was reported to have the attenuated virulence in a mouse model of infection (Chalker *et al.,*

2000). In this study, we hypothesized that SMU.244 encoded a BacA protein that might play an important role in resistance to bacitracin or other cell wall-acting antibiotics. Our first objective of this study was to characterize the functional role of SMU.244 in response to bacitracin and several cell wall-acting antibiotics. We especially focused our study on the molecular mechanism of this gene in response to bacitracin.

In the second objective of this study, we extended our investigation into another genetic locus SMU.862-864 that encodes an ABC transporter (exporter), since our previous study showed that the promoter region of this genetic locus had a well conserved BceR box (a perfect invert repeat) that was directly regulated by the BceABRS four-component system required for bacitracin sensing, responding and resistance in *S. mutans* (Ouyang *et al.,* 2010). However, the true functional role of this ABC transporter was unknown. We therefore set forth the experiments to test the hypothesis that SMU.862-864 encoded an ABC transporter that might play a role in resistance to bacitracin or other cell wall-acting antibiotics.

In the third objective of this study, we investigated the potential involvements of these two genetic loci, SMU.244 and SMU.862-864, in several virulence-associated phenotypes, including bacterial growth, biofilm formation and response to acid or other environmental stresses. We expected that the work from this study would generate new insights into the molecular mechanisms of *S. mutans* in response to bacitracin and other cell wall-acting antibiotics, thereby advancing our understanding of bacterial antibiotic resistance and its roles in promoting the virulence.

Chapter 3. Materials and Methods

3.1 Bacterial Strains and Growth Conditions

3.1.1 The Growth of *Streptococcus mutans*

All bacterial strains, plasmids and their characteristics are listed in Table 3.1. *S. mutans* wild-type (wt) strain UA159 was routinely grown in Todd-Hewitt medium (BD Bacto) plus 0.3% yeast extract (BioShop) (THYE) at 37°C, whereas all the mutant strains that derived from *S. mutans* UA159 were subcultured on THYE containing with 10 μg/ml of erythromycin (Sigma). The complementation strain was grown on THYE containing with both erythromycin (10 μg/ml) and spectinomycin (800 μg/ml). Bacterial growth was monitored by measuring optical density at 590 nm (SPECTRONIC 20+, Thermo Spectronic). All the strains were stored at -80°C as stocks in 25% (w/v) glycerol until use.

3.1.2 The Growth of *Escherichia coli*

E. coli strains were routinely grown on Luria-Bertani (LB) agar plates or in LB broth (BioShop) at 37°C with shaking (240 rpm) (Innova-40, New Brunswick Scientific). For cloning selection, LB medium was supplemented with either spectinomycin (50 μg/ml) or kanamycin (30 μg/ml). For blue/white (*lacZ*) screening of clones, LB medium was supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) (50 μM) (BioShop) and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (80 μg/ml) (BioShop).

Table 3. 1. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source or reference
S. mutans		
UA159	Wild type	(Ajdic *et al.,* 2002)
SmΔbacA	UA159ΔSMU.244::erm	This study
SmΔ862	UA159ΔSMU.862::erm	This study
SmΔ863	UA159ΔSMU.863::erm	This study
SmΔ864	UA159ΔSMU.864::erm	This study
SmΔ862-4	UA159ΔSMU.862-4::erm	This study
Sm-pCpbacA	UA159ΔSMU.244::erm harboring pCpbacA	This study
SmΔbceR	UA159Δ*bceR*::erm	(Ouyang *et al.,* 2010)
E. coli		
DH5α	An *E. coli* strain	New England BioLabs
Plasmids		
pDrive	PCR-cloning vector, *lacZ*, Kan^r, Amp^r	Qiagen
pDrive-CpbacA	pDrive::CpbacA-1089, Kan^r, Amp^r	This study
pCpbacA	pDL278::CpbacA-1089, $Spec^r$	This study
pDL278	*Streptococcus-E. coli* shuttle vector, $Spec^r$	(LeBlanc *et al.,* 1992)

3.2 Genetic Manipulation of *S. mutans* and *E. coli*

3.2.1 Isolation of Genomic DNA from *S. mutans*

The genomic DNA was isolated from *S. mutans* UA159 using the method as described previously (Li *et al.,* 2001b). A single colony of *S. mutans* UA159 was inoculated into 5 ml THYE broth and the culture was incubated at 37°C for overnight. The culture was then centrifuged at 4°C for 15 minutes at 14 000 ×g (Rotanta 460 RS). The pellet was re-suspended with 545 µl TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0) containing 1% (w/v) lysozyme (Sigma), 200 U mutanolysin (Sigma) and 2-5 µl RNase (Qiagen), and incubated for 60 minutes at 37°C. The cells were lysed with addition of 50 µl of 20% (w/v) sodium dodecyl sulfate (SDS) (BioShop) and incubated at 65°C in a heat block (Fisher Scientific Isotemp Digital Heatblock) for 15 minutes. The lysed cells was added with 2 µl of 2% (w/v) proteinase K (33 U/mg; BioShop) and incubated at 37°C for 60 minutes. Next, a 100 µl of 5 M NaCl was added, followed by 80 µl of 10% (w/v) hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide, CTAB) (Sigma) in 0.7 M NaCl and incubated at 65°C in a heat block for 20 minutes. The lysate was extracted by addition of 750 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), followed by centrifugation at 11 000 ×g (SORVALL Pico) for 2 minutes. The aqueous layer was transferred to a new tube and further extracted with 750 µl of chloroform:isoamyl alcohol (24:1, v/v) followed by centrifugation at 11000 ×g for 2 minutes. Again, the aqueous layer was then transferred to a new tube and the DNA was precipitated with 750 µl isopropanol. Finally, the DNA was recovered by washing with 1 ml 70% ethanol followed by 1 ml 95% ethanol and air-dried and dissolved in 20-50 µl TE buffer or ddH_2O. The concentration of the DNA was determined spectrophotometrically at 260 nm (Ultrospec 3100 pro). An absorbance of 1 unit at 260 nm corresponds to 50 µg/ml and that the pure DNA has an OD_{260}/OD_{280} ratio of 1.8. The quality of DNA was evaluated using 1% agarose gel electrophoresis (Sigma).

3.2.2 Isolation of Plasmid DNA from *E. coli*

For plasmid isolation, a single colony of *E. coli* host that harbors a cloned plasmid was first inoculated into 10 ml LB broth supplemented with an appropriate antibiotic, such as spectinomycin (50 µg/ml) or kanamycin (30 µg/ml). The culture was incubated for overnight at 37°C with shaking (240 rpm). The culture was then centrifuged for 10 minutes at 14 000 ×g. The pellet was used for extraction of plasmid DNA using GeneJET plasmid miniprep kit (Fermentas). Following the extraction of plasmid DNA, the concentration was determined spectrophotometrically at 260 nm and the quality of the plasmid DNA was analyzed using 1% agarose gel electrophoresis.

3.2.3 Transformation in *E. coli*

For cloning, constructed DNA was transformed into *E. coli* DH5α competent cells (New England BioLabs) using a transformation assay as recommended by the manufacture. The competent cells (50 µl) were first thawing on ice for 10 minutes and added with an aliquot of cloning DNA or ligation products. The cell suspension was gently mixed and incubated on ice for 30 minutes. The cells were then exposed to heat-shock by incubating the tube at 42 °C for 30 seconds. The tube was then placed on ice for 5 minutes. Then 950 µl of prewarmed SOC broth (super optimal broth or SOB with 20 mM glucose) (New England BioLabs) was added to the cell suspension and the culture was incubated for 60 minutes at 37°C with shaking at 240 rpm. The culture was centrifuged at 10 000 ×g for 5 min, re-suspended in 200 µl of LB medium, and plated on LB agar plates containing an appropriate antibiotic. The agar plates were then incubated for 48h at 37°C aerobically before selection of positive transformants.

3.2.4 Polymerase Chain Reaction (PCR)

All the primers used in this study were designed using MacVector (software 9.0) based on the reference genome sequence of *S. mutans* UA159 (Ajdic *et al.,* 2002). In general, primers were 18-24 nucleotides in length and each pair of primers have compatible melting temperatures (<5°C). All primers designed were tested against the entire *S. mutans* genome and they should be sequence-specific and free of secondary structures or primer-dimers. The primers were commercially synthesized, purified and stored as powders (100 nmole/oligonucleotide) by Alpha DNA (Montreal). The primers were further diluted into 50 nmole working concentration in ddH_2O (double distilled water) before used for PCR reactions.

A typical PCR reaction included 50 µl reaction mixture containing 1 µl of template DNA (50 ng/µl), 0.5 µl of the 50 µM forward primer and 0.5 µl of the 50 µM backward primer, 5 µl 10x PCR buffer (Invitrogen), 4 µl $MgCl_2$ (25 mM) (Invitrogen), 1 µl of the 10 mM dNTP (10 mM dATP, 10 mM dGTP, 10 mM dCTP, 10 mM dTTP) (Invitrogen), 0.25 µl of the 5 U/µl *Taq* DNA polymerase (Invitrogen) and 37.75 µl autoclaved ddH_2O. The PCR reactions were carried out in a T-Gradient Thermocycler (Biometra) and the reaction cycles were set up as the follow: a hot start at 94°C for 5 minutes, followed by 35 cycles of DNA denaturation at 94°C for 30 seconds, annealing at about 50°C for 30 seconds and extension at 72°C for 1 minute/per kb. Following 35 cycles, the PCR reactions were allowed to have a final extension at 72°C for 10 minutes.

The resulting PCR products were analyzed using agarose gel electrophoresis. The 1% (w/v) agarose gel was prepared in TAE (0.04 M Tris-acetate and 0.001 M EDTA) buffer and a final concentration of 0.5 µg/ml of ethidium bromid was added. Then, 5 µl of the PCR products was mixed with 2 µl of 5x loading dye (GelPilot DNA Loading Dye; Qiagen) and then the mixture was loaded into an agarose gel. A 1 kb DNA ladder plus (GeneRuler; Fermentas) was used as a standard to estimate the molecular mass of target DNA bands. The agarose gel was run in TAE buffer with a power supply at 4 voltage/cm for ~1 hour. The gel was then visualized using UV Transilluminator or photographed using FluorChem SP imaging system (Alpha Innotech). The PCR products were purified

either using QIAquick gel extraction kit (Qiagen) or using MinElute PCR purification kit (Qiagen).

3.2.4.1 Colony PCR Method

This method was used for confirmation of gene deletion mutants by a PCR strategy (Lau *et al.,* 2002). In this method, 2 ml of THYE with an appropriate antibiotic was inoculated with the test strain and incubated overnight at 37°C. The culture was centrifuged at 16 000 ×g for 1 minute. The pellet was re-suspended with 50-60 µl TTE buffer (1% Triton X-100, 20 mM Tris HCl, 2 mM EDTA, pH 8.0) and incubated for 10 minutes at 95°C in a heat block. The cell lysate was then centrifuged at 10 000 ×g for 1 minute. The supernatant was transferred to a new tube and used as a template DNA for PCR reactions, which followed the standard method as described above.

3.2.5 Construction of Gene Deletion Mutants

The gene deletion mutants form SMU.244, SMU.862, SMU.863 and SMU.864 were constructed using a PCR-based allelic replacement strategy as described in Figure 3.1 (Lau *et al.,* 2002). For example, the upstream and downstream flanking regions of SMU.244 were amplified from the chromosomal DNA of *S. mutans* UA159 by PCR using specific pairs of primers bacA-P1/P2 with a 5' restriction site of AscI and bacA-P3/P4 with 5' restriction site of FseI (Table 3.2). Both P2 and P3 were chosen to be located in the regions within the first and last 120 bp of the target gene, whereas P1 and P4 were chosen to be located in the regions within 500–1000 bp of the up- and down-stream flanking regions of the target gene, respectively. In addition, an erythromycin resistance cassette (*ermAM,* 876 bps) was also amplified from a previously constructed mutant (SmΔbceR) containing *ermAM* cassette (Ouyang *et al.,* 2010) using primer pair Erm-P1/P2. In this pair, Erm-P1 contains an AscI restriction site added to its 5' end, while Erm-P2 contains an FseI restriction site added to its 5' end.

Table 3. 2. List of primers used in this study

Primer	Nucleotide sequence (5' → 3')	Predicted size of amplicon
	Knockout	
bacA-P1	ATTCATCGTGAGAGAACGG	748
bacA-P2	ATGGCGCGCCTCCACTAAAATCAAATGCCC	
bacA-P3	TAGGCCGGCCTGGCACGATTCTAACTGGTAG	837
bacA-P4	TGTGATAAGCGTCAGACTCC	
Sm.862-P1	CAGTCTTTTCCTTCACCAAG	828
Sm.862-P2	ATGGCGCGCCGGCATAAATCTTCTCCCTC	
Sm.862-P3	TAGGCCGGCCATCTGGTCTTGATAAAGGACAAAC	915
Sm.862-P4	GCTACCCCTATGATAATCCC	
Sm.863-P1	CAGTCTCAAGCGGCTGATTC	607
Sm.863-P2	ATGGCGCGCCTGTTGTGATAAATGATAGGTTCCTG	
Sm.863-P3	TAGGCCGGCCTAGTGAGATTACAGAAGACAGCCAG	686
Sm.863-P4	CGAAGTCGTTCCATAAGTTGC	
Sm.864-P1	GCTAATGAAAAGAGGTGAGTGC	821
Sm.864-P2	ATGGCGCGCCATTATGATAATCCCCAGCATCG	
Sm.864-P3	TAGGCCGGCCCTCTGCTTTTGTTGGGATAGTC	938
Sm.864-P4	GGTGCGACCTTTTTTACCG	
Erm-P1	TAGGCGCGCCCCGGGCCCAAAATTTGTTTGAT	876
Erm-P2	GCTGGCCGGCCAGTCGGCAGCGACTCATAGAAT	
	Complement	
bacA-cp-F	ACGAGTGCTTCAAACTTTTCG	1093
bacA-cp-B	ATTTCCATAGTCTTTACCTCATACGA	

Primer	Nucleotide sequence (5' → 3')	Predicted size of amplicon
RT-PCR		
862-863rt-F	GGTATCTGTAGAGGTAGTCAATGGC	712
862-863rt-B	GCAAATGCTTCATCCGTTC	
863-864rt-F	GCCTTCAGAGTTATCAGGTG	535
863-864rt-B	ACTGCTGTCAGAGGTATCAAC	
qRT-PCR		
gyrA-qRT-F	TTGCGACTATCTGCTATGTG	111
gyrA-qRT-B	CCAAGAATCTGCTGTCCG	
244-qRT-F	CATTGGTCTGTTTCAGGTGCTGTC	104
244-qRT-B	CTGTTGCGACTTGCCGACTG	
bceS-qRT-F	TCTTTATCATCTGCCTCTCGTC	104
bceS-qRT-B	ATTTTTGGTGGAACCGCC	
16S-qRT-F	CGTGGGTAGCGAACAGGATTAG	114
16S-qRT-B	GCGGAGTGCTTATTGCGTTAGC	

* The restriction sites were underlined as GGCGCGCC, *AscI*; GGCCGGCC, *FseI*.

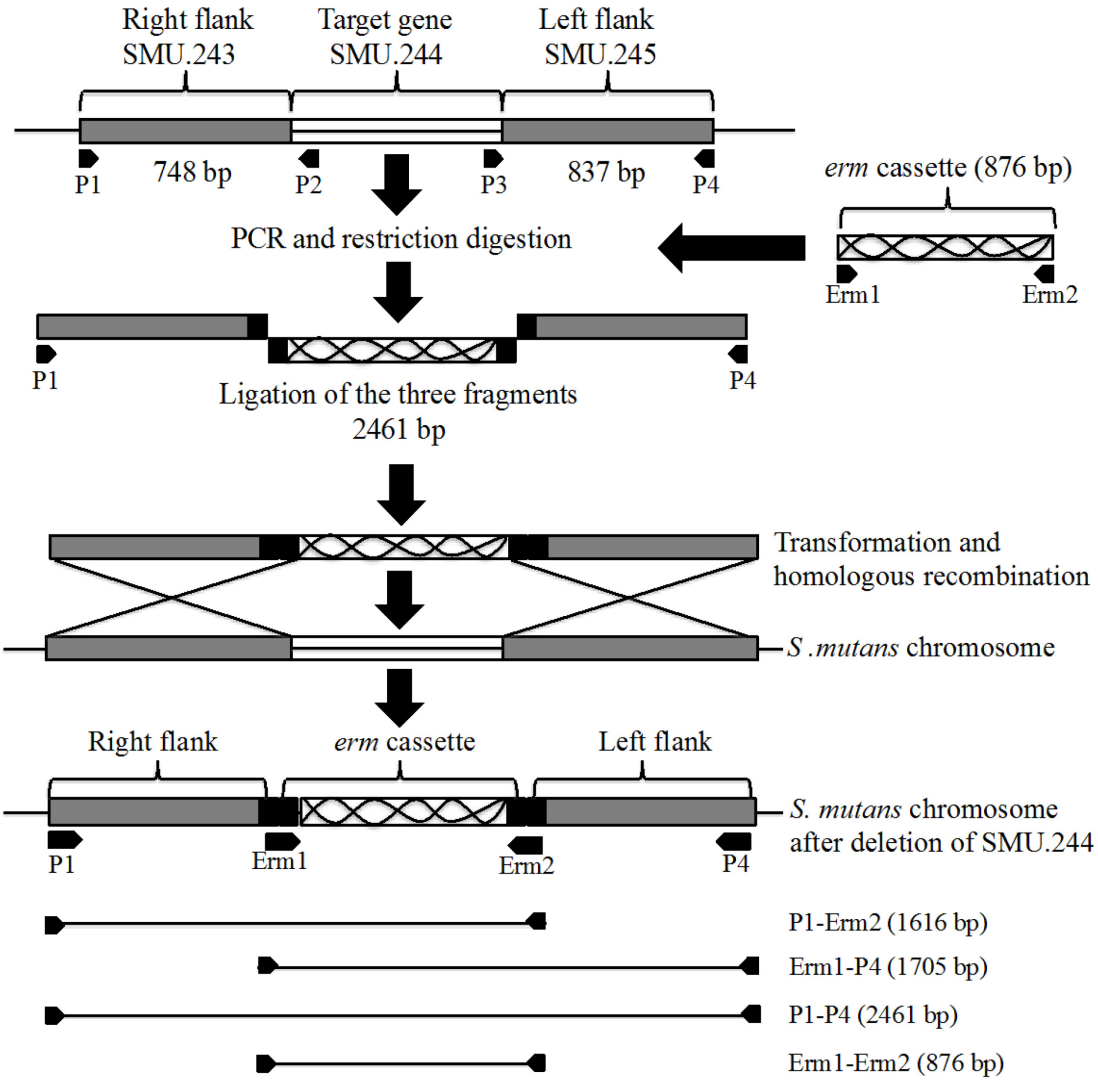

Figure 3. 1. Schematic description of the PCR-ligation mutagenesis strategy used to construct and confirm gene deletion mutans. In this strategy, P1-P2 and P3-P4 were used to amplify the up- and down-stream flanking regions of a target gene, such as SMU.244, respectively. Erm1-Erm2 was used to amplify the *ermAM* cassette (876 bp). The PCR products were then digested, purified and ligated to generate one fragment of ligation product P1-P2::Erm::P3-P4. This fragment was then transformed into *S. mutans* UA159. The mutant was then confirmed genetically by PCR confirmation using a combination of primers (P1-Erm2, Erm1-P4, P1-P4, and Erm1-Erm2) and template DNA from both mutant and the parental strain. Note that the PCR products of P1-Erm2 (1616 bp) and Erm1-P4 (1705 bp) were obtained only if the ligation product was inserted into the target gene. In the parent strain, PCR generated negative results for all primer combinations except primer pair P1-P4.

The PCR products were cut from agarose gel for purification using QIAquick gel extraction kit (Qiagen). The DNA fragments were digested with restriction enzymes, AscI and FseI (New England BioLabs) for overnight at 37°C. The digestion was then stopped by incubating the samples at 65°C for 20 minutes. For ligation, the three DNA fragments were mixed at 1:1:1 ratio in a ligation buffer containing T4 DNA ligase (Invitrogen). The ligation reaction was incubated at 14°C for overnight and the ligation product was transformed into *S. mutans* strain UA159 grown in THYE broth. The culture was incubated for additional two hours before plated on THYE agar plates supplemented with 10 μg/ml of erythromycin. All the plates were incubated at 37°C for two days before examined. After two days, transformants were first screened using primer pairs P1-Erm2 and Erm1-P4. Positive clones were selected for further genetic confirmation using a PCR strategy. The same strategy was used to construct individual deletion mutants of SMU.862, SMU.863, SMU.864 and the three genes deletion mutant SMU.862-4. The primers used for construction of these mutants were listed in Table 3.2.

3.2.6 Genetic Confirmation of the Gene Deletion Mutants

A PCR strategy using primer combinations (P1-Erm2, Erm1-P4, P1-P4, and Erm1-Erm2) was used to confirm all the gene deletion mutants, as described in Figure 3.1. In this strategy, the same primers that were used to construct each gene deletion mutant were also used for confirmation of these mutants. Both transformants and the wild type UA159 genomic DNA were used as templates in the confirmation assay. We considered that the internal region of a target gene was successfully deleted by replacement of the erythromycin cassette if PCR using a specific primer pairs (P1-Erm2, Erm1-P4, and Erm1-Erm2) generated expected sizes of PCR amplicons in the mutant but not in the parent strain. However, the primer pair of P1-P4 combination might generate PCR amplicons in both mutants and parent strain UA159. To distinguish between these two PCR products, designing primers P1 and P4 should allow generation of different sizes of PCR products from the genomic DNA of mutants and the parent strain.

3.2.7 Construction of Sm-pCpbacA Complement Strain

A two-step strategy was used to construct Sm-pCpbacA complement strain. In the first step, SMU.244 (*bacA*) gene together with its promoter region was amplified from the chromosomal DNA of *S. mutans* UA159 by PCR using a specific primer pair bacA-cp-F and bacA-cp-B (Table 3.2). The PCR product was examined using 1% (w/v) agarose gel and purified using QIAquick gel extraction kit. The purified fragment was then ligated with the pDrive-cloning vector using PCR cloning kit (Qiagen). The ligated product was used to transform an *E. coli* DH5α super-competence cells (New England BioLabs). Transformants were selected based upon blue/white screening on LB supplemented with IPTG (50 μM) and X-gal (80 μg/ml) as well as kanamycin (30 μg/ml). The white colonies were selected for PCR screening of positive clones using primer pair bacA-cp-F and bacA-cp-B. The positive clones were selected to prepare plasmid DNA for further confirmation of the insert by restriction digestion analysis using SphI and SacI. The confirmed plasmid was then used for the second step of cloning (Figure 3.2). The newly constructed plasmid pDrive-CpbacA was isolated from a positive clone Ec-pDrive-CpbacA using GeneJET plasmid miniprep kit (Fermentas). The insert (1205 bp) that represents SMU.244 and its promoter region was cut off from the plasmid by restriction digestion using SphI and SacI (New England BioLabs). The insert was then ligated to the same restriction sites (SphI and SacI) of a shuttle victor pDL278. The ligation product was transformed into *E. coli* DH5α super-competence cells. Transformants were grown on LB agar plates supplemented with spectinomycin (50 μg/ml). PCR screening using primer pair bacA-cp-F and bacA-cp-B was performed to determine the positive clones. The plasmid DNA was isolated from positive clones and used for further confirmation by restriction digestion analysis by double digestion using SphI and SacI, and a single digestion of BtgI. The confirmed plasmid, designated as pCpbacA, was then transformed into SmΔbacA mutant. The positive transformants were selected from THYE agar plate containing both erythromycin (10 μg/ml) and spectinomycin (800 μg/ml). The confirmed transformant was designated as Sm-pCpbacA and was ready for use in complementation experiments (Figure 3.3).

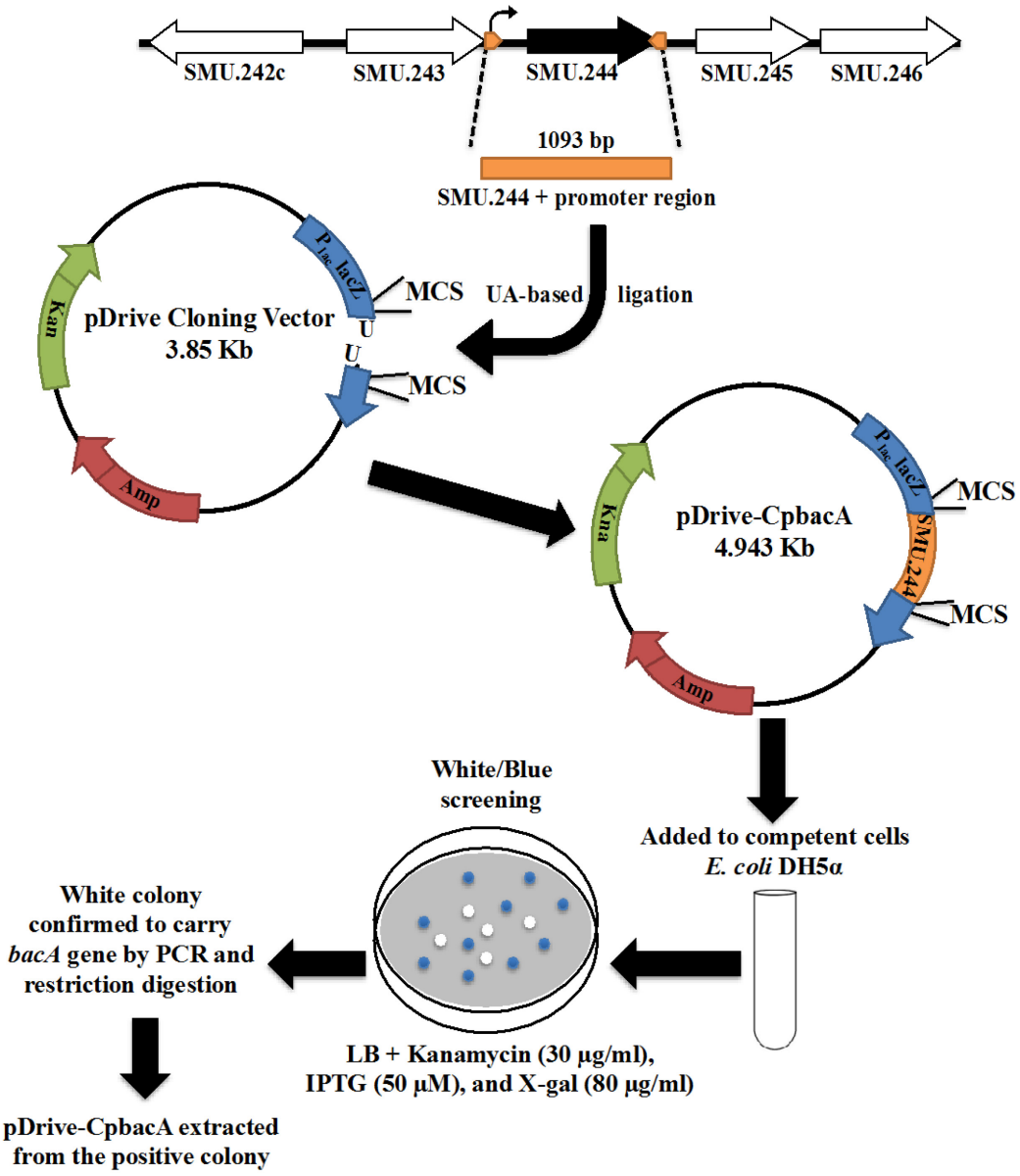

Figure 3. 2. Schematic representation of the first-step in construction of the complement strain Sm-pCpbacA. SMU.244 gene together with its promoter region was first amplified. The amplified products were then cloned into pDrive-cloning vector directly via a UA-based ligation. The ligation product was then transformed to an *E. coli* DH5α competence cells, which was grown on LB plates containing IPTG (50 µM) and X-gal (80 µg/ml) as well as Kanamycin (30 µg/ml). White colonies were selected as positive clones, since the insertion of the target DNA into pDrive-cloning vector interrupted the *lacZ* gene. The positive clones were then confirmed by PCR screening and restriction digestion.

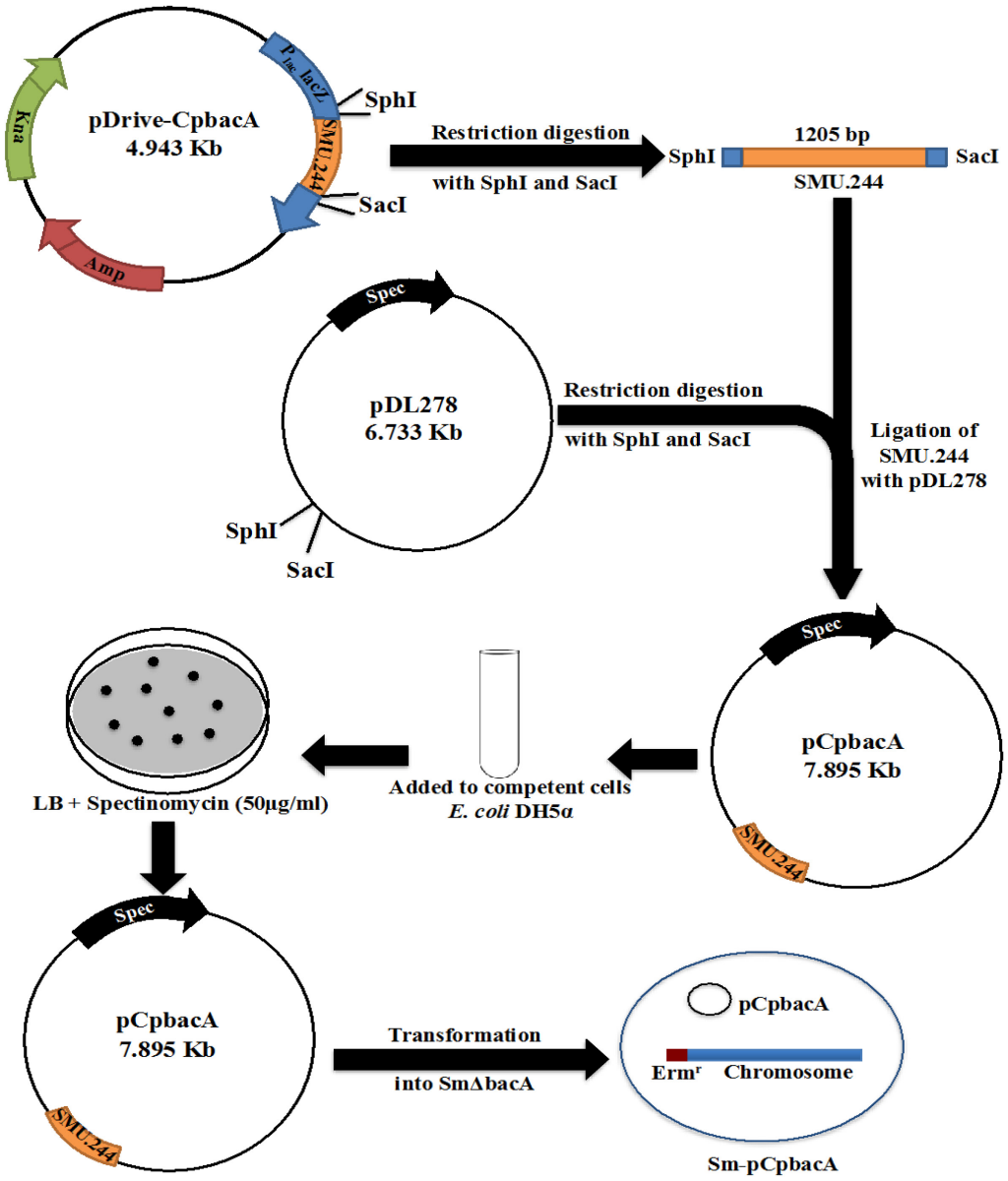

Figure 3. 3. Schematic representation of the second-step in construction of the complement strain Sm-pCpbacA. In this step, pDrive-CpbacA plasmid DNA was isolated from the positive clone and digested with SphI and SacI to release the insert. The insert was ligated to the same restriction sites of a shuttle victor pDL278. The ligation product was transformed into an *E. coli* DH5α and positive transformants selected from LB plate containing spectinomycin (50 µg/ml) were used for confirmation by restriction digestion analysis. The confirmed plasmid, named pCpbacA was then transformed to SmΔbacA mutant. The positive transformants were selected from THYE plates containing both erythromycin (10 µg/ml) and spectinomycin (800 µg/ml) and was designated as Sm-pCpbacA.

3.3 Antibiotics Susceptibility Assays

3.3.1 Broth Dilution Assay

A broth dilution assay was used to determine minimum inhibitory concentration (MIC) of all antibiotics against the newly constructed mutants. Briefly, *S. mutans* strains were cultured to early mid-log phase ($OD_{600} \approx 0.6$) and diluted to the same cell density $OD_{600} \approx 0.2$ ($\approx 10^5$ CFU/ml). Then 100 µl of the diluted strain was added to a 96-well microtiter plate (BD Falcon) containing THYE medium supplemented with two-fold serial dilution of either bacitracin, penicillin G, vancomycin, nisin (Sigma), SDS, kanamycin, chloramphenicol (BioShop), and tetracycline (BioShop), with a starting concentration of 192 U/ml, 0.25 µg/ml, 10 µg/ml, 70 µg/ml, 160 µg/ml, 1600 µg/ml, 16 µg/ml, 16 µg/ml, respectively. The last two wells were used as a positive control, containing a bacterial inoculum only, and a negative control, containing the antibiotic only. The microtiter plates were then incubated for 20 hours and the bacterial growth was spectrophotometrically measured at 590 nm using a microplate reader (The Synergy HT; BioTek). The MIC was defined as the lowest concentration of an antibiotic needed to inhibit the visible growth of microorganism (Suntharalingam *et al.,* 2009).

3.3.2 Plate Dilution Assay

Antibiotic susceptibility of the mutants was further tested using a rapid plate dilution assay with some modification (Wiegand *et al.,* 2008). Briefly, THYE agar plates were prepared that contained bacitracin (2 U/ml) (BioShop), penicillin G (0.03 µg/ml) (BioShop), or vancomycin (0.6 µg/ml) (BioShop) based on the reference concentrations of these antibiotics from *S. mutans* wild type strain UA159 (Ouyang *et al.,* 2010; Suntharalingam *et al.,* 2009). The overnight cultures of the tested strains were adjusted to the same cell density $OD_{600} \approx 1.0$ ($\approx 10^8$ CFU/ml) and a serial dilutions of 1:10 for 6 times were then done for each strain using 10 mM potassium phosphate buffer (pH 7.0). Then 20 µl of the last four dilutions (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) of each tested strain were plated into a THYE plate containing the tested antibiotics and a THYE plate without antibiotic. The plates were then incubated in a candle jar for 24 hours before assessment of antibiotic susceptibility of the mutants.

3.4 Bacterial Growth in the Presence or Absence of Subinhibitory Concentrations of Antibiotics

To assess the growth kinetics, bacterial strains were adjusted to the same cell density $OD_{600} \approx 0.7$ and then cultured 6% (v/v inoculum) in THYE medium at 37°C. The experiment was done using a 96-well plate with 160 µl per well. When required bacitracin (0.48 U/ml), penicillin G (0.00781 µg/ml), vancomycin (0.3125 µg/ml), nisin (4.375 µg/ml), kanamycin (50 µg/ml), chloramphenicol (1 µg/ml), or tetracycline (0.25 µg/ml) were added to the medium. Growth of cultures was monitored turbidimetrically using microplate reader at 600 nm hourly for 16 hours. The doubling time (T_d) was calculated using the following equation [$T_d = (t2 - t1) \times (\log 2 / \log 2N_0 - \log N_0)$], whereas $2N_0$ and N_0 represent the reading of the OD_{600} during the log phase and t2 and t1 represent two-time point at the OD_{600} $2N_0$ and N_0, respectively.

3.5 Assay for the Response of the Mutants to High Salts or Metals

To assess the effect of the presence of different concentrations of salts and metals on the growth of the mutants, a broth dilution assay was carried out. *S. mutans* strains were cultured to mid-log phase ($OD_{600} \approx 0.6$) and diluted to the same cell density $OD_{600} \approx 0.2$ ($\approx 10^5$ CFU/ml). Then 100 µl of the diluted strain was added to a 96-well microtiter plate containing THYE medium supplemented with two-fold serial dilution of either NaCl (BioShop), $ZnCl_2$ (BioShop), $MnCl_2$ (BioShop), $MgCl_2$ (BioShop), $CaCl_2$ (BioShop), or $FeCl_3$ (BioShop) with a starting concentration of 2 M, 64 mM, 2 M, 2 M, 2 M, and 2 M, respectively. The last two wells were used as a positive control (bacterial inoculum only) and a negative control (salts or metals dilution only). The growth of cultures was monitored turbidimetrically using a microplate reader at 600 nm hourly for 16 hours and the doubling time (T_d) was calculated as described previously. Additionally, after 20 hours of incubation the bacterial growth was spectrophotometrically measured at 590 nm to determine MIC.

3.6 Acid Sensitivity Assay

To assay the effect of gene deletion on the ability of *S. mutans* to grow at low pH, an acid sensitivity assay was carried out using the method as described previously (Li *et al.,* 2002a). Briefly, The overnight cultures of the tested strains were adjusted to the same cell density $OD_{600} \approx 1.0$ ($\approx 10^8$ CFU/ml) and a serial dilutions of 1:10 for 6 times was done for each tested strain using 10 mM potassium phosphate buffer (pH 7.0). Then 20 µl of the last four dilutions of each tested strain were plated into pH 7.5 or pH 5.5 THYE agar plates. The plates were then incubated in a candle jar for 24-36 hours and acid sensitivity was evaluated by comparing the growth of the mutants and the parent strain on the same plates.

3.7 Biofilm Formation Assay

Biofilm formation by the mutants was assessed using a method as described previously (Li *et al.,* 2002b). Both 96-well and 24-well microtiter plates (some contained coverslips) (BD Falcon) were used to develop biofilm and to facilitate quantification and microscopy. The overnight cultures of the tested strains were diluted 10% (v/v) in a fresh THYE medium and grow for 2~3 hours. The tested strains were then adjusted to the same cell density $OD_{600} \approx 1.0$ ($\approx 10^8$ CFU/ml) and the biofilm was initiated by inoculating 2.5% (v/v) of the tested strains in either 200 µl (for 96-well plates) or 2 ml (for 24-well plates) of 4 × diluted THYE broth with either 5 mM sucrose or 10 mM glucose. When required bacitracin was added to the medium at a final concentration of 0.48 U/ml. The microtiter plates were then incubated at 37°C for 16 hours without agitation. After 16 hours, liquid was removed from the 96-well plate and air-dried. The biofilm formation was then quantified spectrophotometrically at 600 nm using the microplate reader. Biofilms formed in 24-well plates were photographed after the 16 hours incubation and the cover slides were removed, stained with 0.1% crystal violet for 5 min, examined and photographed by a conventional microscope.

3.8 Competence Assay in *S. mutans*

To determine the effect of inactivation of SMU.244 on the development of genetic competence, mutant was assayed for genetic transformation using a method as described previously (Li *et al.,* 2001b; Li *et al.,* 2002a). A single colony of *S. mutans* was inoculated in 4 ml THYE broth and incubated overnight at 37°C. The strain was then sub-cultured 5% (v/v inoculum) in fresh 1 ml THYE medium and further incubated at 37°C for 1~2 hours $OD_{600} \approx 0.2$ ($\approx 10^5$ CFU/ml). Competence-stimulating peptide (CSP) was then added to the culture (1µg) and further incubated for 15 min. Transforming DNA, pDL278 *Streptococcus–E. coli* shuttle vector that encodes spectinomycin resistance gene, was then added (1 µg) and the culture was incubated for an additional 2 hours. After transformation, an aliquot of 100 µl of cell was spread on THYE plates

containing spectinomycin (800 μg/ml) as well as THYE plates without antibiotics to determine the total recipient cell number. The agar plates were then incubated for 48h at 37°C in a candle jar. Transformation frequency was then calculated using the following equation: transformation frequency = (number of transformants / total recipient cells) × 100.

3.9 Real-Time Quantitative RT-PCR

By using qRT-PCR, we planed to determine if SMU.244 gene was regulated by the BceABRS system and to examine the effect of sub-inhibitory concentrations of bacitracin on the expression of SMU.244 gene.

3.9.1 Isolation and Purification of Total RNA

Total RNA was extracted in cooled trizol (Invitrogen) by a FastPrep method (Gong *et al.,* 2009). The effect of sub-inhibitory concentrations of bacitracin on the expression of SMU.244 gene was examined in UA159. Briefly, the overnight culture of *S. mutans* UA159 was diluted 5% (v/v) in fresh THYE broth and further incubated at 37°C for ~2 hours (early mid-log phase $OD_{600} \approx 0.2$ or $\approx 10^5$ CFU/ml). After 2 hours, the culture was divided into 5 tubes and bacitracin was then added to four tubes at a final concentration of 2, 6, 12, and 24 U/ml respectively. All tubes were further incubated at 37°C and aliquots of 10 ml were taken after 10, 20, and 30 minutes from each tube to collect the cells by centrifugation at 14 000 ×g for 15 minutes at 4°C (Figure 3.4). In addition, total RNA was also extracted from both UA159 and SmΔbceR mutant to determine if SMU.244 gene was regulated by the BceABRS system. Again, the overnight culture of both strains were diluted 5% (v/v) in fresh THYE broth and further incubated at 37°C for ~2 hours $OD_{600} \approx 0.2$ ($\approx 10^5$ CFU/ml). Each strain was divided into 2 tubes and bacitracin was then added to one tube at a final concentration of 0.35 U/ml. All tubes

were further incubated at 37°C for 10 minutes and cell pellet was collected by centrifugation at 14 000 ×g for 15 minutes at 4°C.

The supernatant was discarded, and the cell pellet was resuspended in 1 ml cooled trizol (TRIzol, Invitrogen) and transferred to a new tube containing silica beads (0.1 mm silica beads; BioSpec). The tubes were then mixed vigorously and the cells were broken with a fast-prep machine (FastPrep FP120, Thermo Savant) at 6000 rpm for 28 seconds. After breaking the cells, 200 µl of chloroform were added and mixed by inversion, incubated at room temperature for 3 minutes, and centrifuged at 14 000 ×g for 15 minutes at 4°C (Sorvall Fresco). The upper aqueous phase was then transferred to a new 1.5 ml tube and RNA was precipitated by adding 500 µl isopropanol, which was then mixed, incubated at room temperature for 10 minutes, and centrifuged at 16 000 ×g for 20 minutes at 4°C. After that, RNA was washed with 75% ethanol and 100% ethanol, and then air-dried for 30 minutes. The precipitated RNA was resuspended in 50 µl RNase-free H_2O and stored at -80°C. The concentration of the total RNA was determined spectrophotometrically at 260 nm. An absorbance of 1 unit at 260 nm corresponds to 40 µg/ml and that the pure RNA has an OD_{260}/OD_{280} ratio of 1.9~2.1. The total RNA (5~8 µl) was also analyzed using agarose gel electrophoresis to verify its integrity.

To remove contaminating DNA, each sample was mixed with 2.5 µl DNase I, 10 µl of the RNase-free DNase buffer (RDD), and completed to 100 µl with RNase-free ddH_2O (Qiagen RNase-Free DNase set) and then incubated at room temperature for 10 minutes. After 10 minutes the RNA samples were cleaned up using RNeasy MinElute cleanup kit (Qiagen). Following RNA purification, the concentration of purified RNA was determined spectrophotometrically at 260 nm. The quality of the RNA was first assessed on agarose gel electrophoresis and confirmed by PCR reaction using the RNA as a template and primers specific to *S. mutans* genes such as *gyrA* gene. No detectable PCR products observed on an agarose gel was considered as good RNA samples, which were then used for the following procedures.

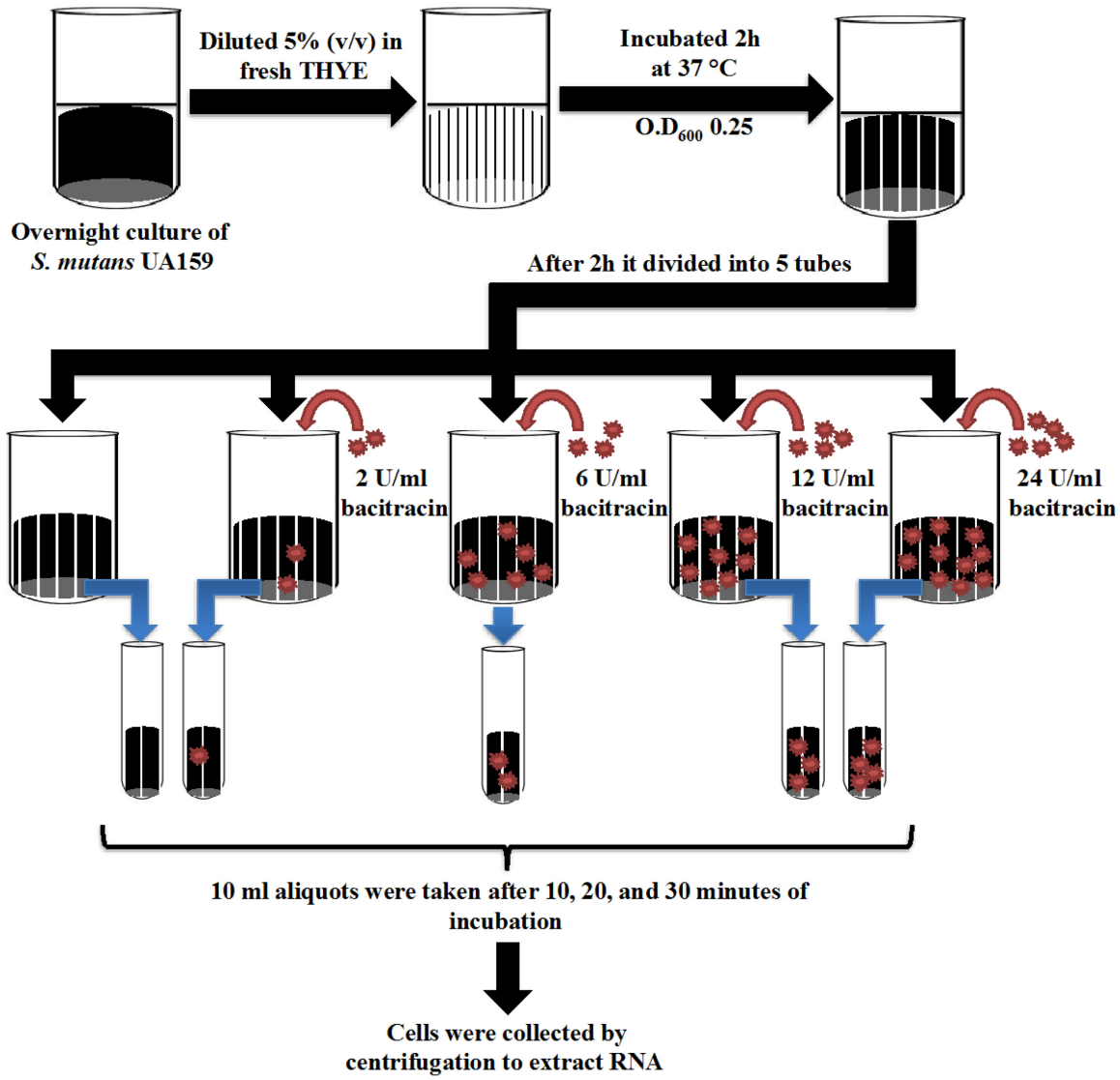

Figure 3. 4. A flowchart of induction of gene expression by bacitracin and RNA extraction from *S. mutans* UA159. The overnight culture was diluted 5% (v/v) in fresh THYE broth and further incubated at 37°C for ~2 hours ($OD_{600} \approx 0.2$). The culture was then divided into 5 tubes and bacitracin was then added to four tubes at a final concentration of 2, 6, 12, and 24 U/ml. All tubes were then incubated at 37°C and aliquots of 10 ml were taken form each tube after 10, 20, and 30 minutes to collect the cells for total RNA extraction.

3.9.2 Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from the purified RNA using QuantiTect Reverse Transcription kit (Qiagen). First, 1 µg of the purified RNA was mixed with 2 µl of gDNA wipeout buffer (Qiagen) and brought the volume of the reaction to 14 µl with RNase-free ddH_2O. The mixture was then incubated for 2 minutes at 42°C. Following incubation, the 14 µl was mixed with 4 µl Quantiscript RT buffer (Qiagen) containing dNTPs, 1 µl RT-primer mix (Qiagen) and 1 µl Quantiscript Reverse Transcriptase (Qiagen). The 20 µl reaction mixture was then incubated for 30 minutes at 42°C to synthesize cDNA. The mixture was then incubated at 95°C for 3 minutes to inactivate Quantiscript Reverse Transcriptase. The newly synthesized cDNA samples were then stored at -80°C.

3.9.3 qRT-PCR

Real time quantitative RT-PCR (qRT-PCR) was performed using QuantiTect SYBR Green PCR kit (Qiagen) in a Cepheid Smart Cycler. The *gyrA* gene of *S. mutans* was selected as an internal standard to normalize the level of expression of the tested genes, since it shows little variation of the expression (Ouyang *et al.,* 2010). The primers used for qRT-PCR for both tested-genes and *gyrA* were listed in Table 3.2. The reaction mixture for qRT-PCR contained 12.5 µl QuantiTect SYBR Green PCR master mix (Qiagen), 1.5 µl of each primer (5 µM), 2 µl cDNA and 7.5 µl ddH_2O. The 25 µl reaction mixture was then incubated in the Cepheid Smart Cycler at 95°C for 15 minutes to activate the HotStartTaq DNA polymerase, followed by 50 cycles of 95°C for 15 seconds, 50~60°C (annealing temperature) for 30 seconds, and 72°C for 30 seconds, followed by a melting curve analysis start at 40°C and end at 95°C. Each sample was performed in duplicates in two independent experiments and a non-template control was also included each time to confirm the absence of contamination. The fold changes in expression levels of the gene of interest were calculated using the $\Delta\Delta C_t$ method as described previously (Ouyang *et al.,* 2010). First ΔC_t was calculated for each cDNA sample to normalize the level of expression of the tested gene using the following

equation: $\Delta C_t = C_t$ (target gene) – C_t (*gyrA* gene); where C_t represents the threshold cycle. Then the $\Delta\Delta C_t$ for the tested gene was calculated by the following equation: $\Delta\Delta C_t = \Delta C_t$ (tested cDNA "e.g. with 2 U/ml of bacitracin") – ΔC_t (reference cDNA "no antibiotic"). Finally, the ratio was calculated as $2^{-\Delta\Delta Ct}$.

3.10 RT-PCR

To determine if SMU.862, SMU.863, and SMU.864 form an operon, reverse transcription PCR (RT-PCR) were used to analyze the transcription products of these three genes by using cross-gene primers 862-863rt-F/B and 863-864rt-F/B (Table 3.2). Total RNA was isolated and purified from *S. mutans* UA159 in the presence of 2 U/ml of bacitracin as described previously. Part of the purified RNA was converted to cDNA by reverse transcription (RT) as described previously. PCR was then performed using the cDNA as a template and the two pairs of cross-gene primers 862-863rt-F/B and 863-864rt-F/B (Table 3.2). The same primers were also used for PCR amplification from *S. mutans* UA159 genomic DNA as a positive control and from purified RNA as a negative control. PCR products were then examined using 1% (w/v) agarose gel.

3.11 Statistical Analysis

All the assays were performed in either duplicate or triplicate. The results were analyzed using a two-tailed student's t-test and a *P* value of ≤ 0.05 was considered statistically significant.

Chapter 4. Results

4.1 Genetic Analysis and Protein Homology

4.1.1 SMU.244

By screening a high-density transposon mutant library constructed previously in our laboratory, we have identified a mutant with transposon insertion in SMU.244 gene that was sensitive to bacitracin (Tian *et al.,* 2007). By *in silico* analysis of SMU.244 gene (http://www.oralgen.lanl.gov/_index.html), we found that SMU.244 encode a putative homolog of bacitracin resistant protein (BacA) from *S. pneumoniae* (Chalker *et al.,* 2000) or undecaprenyl pyrophosphate phosphatase (UppP) from *E. coli* (El Ghachi *et al.,* 2004). SMU.244 is located from 232909 to 233754 in the *S. mutans* genome and is 846 bp in length (Ajdic *et al.,* 2002). The gene upstream of SMU.244 is SMU.243 encoding a conserved hypothetical protein with unknown function, while two genes immediately downstream are *mecA* that encodes a negative regulator of genetic competence, and *rgpG* that encodes an enzyme involved in the biosynthesis of rhamnose-glucose polysaccharide (Figure 4.1).

The sequence alignment of this protein shows that the protein encoded by SMU.244 is highly homologous to BacA (80% similarity and 65% identity) of *S. pneumonia* TIGR4 and to UppP (52% similarity and 35% identity) of *E. coli* K-12 (Figure 4.2), Since it shares high homolog with these proteins, the protein encoded by SMU.244 in *S. mutans* is designated as $BacA_{Sm}$ in our study. The sequence alignment of BacA-like proteins from four genome sequence-completed *S. mutans* strains, UA159, GS-5, NN2025 and LJ23, also shows that these proteins are highly conserved among different *S. mutans* strains (Table 4.1). By using the Promoter Prediction tool at Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001), we identified a promoter-like sequence upstream of SMU.244 as described in Figure 4.1.

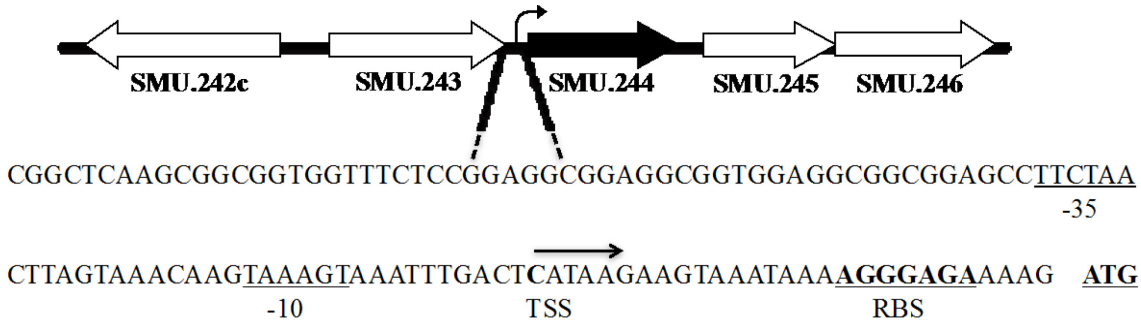

Figure 4. 1. A schematic diagram describing the genetic locus of SMU.244 in the genome of *S. mutans* UA159 (Ajdic *et al.*, 2002). Sequence analysis of this genetic locus indicates a potential promoter-like region upstream of SMU.244 gene. Arrow indicates the location of the transcription start site (TSS) as predicted by the Neural Network Promoter Prediction tool at Berkeley Drosophila Genome Project. The -10 and -35 boxes are underlined. The putative ribosome-binding site (RBS) along with translation start codon (ATG) is also indicated.

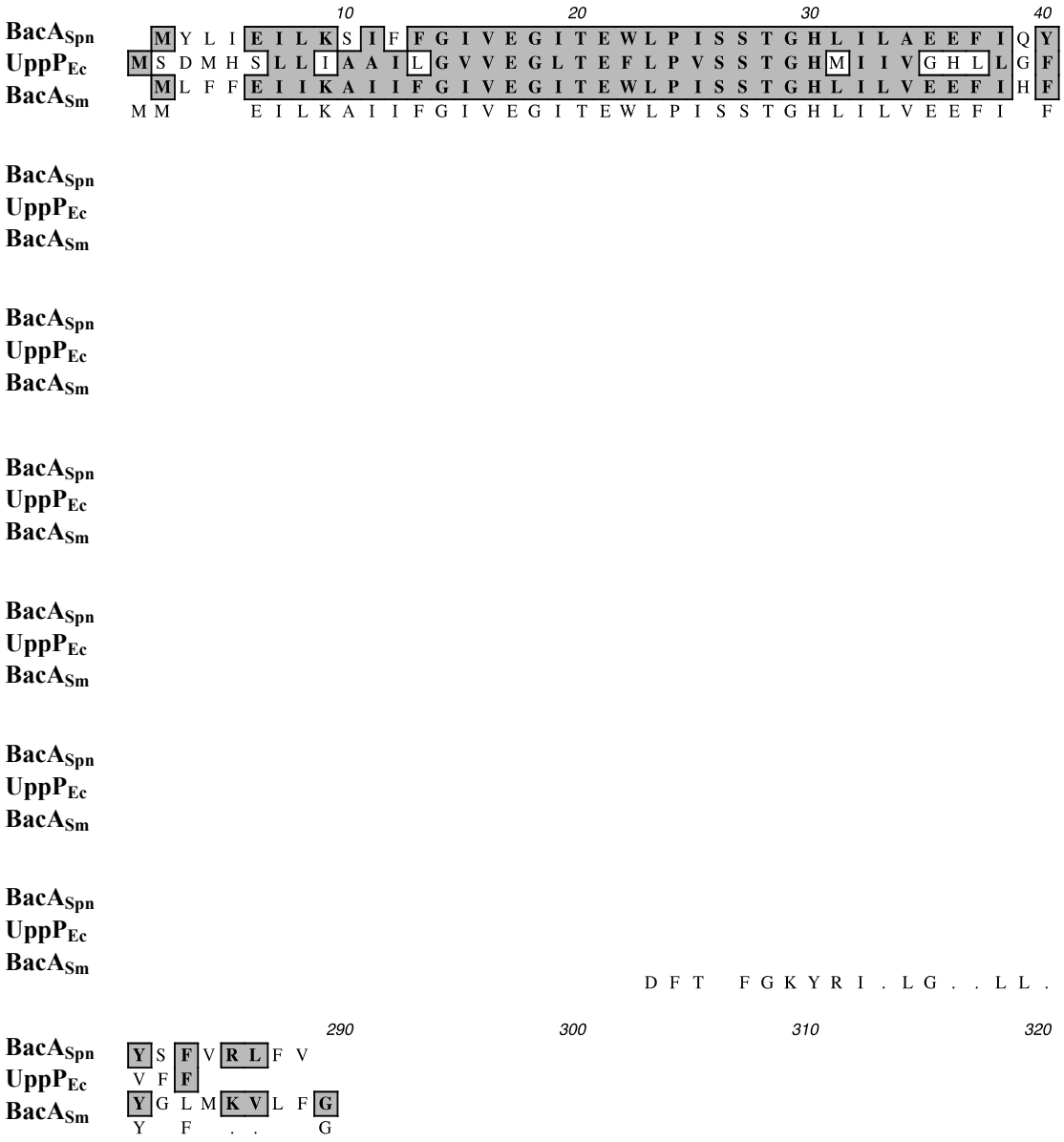

Figure 4. 2. Sequence alignment of the BacA-like protein (BacA$_{Sm}$; 281 amino acid) of *S. mutans* UA159 with BacA of *S. pneumonia* (labeled BacA$_{Spn}$; 281 amino acid) (Chalker *et al.,* 2000) and UppP of *E. coli* (UppP$_{Ec}$; 273 amino acid) (El Ghachi *et al.,* 2004) using ClustalW in the MacVector (MacVector, USA). The conserved residues are highlighted in grey and conserved amino acids are indicated by a dot.

Table 4. 1. The sequence alignment of BacA proteins from four completed genomes of *S. mutans* strains UA159, GS5, NN2025, and LJ23*

Locus tag	Organism	Protein (amino acid)	Homology percentage	Reference
SMU.244	*S. mutans* UA159	Undecaprenyl pyrophosphate phosphatase (281)	100% similarity and 100% identity	(Ajdic *et al.,* 2002)
SMUGS5_ 00950	*S. mutans* GS-5		99% similarity and 99% identity	(Biswas and Biswas, 2012)
SMUNN20 25_1713	*S. mutans* NN2025		99.3% similarity and 99.6% identity	(Maruyama *et al.,* 2009)
SMULJ23 _1724	*S. mutans* LJ23		99.3% similarity and 99.3% identity	(Aikawa *et al.,* 2012)

* All sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/genome/856) and the accession numbers are NP_720708.1, YP_006489515.1, YP_003485631.1, YP_006252001.1, respectively.

4.1.2 SMU.862, SMU.863 and SMU.864 form a Three-Gene Operon

Analysis of the *S. mutans* UA159 genome showed that SMU.862, SMU.863, and SMU.864, appeared to form a three-gene operon that encoded an ABC transporter (exporter). SMU.862 encodes a conserved hypothetical protein that appears to be membrane-associated. SMU.863 encodes an ATP-binding protein and SMU.864 encodes a permease protein of an ABC transporter. The gene upstream from SMU.862 is *carB*, which encodes a carbamoyl-phosphate synthase involved in purines and pyrimidines biosynthesis, while the gene downstream from SMU.864 is *rpsP*, which encodes a 30S ribosomal protein (Figure 4.3).

By using the Neural Network Promoter Prediction tool at Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001), we have identified a promoter-like sequence located upstream of SMU.862-864 locus, but no such promoter-like sequence was identified at the upstream of SMU.863 or SMU.864 (Figure 4.3). By using a transcription terminator prediction tool (http://transterm.cbcb.umd.edu/index.php) (Kingsford *et al.,* 2007), we also identified a Rho-independent transcription terminator-like sequence located downstream of SMU.864. Thus, the sequence information strongly suggests that these three genes likely form an operon that is co-transcripted in a single polycistronic transcript by the same regulatory elements. Interestingly, a well-conserved invert repeat or BceR box ($TTACAA_{TT}TTGTAA$) was also identified at the promoter region of SMU.862-864 (Figure 4.3), suggesting that this operon might be regulated directly by the BceABRS four-component system that is required for bacitracin sensing and resistance (Ouyang et al, 2010). Therefore, we included this genetic locus for further investigation in this study.

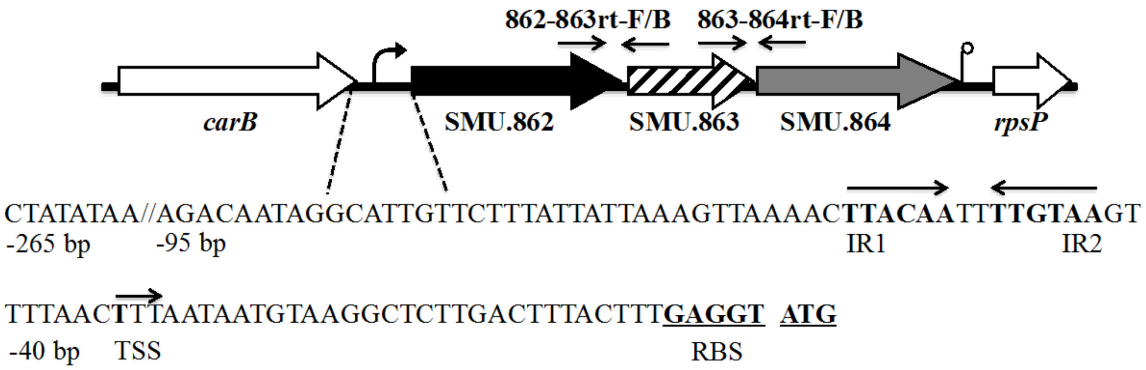

Figure 4. 3. The genetic locus of SMU.862, SMU.863, and SMU.864 together with the adjacent genes in *S. mutans* UA159 (Ajdic *et al.,* 2002)**.** Sequence analysis of this genetic locus indicates a potential promoter-like region upstream of SMU.862 gene and a potential Rho-independent transcription terminator-like sequence downstream of SMU.864. Arrows indicate the location of the transcription start site (TSS) and the invert repeats or the BceR box. The putative ribosome-binding site (RBS) along with translation start codon (ATG) is also indicated.

To test the hypothesis that these three genes (SMU.862-864) might form an operon that was co-transcripted, we used RT-PCR to analyze the transcription products of these genes using cross-gene primer pairs as indicated in Figure 4.3. Two pairs of cross-gene primers were used for RT-PCR, including 862-863rt-F (located in SMU.862) and 862-863rt-B (located in SMU.863) with an anticipated product size of 712 bp, and 863-864rt-F (located in SMU.863) and 863-864rt-B (located in SMU.864) with an anticipated product size of 535 bp.

It was anticipated that if these three genes were cotranscripted from the same promoter, we should get the anticipated transcripts using the cross-gene primers. Otherwise, we might have negative results (no products). Indeed, the RT-PCR results confirmed that all three genes were cotranscripted as a single polycistronic transcript with an anticipated amplicons between SMU.862-SMU.863 and SMU.863-SMU.864 (Figure 4.4 lanes 3 and 6). The same primers could not generate any products when the purified RNA was used as a template (Figure 4.4 lanes 4 and 7), indicating the RNA samples had no contamination with the genomic DNA.

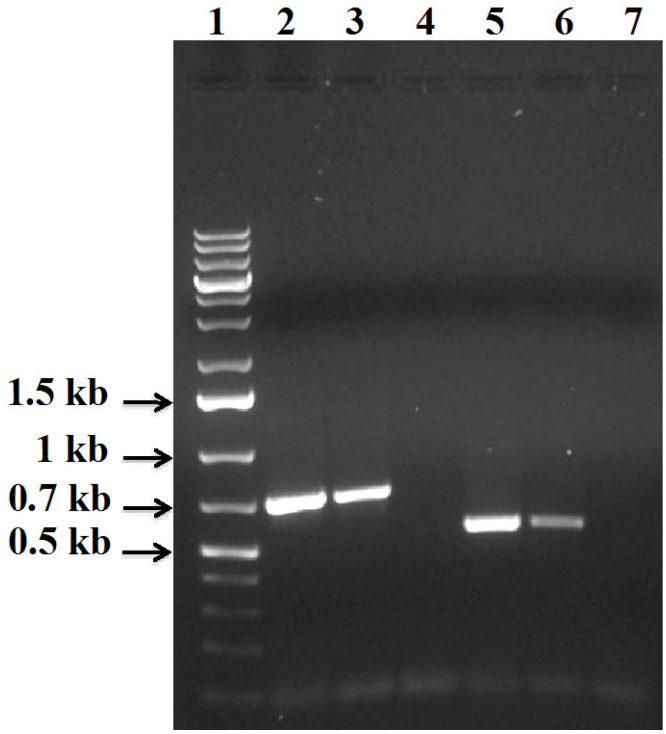

Figure 4. 4. RT-PCR analysis of transcripts from the SMU.862-864 operon in *S. mutans* UA159. Two pairs of cross-gene primers (indicated in Figure 4.3) were used for RT-PCR included 862-863rt-F/B (lanes 2 to 4) with an anticipated product size of 712 bp, and 863-864rt-F/B (lanes 5 to 7) with an anticipated product size of 535 bp. Lane 1 represents 1 kb+ ladder. Lanes 2 and 5 represent positive controls amplified from the genomic DNA of UA159. Lanes 3 and 6 represent RT-PCR products amplified from the cDNA. Lanes 4 and 7 represent negative controls amplified from purified RNA.

4.2 Genetic Confirmation of the Gene Deletion Mutants

To determine the role of SMU.244 (*bacA*), SMU.862, SMU.863, and SMU.864 genes on antibiotics resistance and other virulence factors, we constructed individual gene deletion mutants of SMU.244, SMU.862, SMU.863, and SMU.864 by allelic replacement strategy as described in Materials and Methods (Section 3.3.6; Figure 3.1). This method enables the deletion of a target gene and insertion of the erythromycin-resistance cassette (*ermAM*) without interruption of the downstream genes. The correct replacement of the target gene with the *ermAM* cassette was verified using PCR strategy and four primers combinations (P1-Erm2, Erm1-P4, P1-P4, and Erm1-Erm2) on the mutants in comparison with the parent genomic DNA. The agreement of product sizes predicted by the MacVector software ‘‘PCR Primer Pair’’ function (Table 4.2) with actual amplicons length (Figure 4.5, 4.6, and 4.7) provided positive identification for the gene deletion mutants.

For example, deletion of the 551-bp internal region of SMU.244 gene took place through an allelic exchange mechanism by the replacement of the 876-bp *ermAM* cassette during the double-crossover recombination. Therefore, the PCR reactions using the primer pairs P1-Erm2, Erm1-P4, and Erm1-Erm2 against the mutant template DNA would generate predictable sizes of the PCR products of 1616 bp, 1705 bp, and 876 bp, respectively. However, these primer pairs did not generate any PCR products when the parent genomic DNA was used as the template due to its lack of the insertion of the *ermAM* cassette (Figure 4.5 and Table 4.2). Although PCR with primer pair P1-P4 could generate products from both mutant and the parent template DNA, the size of the PCR product of the mutant (2461 bp) different from that of the parent (2136 bp), indicating that the *ermAM* cassette (876 bp) was 325 bp bigger than its replaced internal region (551 bp) of SMU.244 (Figure 4.5 and Table 4.2). The same strategy was also used to confirm the gene deletion of SMU.862, SMU.863, and SMU.864. The results showed that the three genes of SMU.862, SMU.863, and SMU.864 were successfully deleted (Figure 4.6 A and B; Figure 4.7 A; Table 4.2).

We also constructed a mutant with deletion of the three genes of SMU.862-864 to rule out the possibility that the inactivation of individual gene might not be sufficient to inactivate the function of this ABC transporter. The mutant was confirmed to have a deletion of the internal region (3176 bp) of SMU.862 to SMU.864, which was completely replaced by the *ermAM* cassette (Figure 4.7 B and Table 4.2). The result confirmed that we successfully constructed this mutant, designated as SmΔSMU.862-4.

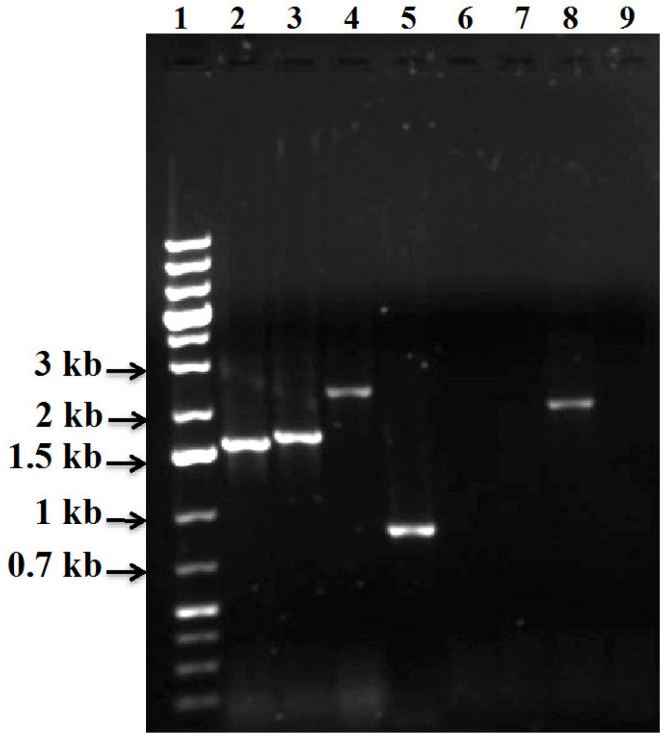

Figure 4. 5. Confirmation of the gene deletion mutant for SMU.244 (*bacA*) using PCR strategy. The mutant was genetically confirmed by PCR using a combination of the primers described in materials and methods and templates of DNA from both the mutant and the parent strain. Note that the PCR products of P1-Erm2 and Erm1-P4 were obtained only if the ligation product was inserted into the correct location at correct orientation. Lane 1 represents 1 kb+ ladder; lane 2-5 (mutant template); lane 2: P1–Erm2 PCR fragment (1616 bp); lane 3: Erm1–P4 PCR fragment (1705 bp); lane 4: P1-P4 PCR fragment (2461 bp); lane 5: Erm1-Erm2 PCR fragment (876 bp); lane 6-9 (parent strain template); lane 6: P1–Erm2 PCR fragment (no product); lanes 7: Erm1–P4 PCR fragment (no product); lane 8: P1-P4 PCR fragment (2136 bp); lane 9: Erm1-Erm2 PCR fragment (no product).

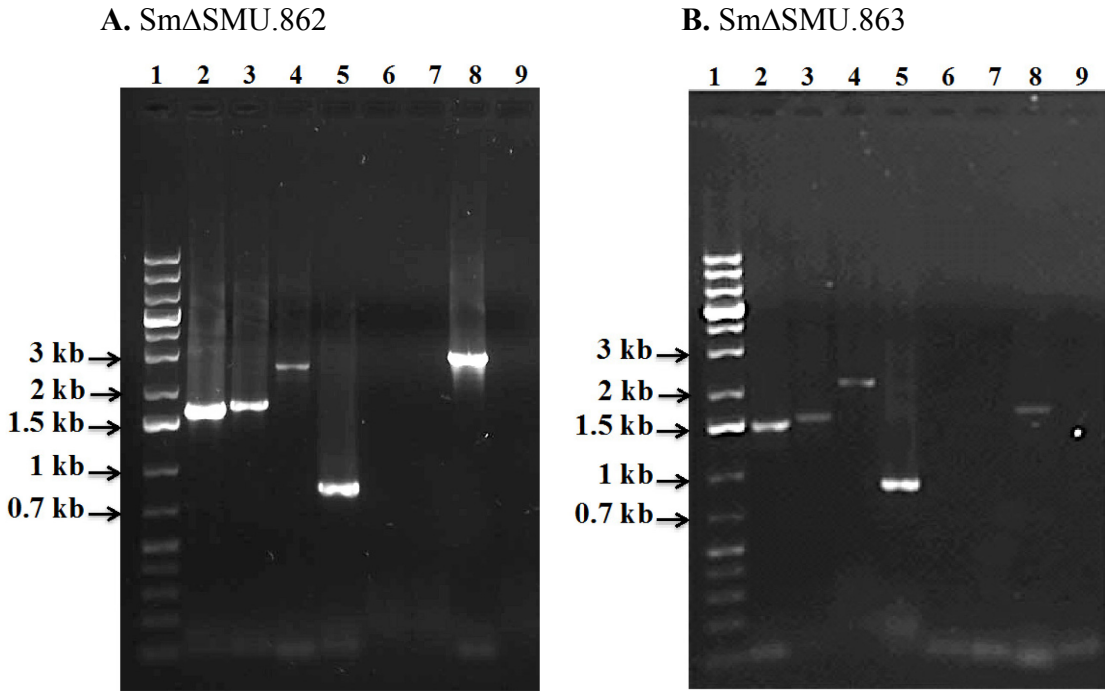

Figure 4. 6. Confirmation of the gene deletion mutants for SMU.862 and SMU.863 using PCR strategy. The mutant was genetically confirmed by PCR using a combination of the primers described in materials and methods and templates of DNA from both the mutant and the parent strain. Note that the PCR products of P1-Erm2 and Erm1-P4 were obtained only if the ligation product was inserted into the correct location at correct orientation. **A.** Confirmation of SMU.862 mutant; lane 1 represents 1 kb+ ladder; lane 2-5 (mutant template); lane 2: P1–Erm2 PCR fragment (1696 bp); lane 3: Erm1–P4 PCR fragment (1783 bp); lane 4: P1-P4 PCR fragment (2619 bp); lane 5: Erm1-Erm2 PCR fragment (876 bp); lane 6-9 (parent strain template); lane 6: P1–Erm2 PCR fragment (no product); lanes 7: Erm1–P4 PCR fragment (no product); lane 8: P1-P4 PCR fragment (2936 bp); lane 9: Erm1-Erm2 PCR fragment (no product). **B.** Confirmation of SMU.863 mutant; lane 1 represents 1 kb+ ladder; lane 2-5 (mutant template); lane 2: P1–Erm2 PCR fragment (1475 bp); lane 3: Erm1–P4 PCR fragment (1554 bp); lane 4: P1-P4 PCR fragment (2169 bp); lane 5: Erm1-Erm2 PCR fragment (876 bp); lane 6-9 (parent strain template); lane 6: P1–Erm2 PCR fragment (no product); lanes 7: Erm1–P4 PCR fragment (no product); lane 8: P1-P4 PCR fragment (1739 bp); lane 9: Erm1-Erm2 PCR fragment (no product).

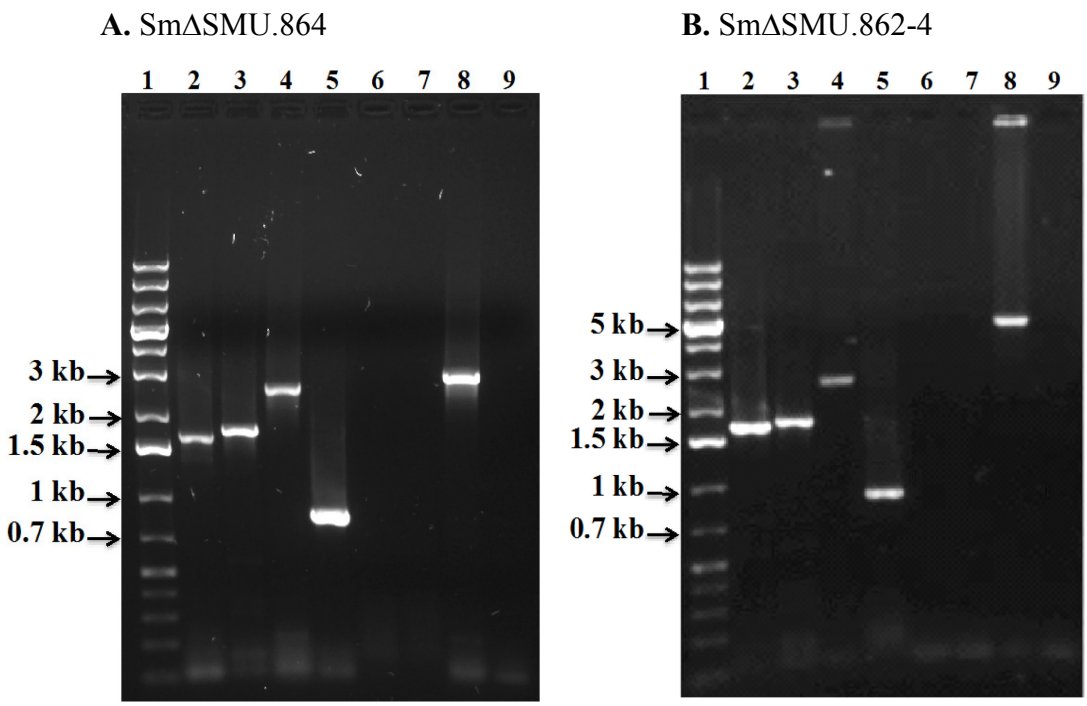

Figure 4. 7. Confirmation of the gene deletion mutants for SMU.864 and SMU.862-4 using PCR strategy. The mutant was genetically confirmed by PCR using a combination of the primers described in materials and methods and templates of DNA from both the mutant and the parent strain. Note that the PCR products of P1-Erm2 and Erm1-P4 were obtained only if the ligation product was inserted into the correct location at correct orientation. **A.** Confirmation of SMU.864 mutant; lane 1 represents 1 kb+ ladder; lane 2-5 (mutant template); lane 2: P1–Erm2 PCR fragment (1689 bp); lane 3: Erm1–P4 PCR fragment (1806 bp); lane 4: P1-P4 PCR fragment (2635 bp); lane 5: Erm1-Erm2 PCR fragment (876 bp); lane 6-9 (parent strain template); lane 6: P1–Erm2 PCR fragment (no product); lanes 7: Erm1–P4 PCR fragment (no product); lane 8: P1-P4 PCR fragment (2835 bp); lane 9: Erm1-Erm2 PCR fragment (no product). **B.** Confirmation of SMU.862-4 mutant**;** lane 1 represents 1 kb+ ladder; lane 2-5 (mutant template); Lane 2: P1–Erm2 PCR fragment (1696 bp); lane 3: Erm1–P4 PCR fragment (1806 bp); lane 4: P1-P4 PCR fragment (2642 bp); lane 5: Erm1-Erm2 PCR fragment (876 bp); lane 6-9 (parent strain template); lane 6: P1–Erm2 PCR fragment (no product); lanes 7: Erm1–P4 PCR fragment (no product); lane 8: P1-P4 PCR fragment (4942 bp); lane 9: Erm1-Erm2 PCR fragment (no product).

Table 4. 2. Anticipated results using a PCR confirmation strategy

Strains	Primers		Anticipated amplicon (bp)	
	Forward	Backward	Mutant	Parent
SmΔbacA	bacA-P1	Erm-P2	1616	-
	Erm-P1	bacA-P4	1705	-
	bacA-P1	bacA-P4	2461	2136
	Erm-P1	Erm-P2	876	-
SmΔSMU.862	Sm.862-P1	Erm-P2	1696	-
	Erm-P1	Sm.862-P4	1783	-
	Sm.862-P1	Sm.862-P4	2619	2936
	Erm-P1	Erm-P2	876	-
SmΔSMU.863	Sm.863-P1	Erm-P2	1475	-
	Erm-P1	Sm.863-P4	1554	-
	Sm.863-P1	Sm.863-P4	2169	1739
	Erm-P1	Erm-P2	876	-
SmΔSMU.864	Sm.864-P1	Erm-P2	1689	-
	Erm-P1	Sm.864-P4	1806	-
	Sm.864-P1	Sm.863-P4	2635	2835
	Erm-P1	Erm-P2	876	-
SmΔSMU.862-4	Sm.862-P1	Erm-P2	1696	-
	Erm-P1	Sm.864-P4	1806	-
	Sm.862-P1	Sm.864-P4	2642	4942
	Erm-P1	Erm-P2	876	-

-: No product.

4.3 Genetic Confirmation of Sm-pCpbacA Complementation Strain

To help to confirm if the defective phenotype of SmΔbacA mutant was truly because of *bacA* deletion, we constructed a complement strain, designated as Sm-pCpbacA, by re-introducing a wild-type copy of *bacA* gene (1093 bp) into SmΔbacA mutant using a low copy number of shuttle vector, pDL278 (LeBlanc *et al.,* 1992). The newly constructed plasmid pCpbacA was initially confirmed by PCR and restriction digestion analysis (Figure 4.8). The results of the restriction digestion analysis with both SacI and SphI enzymes showed the release of the insert (1205 bp) from pCpbacA plasmid. By further restriction digestion analysis using another enzyme BtgI, we again confirmed that the *bacA*-related fragment was successfully inserted into shuttle vector pDL278 at the correct location (Figure 4.8). The confirmed plasmid, named as pCpbacA, was then transformed to SmΔbacA mutant. The transformants that grew on THYE agar plates containing both erythromycin (10 μg/ml) and spectinomycin (800 μg/ml) were considered as correct strain, named Sm-pCpbacA, which could be used for complementation experiments.

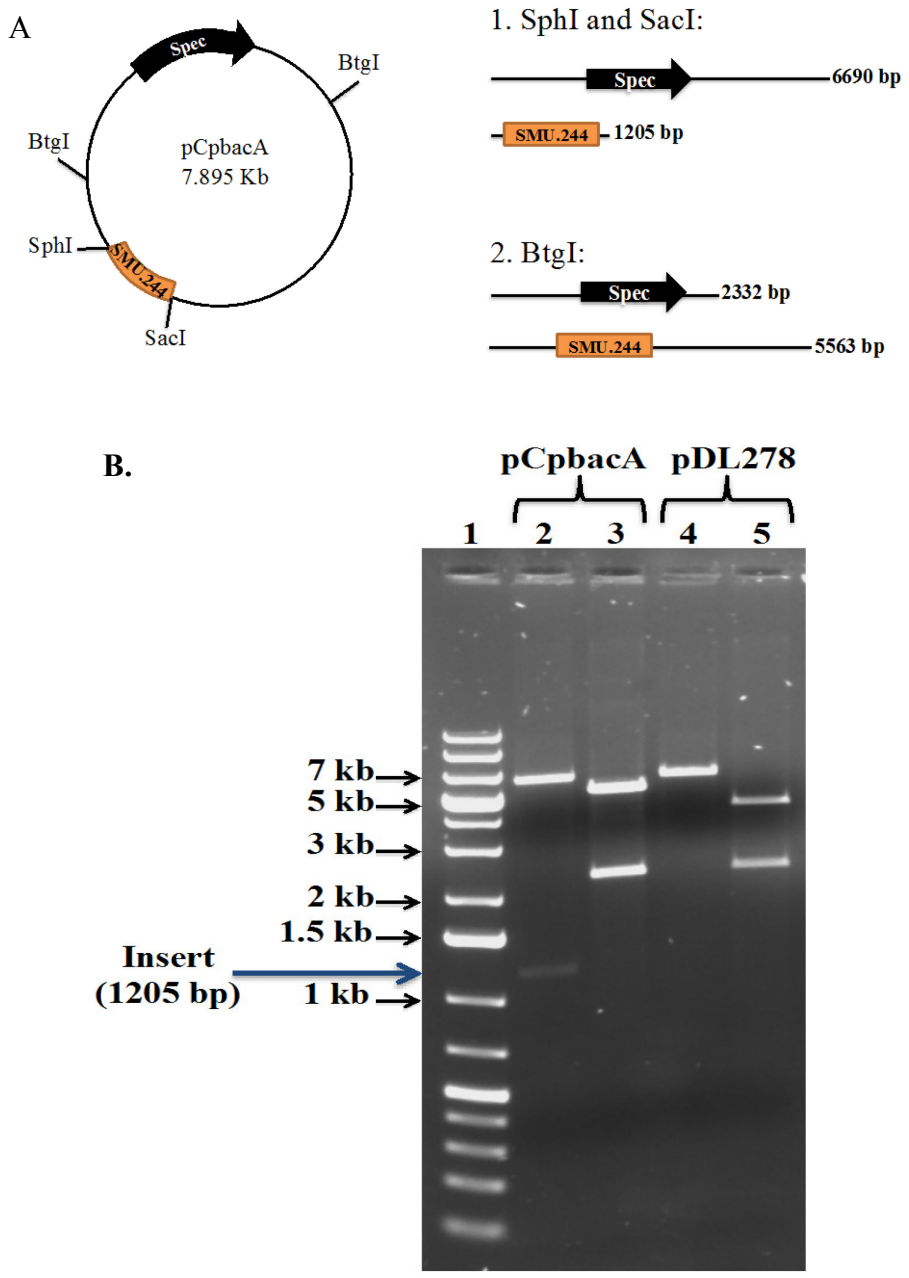

Figure 4. 8. Confirmation of the construction of pCpbacA using two restriction digestion reactions. A. Map of pCpbacA plasmid with the restriction digestion sites. **B.** Results of the restriction digestion reactions. Lane 2: restriction digestion of pCpbacA plasmid DNA with both SphI and SacI, showed linearized plasmid (6690 bp) and release of insert (1205 bp); lane 3: restriction digestion of pCpbacA plasmid DNA with BtgI, gave two fragments 5563 bp (plasmid with insert) and 2332 bp (plasmid DNA); lane 4: restriction digestion of a control pDL278 plasmid DNA (no insert) with both SphI and SacI, showed linearized plasmid (6690 bp); lane 5 restriction digestion of wild type pDL278 plasmid DNA (no insert) with BtgI, showed cut of plasmid DNA into two fragments with 4401 bp and 2332 bp.

4.4 Phenotypic Characteristics of SMU.244 Deletion Mutant

4.4.1 SmΔbacA Mutant is Sensitive to Bacitracin

Antibiotic susceptibility test showed that deletion of SMU.244 or *bacA* gene resulted in a mutant (SmΔbacA) that was more sensitive (32-folds) to bacitracin than the parent strain UA159. This mutant could not even grow in the presence of as low as 2 U/ml of bacitracin (Figure 4.9). However, this phenotypic defect in bacitracin resistance could be completely restored by re-introducing an intact copy of bacA gene into the mutant (complement strain Sm-pCpbacA) (Figure 4.9 and Table 4.3).

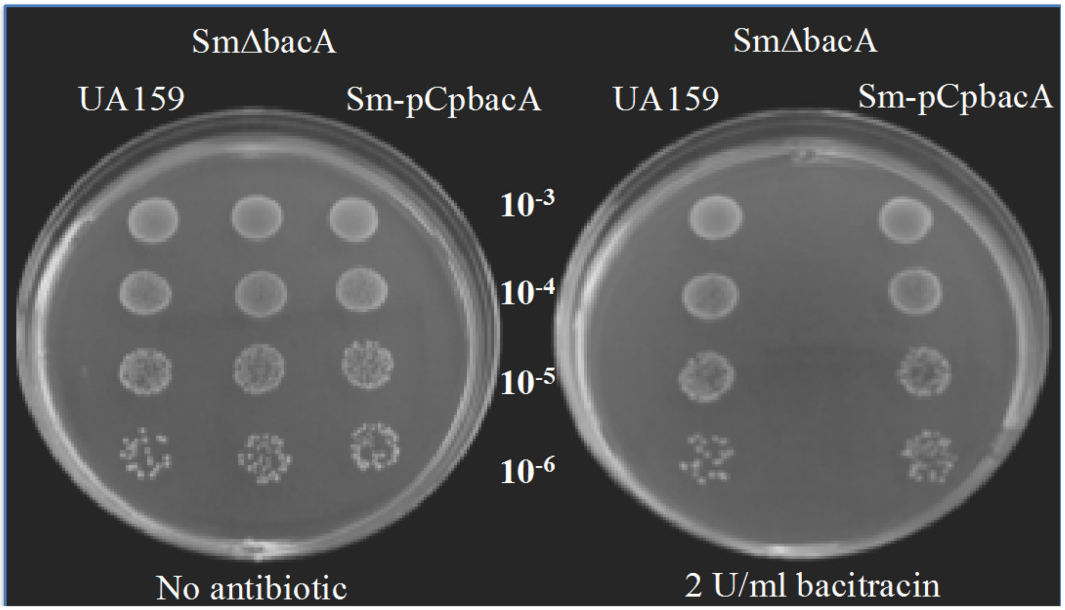

Figure 4. 9. Bacitracin susceptibility result for SmΔbacA mutant. The wild type UA159, SmΔbacA mutant, and complement strain Sm-pCpbacA were grown in the presence or absence of sub-inhibitory concentration of bacitracin 2 U/ml. All the plates were incubated at 37 °C for two days.

Table 4. 3. Minimum inhibitory concentration (MIC)-based determination of sensitivities of *S. mutans* strains to selected antimicrobials

Antimicrobial	MIC*		
	UA159	SmΔbacA	Sm-pCpbacA
Bacitracin (U/ml)	48	1.5	48
Penicillin G (μg/ml)	0.063	0.0313	0.063
Vancomycin (μg/ml)	1.25	0.625	1.25
Nisin (μg/ml)	17.5	8.75	17.5
SDS (μg/ml)	40	40	40
Kanamycin (μg/ml)	100	100	100

* Results are averages from duplicate samples of two independent experiments.

4.4.2 SmΔbacA Mutant is also Sensitive to Other Cell Wall-Acting Antibiotics

In addition to bacitracin, SmΔbacA mutant was also more sensitive to penicillin G (0.03 μg/ml) and vancomycin (0.6 μg/ml) than the parent strain UA159 (Figure 4.10 A and B). Based on the MIC test (Table 4.3), SmΔbacA mutant was about 2-fold more sensitive to penicillin G, vancomycin, and nisin than the parent strain UA159. Again, the defects in resistance to these antibiotics could completely be restored in the complement strain Sm-pCpbacA. Moreover, we also examined the effect of inactivation of SMU.244 on a detergent SDS and the protein-inhibiting antibiotic, kanamycin. However, the results showed little difference between SmΔbacA mutant and the parent strain, suggesting that the deletion of *bacA* gene might not affect its susceptibility to protein synthesis inhibitors or detergent (Table 4.3).

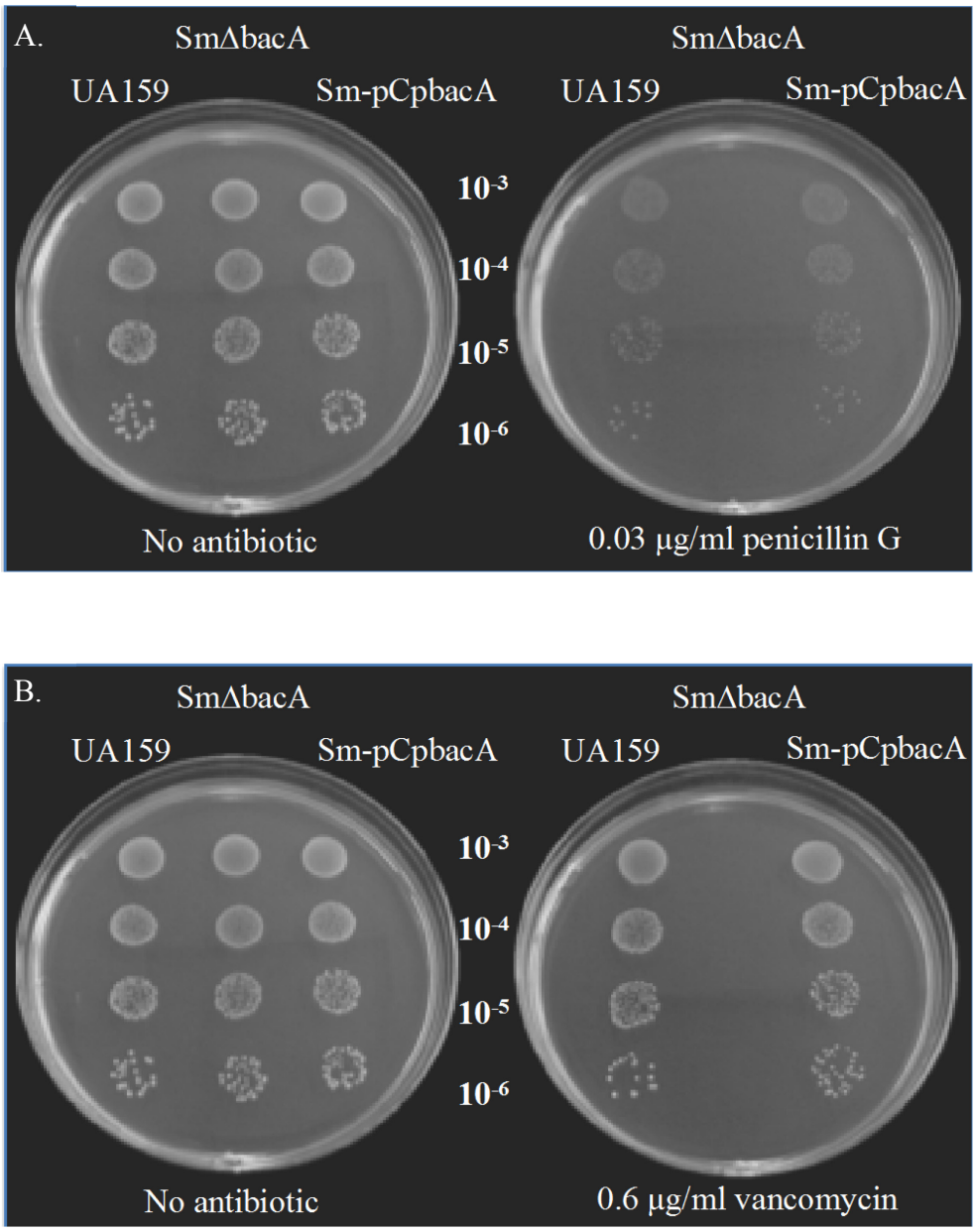

Figure 4. 10. Antibiotics susceptibility result for SmΔbacA mutant. The wild type UA159, SmΔbacA mutant, and complement strain Sm-pCpbacA were grown in the presence or absence of sub-inhibitory concentration of penicillin G 0.03 µg/ml (A), and vancomycin 0.6 µg/ml (B). All the plates were incubated at 37 °C for two days.

4.4.3 Deletion of SMU.244 also Affects the Growth of *S. mutans*

To determine the effect of deletion of SMU.244 on the growth kinetics of *S. mutans*, we grew UA159, SmΔbacA mutant and complement strain Sm-pCpbacA in THYE broth with or without a cell wall-acting antibiotic. The results revealed that even in the absence of antibiotics the growth of SmΔbacA mutant was affected with a longer doubling time (T_d) of 1:22 h^{-1} than the parent strain (T_d = 1:05 h^{-1}), suggesting that SmΔbacA mutant had a slower growth rate than the parent strain UA159. However, the final growth yield of SmΔbacA mutant appeared to be the same as that of the parent strain UA159 after the growth for 16 hours. Again, the growth arrest of SmΔbacA mutant could be completely restored by introducing a wild-type copy of the *bacA* gene in this mutant or the complement strain Sm-pCpbacA (Figure 4.11 A and Table 4.4).

In the presence of 0.48 U/ml (100-fold lower than MIC of the parent strain) of bacitracin, the growth of SmΔbacA was dramatically affected and its growth nearly ceased until 12 hours, when SmΔbacA mutant began to grow based on the optical density reading. Also, the growth yield of this mutant was much lower than that of the wild type UA159 (Figure 4.11 B and Table 4.4). In addition, SmΔbacA also showed a delay in growth in the presence of sub-inhibitory concentration of penicillin G (0.00781 μg/ml) and vancomycin (0.3125 μg/ml), with a longer doubling time than the wild type UA159 (Figure 4.12 A and B, and Table 4.4). Furthermore, a similar growth arrest of SmΔbacA with a longer doubling time than the wild type UA159 was also observed when it was grown in the presence of sub-inhibitory concentration of nisin (4.375 μg/ml). However, the final growth yield of SmΔbacA after 16 h of growth in the presence of nisin (4.375 μg/ml) appeared to be the same as that of the parent strain UA159 (Figure 4.13 and Table 4.4).

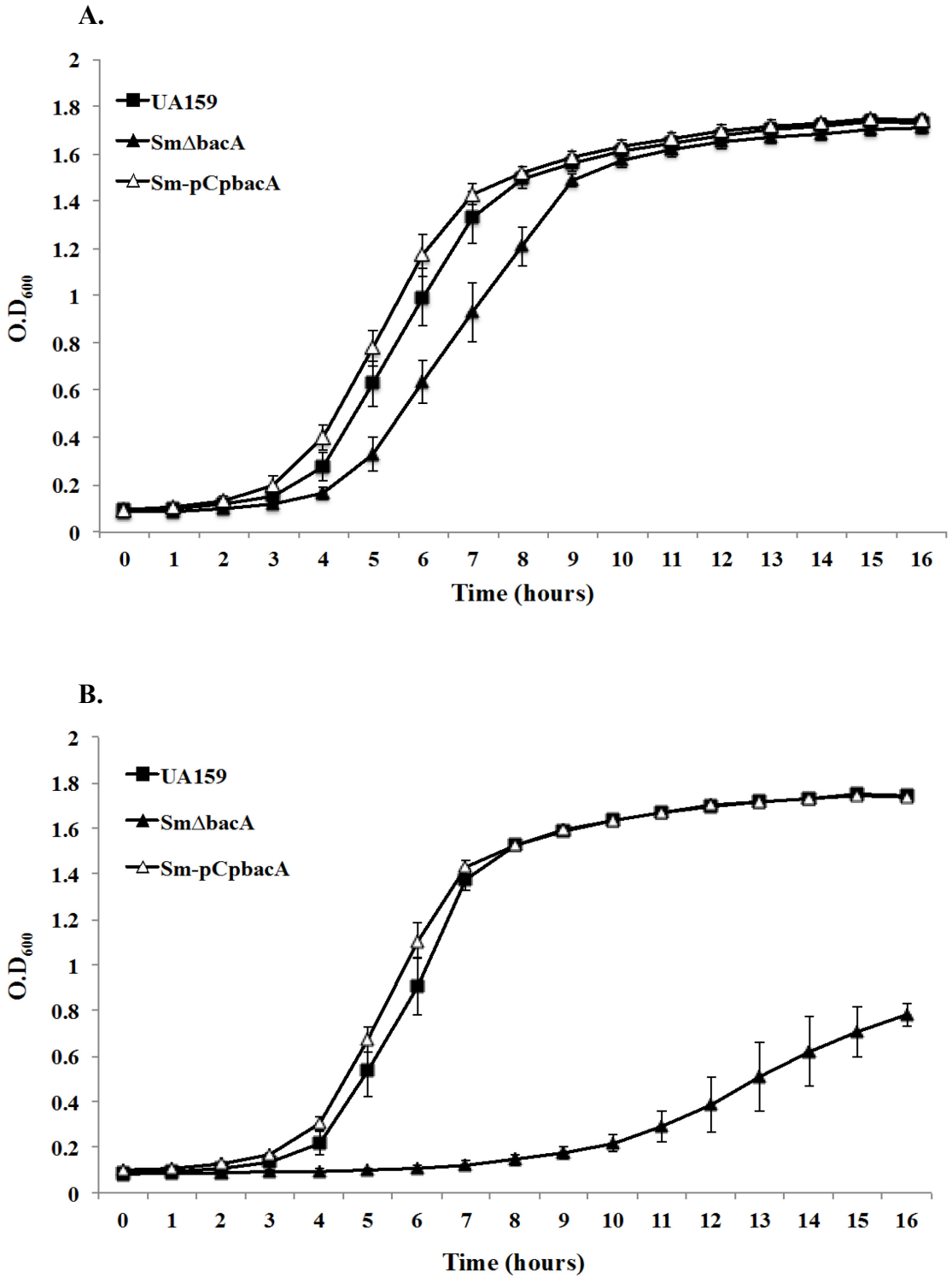

Figure 4. 11. The growth curves of *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA in THYE only (A) or with addition of bacitracin (0.48 U/ml) (B). The growth was monitored for 16 hours and the results represent the means of duplicates from three independent experiments.

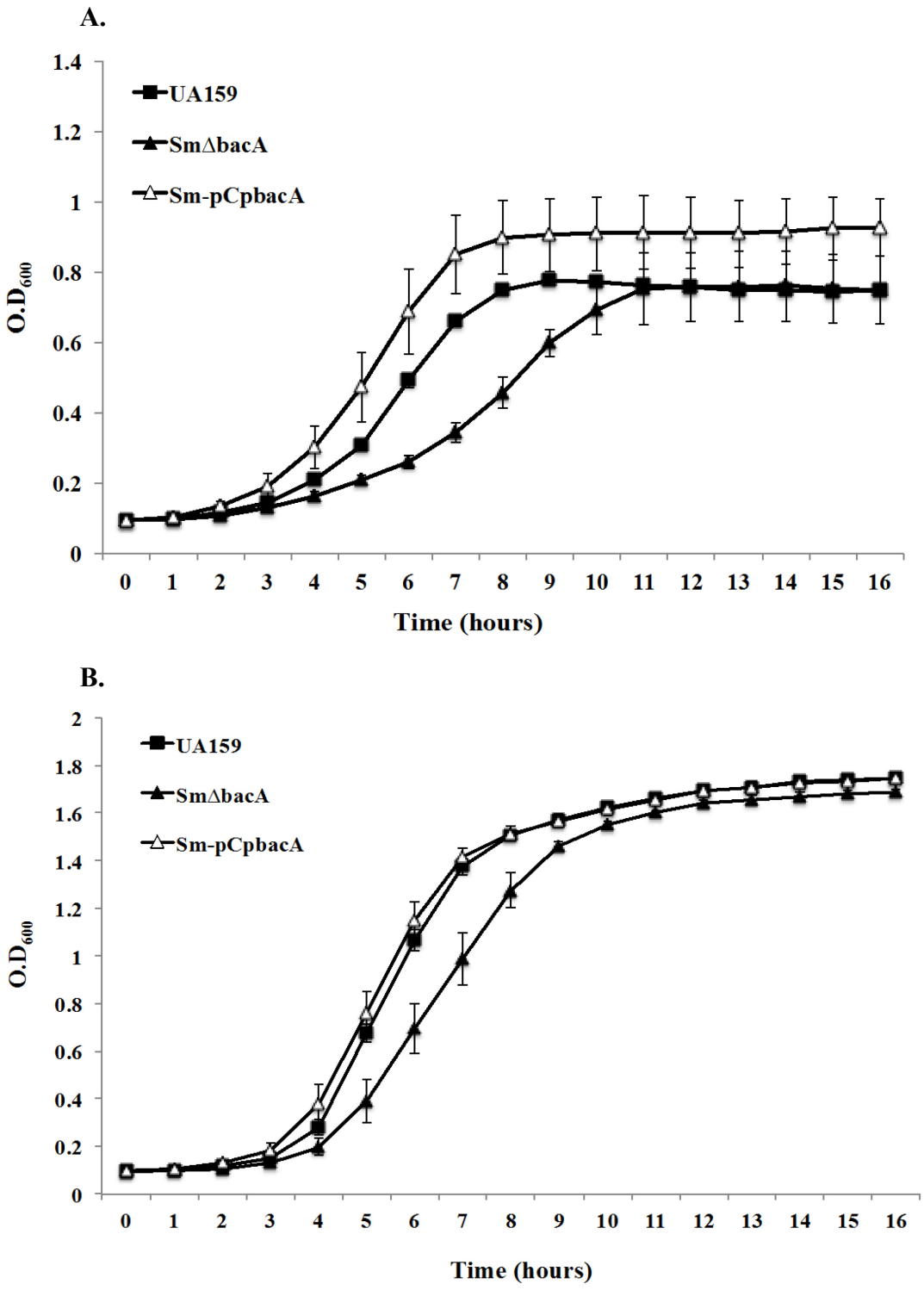

Figure 4. 12. The growth curves of *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA in THYE with 0.00781 µg/ml of penicillin G (A) and with 0.3125 µg/ml of vancomycin (B). The growth was monitored for 16 hours and the results represent the means of duplicates from two independent experiments**.**

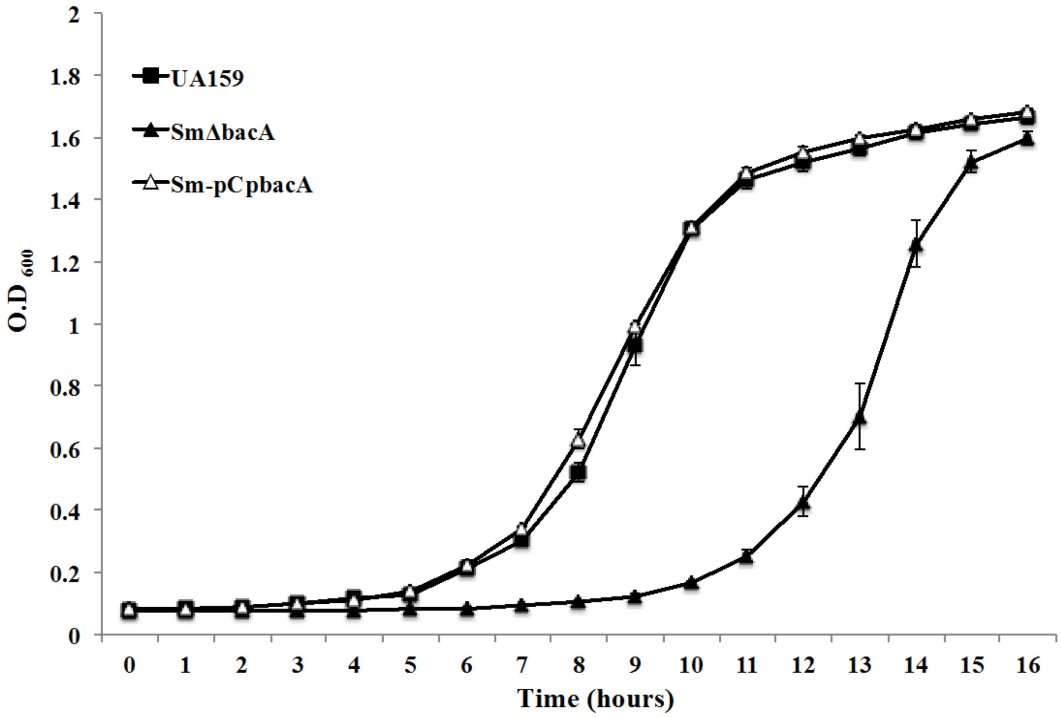

Figure 4. 13. A growth curve of *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA in THYE with 4.375 µg/ml of nisin. The growth was monitored for 16 hours and the results represent the means of duplicates from two independent experiments.

Table 4. 4. The doubling times (T_d) of *S. mutans* strains grown in the presence or absence of cell wall-acting antibiotics

Growth condition	Doubling time (h^{-1})		
	UA159	SmΔbacA	Sm-pCpbacA
THYE only	1:05	1:22	1:00
Bacitracin (0.48 U/ml)	1:00	4:09	1:05
Penicillin G (0.0078 μg/ml)	1:37	3:01	1:32
Vancomycin (0.3125 μg/ml)	1:02	1:29	0:59
Nisin (4.375 μg/ml)	1:14	1:30	1:17

***** The results were presented as (hours : minutes) and the doubling times were calculated using the following equation [$Td= (t_2 – t_1) \times (\log2/ \log2N_0-\log N_0)$] whereas $2N_0$ and N_0 represent two reading of the OD_{600} during the log phase and t_2 and t_1 represent the two-time point at the OD_{600} $2N_0$ and N_0, respectively.

4.4.4 Deletion of SMU.244 Affects Biofilm Formation of *S. mutans*

A previous study showed that inactivation of the *bacA* gene in *Streptococcus gordonii,* a pioneer colonizer in dental plaque biofilms, resulted in a defect in biofilm formation with a reduced biomass (Loo *et al.,* 2000). *S. mutans* is also a primary colonizer in dental biofilms and uses a number of mechanisms to initiate biofilm formation (Islam *et al.,* 2007; Mitchell, 2003). Therefore, we set forth an experiment to determine if deletion of SMU.244 affects biofilm formation of *S. mutans* in the presence or absence of subinhibitory concentrations of bacitracin.

The results showed that in the absence of bacitracin SmΔbacA formed a biofilm that showed no significant difference from those formed by the parent UA159 or the complement strain Sm-pCpbacA grown in 4X diluted THYE medium supplemented with either glucose (10 mM) or sucrose (5 mM). This suggested that deletion of SMU.244 did not significantly affect biofilm formation by *S. mutans*. In the presence of a sub-inhibitory concentration of bacitracin (0.48 U/ml) however, SmΔbacA formed a biofilm with significantly reduced biomass ($P < 0.005$) compared to the parent strain UA159. The formation of reduced biofilms by the mutant could be readily visualized or measured by optical density reading (Figure 4.14 and 4.15). We then examined the biofilms formed by these strains using conventional microscopy. We found that even in the absence of bacitracin, the biofilm formed by SmΔbacA appeared to be slightly unstructured compared to the parental UA159. In the presence of 0.48 U/ml of bacitracin, the biofilm of SmΔbacA formed a sponge-like or net-like structure with formation of cell aggregations (Figure 4.16 and Appendix1.1). Clearly, the presence of a sub-lethal concentration of bacitracin was a major factor influencing the biofilm formation by SmΔbacA mutant. However, The defect of the biofilm formed by SmΔbacA mutant was independently from the use of either glucose or sucrose.

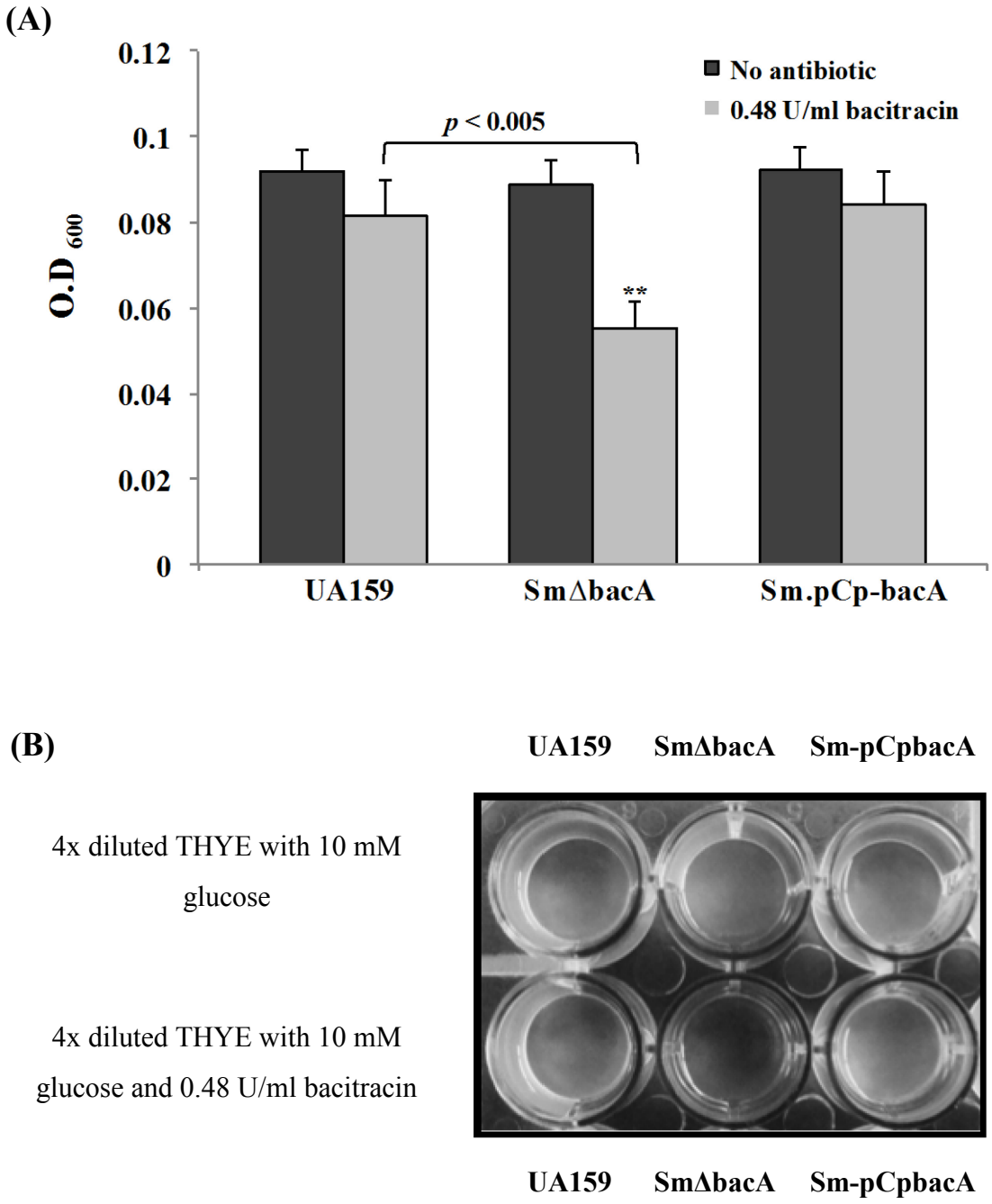

Figure 4. 14. Biofilm formation by SmΔbacA mutant, Sm-pCpbacA, and parent strain UA159 in 4x diluted THYE plus 10 mM of glucose. A. Optical density reading of biofilms formed by SmΔbacA, Sm-pCpbacA, and UA159. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation **B.** Biofilm formed by SmΔbacA, Sm-pCpbacA, and UA159 on the surface of a polystyrene 24-well plate.

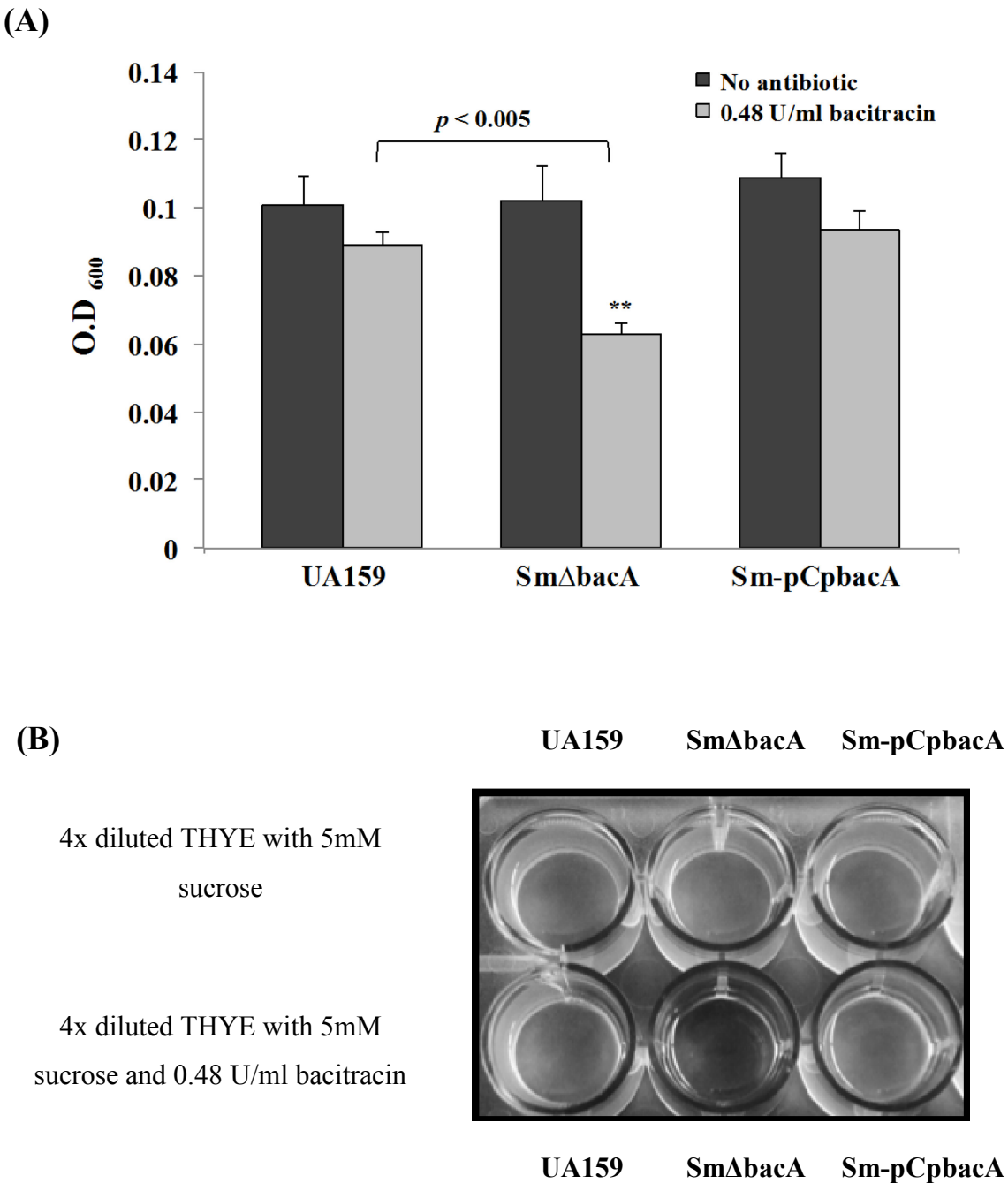

Figure 4. 15. Biofilm formation by SmΔbacA mutant, Sm-pCpbacA, and parent strain UA159 in 4x diluted THYE plus 5 mM sucrose. A. Optical density reading of biofilms formed by SmΔbacA, Sm-pCpbacA, and UA159. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation **B.** Biofilm formed by SmΔbacA, Sm-pCpbacA, and UA159 on the surface of a polystyrene 24-well plate.

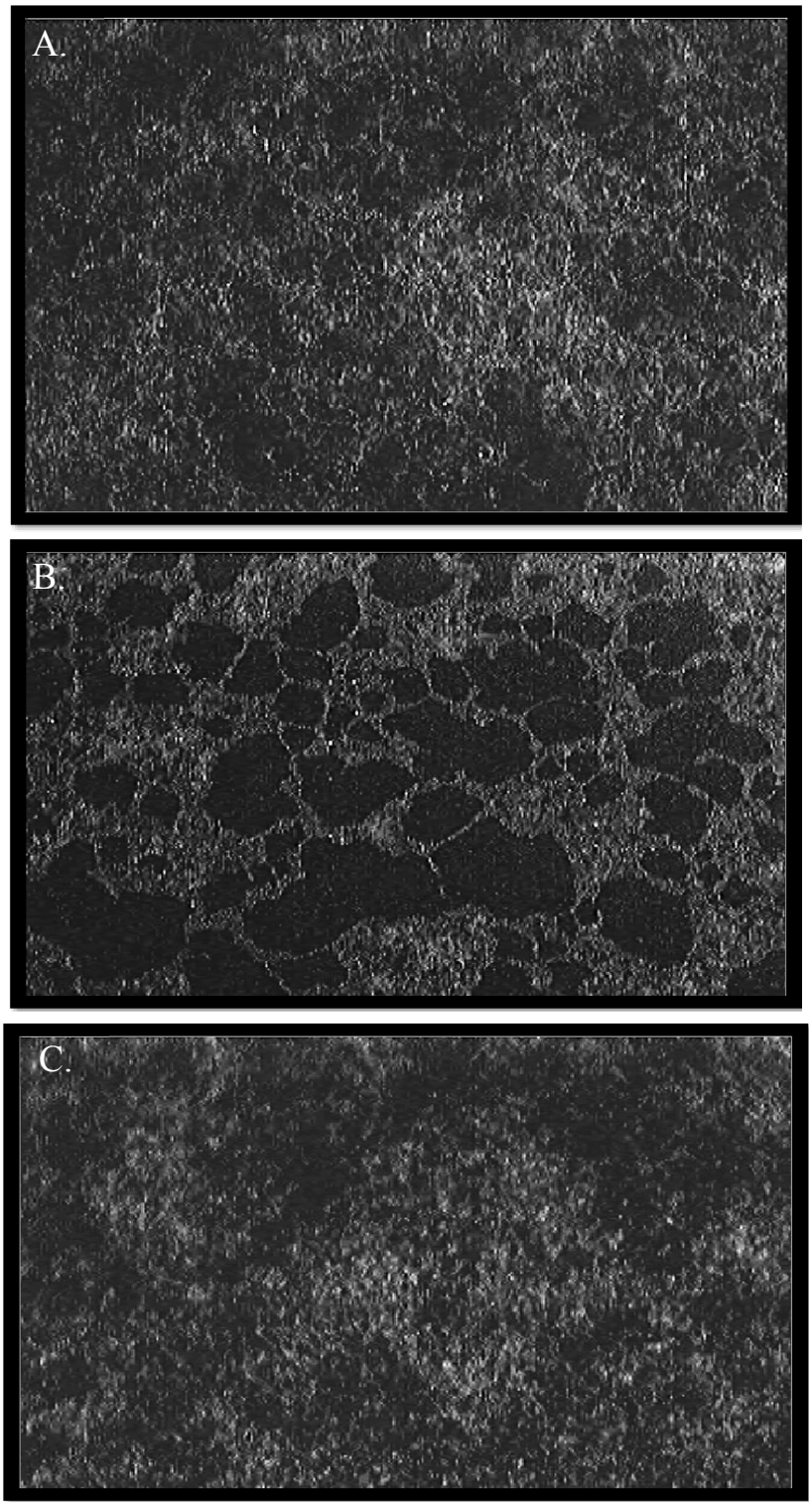

Figure 4. 16. Conventional microscopy showed 16 hours biofilm formed by *S. mutans* strains. Wild type *S. mutans* UA159 (A), SmΔbacA mutant (B), and Sm-pCpbacA (C) strains were cultured in 4x diluted THYE plus 5 mM sucrose with 0.48 U/ml of bacitracin. Representative fields were viewed at 1000X.

4.4.5 Deletion of SMU.244 Gene did not Affect Acid Sensitivity of *S. mutans*

The ability of *S. mutans* to grow at a low pH environment is known to be an important virulence factor for this organism to cause dental caries (Matsui and Cvitkovitch, 2010). To determine if deletion of SMU.244 could affect the ability of *S. mutans* to grow in low pH environment, we used a rapid acid resistant assay to examine the growth of *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA on THYE agar plates with either pH 7.5 or pH 5.5. The result showed that deletion of SMU.244 gene did not affect the growth of SmΔbacA at pH5.5 on agar plates compared to the parent strain UA159, although the size of colonies on THYE plate at pH 5.5 were smaller than those at pH 7.5 after the same incubation (Figure 4.17).

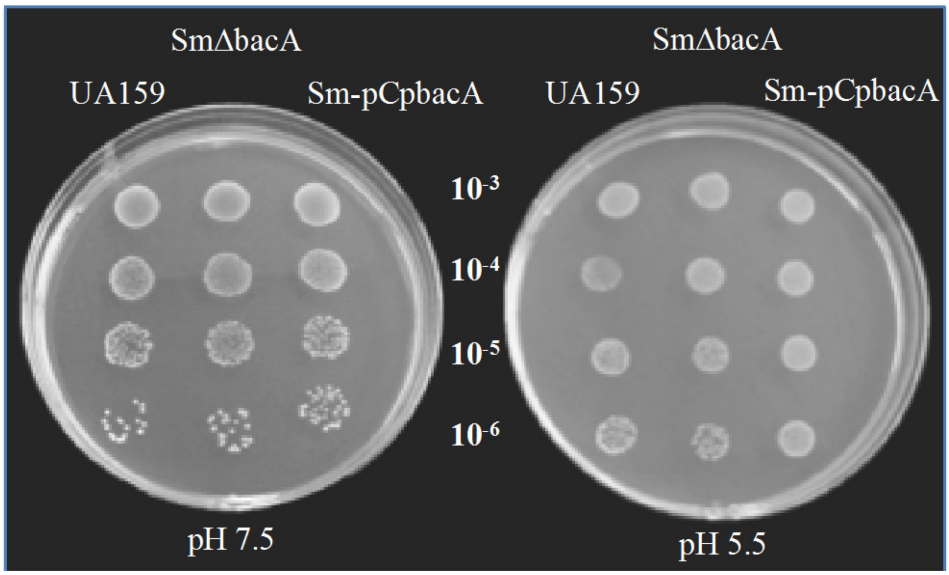

Figure 4. 17. Effects of low pH on the growth of *S. mutans* SmΔbacA strain. Both parent strain UA159 and SmΔbacA mutant strain were grown at pH 7.5 and 5.5 for 24-36 hours.

4.4.6 Deletion of SMU.244 did not Affect Genetic Competence of *S. mutans*

S. mutans is an organism that is naturally transformable (Li *et al.,* 2001b). Antibiotics can be a factor that induces or promotes the transformability of many bacteria, including *S. pneumonia* and probably *S. mutans* (Prudhomme *et al.,* 2006). However, this requires the existence of all intact machinery, such as the early and late competence genes, which allow the bacteria to transport, take up and integrate foreign DNA (Li *et al.,* 2001b). It was unknown whether SMU.244 gene was required for genetic competence in *S. mutans*. With this curiosity, we therefore examined the effect of deletion of SMU.244 gene on genetic competence of *S. mutans*. The result revealed that deletion of SMU.244 gene did not affect the transformation frequency of SmΔbacA mutant compared to the parent strain UA159 (Figure 4.18).

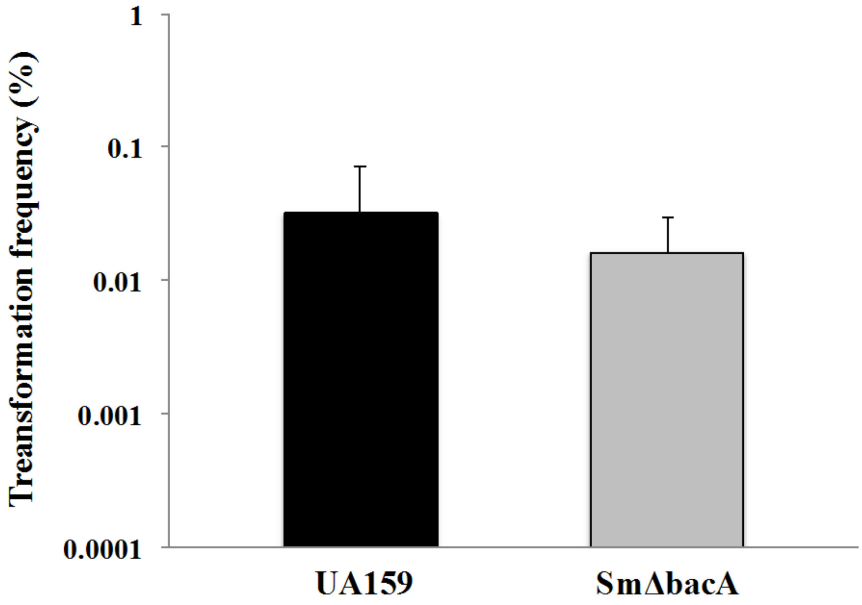

Figure 4. 18. Natural genetic transformation of SmΔbacA mutant and the parent strain UA159. The transformation frequency is expressed as the percentage of viable cells transformed to spectinomycin resistance. The results represent the average from two independent experiments and the error bars represent the standard deviation.

4.5 Regulation of SMU.244 Gene

A previous study showed that the BceABRS four-component system along with its controlled genes, such as SMU.862-864, SMU.302 and SMU.1856, was suggested to constitute the BceABRS regulon that played an important role in regulation of sensing, response and resistance to lipid II antibiotics, such as bacitracin in *S. mutans* (Ouyang *et al.,* 2010). However, it was unknown whether *bacA* was a part of the BceABRS regulon. To explore this question, we used quantitative real-time RT-PCR (qRT-PCR) to examine the expression of *bacA* gene in the genetic background of both wild-type UA159 and SmΔbceR mutant in response to a sub-inhibitory concentration of bacitracin (0.35 U/ml). The *bceS* gene was selected as a positive control, since this gene was directly controlled by BceABRS system and known to be up-regulated by induction of bacitracin (Ouyang *et al.,* 2010). We also included a *16S rRNA* gene (encoding 16S ribosome RNA subunit) as a positive control, since it is constitutively expressed in *S. mutans* (Tremblay *et al.,* 2009). The results revealed that in the wild type UA159 the expression of *bceS* gene was indeed induced for more than 2-fold by as low as 0.35 U/ml of bacitracin. In contrast, there was no significant difference in the expression of *bacA* before and after addition of bacitracin (Figure 4.19). In the strain of SmΔbceR mutant, however, both *bceS* and *bacA* genes showed no difference in their expression, no matter whether bacitracin was added or not (Figure 4.19). These results suggest that the *bacA* gene may not be controlled directly by the BceABRS signal transduction pathway.

We next examined the expression of *bacA* gene in the wild type strain of UA159 in response to increasing (subinhibitory) concentrations of bacitracin (2 U/ml, 6 U/ml, 12 U/ml, and 24 U/ml) over different time points (10 min, 20 min, and 30 min). The results revealed that the positive control *bceS* gene was highly induced in response to different sub-inhibitory concentrations of bacitracin over times. In contrast, there was no significant difference in the levels of expression of *bacA* gene in response to bacitracin at the same time points (Figure 4.20 and Appendix 1.3, 1.4, and 1.5). Similarly, the expression of the positive control gene (16s rRNA), which is a constitutively expressed gene, remained unchanged in *S. mutans* (data not shown). These results suggest that the expression of *bacA* gene may not be induced in response to bacitracin.

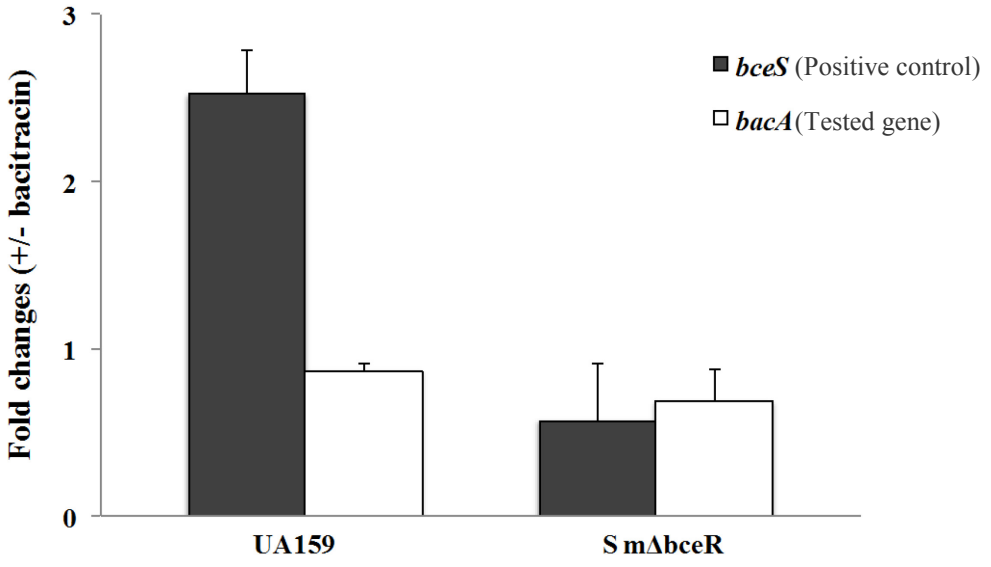

Figure 4. 19. qRT-PCR showed the relative level of expression of *bacA* gene in UA159 and SmΔbceR mutant in response to 0.35 U/ml of bacitracin. The results represent the average of duplicates from two independent experiments and the error bars represent the standard deviation.

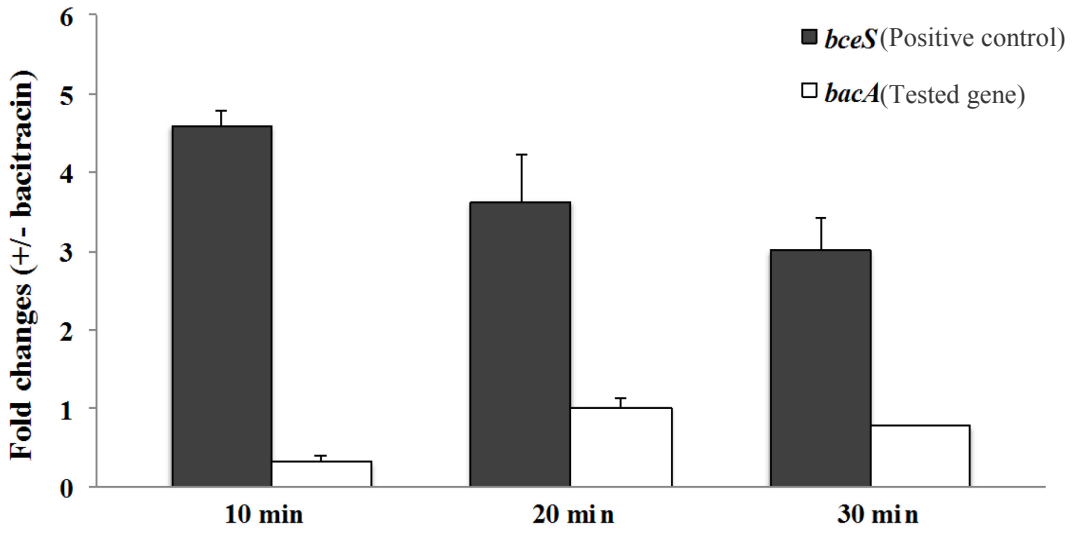

Figure 4. 20. The relative levels of expression of *bacA* and *bceS* (positive control) of *S. mutans* UA159 in response to 12U/ml of bacitracin. The results represent the average of duplicates from two independent experiments and the error bars represent the standard deviation.

4.6 Phenotypic Characterization of SMU.862-864 Deletion Mutants

4.6.1 Deletion of SMU.862-864 Genes did not Affect Antibiotics Sensitivity or Growth of *S. mutans*

In *S. mutans,* the BceABRS four-component system is known to positively regulate the expression of three downstream genetic loci, SMU.862, SMU.302, and SMU.1856 in response to bacitracin (Ouyang *et al.,* 2010). One of these genetic loci is a three-gene operon, SMU.862-864, which encodes an ABC transporter (exporter) but the role of this ABC transporter in antibiotics resistance in *S. mutans* is unknown. Therefore, we examined the effect of inactivation of SMU.862, SMU.863, SMU.864, and SMU.862-864 on antibiotic resistance, growth, and the virulence factors of *S. mutans*.

We first constructed individual mutants of SMU.862, SMU.863, and SMU.864 genes, and a mutant with the deletion of all three genes (SMU.862-4). We then examined the effect of deletion of these genes on cell-wall acting antibiotics, including bacitracin (2 U/ml), vancomycin (0.6 μg/ml) and penicillin G (0.03 μg/ml). The results showed that deletion of any one of SMU.862, SMU.863 or SMU.864 or the deletion of all three genes did not affect the susceptibility of *S. mutans* to these cell wall-acting antibiotics (Figure 4.21, 4.22, and Appendix 2.1-2.4). All the mutants showed no differences in their MICs against the antibiotics tested compered to the parent strain UA159 (Table 4.5).

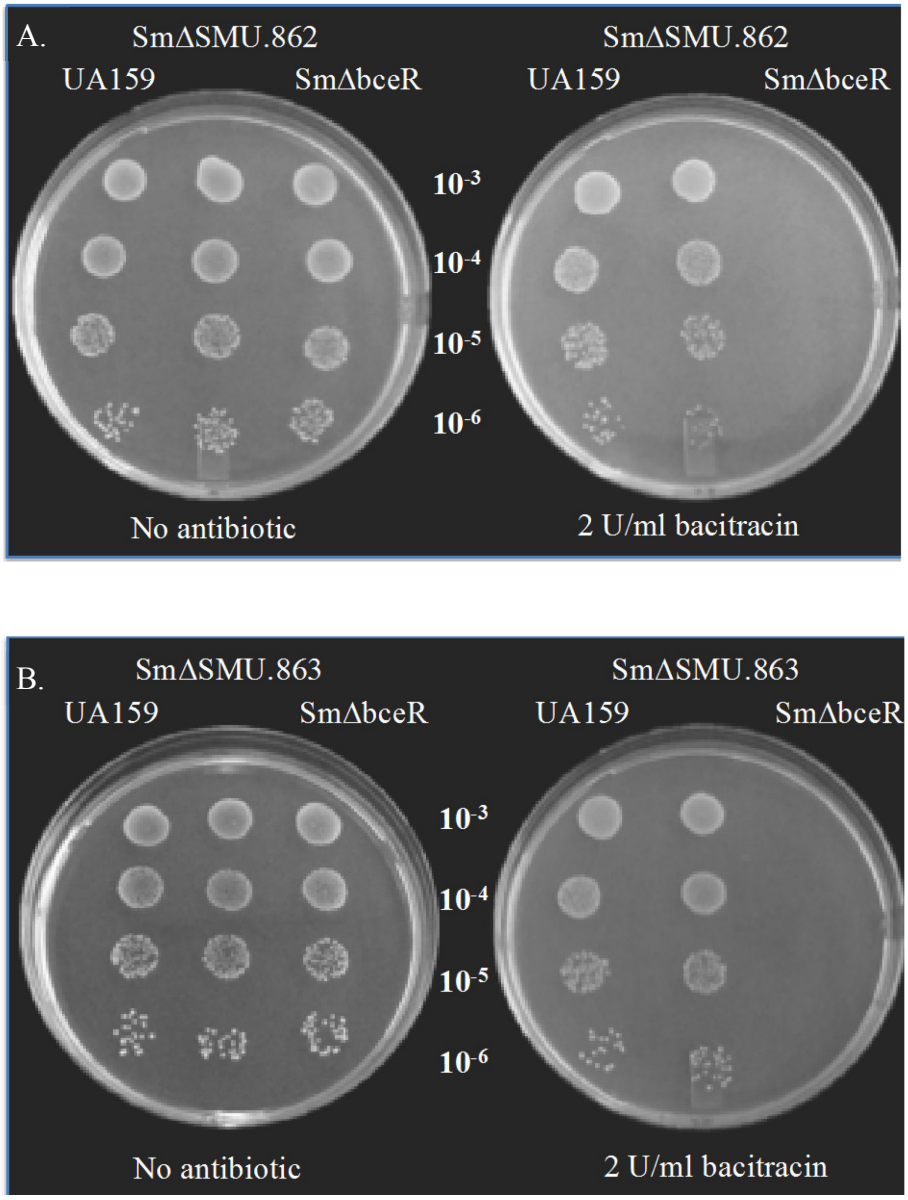

Figure 4. 21. Antibiotics sensitivity assay (plate dilution method) for SmΔSMU.862 and SmΔSMU.863. The wild type UA159 with either SmΔSMU.862 (A), or SmΔSMU.863 (B) were grown in the presence or absence of sub-inhibitory concentration of bacitracin 2 U/ml. The SmΔbceR strain was also used as a positive control.

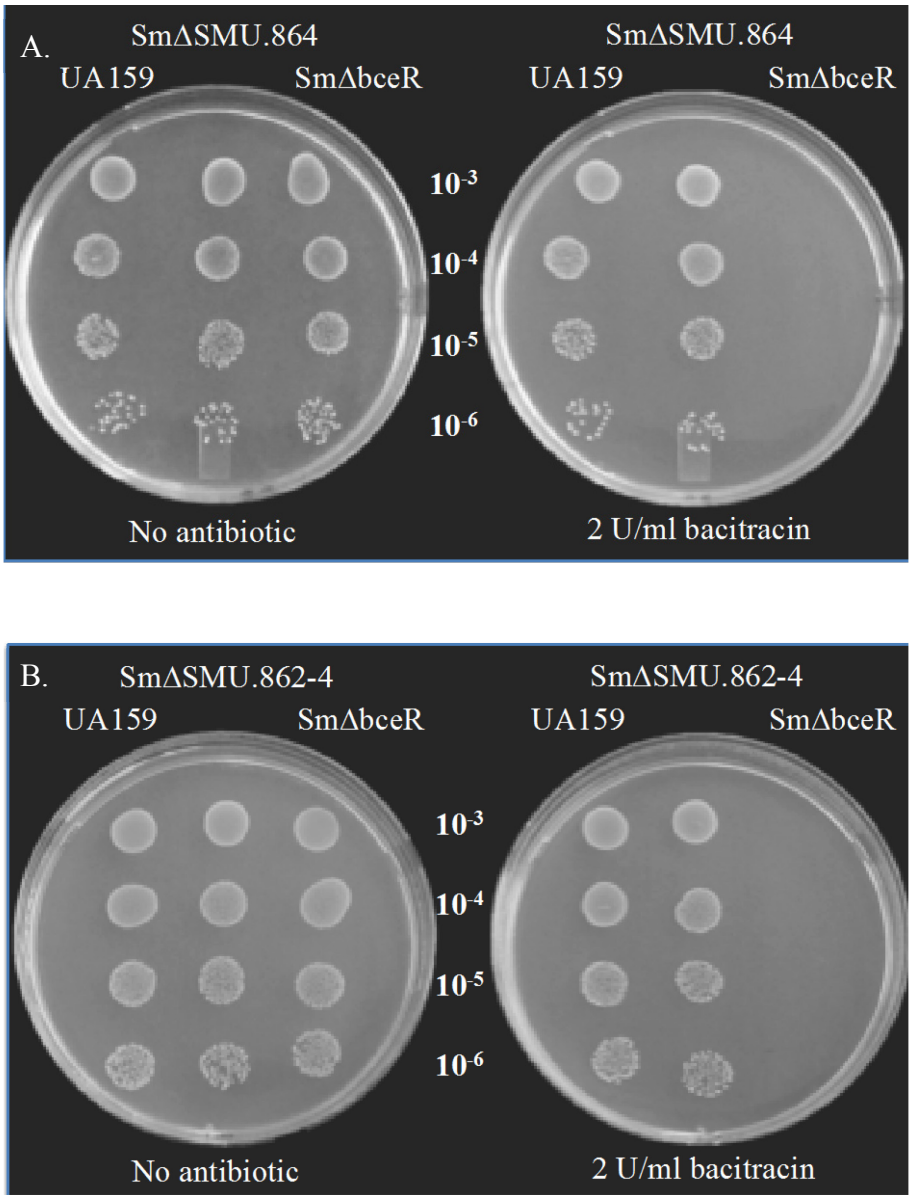

Figure 4. 22. Antibiotics sensitivity assay (plate dilution method) for SmΔSMU.864 and SmΔSMU.862-4. The wild type UA159 with either SmΔSMU.864 (A), or SmΔSMU.862-4 (B), were grown in the presence or absence of sub-inhibitory concentration of bacitracin 2 U/ml. The SmΔbceR strain was also used as a positive control.

Table 4. 5. Minimum inhibitory concentration (MIC) of *S. mutans* strains against selected antibiotics

Antimicrobial	MIC*				
	UA159	SmΔSMU.862	SmΔSMU.863	SmΔSMU.864	SmΔSMU.862-4
Cell wall-acting antibiotics					
Bacitracin (U/ml)	48 ± 0	48 ± 0	48 ± 0	48 ± 0	48 ± 0
Penicillin G*	0.062 ± 0	0.062 ± 0	0.062 ± 0	0.062 ± 0	0.062 ± 0
Vancomycin*	1.25 ± 0	1.25 ± 0	1.25 ± 0	1.25 ± 0	1.25 ± 0
Nisin*	17.5 ± 0	17.5 ± 0	17.5 ± 0	17.5 ± 0	17.5 ± 0
Protein-inhibitor antibiotics					
Kanamycin*	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Chloramphenicol*	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Tetracycline*	0.25 ± 0	0.25 ± 0	0.25 ± 0	0.25 ± 0	0.25 ± 0

* The concentration of penicillin G, vancomycin, nisin, kanamycin, chloramphenicol, and tetracycline were in μg/ml and the results represent averages ± SD for duplicates from two independent experiments.

The growth of the ABC transporter's mutant strains was also measured and compared to the wild-type *S. mutans* UA159. The results showed that in the absence of stress all the mutant strains were grown similar to the wild type UA159 with overlapped growth curves (similar double times and growth yields). No matter in the presence or in the absence of sub-inhibitory concentration of antibiotics, including bacitracin (0.48 U/ml), penicillin G (0.00781 μg/ml), vancomycin (0.3125 μg/ml) and nisin (4.375 μg/ml), all the mutant strains grew similarly compared to the parent UA159. There were no notable differences in the growth between four mutants and the parent UA159 (Table 4.6 and Appendix 2.5-2.9). The results suggest that deletion of these genes encoding the ABC transporter does not appears to affect their susceptibility or the growth in the presence of cell wall-acting antibiotics tested in this study.

Considering that this ABC transporter may be involved in export of protein-inhibitor antibiotics, we examined the MIC of these mutants against three protein inhibitory antibiotics, including kanamycin, chloramphenicol, and tetracycline. Again, the results revealed no differences in their susceptibility or the growth between these mutants and the parent UA159 (Table 4.5). All the strains showed similar doubling times with a slightly lower cell density in the presence of these protein synthesis inhibitors (Table 4.6 and Appendix 2.10-2.12).

We also examined the effects of deletion of SMU.862, SMU.863, SMU.864 individually or the deletion of all three genes on the growth and response of *S. mutans* in the presence of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $MnCl_2$, NaCl, and $FeCl_3$. The results showed no differences in the viability, growth, and response between all the mutants and the parent UA159. However, all the mutants and the parent UA159 showed longer doubling times but lower growth yields in the presence of higher concentrations of these salts (Table 4.7, 4.8 and Appendix 2.13-2.18).

Table 4. 6. Summary of the doubling time of *S. mutans* strains in the presence or absence of different antibiotics

Growth condition	UA159	SmΔSMU.862	SmΔSMU.863	SmΔSMU.864	SmΔSMU.862-4
THYE only	1: 05	1:02	1:00	1:02	1:02
Cell wall-acting antibiotics					
Bacitracin (0.48 U/ml)	1:00	0:55	0:58	0:59	0:57
Penicillin G (0.0078 µg/ml)	1:37	1:28	1:29	1:27	1:33
Vancomycin (0.3125 µg/ml)	1:02	1:11	1:02	1:04	1:04
Nisin (4.375 µg/ml)	1:14	1:12	1:20	1:20	1:14
Protein-inhibitor antibiotics					
Kanamycin (50 µg/ml)	3:11	3:16	3:06	3:04	3:05
Tetracycline (0.25 µg/ml)	3:36	3:45	3:38	3:29	3:46
Chloramphenicol (1 µg/ml)	3:00	3:10	3:12	3:12	3:09

The results were presented as (hours : minutes).

Table 4. 7. Minimum inhibitory concentration of *S. mutans* strains in the presence of metals or salts

Salts	Minimum inhibitory concentration*				
	UA159	**SmΔSMU.862**	**SmΔSMU.863**	**SmΔSMU.864**	**SmΔSMU.862-4**
NaCl (M/ml)	2 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0
$CaCl_2$ (mM/ml)	125 ± 0	125 ± 0	125 ± 0	125 ± 0	125 ± 0
$MgCl_2$ (M/ml)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
$ZnCl_2$ (mM/ml)	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0
$MnCl_2$ (mM/ml)	62.5 ± 0	62.5 ± 0	62.5 ± 0	62.5 ± 0	62.5 ± 0
$FeCl_3$ (mM/ml)	15.6 ± 0	15.6 ± 0	15.6 ± 0	15.6 ± 0	15.6 ± 0

* The results represent averages ± SD for duplicates from two independent experiments.

Table 4. 8. Summary of the doubling time of *S. mutans* strains in the presence or absence of different salts

Growth condition	UA159	SmΔSMU.862	SmΔSMU.863	SmΔSMU.864	SmΔSMU.862-4
THYE only	1: 05	1:02	1:00	1:02	1:02
NaCl (0.5 M)	1:36	1:36	1:40	1:44	1:41
MgCl (125 mM)	2:58	2:52	2:59	2:47	2:49
CaCl (62.5 mM)	2:45	2:53	2:46	2:48	2:44
ZnCl (2 mM)	2:12	2:01	2:00	2:01	2:13
MnCl (15.6 mM)	4:14	4:20	4:20	4:10	4:11
FeCl (3.9 mM)	1:38	1:32	1:36	1:39	1:34

The results were presented as (hours : minutes).

4.6.2 Deletion of SMU.862-864 Genes did not Affect the Acid Sensitivity or Biofilm Formation of *S. mutans*

All the mutants of SMU.862, SMU.863, and SMU.864 were also examined for their growth or resistance to acidic pH and biofilm formation in this study. The results showed no significant differences in acid sensitivity and biofilm formation between SmΔSMU.862, SmΔSMU.863, SmΔSMU.864, and SmΔSMU.862-4 mutants and the parent strain UA159 (Appendix 2.19-2.22).

Chapter 5. Discussion

S. mutans is well known to be resistant to bacitracin and this property is often exploited in the isolation of this organism from the highly diverse oral microflora (Liljemark *et al.,* 1976). Several mechanisms involved in bacitracin resistance have been identified in *S. mutans* (Lis and Kuramitsu, 2003; Ouyang *et al.,* 2010; Tsuda *et al.,* 2002). These mechanisms probably help *S. mutans* to cope with bacitracin or other peptide antibiotics produced not only by bacitracin-producers in the environments but also by competing species in dental plaque and by the host (Ouyang *et al.,* 2010; Tsuda *et al.,* 2002). To fully understand the molecular mechanisms how *S. mutans* senses, responds and resists to bacitracin, we had launched a genome-wide investigation of *S. mutants* to identify bacitracin-resistant genes. By screening a high-density transposon mutant library, we identified several transposon insertion mutants that were sensitive to bacitracin (Tian *et al.,* 2007). Among these mutants, two transposon insertion mutants were found to be in the same genetic locus of SMU.1006, and SMU.1008. We confirmed that these two genes along with SMU.1007 and SMU.1009 were organized as a four-gene operon that encodes the BceABRS four-component system required for bacitracin sensing and resistance in *S. mutans* (Ouyang *et al.,* 2010).

In this study, we further investigated a new transposon insertion mutant with an insertion within genetic locus SMU.244. We also extended our study into another genetic locus, SMU.862-864, since the promoter region of these genes carried a well-conserved BceR box that was directly controlled by the BceABRS four-component system required for bacitracin sensing, response and resistance (Ouyang *et al.,* 2010). We focused our investigation on the effects of deletion of these genes on the susceptibility of cell wall-acting antibiotics, biofilm formation and stress response.

5.1 Analysis of SmΔbacA Mutant Phenotypes

One of the most important finding from this study was that deletion of SMU.244 gene resulted in SmΔbacA mutant that was unable to grow in the presence of as low as 1.5 U/ml of bacitracin, which was 32-fold more sensitive to bacitracin than the parent strain UA159 (MIC: 48 U/ml). Re-introducing the wild-type copy of SMU.244 into SmΔbacA mutant completely restored the defective phenotype. These findings demonstrate that SMU.244 (*bacA*) play an important role in bacitracin resistance in *S. mutans*, which is highly consistent with the previous report from *S. pneumonia* and *S. aureus*. In these organisms, the deletion of *bacA* homologs also results in mutants that are defective in the cell wall synthesis and sensitive to bacitracin (Chalker *et al.,* 2000). Inactivation of *bacA* in *S. pneumoniae* resulted in a mutant that was not only more sensitive to bacitracin but also had a reduced virulence in a mouse model of infection (Chalker *et al.,* 2000). A similar observation is also reported with another *bacA* homolog, *bcrC* gene encoding undecaprenyl pyrophosphate phosphatase in *B. subtilis* (Bernard *et al.,* 2005; Ohki *et al.,* 2003b).

Previous studies show that *uppP* in *E. coli* encoded an undecaprenyl pyrophosphate phosphatase that is responsible for the biosynthesis and recycling of the lipid carrier by catalyzing the dephosphorylation of undecaprenyl pyrophosphate (Upp) to undecaprenyl phosphate (Up) in this organism (Figure 5.1) (El Ghachi *et al.,* 2004; Tatar *et al.,* 2007). The lipid carrier undecaprenyl phosphate (Up) is a key component in the cell wall biosynthesis of bacteria (Navarre and Schneewind, 1999). It arises from either dephosphorylation of the Upp via UppP enzyme or from the phosphorylation of undecaprenol (U-OH) via undecaprenol kinase (UK) enzyme. The Upp in turn results from either *de novo* synthesis by *cis*-prenyl pyrophosphate synthase (UppS) or release after the transfer of the peptidoglycan precursor (Coker and Palittapongarnpim, 2011; Navarre and Schneewind, 1999). Thus, we speculate that the increased susceptibility to bacitracin in SmΔbacA mutant might be due to both the reduction in the availability of free Up (lipid carrier) and the increase in the availability of Upp (target site for bacitracin) (Figure 5.1). This speculation is supported by the finding from *B. subtilis*, in which overexpression of *uppS* resulted in an increased susceptibility to bacitracin, while

inactivation of *uppS* in *B. subtilis* resulted in an increased resistance to bacitracin (Inaoka and Ochi, 2012). In addition, overexpression of *uppP* gene in *E. coli* or *bcrC* gene in *B. subtilis* (both genes encode UppP enzyme) resulted in increased bacitracin resistance in these organisms (Bernard *et al.,* 2005; Cain *et al.,* 1993; El Ghachi *et al.,* 2004).

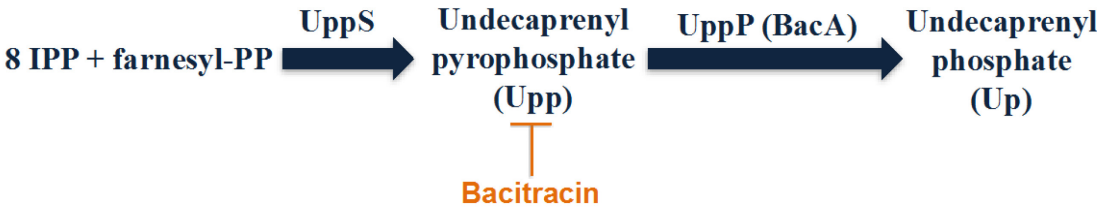

Figure 5. 1. Chemical reactions and enzymes involved in the biosynthesis of lipid carrier undecaprenyl phosphate (Up). UppS (*cis*-prenyl pyrophosphate synthase) enzyme catalyzes the synthesis of undecaprenyl pyrophosphate (Upp) from farnesyl pyrophosphate (farnesyl-PP) and eight molecule of isopentenyl pyrophosphate (IPP). Undecaprenyl pyrophosphate phosphatase (UppP or BacA) enzymatically convert Upp into the lipid carrier undecaprenyl phosphate (Up). Adapted from El Ghachi and colleagues (2004).

Since BacA or UppP plays a role in the cell wall biosynthesis, it is reasonable to assume that inactivation or deletion of SMU.244 might affect the cell wall synthesis and the susceptibility of this organism to cell-wall acting antibiotics other than bacitracin. Therefore, we investigated the susceptibility of SmΔbacA to several other cell-wall acting antibiotics. The results showed that SmΔbacA mutant was unable to grow in the presence of 0.03 μg/ml and 0.6 μg/ml of penicillin and vancomycin, respectively. Also, this mutant was 2-fold more sensitive to penicillin G, vancomycin, and nisin compared to the parent strain UA159. In *S. pneumonia* and *S. aureus*, however, deletion of *bacA* gene appears to have little effect on the susceptibility to vancomycin or amoxicillin (Chalker *et al.,* 2000). This divergence is unclear. One possible explanation is that these bacteria may have an alternative pathway for Up production that does not require the dephosphorylation Upp. This might occur through the phosphorylation of undecaprenol by undecaprenol kinase (Coker and Palittapongarnpim, 2011; Lis and Kuramitsu, 2003).

Another explanation is that these bacteria might have more than one gene that encodes UppP-like proteins, so that they might compensate the deletion of *bacA* gene. In *E. coli,* for example, four homologous proteins (UppP, YbjG, YeiU and PgpB) with the UppP activity were identified, among which UppP accounts for 75% of the total cellular UppP-activity in *E. coli* (El Ghachi *et al.,* 2005). Inactivation of all these genes *uppP*, *ybjG*, and *pgpB* (triple mutant) resulted in a significant growth defect and abnormal cell morphology in *E. coli* (El Ghachi *et al.,* 2005; Tatar *et al.,* 2007). In *S. mutans*, we do not know if any gene other than *bacA* might have UppP-like activity. This might explain why deletion of *bacA* in *S. mutans* resulted in such dramatic defects in resistance to bacitracin.

Remarkably, deletion of SMU.244 (*bacA*) gene also affected the growth of SmΔbacA mutant with a doubling time (T_d) of 1:22 h^{-1} compared to the parent UA159 (T_d = 1:05 h^{-1}). The growth of SmΔbacA mutant was drastically affected when grown in the presence of even as low as 0.48 U/ml of bacitracin (100-fold lower than the MIC for the parent strain UA159). The mutant showed an extremely long doubling time (T_d: 4:09 h^{-1}) when compared to the parent strain UA159 (T_d: 1:00 h^{-1}). The SmΔbacA mutant also showed a notable delay in the growth compared to the parent strain in the presence of a

sub-inhibitory concentration of penicillin G (0.00781 μg/ml), vancomycin (0.3125 μg/ml), or nisin (4.375 μg/ml). This finding suggests that SMU.244 is required for maintaining the normal growth rate of *S. mutans*. It is not surprising, because that the BacA protein appear to be the only enzyme with the UppP activity in *S. mutans*. The inability of the BacA deletion mutant to catalyze the reaction for the recycling of Upp to Up resulted in the unavailability of the lipid carrier (Up), therefore, blocking the following reactions required for the biosynthesis of peptidoglycan (Coker and Palittapongarnpim, 2011; Navarre and Schneewind, 1999). This defect might further affect the assembly of macromolecules in the biosynthesis of the cell wall, even cell growth and division (Vollmer *et al.,* 2008). However, inactivation of *bacA* gene in *S. aureus* and *S. pneumonia*e does not appear to significantly affect their growth rate (Chalker *et al.,* 2000). Again, this difference might be due to the presence of more than one UppP-like protein in these bacteria.

5.2 Regulation of SMU.244 (*bacA*) Gene Expression in Response to Bacitracin

Since deletion of *bacA* gene resulted in increased sensitivity of *S. mutans* to bacitracin, it was reasonable to assume that the expression of *bacA* (SMU.244) gene might be inducible by bacitracin. We examined relative levels of expression of *bacA* of *S. mutans* UA159 in response to increasing concentrations of bacitracin at different time points. Surprisingly, bacitracin at all concentrations tested did not significantly induce the expression of *bacA* gene, but rather the cells maintained relatively stable levels of expression of *bacA* during the growth at all the times tested. However, the expression of the positive control, *bceS* gene, was highly induced by bacitracin, suggesting that no significant induction of *bacA* gene in the presence of bacitracin was not because the concentrations of bacitracin tested were too low. Interestingly, the level of expression of the positive control, *16S rRNA* gene, was similar to that of *bacA* gene. Based on the available data from this study, we speculate that *bacA* gene in *S. mutans* may be constitutively expressed, independent on the presence of bacitracin or any other antibiotics. It is likely that *bacA* encoding an enzyme, undecaprenyl pyrophosphate phosphatase or UppP, which is always required for the cell wall synthesis and its integrity during the growth of *S. mutans*, no matter whether or not there is a lipid II inhibitory antibiotics in the environment.

5.3 A Proposed Model to Describe the Roles of SMU.244 (*bacA*) in the Cell Wall Synthesis and Recycling of the Lipid Carrier in *S. mutans*

Based on the findings from this study, we propose that SMU.244 in *S. mutans* encodes a BacA protein with an enzymatic activity involved in the synthesis and recycling of the lipid carrier during the cell wall biosynthesis in *S. mutans* (Figure 5.2). The evidence from this study strongly suggests that the BacA protein in *S. mutans* most likely functions as an enzyme that has the activity parallel to undecaprenyl pyrophosphate phosphatase or UppP in *E. coli*, which catalyzes the reaction of dephosphorylation to convert its substrate Upp to Up, a step of biochemical reactions essential for the synthesis and recycling of the lipid carrier during the cell wall biosynthesis (Tatar *et al.,* 2007).

Consistent with the previous studies in both Gram-positive and Gram-negative bacteria (Bernard *et al.,* 2005; Chalker *et al.,* 2000; El Ghachi *et al.,* 2004), the BacA in *S. mutans* appears to function as an enzyme to catalyze the dephosphorylation of Upp to Up. The BacA protein encoded by SMU.244 in *S. mutans* might be the only enzyme that plays an essential role in the recycling of the lipid carrier essential for the biosynthesis of the cell wall peptidoglycan in this organism. Because it catalyzes a key reaction involved in the recycling of the lipid carrier, the BacA protein in *S. mutans* appears to be absolutely required for the cell wall biosynthesis, the growth, and resistance to lipid II inhibitory antibiotics such as bacitracin. However, further investigation may be required to confirm the enzymatic activity of BacA in the proposed reaction and the regulatory mechanism in *S. mutans*.

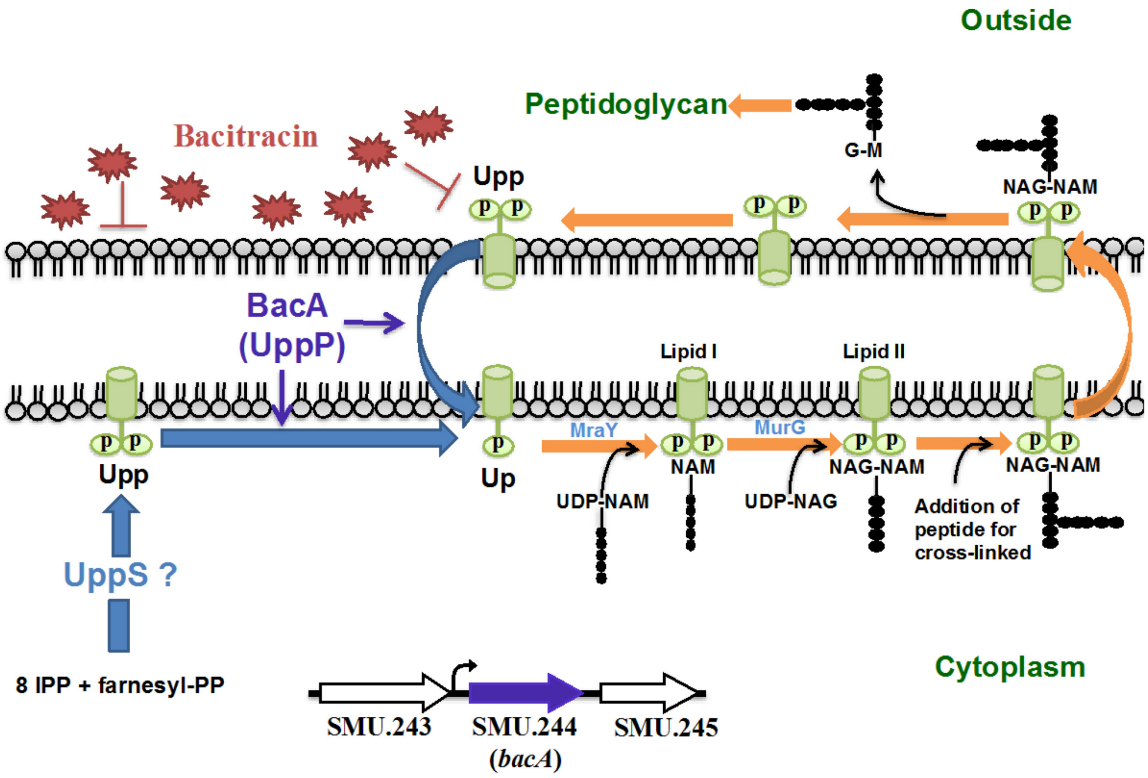

Figure 5. 2. A proposed model of BacA protein and its target site to catalyze the reaction in the biosynthesis and recycling of the lipid carrier in *S. mutans.* In this model, we propose that BacA in *S. mutans* function as UppP enzyme that catalyze the reaction to convert Upp to Up (lipid carrier). The lipid carrier is then used for the translocation of peptidoglycan precursor across cell membrane. Blue arrows indicate the process of lipid carrier recycling and biosynthesis including the role of SMU.244 (BacA or UppP). Orange arrows indicate the process of cell wall biosynthesis.

5.4 Is SMU.244 (*bacA*) a Part of the BceABRS Regulon in *S. mutans*?

In *S. mutans*, a four-component system, BceABRS is found to regulate bacitracin sensing, response and resistance (Ouyang *et al.,* 2010). This signal transduction pathway together with its controlled genes appears to form a cell wall-acting antibiotic sensing and detoxification regulon (Ouyang *et al.,* 2010). This is also the case for *Bacillus subtilis*, in which the BceABRS system directly regulates the activity of downstream genes in response to bacitracin or other cell wall-acting antibiotics (Jordan *et al.,* 2008; Rietkotter *et al.,* 2008).

In this study, we explored the question whether SMU.244 could be a part of the BceABRS regulon in *S. mutans*. We used qRT-PCR to examine the expression profiles of *bacA* gene in wild type UA159 and its isogenic mutant SmΔbceR in response to sub-lethal concentration of bacitracin (0.35 U/ml). We anticipated that genes that directly controlled by the BceABRS pathway might not be induced for expression in the *bceR* deletion mutant (SmΔbceR) even in the presence of bacitracin, because of the BceABRS signal transduction pathway was blocked in the *bceR* deletion mutant (SmΔbceR). The results showed that there was no significant difference in the relative levels of expression of *bacA* gene between SmΔbceR mutant and the parent UA159 in response to bacitracin (0.35 U/ml). The result suggests that *bacA* gene may not be a part of the BceABRS regulon in *S. mutans*. The evidence from this study suggests that the gene product and its functional activity of SMU.244 appears to represent a new ‘stand along’ mechanism for *S. mutans* to resist bacitracin and other cell-wall acting antibiotics.

5.5 Roles of SMU.244 (*bacA*) in Virulence-Associated Factors in *S. mutans*

In this study we examined if deletion of *bacA* in *S. mutans* had an effect on biofilm formation, especially when the SmΔbacA mutant was grown in the presence of low concentration of antibiotic such as bacitracin. In the absence of bacitracin, we found no difference in biofilm formation between SmΔbacA and the parent UA159. In the presence of a sub-inhibitory concentration of bacitracin (0.48 U/ml) SmΔbacA mutant formed a biofilm with significantly reduced biomass. The biofilm cells appeared to be aggregated, forming a sponge or net-like architecture compared to the parent strain. The defective biofilm could be readily visualized by conventional microscope. Such a biofilm phenotype was previously reported to be a kind of stress response of biofilm cells such as under acidic stress (Li *et al.,* 2002a).

It is reasonable to assume that BacA protein may be required for the maintenance of the cell wall integrity, since bacitracin at a concentration of as low as 0.48 U/ml (100-fold lower than the MIC of the parent strain) could affect the growth of biofilm of the mutant. The results from this study appear to favor the suggestion that the defective biofilm formed by SmΔbacA mutant primarily results from the growth arrest of the mutant cells due to the presence of bacitracin stress.

S. mutans is known to be a major bacterium to form dental biofilm, especially after the continuous consumption of carbohydrates rich-diet. The formation of biofilm by *S. mutans* is known to be important virulence factor for this pathogen to cause dental caries (Banas, 2004). In dental biofilm, many organisms produce antimicrobial peptides termed bacteriocins. In addition, the host also produce a number of antimicrobial compounds such as α- and β-defensin that can kill susceptible organisms (Kuramitsu *et al.,* 2007). These antimicrobial compounds are known to affect the cell envelope integrity, which is important for proper biofilm formation (Kolenbrander *et al.,* 2010; Loo *et al.,* 2000). The sub-lethal concentrations of these antimicrobial compounds could also act as a signal to altered biofilm formation (Kaplan, 2011; Marsh, 2004).

Thus, it is reasonable to assume that inhibiting SMU.244 gene product in this environment could probably affect the ability of *S. mutans* to form a proper biofilm. We speculate that SmΔbacA mutant would be likely much less competitive to grow in multi-species dental biofilms if this mutant were introduced into the oral cavity in an animal model. We also speculate that the abilities of *S. mutans* to persist in dental biofilm and to initiate dental caries would be likely attenuated in natural dental plaque biofilms. This speculation is supported by the finding from *S. aureus* and *S. pneumonia*, in which deletion of *bacA* gene in both pathogens resulted in an attenuation of virulence in a mouse model of infection (Chalker *et al.,* 2000). Further study using an animal caries model may be very useful to answer this question in *S. mutans*.

The ability of *S. mutans* to tolerate acidic environment is also considered to be an important virulence factor for this pathogen to cause dental caries (Banas, 2004). The integrity of the cell envelope is important for *S. mutans* to cope with various stresses including acids (Lemos and Burne, 2008; Matsui and Cvitkovitch, 2010). We examined the growth of SmΔbacA on THYE agar plate with lower pH (5.5) to determine if deletion of SMU.244 could affect the ability of *S. mutans* to tolerate acids. The results showed that although the mutant grew more slowly at pH 5.5 than pH 7.5, but it showed little difference in acid resistance from the parent strain UA159. The result suggests that deletion of SMU.244 did not affect the growth of *S. mutans* at lower pH (pH 5.5).

S. mutans is also known to take up foreign DNA from its environment when becoming genetically competent (Perry and Kuramitsu, 1981). This enables the recipient cells to acquire novel genes or heritable traits, promoting the emergence of genetic variations, antibiotic resistance, and rapid evolution of virulence factors (Lorenz and Wackernagel, 1994). In this study, we examined the effect of deletion of SMU.244 gene on genetic competence of *S. mutans*. However, the result revealed little difference in the transformation efficiency between SmΔbacA and the parent UA159, suggesting that inactivation of SMU.244 did not affect genetic competence of *S. mutans.* Collectively, the evidence from this study suggests that BacA is an important defense mechanism against bacitracin and other related antibiotics in *S. mutans*.

5.6 Analysis of the ABC Transporter (SMU.862-4) Mutants Phenotypes

In this study, we have extended our investigation into another genetic locus of SMU.862-864 that encodes an ABC transporter in *S. mutans*. One of the major reasons was that we previously identified a well-conserved invert repeat or BceR box ($TTACAA_{TT}TTGTAA$) at the promoter region upstream of SMU.862-864 (Figure 4.3). The initial work further confirmed that these three genes appeared to be up-regulated by the BceABRS four-component system in response to bacitracin (Ouyang *et al.,* 2010). Therefore, we hypothesized that these three genes of SMU.862-864 encoded an ABC transporter that might be involved in resistance of bacitracin or cell wall-acting antibiotics or potentially in response to some stressful conditions.

Our work first confirmed that three genes of SMU.862, SMU.863, and SMU.864 were indeed organized as an operon, and their expression were most likely regulated by the same regulatory elements. To further investigate functional roles of this three-gene operon, especially their involvement in bacitracin resistance in *S. mutans*, we constructed individual gene deletion mutants of SMU.862, SMU.863, and SMU.864 as well as a mutant with deletion of the three genes to examine their phenotypes in response to bacitracin and other antibiotics. Surprisingly, our results did not appear to support the original hypothesis, since we found no significant differences in the susceptibility to various antibiotics tested between these mutants and the parent UA159. Neither did we find that this ABC transporter was involved in biofilm formation and response to several stressful conditions.

Currently, we do not know which substrate that this ABC transporter transports. The positive outcome from these results is that we excluded the involvement of this ABC transporter in resistance to bacitracin and other tested antibiotics. It may need to first develop a new rationale to probe the question about what this ABC transporter might transport using a high-throughput screening technology. It may also need to investigate the involvement of this ABC transporter in stress response and the virulence again under more defined conditions after identification of its substrate and growth conditions of this ABC transport.

5.7 General Conclusion

The results obtained from this study showed that SMU.244 gene encodes a protein that share high homology with UppP enzyme in *E. coli* and BacA in *S. pneumoniae*. Deletion of this gene (SMU.244) resulted in a dramatic defects in the growth, resistance to bacitracin and several other cell wall-acting antibiotics. This mutant also formed a defective biofilm in the presence of a subinhibitory concentration (100-fold lower than the MIC for the parent strain) of bacitracin. We speculate that this mutant may be significantly attenuated with its ability to survive in natural dental biofilms and its cariogenic potential, which could be further examined in a rat caries model. The evidence from this study suggests that SMU.244 protein may have enzymatic activity in a similar manner to UppP in *E. coli* and BacA in *S. pneumoniae* (Chalker *et al.,* 2000; El Ghachi *et al.,* 2004). Therefore, further study may be necessary to investigate the functional activity of SMU.244 protein as an enzyme UppP.

In this study, we also investigated another genetic locus that encodes ABC transporter (exporter). Based on the methods used in this study, we are not able to draw the conclusion that this ABC transporter is directly involved in resistance of bacitracin or other related antibiotics in *S. mutans*. We also excluded the involvement of this ABC transporter in biofilm formation and response to several stressful conditions. Further study may be necessary to investigate functional roles of this ABC transporter in physiology and virulence of *S. mutans* under more defined conditions.

5.8 Future Direction

The work presented in this study provides new insights into the mechanism involved in bacitracin resistance in *S. mutans*. In this study, we have also identified some interesting questions that require further investigation. In the case of SMU.244 (*bacA*), this gene encodes an enzyme termed undecaprenyl pyrophosphate phosphatase. Thus, analysis of enzymatic activity of BacA protein may offer a better understanding of its functional role in the cell wall biosynthesis of *S. mutans*. This can be achieved using recombinant protein expression technology and enzymatic analysis techniques.

In addition, the *S. mutans* genome contains an open reading frame of SMU.1786c that encodes a homolog of UppS enzyme, which may also involved in the synthesis of the lipid carrier in *S. mutans*. Therefore, determining the role of this gene product in cell wall biosynthesis and bacitracin resistance may also help our understanding of BacA function in *S. mutans*. This can be achieved by analyzing the effect of inactivation of SMU.1786c gene on antibiotic resistance (e.g. bacitracin).

To test the hypothesis that SMU.244 (*bacA*) gene product might also be involved in the virulence and cariogenic potential in *S. mutans*, a specific-pathogen-free rat model can be used to test the effect of inactivation of SMU.244 gene on oral colonization and cariogenic potential of *S. mutans*.

In the case of ABC transporter encoded by SMU.862-864, further experiments could be carried out to search for substrates that might be transported via this system. The first step is to develop a rationale about what this ABC might transport before selection of a high-throughput screening method.

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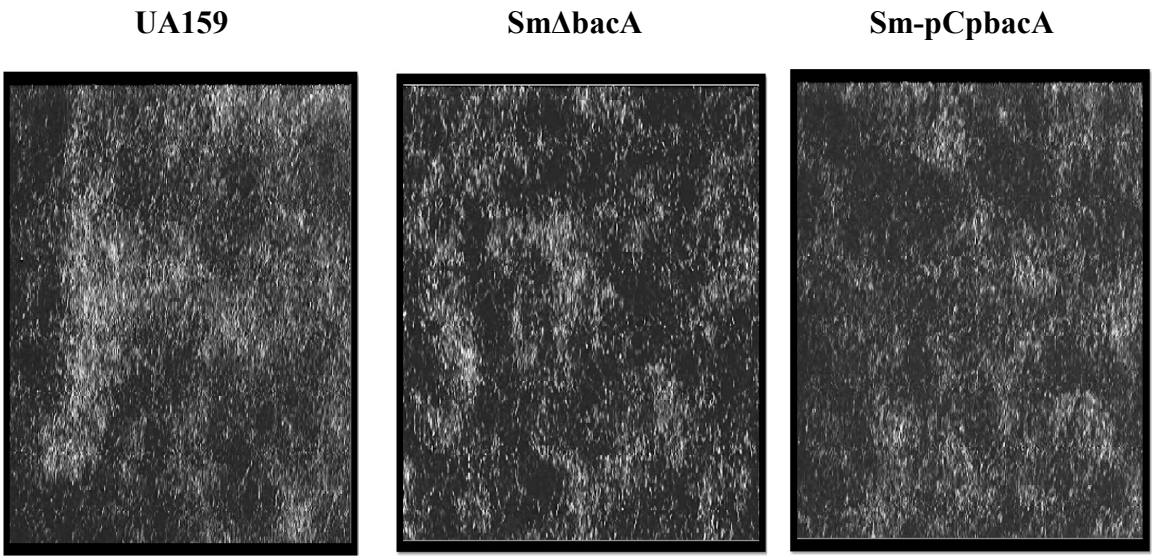
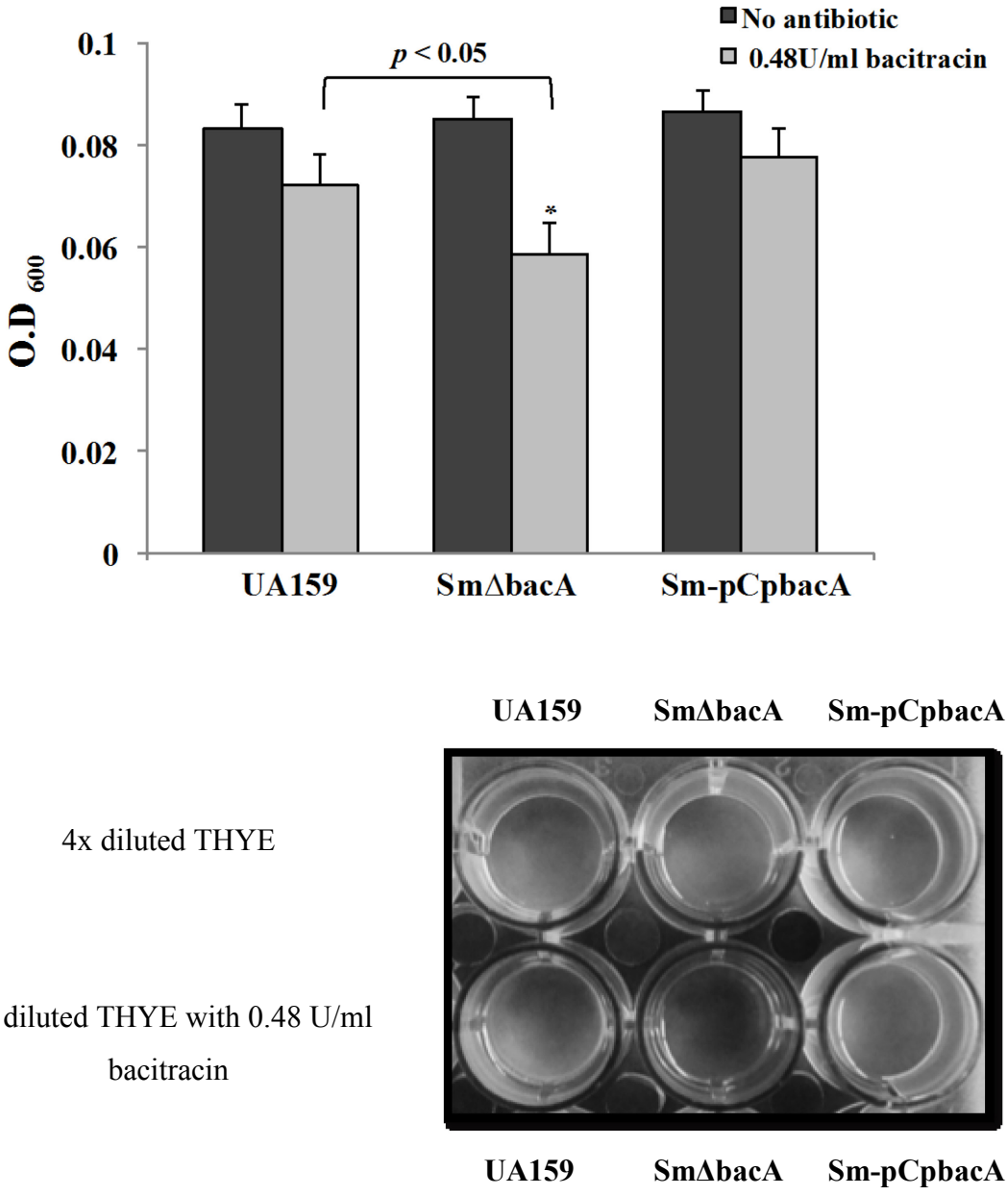
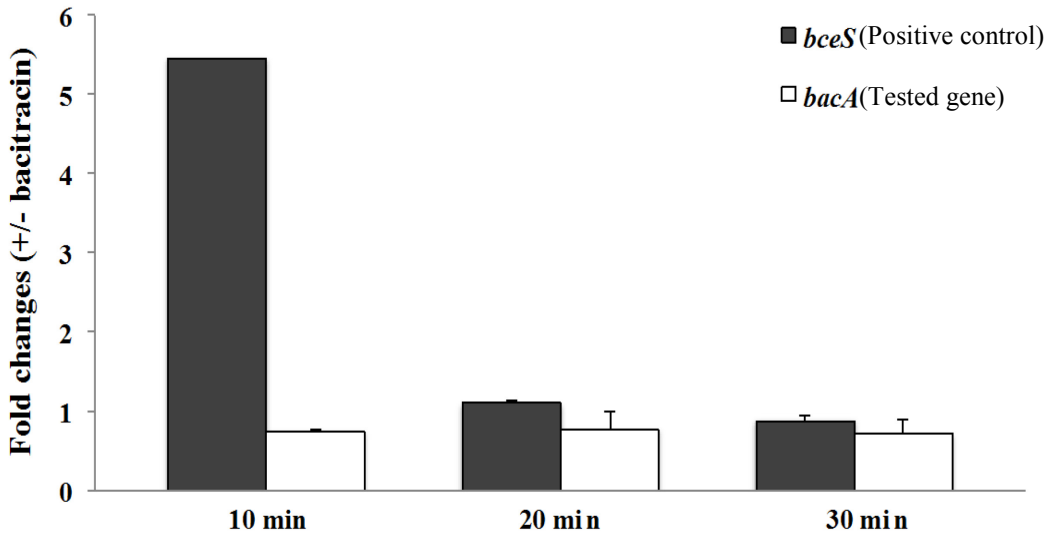
Appendix 1. SMU.244 (BacA)



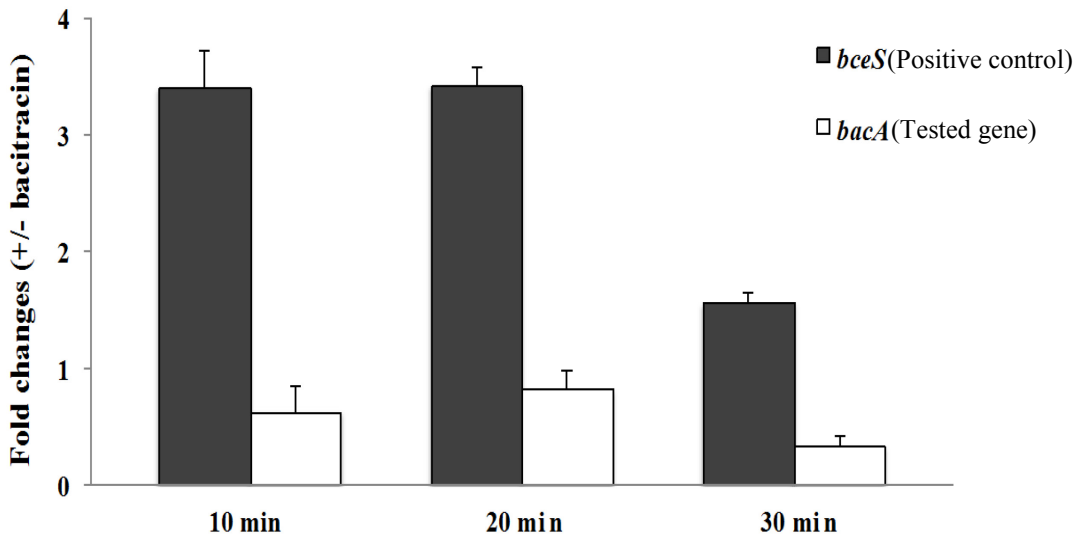
Appendix 1. 1. Conventional microscopy showed 16 hours biofilm formed by wild type *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA. The strain was cultured in 4x diluted THYE plus 5 mM sucrose without bacitracin. Representative fields were viewed at 1000X.



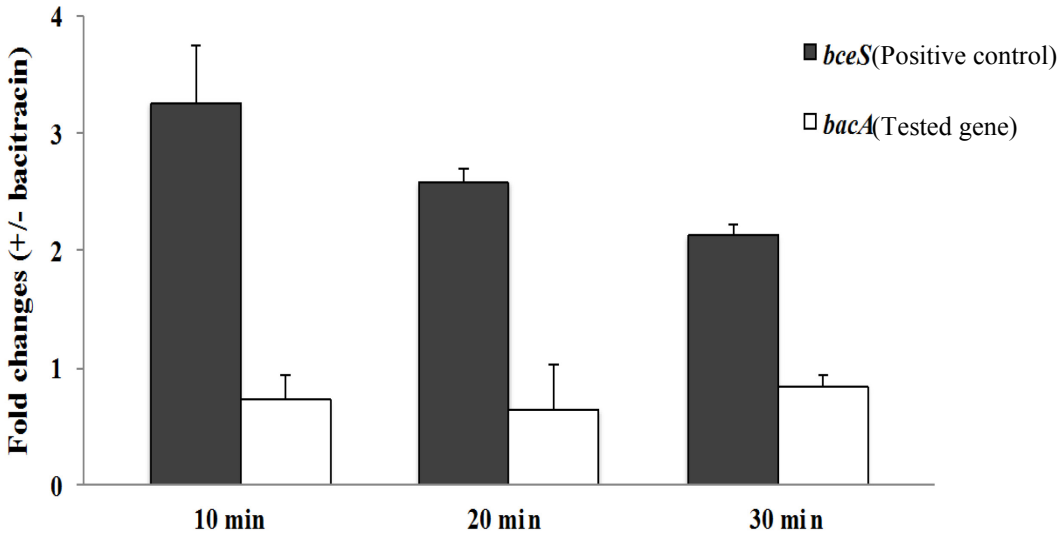
Appendix 1. 2. Biofilm formation by *S. mutans* UA159, SmΔbacA, and Sm-pCpbacA in 4x diluted THYE with or without 0.48 U/ml of bacitracin. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation.



Appendix 1. 3. The relative levels of expression of *bacA* and *bceS* (positive control) of *S. mutans* UA159 in response to 2 U/ml of bacitracin. The results represent the average of duplicates from two independent experiments and the error bars represent the standard deviation.

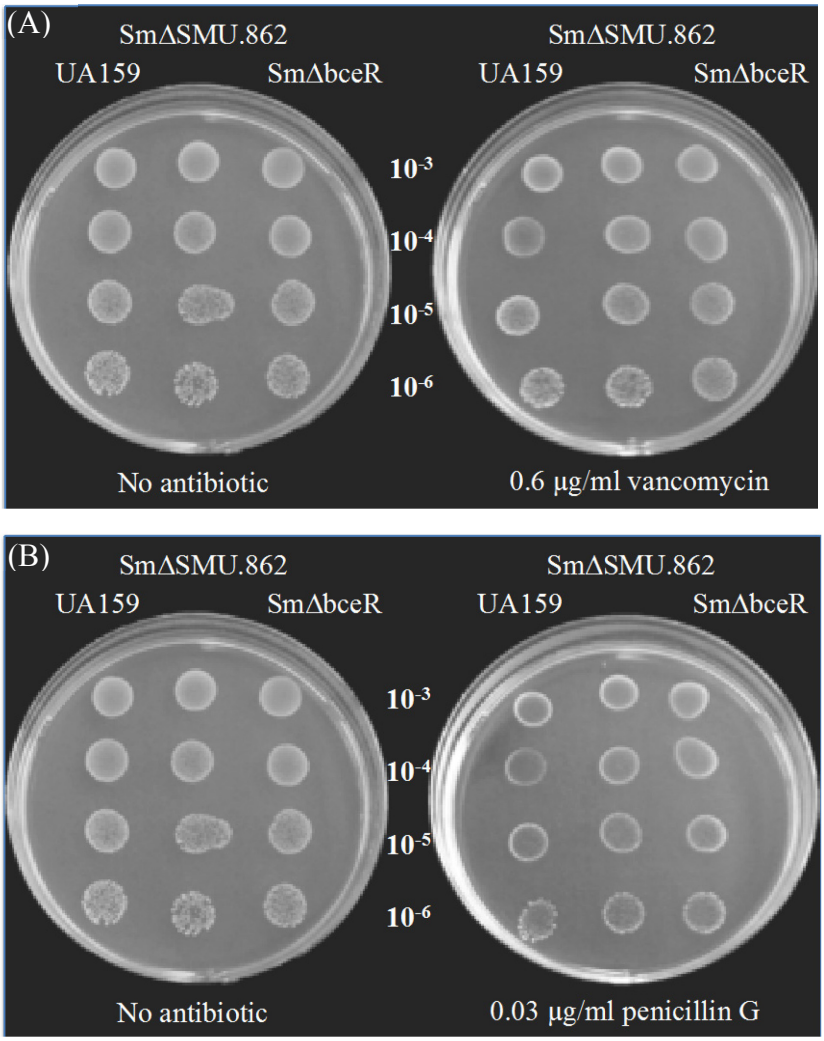


Appendix 1. 4. The relative levels of expression of *bacA* and *bceS* (positive control) of *S. mutans* UA159 in response to 6 U/ml of bacitracin. The results represent the average of duplicates from two independent experiments and the error bars represent the standard deviation.

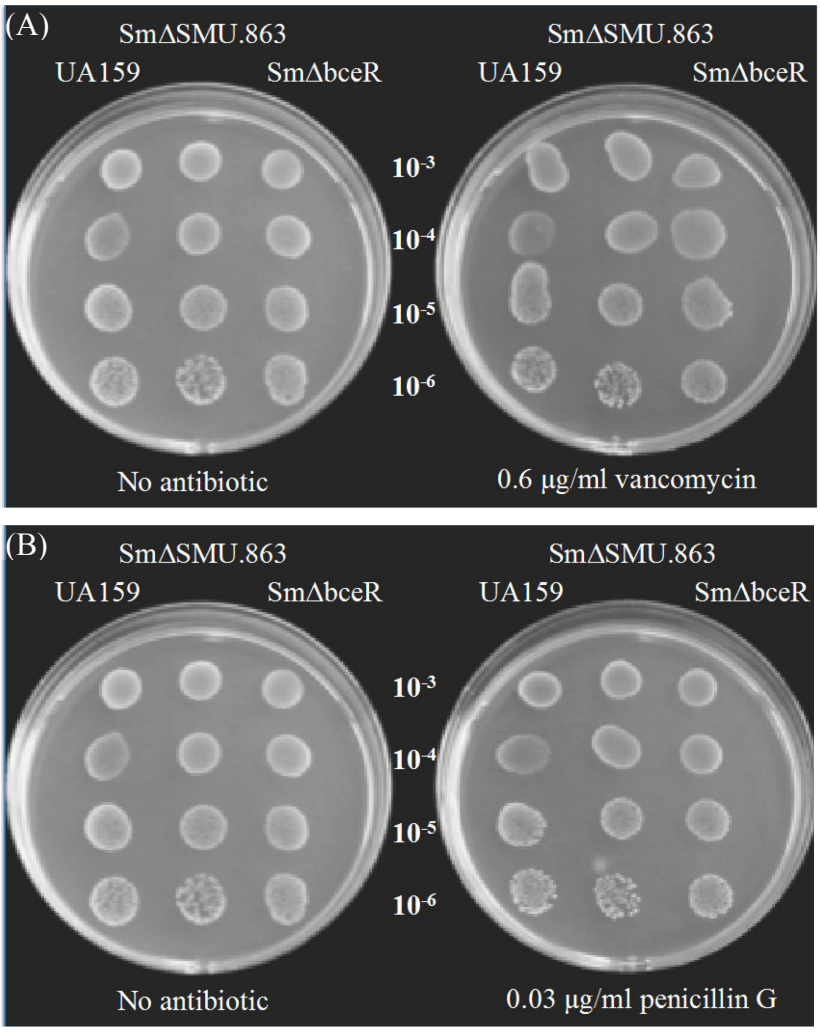


Appendix 1. 5. The relative levels of expression of *bacA* and *bceS* (positive control) of *S. mutans* UA159 in response to 24 U/ml of bacitracin. The results represent the average of duplicates from two independent experiments and the error bars represent the standard deviation.

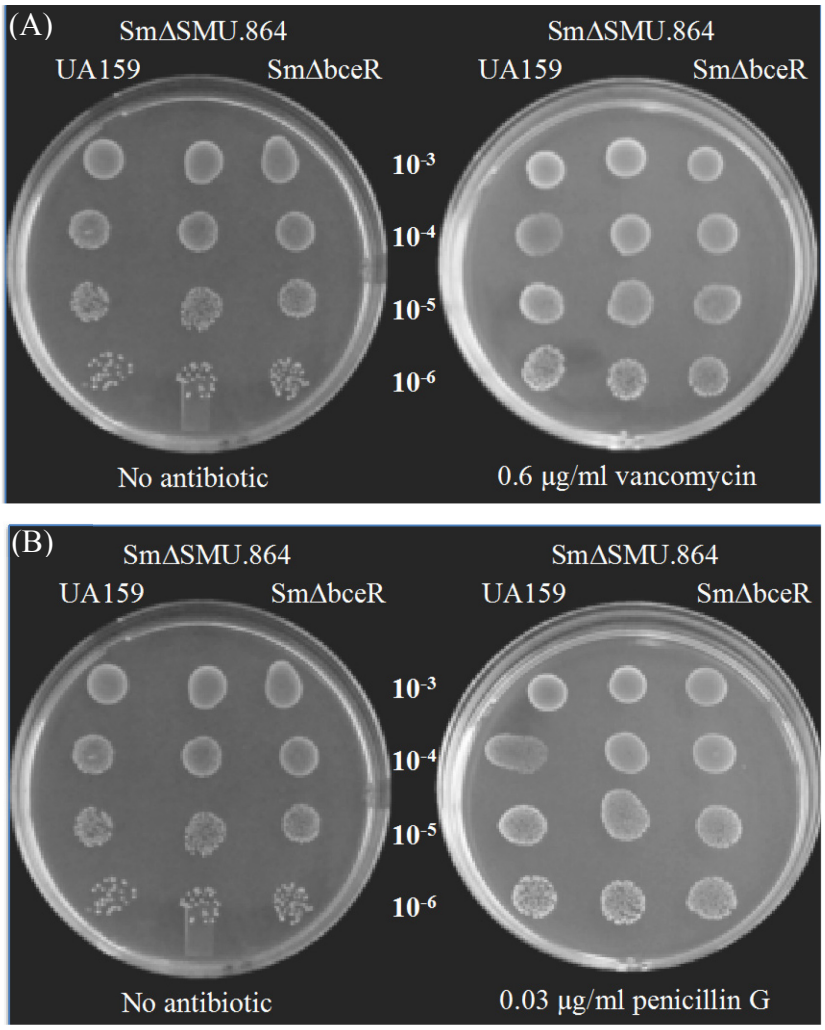
Appendix 2. SMU.862-864



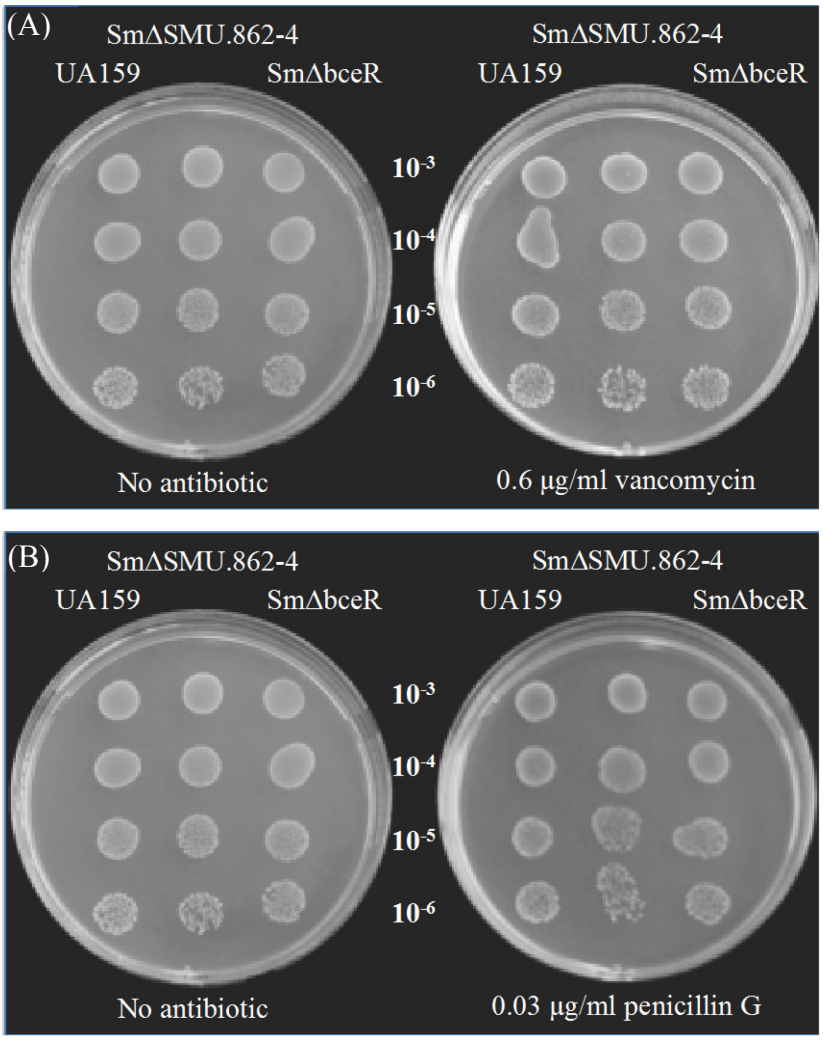
Appendix 2.1. Antibiotic sensitivity of *S. mutans* UA159 and SmΔSMU.862 to 0.6 μg/ml of vancomycin (A) and 0.03 μg/ml penicillin G (B).



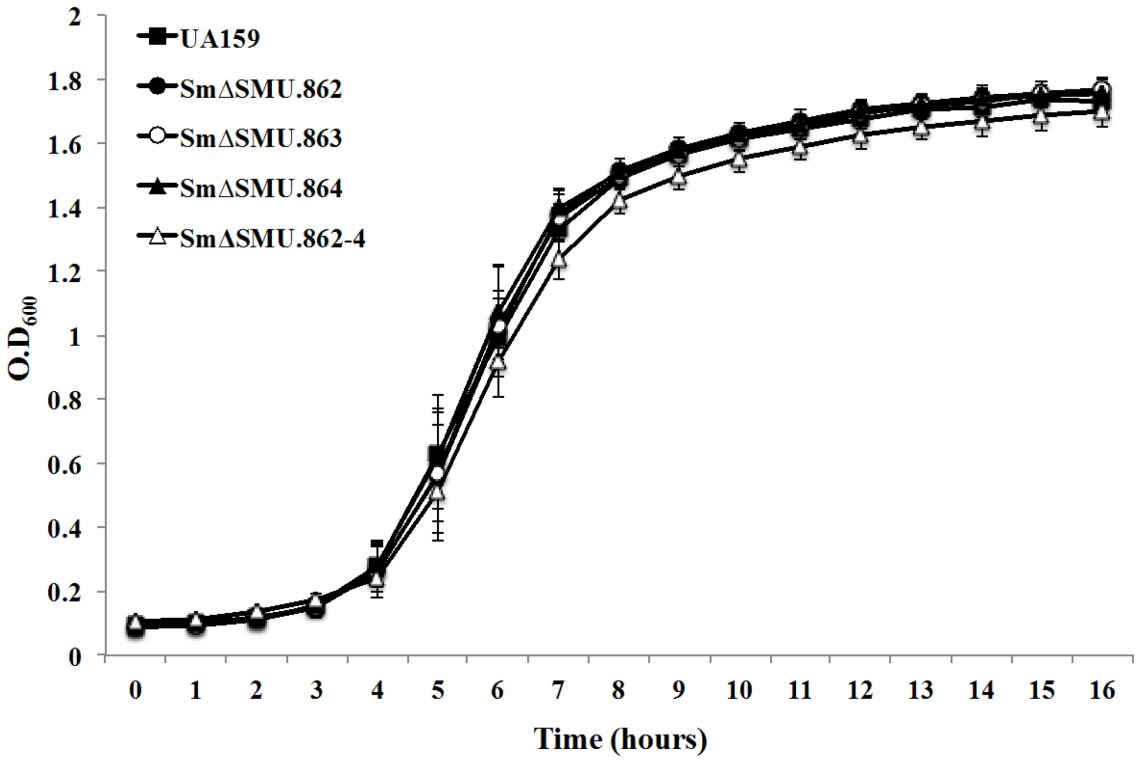
Appendix 2.2. Antibiotic sensitivity of *S. mutans* UA159 and SmΔSMU.863, to 0.6 µg/ml of vancomycin (A) and 0.03 µg/ml penicillin G (B).



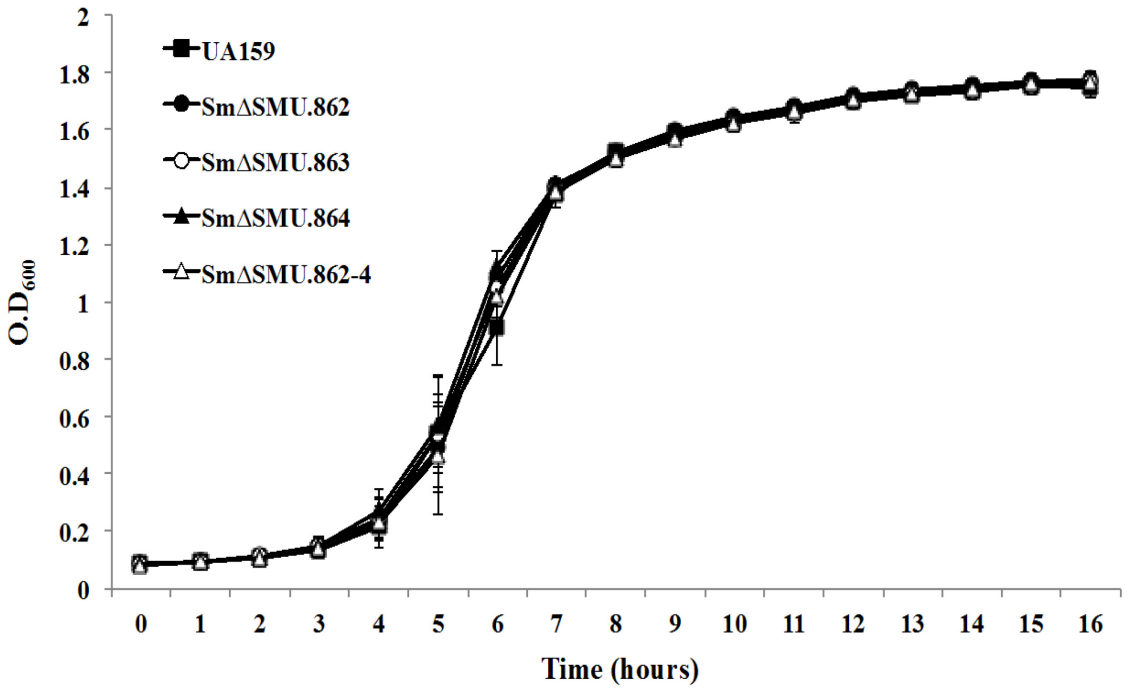
Appendix 2.3. Antibiotic sensitivity of *S. mutans* UA159 and SmΔSMU.864 to 0.6 µg/ml of vancomycin (A) and 0.03µg/ml penicillin G (B).



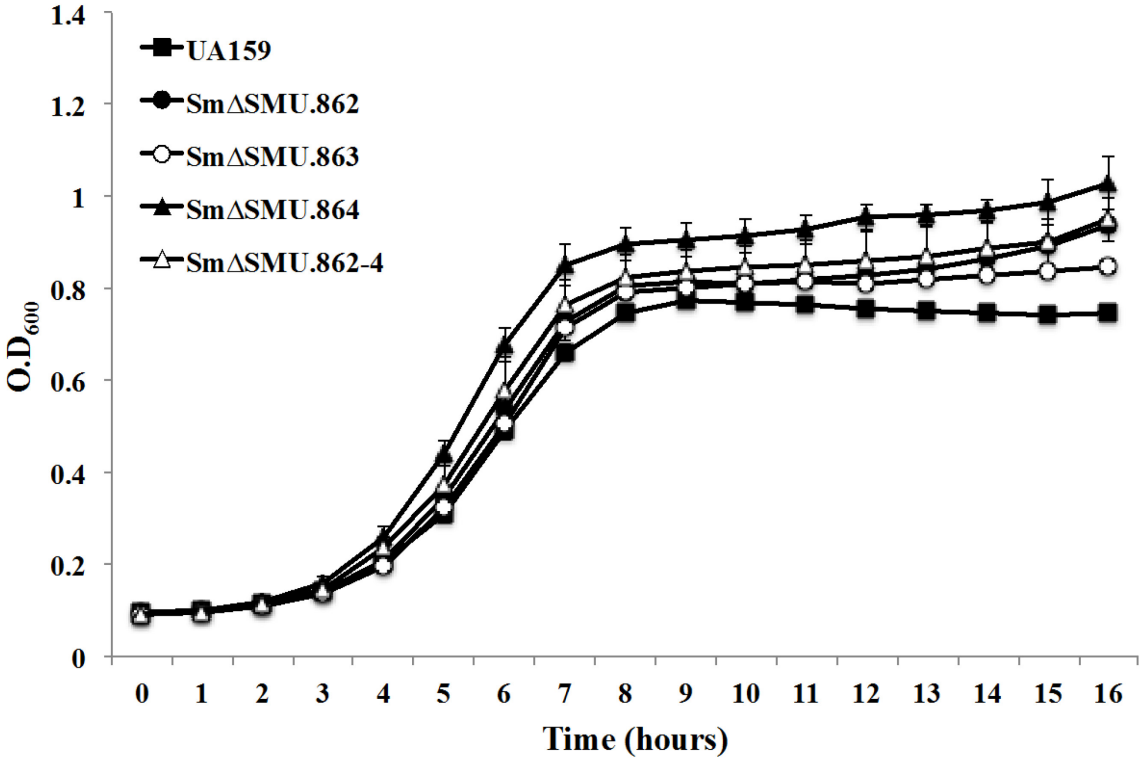
Appendix 2.4. Antibiotic sensitivity of *S. mutans* UA159 and SmΔSMU.862-4 strains to 0.6 μg/ml of vancomycin (A) and 0.03 μg/ml penicillin G (B).



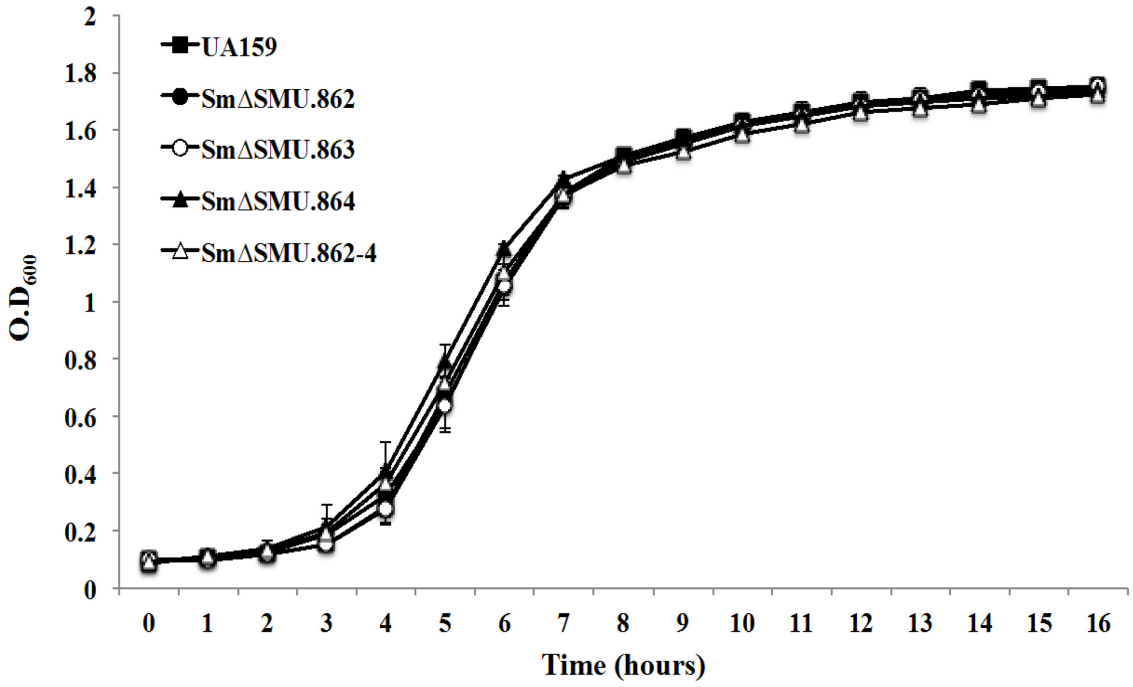
Appendix 2.5. Growth of *S. mutans* strains in THYE broth only. The results represent the means of duplicates from two independent experiments.



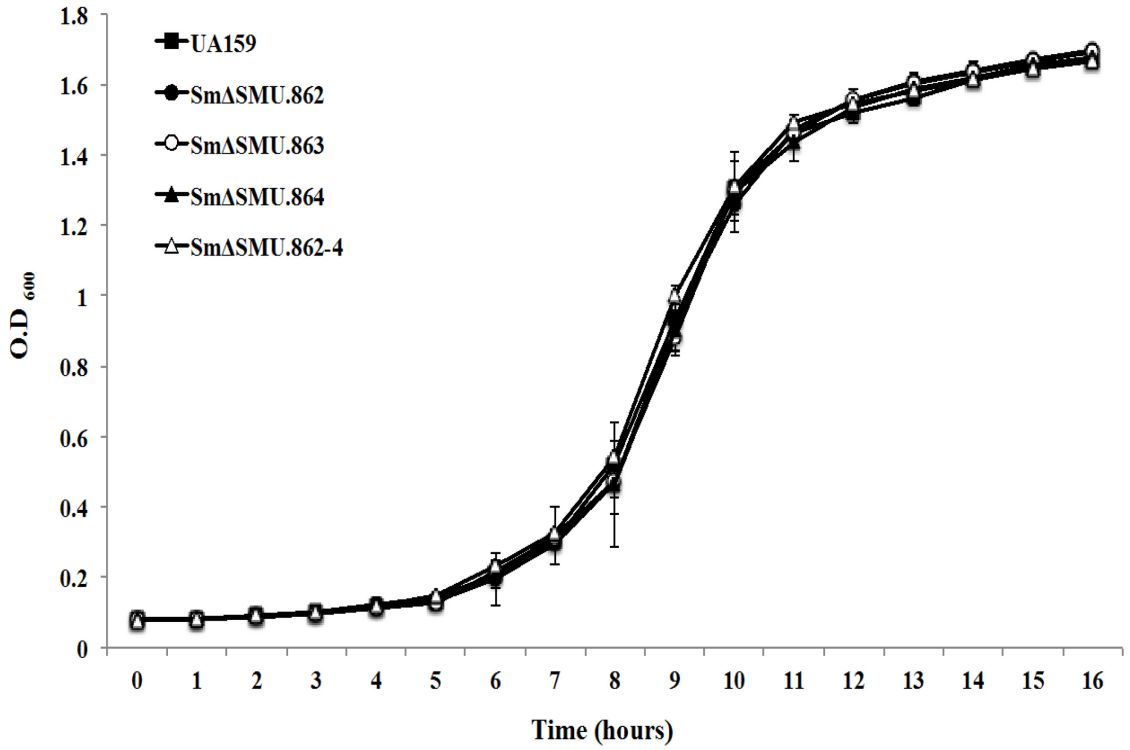
Appendix 2.6. Growth of *S. mutans* strains in THYE with 0.48 U/ml of bacitracin. The results represent the means of duplicates from two independent experiments.



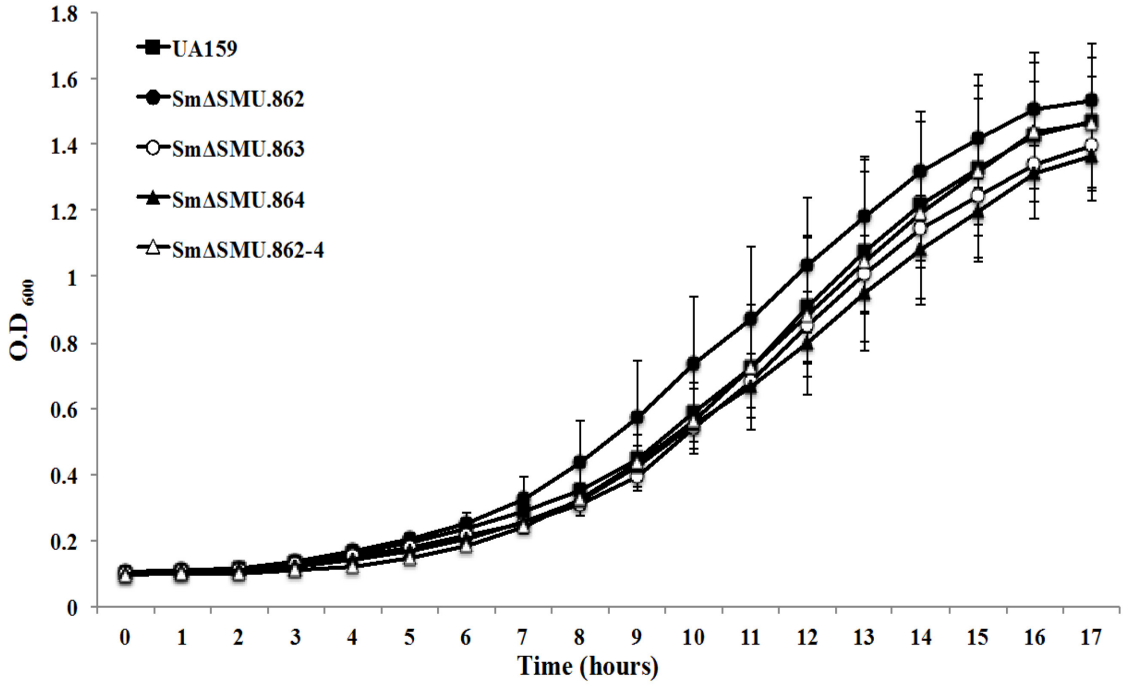
Appendix 2.7. Growth of *S. mutans* strains in THYE with 0.00781 µg/ml of penicillin G. The results represent the means of duplicates from two independent experiments.



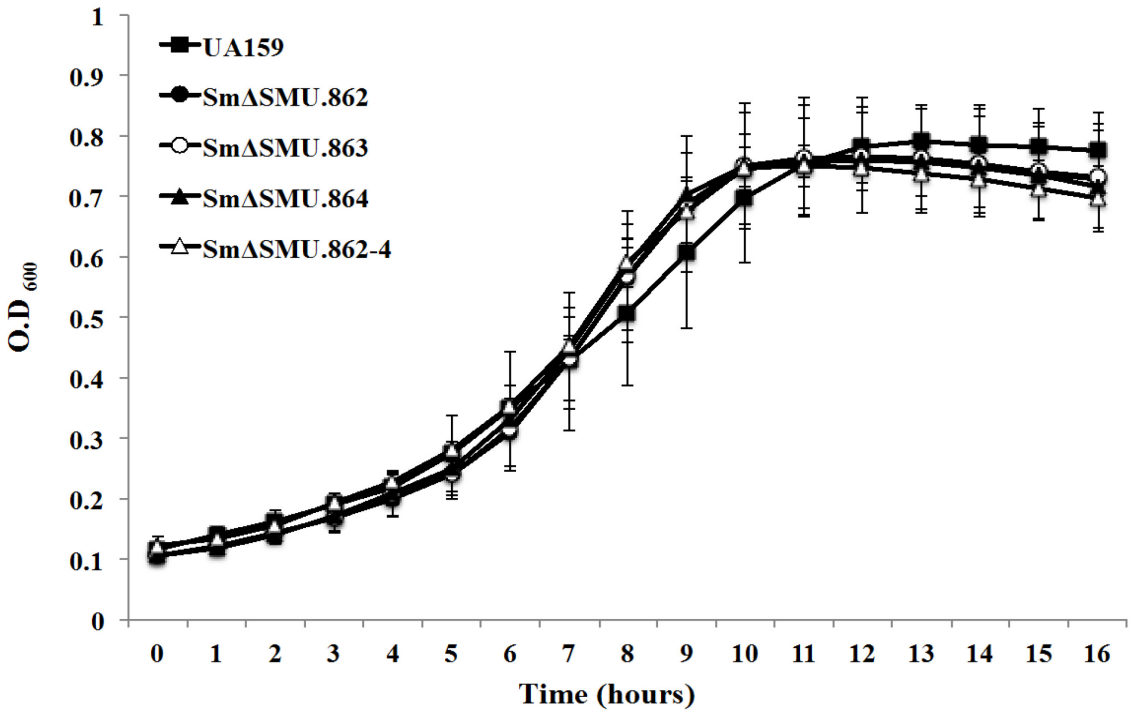
Appendix 2.8. Growth of *S. mutans* strains in THYE with 0.3125 μg/ml of vancomycin. The results represent the means of duplicates from two independent experiments.



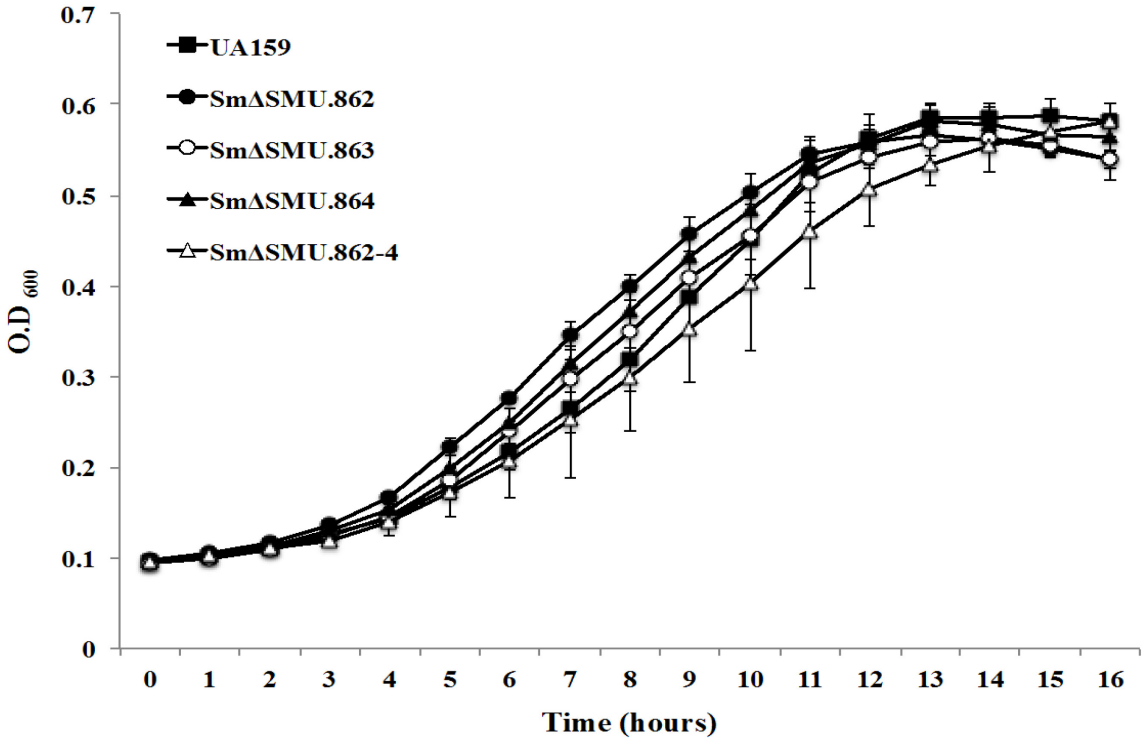
Appendix 2.9. Growth of *S. mutans* strains in THYE with 4.375 µg/ml of nisin. The results represent the means of duplicates from two independent experiments.



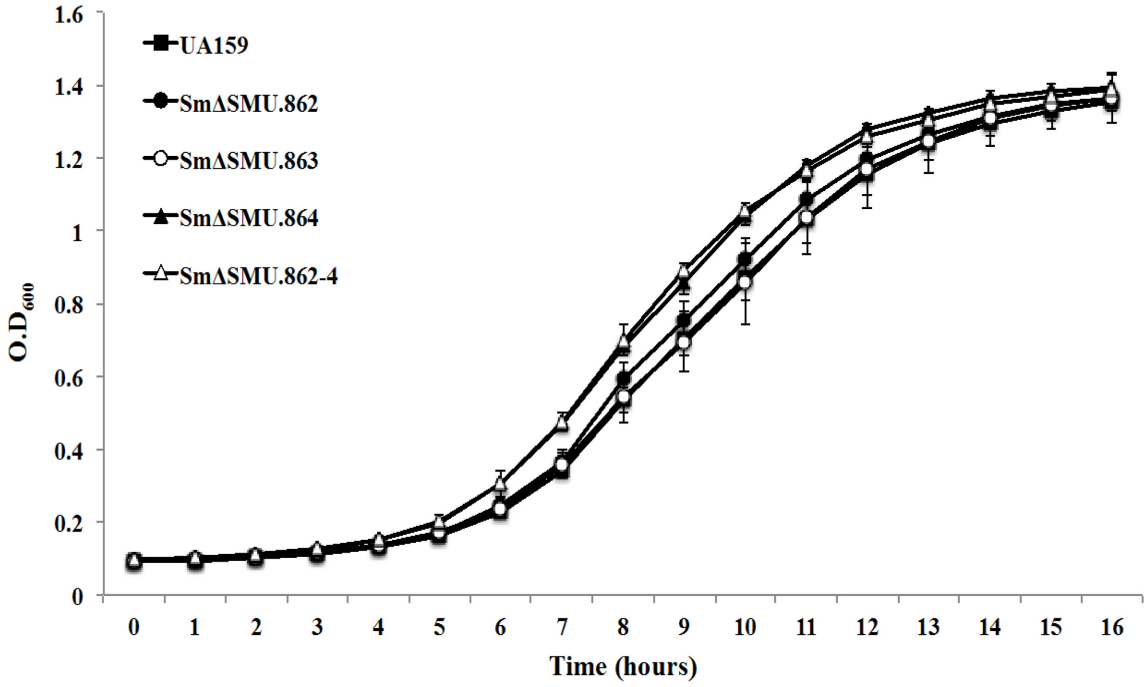
Appendix 2.10. Growth of *S. mutans* strains in THYE with 50 μg/ml of kanamycin. The results represent the means of duplicates from two independent experiments.



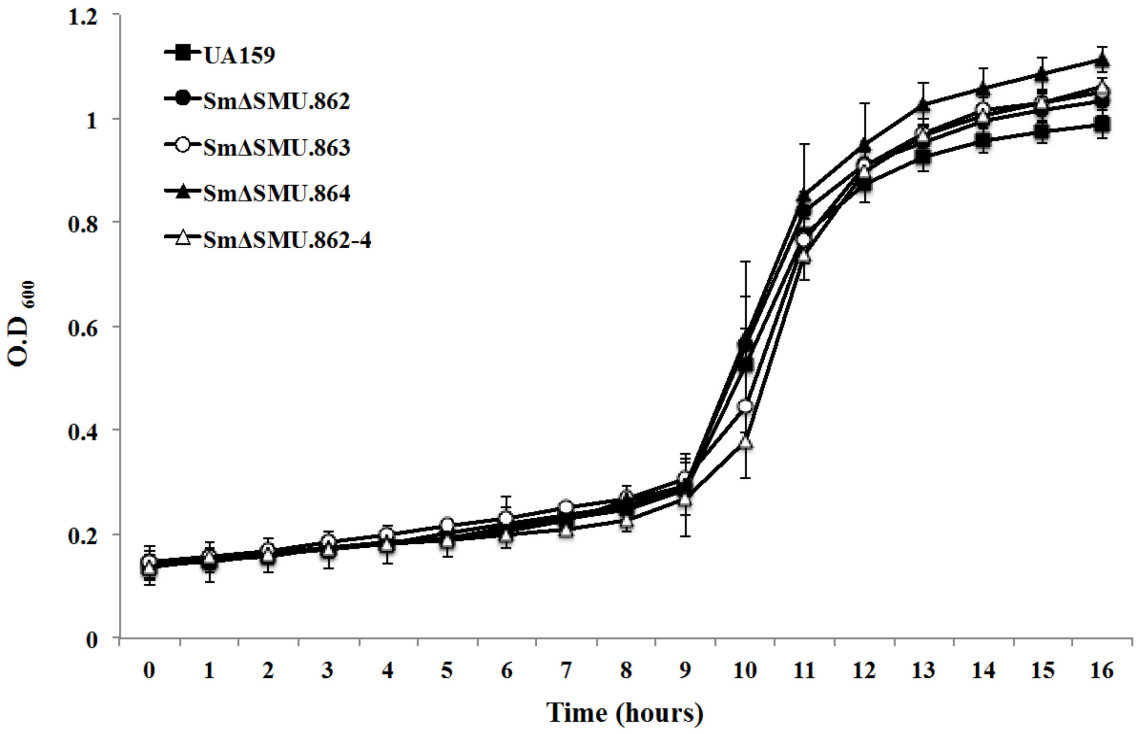
Appendix 2.11. Growth of *S. mutans* strains in THYE with 1 μg/ml of chloramphenicol. The results represent the means of duplicates from two independent experiments.



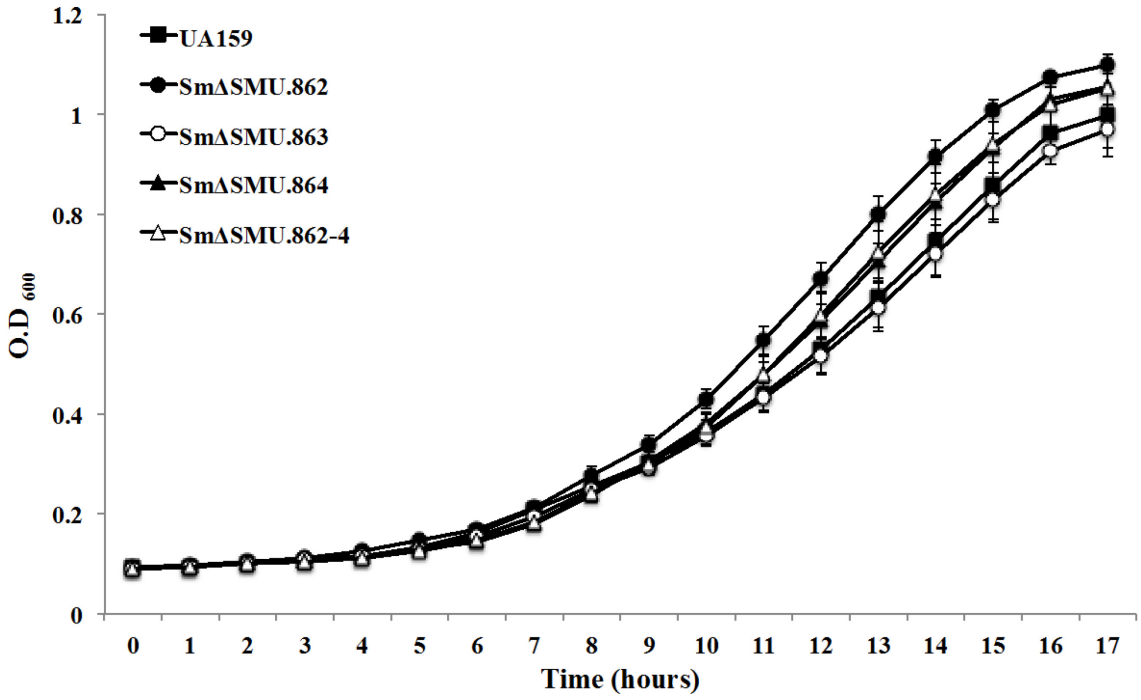
Appendix 2.12. Growth of *S. mutans* strains in THYE with 0.25 µg/ml of tetracycline. The results represent the means of duplicates from two independent experiments.



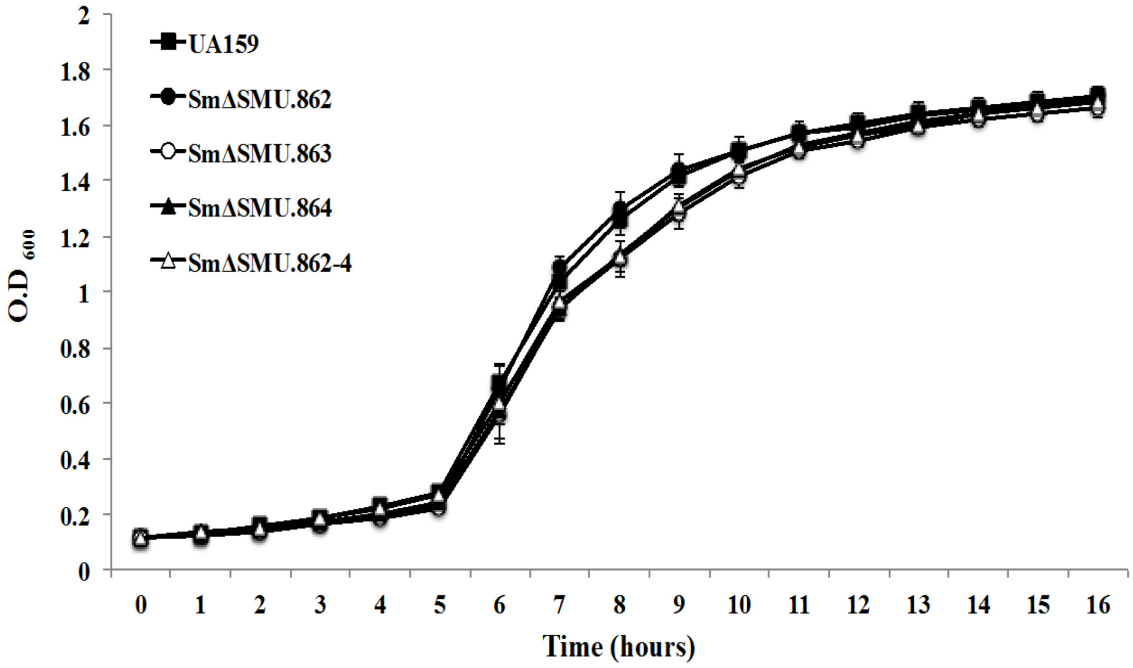
Appendix 2.13. Growth of *S. mutans* strains in THYE with 0.5 M/ml of NaCl. The results represent the means of duplicates from two independent experiments.



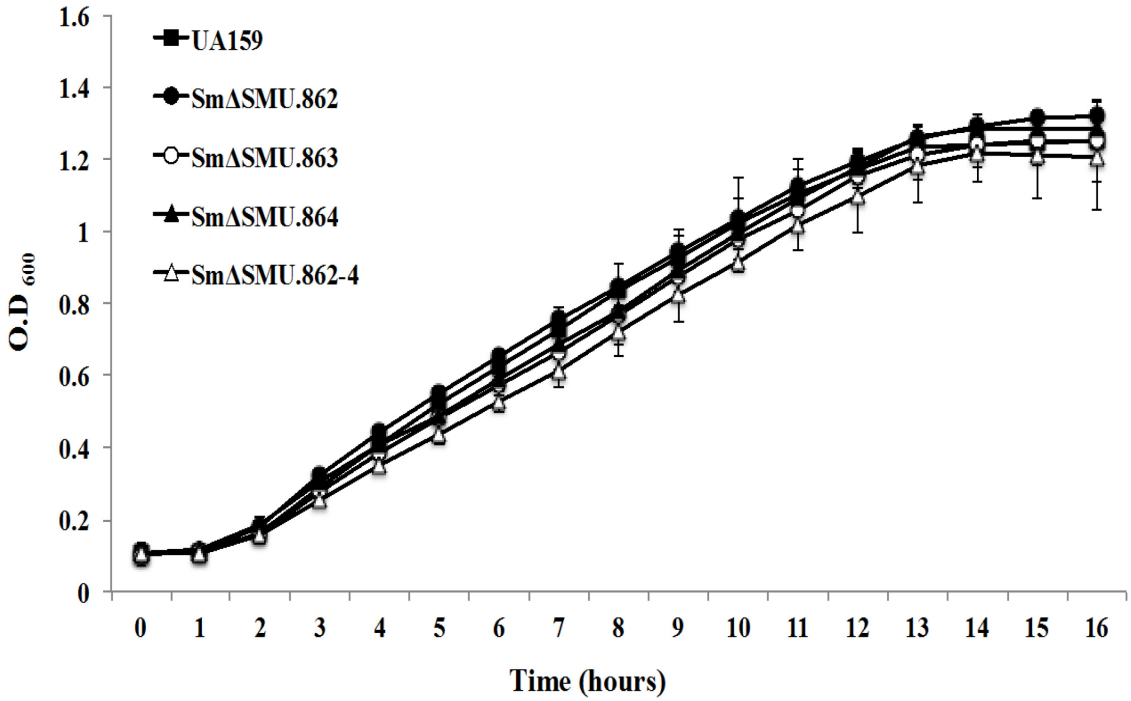
Appendix 2.14. Growth of *S. mutans* strains in THYE with 62.5 mM/ml of $CaCl_2$. The results represent the means of duplicates from two independent experiments.



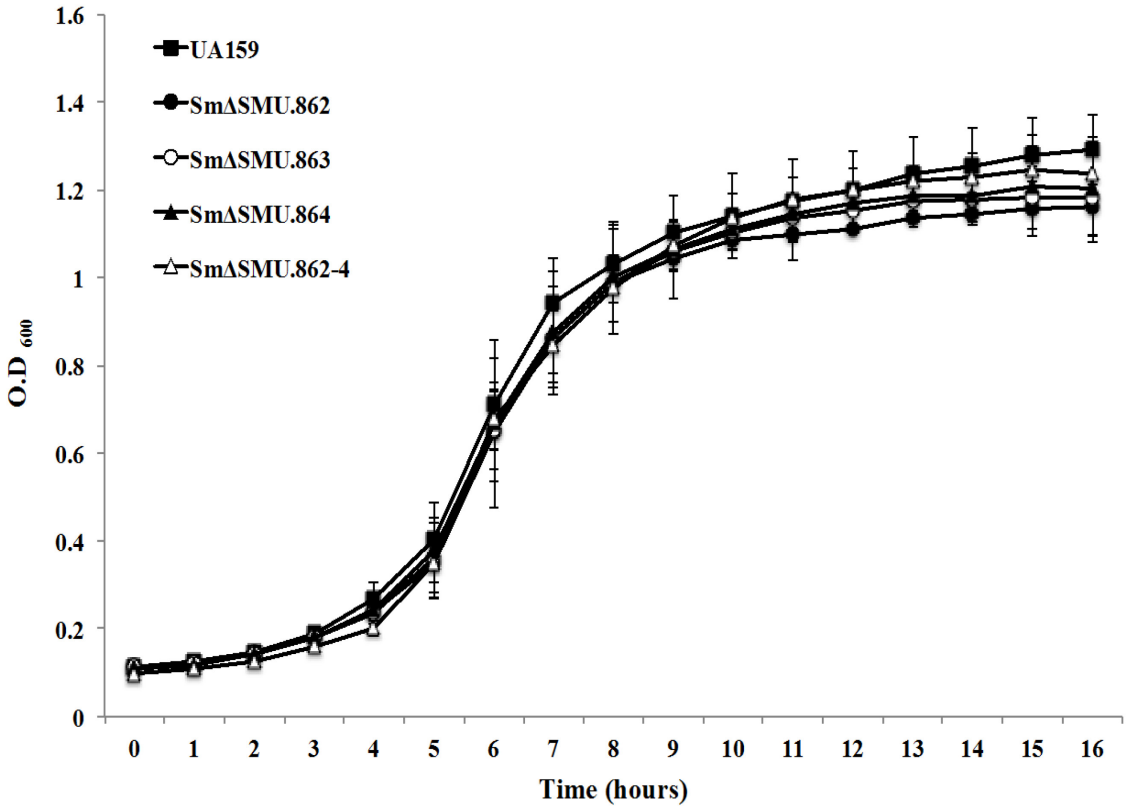
Appendix 2.15. Growth of *S. mutans* strains in THYE with 125 mM/ml of $MgCl_2$. The results represent the means of duplicates from two independent experiments.



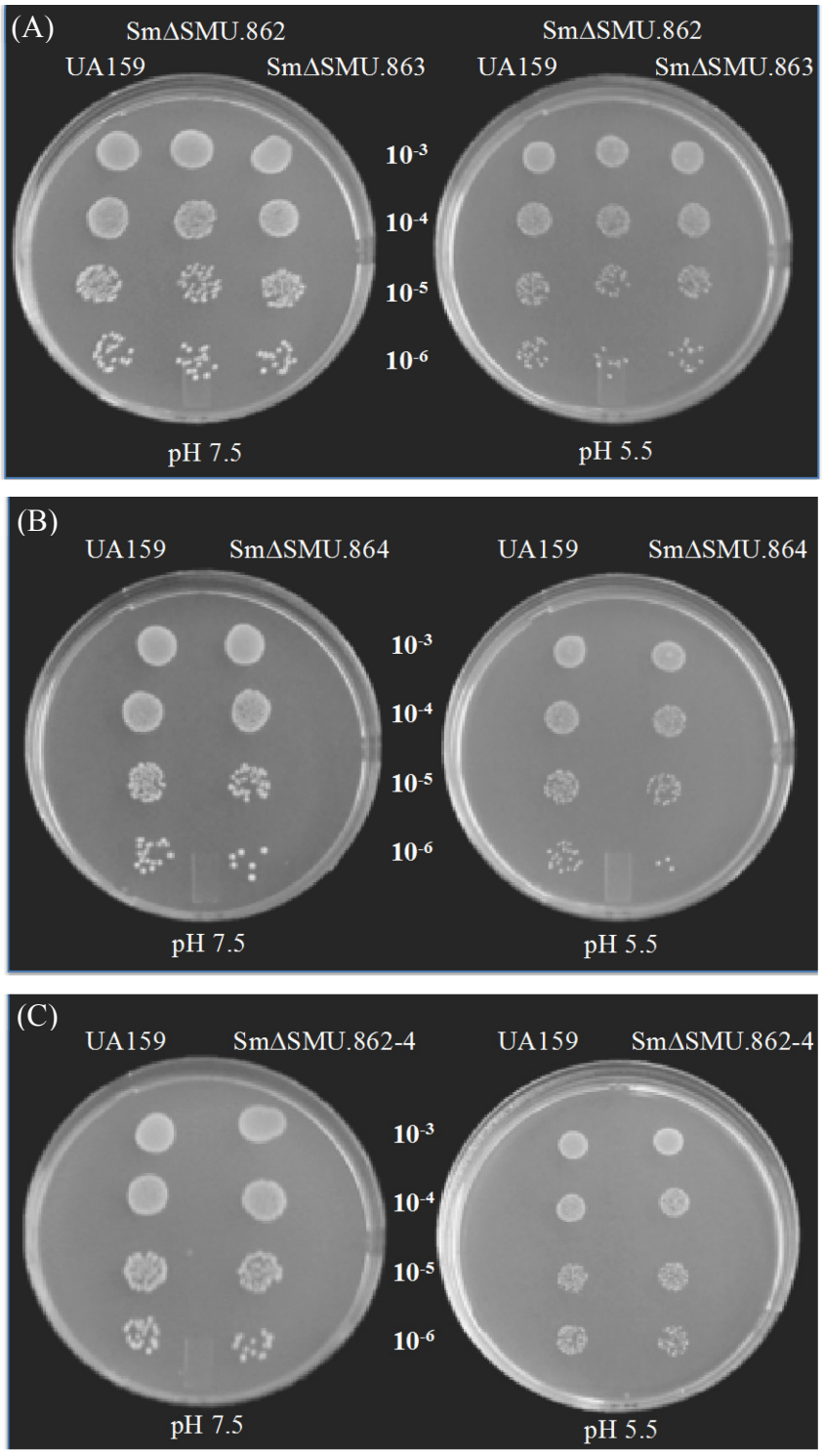
Appendix 2.16. Growth of *S. mutans* strains in THYE with 2 mM/ml of $ZnCl_2$. The results represent the means of duplicates from two independent experiments.



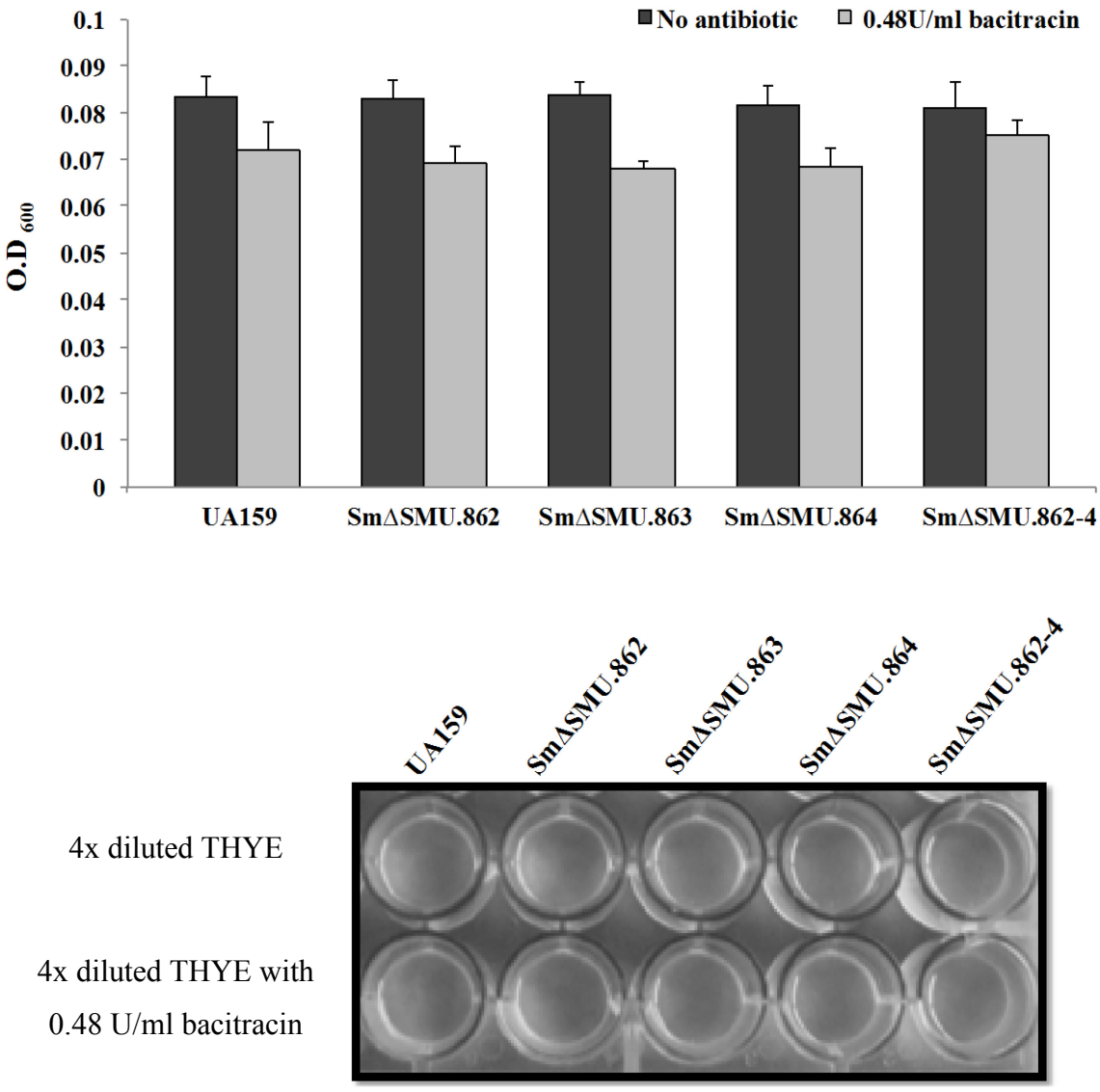
Appendix 2.17. Growth of *S. mutans* strains in THYE with 15.6 mM/ml of $MnCl_2$. The results represent the means of duplicates from two independent experiments.



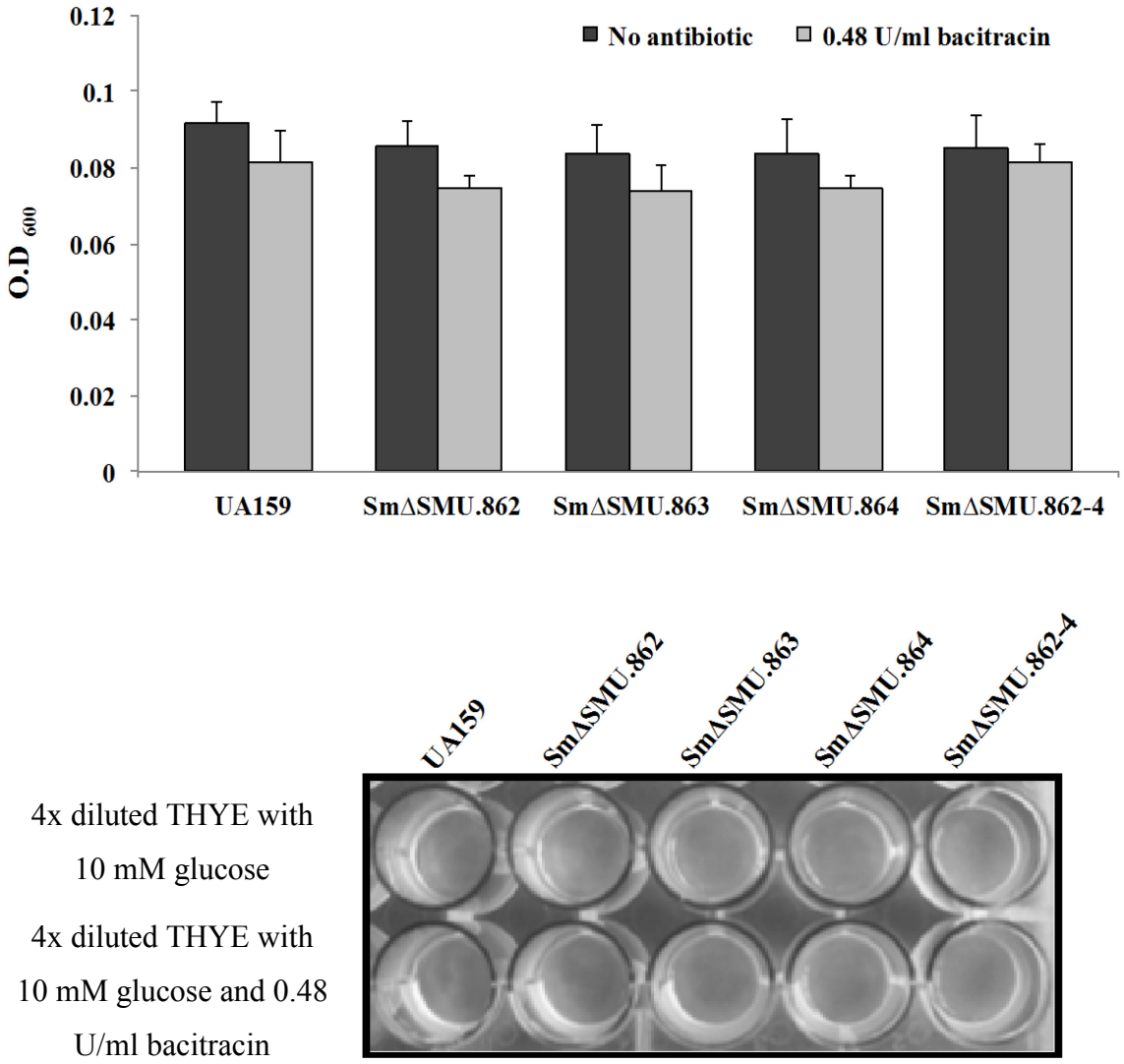
Appendix 2.18. Growth of *S. mutans* strains in THYE with 3.9 mM/ml of $FeCl_3$. The results represent the means of duplicates from two independent experiments.



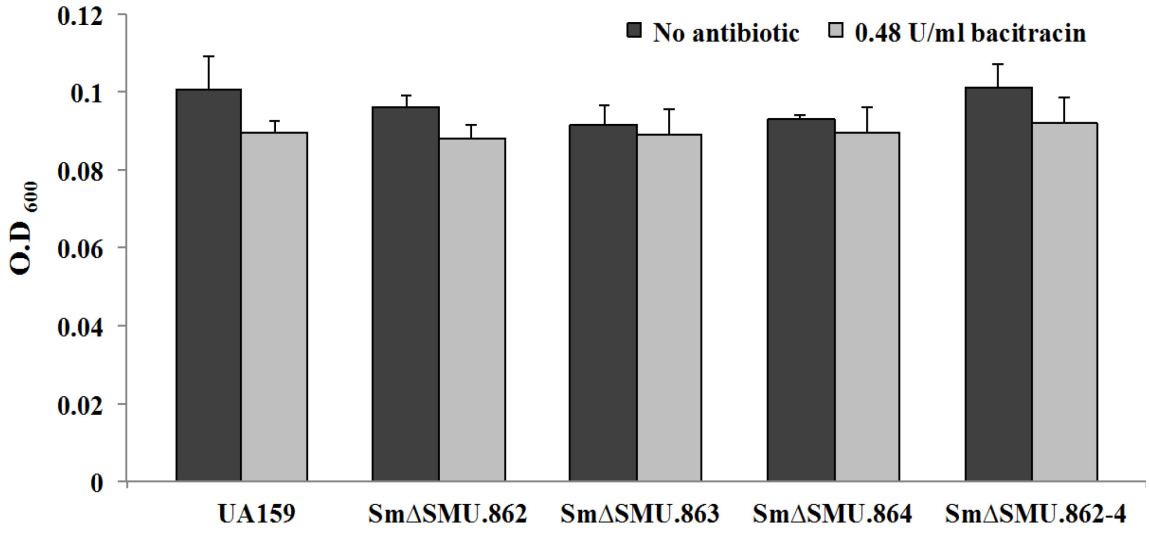
Appendix 2.19. Acid sensitivity of *S. mutans* strains UA159, SmΔSMU.862 (A), SmΔSMU.863 (A), SmΔSMU.864 (B), and SmΔSMU.862-4 (C) grown at pH 7.5 and 5.5.

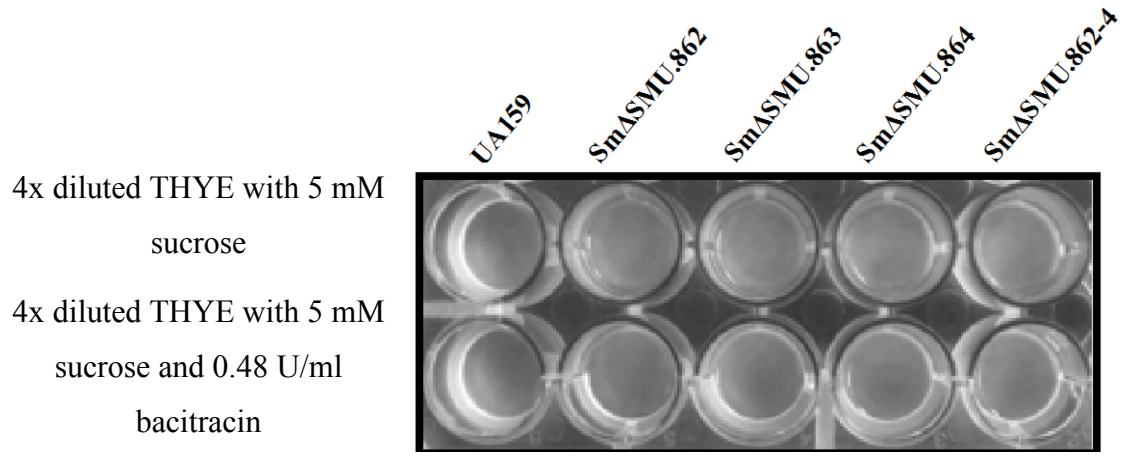


Appendix 2.20. Biofilm formation by *S. mutans* strains in 4x diluted THYE with or without 0.48 U/ml of bacitracin. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation.



Appendix 2.21. Biofilm formation by *S. mutans* strains in 4x diluted THYE plus 10 mM glucose with or without 0.48 U/ml of bacitracin. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation.





Appendix 2.22. Biofilm formation by *S. mutans* strains in 4x diluted THYE plus 5 mM sucrose with or without 0.48 U/ml of bacitracin. The results represent the average of duplicates from three independent experiments and the error bars represent the standard deviation.