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A conserved Inner Membrane Protein of Aggregatibacter actinomycetemcomitans is integral for membrane function

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A CONSERVED INNER MEMBRANE PROTEIN OF *AGGREGATIBACTER
ACTINOMYCETEMCOMITANS* IS INTEGRAL FOR MEMBRANE FUNCTION

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ABSTRACT

The cell envelope of *Aggregatibacter actinomycetemcomitans*, a Gram-negative pathogenic bacterium implicated in human oral and systemic disease, plays a critical role in maintenance of cellular homeostasis, resistance to external stress, and host–pathogen interactions. Our laboratory has identified a novel gene product, morphogenesis protein C (MorC), deletion of which leads to multiple pleotropic effects pertaining to membrane structure and function. The MorC sequence was determined to be conserved in Gammaproteobacteria. Based on this bioinformatic analysis, the functional conservation of this protein was investigated utilizing an *A. actinomycetemcomitans morC* mutant as a model system to express homologs from four phylogenetically diverse representatives of the Gammaproteobacteria: *Haemophilus influenzae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Moraxella catarrhalis*. MorC from all organisms restored at least one of the *A. actinomycetemcomitans* mutant phenotypes, implying that the protein is functionally conserved across Gammaproteobacteria. Further, deletion mutagenesis indicated that the last 10 amino acids of the carboxyl terminus were necessary to maintain the integrity of the membrane. The observed pleiotropic effects suggested alterations in the membrane protein composition of the *morC* mutant. Stable isotope dimethyl labeling in conjunction with mass spectrometry was employed to quantitatively determine the differences in the abundance of membrane proteins of the isogenic mutant and wild-type strains. A total of 665 envelope associated proteins were identified and functionally annotated using bioinformatic tools. All proteins, except MorC, were detected in the mutant strain. However, 12 proteins were found in lesser (10) or greater (2) abundance in the membrane preparation of the mutant strain. These proteins were ascribed functions associated with protein quality control, oxidative stress response, and protein secretion systems.

One protein found to be reduced was a component of the fimbrial secretion system of *A. actinomycetemcomitans*. The significance of this finding was unclear due to the afimbriated nature of the laboratory strain used in the study. Therefore, the defect in fimbriation was identified and complemented in *trans*. The transformed strain displayed all of the hallmarks of a naturally fimbriated strain including: a distinct star-like colony morphology; robust biofilm formation; and presence of fimbriae as detected by electron microscopy. The isogenic *morC* mutant strain transformed with an identical plasmid did not display any fimbriated phenotypes. The role of MorC in fimbriae production of a naturally fimbriated strain was investigated by inactivation of *morC* in a clinical isolate. The mutant strain displayed phenotypes typically associated with inactivation of *morC*. However, fimbriae were still observed on the surface, although in lesser amounts on some individual bacteria, and this strain formed a biofilm with volume similar to the parent. Interestingly, significant changes in microcolony architecture of the biofilm were observed by confocal microscopy.

MorC plays a critical role in maintaining secretion of major virulence determinants of *A. actinomycetemcomitans*. Specific changes in the protein composition of the cell envelope indicate a direct or compensatory role of these proteins in maintaining membrane physiology. The functional conservation of MorC also implies an important role for this protein in other Gram-negative bacteria. This work suggests a role of MorC as an accessory or a scaffold protein involved in secretion.

CITATIONS

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CHAPTER 1: Introduction

1. Oral Microbiology and Disease

It has long been appreciated that the soft and hard tissues of the human mouth are colonized by microorganisms. A diverse array of shapes, sizes, and spatial orientations of bacteria are found in micrographs of dental plaque, a biofilm composed of salivary proteins and microorganisms. As the field of bacteriology progressed, some of these organisms were successfully isolated in pure culture, allowing scientists to study their physiology. It quickly became evident that many of the microscopically observed bacteria in samples of plaque and oral fluids cannot be cultured on standard media. This discrepancy is known as "the plate count paradox", in which the numbers of directly observed bacteria in a sample do not reflect the numbers of bacteria grown *in vitro* (Paster *et al.* 2006).

The plate count paradox led to an interest in identifying the microbes that comprise the human oral microbiome, a census of all organisms present in the mouth. This effort was greatly aided by the advent of DNA technologies, including sequencing and fluorescent *in situ* hybridization, which allowed scientists to taxonomically classify non-cultivable organisms for the first time (Al-Ahmad *et al.* 2007, Dewhirst *et al.* 2010). Today, next-generation DNA sequencing technology has revealed that the microbiota of the human oral cavity is extremely complex, representing over 700 species of bacteria from 16 phyla (Human Oral Microbiome Project, www.homd.org). As our understanding of the oral microbiome grows, so does our understanding of the role of bacteria in oral disease.

Maintenance of oral health is an important component of overall health from both a medical and a psychological perspective (Gift and Atchison 1995). Bacterial diseases of the oral cavity, many of which are polymicrobial in etiology, are among the most common infectious diseases in humans and can lead to chronic lesions, inflammation, or tooth loss (Petersen *et al.* 2005). Nearly all adults will experience some form of oral disease in their lifetime and the most common causes of tooth loss are dental caries (known colloquially as cavities) and periodontal disease (Phipps and Stevens 1995, Eke *et al.* 2012). Dental caries result when specific Gram-positive microorganisms such as *Streptococcus mutans* colonize the tooth surface and produce acid. The acid leads to tooth decay (degradation of the minerals of the tooth enamel) and may expose internal tissues of the tooth. This results in damage to the living portion of the tooth, causing tooth death and ultimately tooth loss (Selwitz *et al.* 2007).

2. Periodontal Disease

Periodontal disease has a pathogenesis distinct from dental caries. The infection affects the tissue surrounding the tooth rather than causing direct destruction of the tooth. Periodontitis is a chronic inflammatory disease mediated by the immune response to pathogenic microorganisms below the gumline (Darveau 2010). This inflammation leads to activation of host matrix metalloproteinases that degrade the extracellular matrix proteins collagen and fibronectin, critical components of the ligaments anchoring the tooth to the jawbone (Golub *et al.* 1997, Hernandez *et al.* 2006). Increased protease activity also leads to repression of osteoblast differentiation, effectively limiting the formation of new bone

(Joseph *et al.* 2010). Osteoblasts are important in maintaining bone homeostasis and when reduced, result in bone loss. Conversely, osteoclasts are cells involved in normal degradation of bone. Differentiation of osteoprogenitor cells into osteoclasts is inhibited by proteins secreted by periodontal ligament cells (Wada *et al.* 2001). Destruction of these cells may increase numbers of osteoclasts, increasing bone resorption. Bone loss is an irreversible process that combined with degradation of the periodontal ligament, leads to loss of teeth (Darveau 2010). Periodontal disease is a factor in half of all tooth extractions in the United States and is likely to involve multiple teeth (Phipps and Stevens 1995).

2.1 Microbiology of Periodontal Disease

Periodontal disease is a polymicrobial, biofilm-mediated infection with an unknown number of causative organisms. The identification of periodontal pathogens is complicated by the fact that up to 60% of the species residing in the oral cavity have not yet been cultivated in the laboratory (Paster *et al.* 2006). Traditional techniques such as Koch's postulates cannot be applied to periodontal disease due to the polymicrobial nature of the infection. Therefore, new technologies, specifically high-throughput sequencing, have been employed to identify uncultivable species and novel pathogens (Siqueira and Rocas 2013, Aruni *et al.* 2014, Perez-Chaparro *et al.* 2014). Bacteria colonize the tooth surface below the gumline as a polymicrobial biofilm known as subgingival plaque. The subginvigal is composed of hundreds of different species of microbes, which may be associated with health or disease (Abusleme *et al.* 2013). In health, stable populations of mostly Gram-positive organisms such as *Actinomyces* and *Streptococcus* predominate.

However others such as *Veillonella*, an anaerobic, Gram-negative staining member of the Firmicutes, may also be found (Ximenez-Fyvie *et al.* 2000, Kumar *et al.* 2006). During the transition to disease, the species composition of this population shifts dramatically from mostly Gram-positive aerobic bacteria to Gram-negative anaerobes (Ximenez-Fyvie *et al.* 2000).

The population shift proceeds by a well ordered ecological succession mediated by co-aggregation between bacteria. *Streptococcus* spp., are early colonizers indicative of health and establish the community by binding to polysaccharides or proteins that coat the tooth surface using specific adhesins (Schilling and Bowen 1992, Crowley *et al.* 1993). These bacteria act as scaffolds and co-aggregate with other bacteria such as *Actinomyces* spp., *Veillonella* spp.*,* and *Eikenella* spp. (Cisar *et al.* 1979, Weerkamp and McBride 1981, Ebisu *et al.* 1992). The Gram-negative bacterium *Fusobacterium nucleatum* is a major intermediary species and interacts with early-colonizing Streptococci via an outer membrane receptor (Kaplan *et al.* 2009). This organism also interacts with Gram-negative bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans,* via a specific adhesin (Shaniztki *et al.* 1997). Thus, *Fusobacterium* creates a "bridge" between early colonizing Gram-positives and late colonizing Gram-negative species (Kolenbrander *et al.* 2006). The subgingival environment eventually becomes anaerobic, promoting growth of periodontal pathogens (Loesche *et al.* 1983). Further growth of these organisms results in deepening of the space between the tooth and gum, which in the disease state is known as the periodontal pocket.

Although there are hundreds of bacteria in the periodontal pocket during the course of disease, only a few are considered pathogens based on their consistent association with periodontitis. These include the Gram-negative obligate anaerobes *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Tannerella forsythia* of the phylum Bacteroidetes and *Treponema denticola* of the Spirochaetes. Another important periodontal pathogen is the facultative anaerobe *Aggregatibacter actinomycetemcomitans*, an organism physiologically and phylogenetically unique from any other periodontal pathogen (Socransky *et al.* 1998).

3. *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a Gram-negative bacterium of the Pasteurellaceae family of the class Gammaproteobacteria. It is the only member of the Proteobacteria known to cause periodontitis (Socransky *et al.* 1998). In fact, *A. actinomycetemcomitans* is evolutionarily far-removed from other periodontal pathogens such as *P. gingivalis* or *T. denticola* which belong to different phyla. *A. actinomycetemcomitans* is in the same family (Pasteurellaceae) as *Haemophilus influenzae, Pasteurella multocida,* and *Mannheimia haemolytica* and the same class (Gammaproteobacteria) as the model organism *Escherichia coli.*

Originally isolated in 1912 from an oral co-infection with *Actinomyces* spp. *A. actinomycetemcomitans* was initially named *Actinobacillus actinomycetemcomitans* to

reflect this association (Klinger 1912). The epithet *actinomycetemcomitans* literally means "occurring with *Actinomyces*", as it was then believed that *A. actinomycetemcomitans* was avirulent without the presence of an Actinomycete (Klinger 1912). It has since been shown that *A. actinomycetemcomitans* is a pathogen in its own right and the species name remains. However, in 2006, it was assigned to a novel genus based on conservation of DNA sequence and physiological properties (Norskov-Lauritsen and Kilian 2006). The new genus, *Aggregatibacter*, reflects this organism's propensity to adhere to neighboring cells and form tenacious biofilms.

A. actinomycetemcomitans is capnophilic and nutritionally fastidious, requiring a 10% CO_2 atmosphere and a rich medium for propagation in the laboratory (Mandell and Socransky 1981). The organism is highly adapted to human body temperature and loses viability rapidly below 37°C. Under ideal conditions doubling times range between 150 and 180 minutes, which is quite slow compared to other closely related model organisms (such as *E. coli* or *H. influenzae*) with doubling times of 20-30 minutes. The cells are coccobacillus in shape with individuals ranging from nearly circular (0.5µm x 0.6µm) to rod shaped (0.5µm x 1µm), depending on culture conditions and growth phase. As observed by electron microscopy, the bacterial outer membrane displays a unique convoluted morphology (Gallant *et al.* 2008, Azari *et al.* 2013) (Figure 1.1). These convolutions are not mirrored by the inner membrane, which remains flat (Azari *et al.* 2013).

Clinical isolates form small colonies (1-2mm) with rough edges that possess a unique star or cigar-shaped interior. This colony morphology gave the organism its historical genus

name *Actinobacillus* (the root word actino refers to a star shape). Colonies adhere strongly to agar, glass, and plastic and are refractory to disruption with a microbiological loop (Rosan *et al.* 1988, Inouye *et al.* 1990). This phenotype corresponds to the presence of fimbriae, which can be rapidly lost upon subculture in liquid media. Loss of fimbriae typically results from mutations in the promoter region of the *tad* operon that encodes the fimbrial secretion system (Wang *et al.* 2005). The afimbriated bacteria form larger (3-4mm), slightly convex colonies with smooth edges. These colonies lack internal structures and are easily disrupted (Inouye *et al.* 1990) (Figure 1.1). Afimbriated strains have been integral to the study of *A. actinomycetemcomitans* protein structure, cell adhesion and physiology in part due to their ease of manipulation in the laboratory as compared to their fimbriated counterparts (Mintz and Fives-Taylor 1994a, Gallant *et al.* 2008, Yu *et al.* 2009, Azari *et al.* 2013).
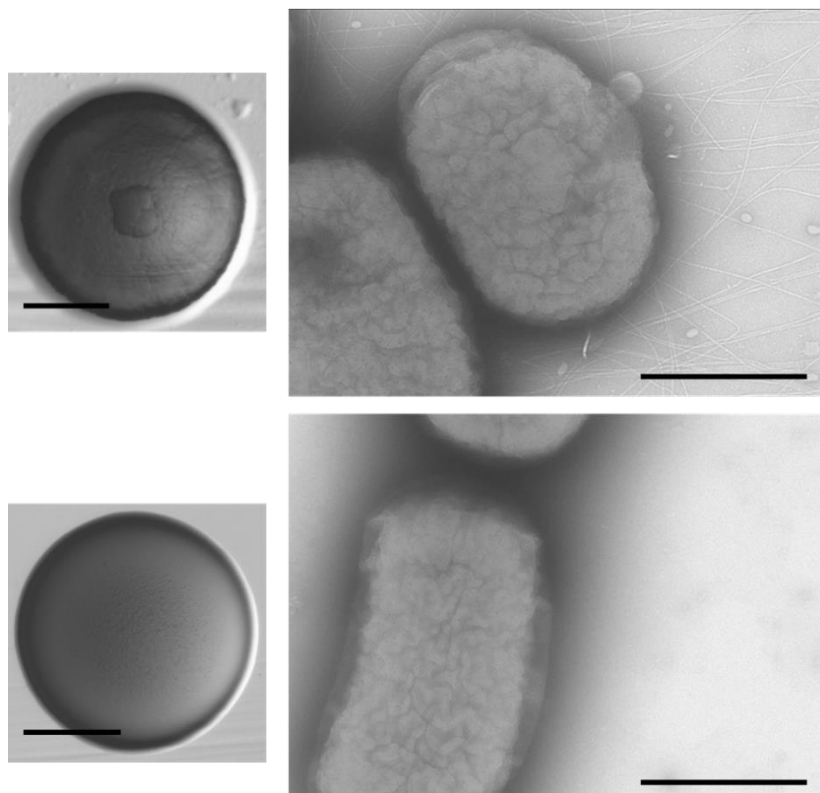
Figure 1.1 Colonies and electron micrographs of *A. actinomycetemcomitans* **fimbriated and afimbriated strains.** Upper Panel: Fimbriated strain, colony scale bar = 0.5 mm. Lower Panel: Afimbriated strain, colony scale bar = 1 mm. Electron micrograph scale bars = 0.5 μm.

A. actinomycetemcomitans is strongly associated with a form of periodontal disease called localized aggressive periodontitis. This disease typically strikes adolescents, affects specific teeth and progresses rapidly, causing tooth loss within months as opposed to decades as seen in other forms of periodontal disease (Mandell and Socransky 1981). In addition to oral disease, *A. actinomycetemcomitans* may escape the oral cavity and cause systemic infections, most commonly of the heart valves (infective endocarditis). *A. actinomycetemcomitans* is the most commonly isolated member of the HACEK group of pathogens (*Haemophilus* spp.*, A. actinomycetemcomitans*, *Cardiobacterium hominis*,

Eikenella corrodens, and *Kingella* spp.). These organisms are causes of "culture-negative"
endocarditis, an infection from which no viable cells can be isolated on laboratory media
(Paturel *et al.* 2004). *A. actinomycetemcomitans* is also capable of causing infections of the
lung, soft tissue, bone, and even the nervous system (Scannapieco *et al.* 2003, Rahamat-
Langendoen *et al.* 2011). Pathogenesis of *A. actinomycetemcomitans* depends on
interaction with host tissues. These interactions are mediated by molecules embedded in
the membrane system of this bacterium, termed the cell envelope.

4. The Gram-negative Cell Envelope

Gram-negative bacteria have two spatially and physiologically distinct membranes, an
inner membrane and an outer membrane, separated by a narrow space called the periplasm,
which contains the peptidoglycan cell wall. These membranes serve as anchoring points
for a wide variety of proteins involved in energy generation, synthesis of biologically
important molecules, stress tolerance, and pathogenesis. The membrane system itself also
acts as a permeability barrier, allowing cells to resist physical and chemical insults from
the environment.

4.1 Inner Membrane

The inner membrane is analogous to the cytoplasmic membrane of eukaryotic organisms.
It is a symmetric phospholipid bilayer with specific lipid composition varying by bacterial
species (Silhavy *et al.* 2010). This membrane is permeable only to small or hydrophobic
compounds. Multiple transport proteins are present to import nutrients and export toxins.

In addition, the membrane serves as an anchor point for the chromosome as well as DNA and protein synthesis machinery (Silhavy *et al.* 2010).

4.2 Periplasmic Space and Cell Wall

External to the inner membrane is the periplasmic space. This aqueous compartment is more viscous than the cytoplasm, resulting from a high concentration of proteins, many of which are involved in enzymatic degradation, such as phosphatases and RNAses (Hobot *et al.* 1984). Sequestration of these enzymes in the periplasm, combined with the lack of available energy in the form of ATP in this compartment, helps prevent nonspecific degradation of important molecules (Silhavy *et al.* 2010). Another class of proteins in the periplasm are known as chaperones. These proteins have many functions including the assistance in the proper folding of proteins and degradation of misfolded proteins. As proteins destined for the outer membrane or extracellular environment must pass the periplasm, chaperones are critical for proper localization of these proteins (Wulfing and Pluckthun 1994, Silhavy *et al.* 2010).

The periplasm also houses the peptidoglycan cell wall and enzymes requisite for its synthesis and maintenance. Peptidoglycan is a mesh-like structure composed of polysaccharides cross-linked with peptide bridges (Vollmer *et al.* 2008). Gram-negative bacteria are defined by their thin layer of peptidoglycan confined to the periplasmic space. The cell wall serves to maintain rigidity and structure of the cell. As the external

environment is rarely isotonic to the cytoplasm, the cell wall is also critical in maintaining turgor pressure (Vollmer *et al.* 2008).

4.3 Outer Membrane

The Gram-negative outer membrane is an asymmetric lipid bilayer with phospholipids on the inner leaflet (facing the periplasm) and lipopolysaccharides (LPS) forming the outer leaflet (facing the external environment). The lipid portion of the LPS consists of lipid A, a potent stimulator of inflammation and a major driver of periodontal disease (Taubman *et al.* 2005). A chain of sugars (O-polysaccharide), the identity and arrangement of which are used to define the serotype of the bacterium, is covalently linked to lipid A. *A. actinomycetemcomitans* has seven serotypes, designated a-f, of which strains of serotype b are the most often isolated (Yang *et al.* 2004). As it contains both hydrophilic and hydrophobic portions, the outer membrane serves as an excellent permeability barrier for toxic compounds including antibiotics (Nikaido 2003). Proteins may be anchored in the inner leaflet of this membrane via a lipid moiety or embedded into the membrane by a beta-barrel structure.

5. Protein Secretion Systems in *A. actinomycetemcomitans*

The multiple layers of the cell envelope present a challenge to the bacterium since envelope and extracellular proteins must traverse at least one membrane to arrive at their destination. To solve this problem, organisms utilize multiple pathways to ensure that these proteins are accurately targeted to the correct compartment (Pugsley 1993, Delepelaire 2004, Leo

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et al. 2012). These pathways, termed secretion systems, are composed of protein complexes responsible for facilitating the passage of proteins across cell membranes and the periplasmic space. Specific mechanisms for secretion of membrane and periplasmic proteins (general secretory pathway), outer membrane proteins (beta barrel assembly module, BAM) and lipoproteins (localization of lipoproteins system, LoL) exist. In the *A. actinomycetemcomitans* genome, three prototypic secretion systems (type I, II, and V) for transport of specific protein substrates are also predicted (Zijnge *et al.* 2012).

5.1 General Secretory Pathway

The general secretory pathway is ubiquitous in prokaryotic and eukaryotic organisms. In Gram-negative bacteria, this pathway is responsible for transporting proteins across or the inner membrane (Pugsley 1993). Proteins secreted by this complex are inserted into the membrane or released into the periplasmic space. From the periplasm, proteins are exported to the outer membrane or the extracellular environment by other secretion mechanisms, type II or type V secretion systems. The general secretory pathway initiates with either the signal recognition particle (SRP, a protein-ribonucleic acid complex) or binding of SecB to specific hydrophobic regions of proteins emerging from the ribosome (Bernstein *et al.* 1989, Watanabe and Blobel 1989).

For membrane proteins destined for the inner membrane, the SRP recognition sequence is the first hydrophobic transmembrane helix (Silhavy *et al.* 2010). Interaction with SRP protects these hydrophobic sequences from aggregating in the aqueous environment of the

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cytoplasm. Protein-SRP complexes interact with the inner membrane protein FtsY, anchoring the complex to the inner membrane (Miller *et al.* 1994, Parlitz *et al.* 2007). FtsY delivers the protein-SRP complex to the Sec (secretory) translocon, an inner membrane secretion machine composed of the proteins SecYEG and the accessory protein YidC, through direct interaction with SecY (Angelini *et al.* 2005). Hydrophobic membrane-spanning regions are released directly into the membrane by opening of a lateral gate in the translocon formed by YidC, which provides access to the lipid bilayer (Samuelson *et al.* 2000).

Proteins targeted to the periplasm, outer membrane, or extracellular environment contain a specific sequence of amino acids called a signal sequence. This sequence is composed of positively charged amino acids at the N-terminus, followed by a hydrophobic region, and finally, a cleavage site for a signal peptidase (Driessen and Nouwen 2008). Concomitant with transport, the cleavage site is recognized and cut by the signal peptidase LepB after which the protein is then released to the periplasmic space (Zwizinski and Wickner 1980). From the periplasm, it may be transported to the outer membrane or extracellular environment by specific secretion complexes.

5.2 Beta-barrel Assembly Module (BAM) Complex

The Sec translocon is responsible for transporting proteins across the inner membrane, after which the BAM complex localizes beta-barrel containing proteins to the outer membrane. In the periplasm, proteins interact with chaperones SurA or Skp/DegP for transport to the

BAM complex an essential secretion system consisting of the integral outer membrane protein BamA and the associated periplasmic proteins BamBCDE (Webb *et al.* 2012). Specific repeats termed polypeptide transport associated domains are found in the amino acid sequence of BamA and have been shown to be responsible for transport of nascent outer membrane proteins (Workman *et al.* 2012).

5.3 Localization of Lipoproteins (Lol) System

A specific subset of proteins utilize post-translationally added lipid tails for anchorage to the periplasmic face of either the inner or outer membrane (Tokuda and Matsuyama 2004). Initial lipidation of the apolipoprotein occurs by transfer of diacylglycerol from phosphatidylglycerol to a specific cysteine residue within a recognition motif in a single-step reaction mediated by the enzyme Lgt (Sankaran and Wu 1994). The modified protein is translocated across the inner membrane by the Sec translocon with simultaneous cleavage of its signal peptide by the lipoprotein signal pepditase Lsp (also known as signal peptidase II) (Yamagata *et al.* 1983). In Gram-negative bacteria, a second acylation step, mediated by the inner-membrane associated enzyme Lnt attaches another acyl group to the N-terminus of the peptide (Gupta and Wu 1991). This results in a mature lipoprotein anchored to either leaflet of the inner membrane.

Lipoproteins destined for the outer membrane contain a specific sequence that allows for recognition by LolCDE, an ABC transporter that releases lipoproteins from the inner membrane (Tokuda and Matsuyama 2004). Release depends on the periplasmic chaperone

LolA, which binds to the protein and delivers it to the outer membrane. At the outer membrane, LolB inserts the lipoprotein into the inner leaflet. Lipoproteins that remain in the inner membrane contain a specific amino acid sequence called a Lol avoidance sequence. This sequence inhibits recognition by the LolA chaperone and prevents release from the inner membrane (Hara *et al.* 2003).

5.4 Type I Secretion

The *A. actinomycetemcomitans* genome encodes a single type I secretion system. This three-component complex is used exclusively to secrete leukotoxin (LtxA) (Kachlany *et al.* 2000a). Leukotoxin is a soluble toxin of the repeats in toxin (RTX) family, a designation based on repetitive elements in the primary amino acid structure which are responsible for binding Ca^{2+} ions (Lally *et al.* 1989a). The toxin was first identified for the specific activity towards white blood cells (Baehni *et al.* 1981). More recently, the toxin has been demonstrated to lyse specific types of red blood cells (Balashova *et al.* 2006). Toxin activity is dependent on acylation of specific lysine residues within the polypeptide chain. This activity is attributed to LtxC and occurs in the cytoplasm (Fong *et al.* 2011). Following acylation of lysines, the toxin is secreted to the extracellular environment by a three-component secretion system homologous to the type I secretion of hemolysin (HlyA) in *E. coli* (Kachlany *et al.* 2000a).

The secretion machine is composed of three proteins: an inner membrane ATPase, a periplasmic channel protein, and an outer membrane pore (Figure 1.2). The inner

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membrane ATPase (LtxB) provides energy for toxin export (Lally *et al.* 1991). Typical of ATPases involved in protein secretion, LtxB functions as a dimer, in which each monomer contains a membrane spanning domain to facilitate transport of LtxA across the inner membrane and an ATPase domain to provide energy for this process (Lally *et al.* 1991). In homologous secretion systems, the ATPase is responsible for recognition of the toxin substrate via specific amino acid sequences (Kenny *et al.* 1994). This triggers a cascade of protein-protein interactions that ultimately form a complete secretion complex (Letoffe *et al.* 1996).

Interaction of the toxin and LtxB induces a conformational change of LtxB resulting in an interaction with the periplasmic channel protein LtxD (Lally *et al.* 1991). The channel functions to transport the toxin across the periplasm, protecting it from degradation by periplasmic enzymes (Crosby and Kachlany 2007). To cross the outer membrane, the periplasmic channel protein recruits the outer membrane protein TolC (TdeA in *A. actinomycetemcomitans*) (Crosby and Kachlany 2007). TolC forms a trimeric pore that allows the toxin to cross the outer membrane (Werner *et al.* 2003). The transient nature of this interaction allows TolC to act as a component of other secretion apparatuses such as small molecule efflux pumps (Crosby and Kachlany 2007).

Prototypical type I secretion systems require only the three proteins described above: an inner membrane ATPase, periplasmic channel, and outer membrane pore. In *A. actinomycetemcomitans* however, type I secretion has been shown to rely on other factors

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including the O-antigen component of LPS and the inner membrane protein morphogenesis protein C (MorC) (Gallant *et al.* 2008, Tang *et al.* 2012a). However, the mechanism by which these factors modulate secretion independently of the canonical secretion system is unknown.
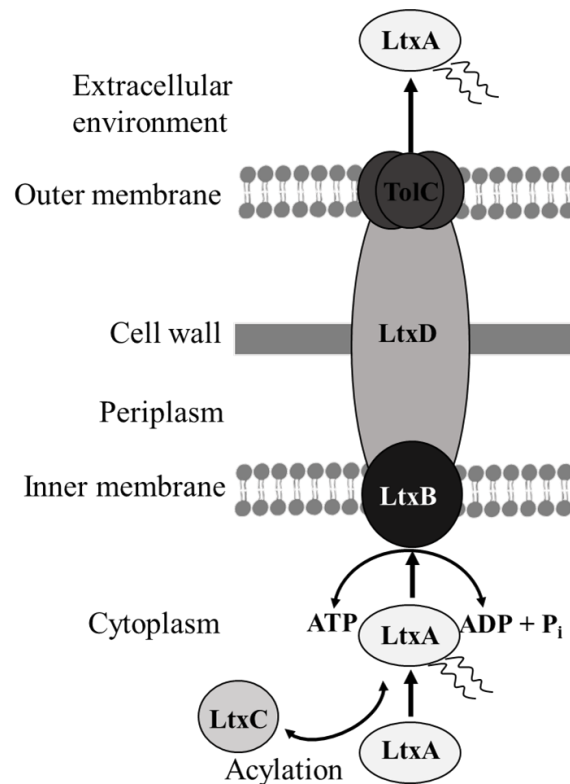
Figure 1.2 Type I secretion of leukotoxin. Toxin is synthesized in the cytoplasm and recognized by the ATPase LtxB, which provides energy for transport. After toxin recognition, LtxD and TolC assemble a transenvelope channel that allows export to the environment.

5.5 Type II Secretion

Prototypical type II secretion machines transport proteins across the outer membrane from the periplasm to the extracellular environment. This system can only secrete substrates that are already present in the periplasm. Thus, it is a two-step system in which substrates are first translocated across the inner membrane by the Sec or twin-arginine translocon and then across the outer membrane by the type II secretion complex (Voulhoux *et al.* 2001, Lee *et al.* 2006). Because of this dependency on SecYEG the type II secretion system is commonly called the main terminal branch of the general secretory pathway. The type II secretion complex consists of proteins localized to the inner membrane, periplasm, and outer membrane. This system is well characterized in *E. coli*. In this organism, energy is provided by the ATPase GspE which interacts with membrane proteins GspF, L, and M form a platform for the assembly of the other components of the secretion complex (Py *et al.* 2001). A periplasmic channel is formed by the GspG, H, I, and J proteins which guides substrates to the outer membrane pore GspD (Hardie *et al.* 1996).

The genome of *A. actinomycetemcomitans* does not contain any genes coding for prototypical type II secretion system proteins. However, fimbrial secretion in this organism depends on a modified type II secretion system known as the Tad complex (Figure 1.3). The Tad complex is encoded in a single operon called the *tad* locus, which contains 14 genes (Kachlany *et al.* 2000b). The functions of many of these proteins remain unknown, however, mutational analyses have demonstrated their importance in fimbrial secretion and

function (Wang and Chen 2005). Currently, there are no known proteins outside of this locus involved in Tad complex formation.

Like the prototypical type II secretion system, this complex is composed of proteins localized to the inner membrane, periplasm, and outer membrane. Energy is provided by the ATPase TadA, localized to the cytoplasmic face of the inner membrane, likely through interactions with an integral membrane protein (Bhattacharjee *et al.* 2001). A periplasmic channel is formed by TadE and F (Tomich *et al.* 2006). The outer membrane pore RcpA allows substrate access to the external environment (Haase *et al.* 1999).

Unlike prototypical type II secretion systems, the Tad complex is known to secrete only a single substrate, the fimbrial subunit Flp1. Flp1 is produced as a pre-protein with an N-terminal hydrophobic region. Upon secretion across the inner membrane, this hydrophobic tail embeds the protein in the lipid bilayer. The tail is then cleaved resulting in a secretion competent form by the protease TadV (Tomich *et al.* 2006). Subunits are secreted in a polymeric form to create long fimbriae that extends several microns from the cell surface (Tomich *et al.* 2007).
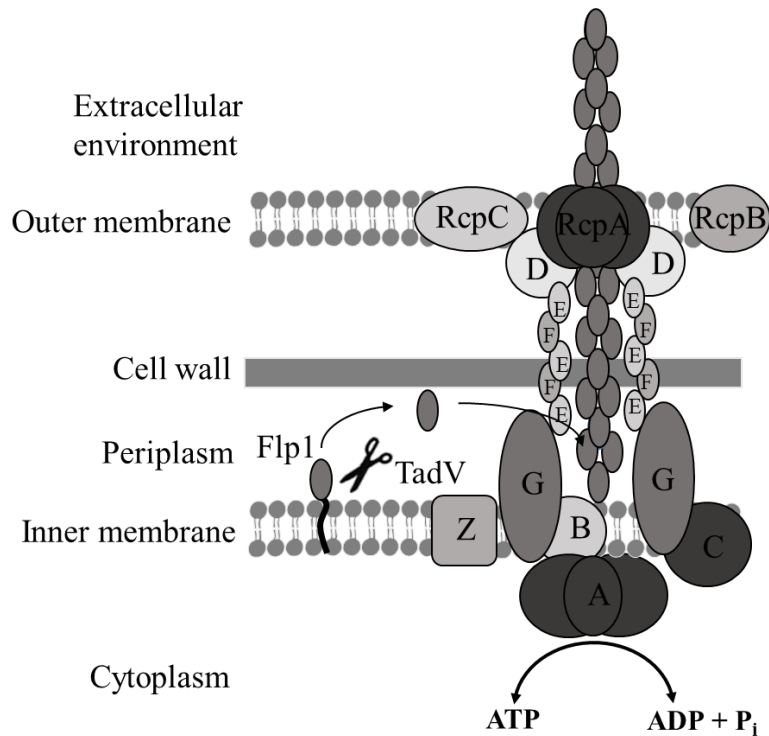
Figure 1.3 Type II Secretion. The prepilin Flp1 is embedded in the inner membrane and released to the periplasm upon cleavage by TadV. Secretion of Flp1 is accomplished via the Tad complex of 10 proteins. Tad proteins are designated by letters, Rcp proteins by their full designation. Adapted from Tomich *et al.* (2007).

5.6 Type V Secretion

The type V secretion system is used by *A. actinomycetemcomitans* to insert adhesins into the outer membrane. These proteins are transported across the inner membrane by the Sec translocon and contain a signal peptide that indicates they are to be released into the periplasm (Pugsley 1993). Concomitant with translocation to the periplasm, the signal peptide is cleaved by a signal peptidase (Zwizinski and Wickner 1980). Periplasmic chaperones protect the protein from degradation on its way to the outer membrane (Silhavy *et al.* 2010) (Figure 1.4).

Proteins secreted via the type V secretion system encode all of the information necessary to catalyze transport across the outer membrane, giving them the name "autotransporters" (Leo *et al.* 2012). This is accomplished by two main domains: the translocator domain (also known as the beta domain) and the passenger domain. The translocator domain inserts into the outer membrane with assistance from the BAM complex (Rossiter *et al.* 2011, Leo *et al.* 2012). This domain may be composed of a single polypeptide (as is the case for monomeric autotransporters) or three polypeptides in the case of trimeric autotransporters (Leyton *et al.* 2012). In both cases, the translocator domain catalyzes the transport of the passenger domain through the outer membrane where it will be exposed to the extracellular environment. This process requires no energy and is independent of any other protein factors (Leo *et al.* 2012).
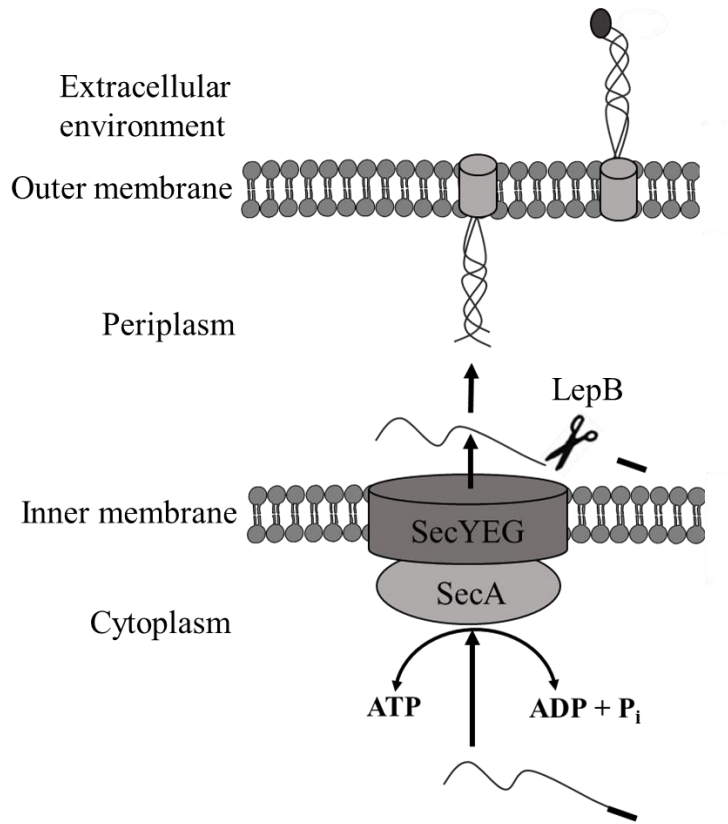
Figure 1.4 Type V secretion of a trimeric autotransporter. Autotransporter proteins are produced with a signal peptide (dark line) which, along with cytoplasmic chaperones (not shown) guide it to the Sec translocon. After crossing the inner membrane the signal peptide is cleaved by LepB. Specific periplasmic chaperones (not shown) guide the protein to the outer membrane where it trimerizes and forms a beta-barrel structure aided by the BAM complex. The protein then transports the passenger domain through the outer membrane independent of other protein factors.

6. Membrane Proteins of *A. actinomycetemcomitans*

All of the protein secretion systems listed above are important for cell survival. This is because membrane proteins of *A. actinomycetemcomitans* play a critical role in macromolecule biosynthesis, metabolism, resistance to environmental insults, and interaction with the host. Despite their importance, relatively few membrane proteins have been characterized in this organism. The following represents a catalog of well-

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characterized membrane proteins of *A. actinomycetemcomitans*. Integral membrane components of type I and II secretion systems have already been discussed and will not be included here.

7. Outer Membrane Proteins of *A. actinomycetemcomitans*

The outer membrane of Gram-negative bacteria is attached to the cell wall via lipoproteins in the inner leaflet, which are linked to diaminopimelic acid in peptidoglycan by a terminal lysine residue (Braun and Sieglin 1970). The secondary structure of outer membrane proteins that span both (inner and outer) leaflets and is composed of multiple beta-strands formed into a ring termed a beta-barrel (Koebnik *et al.* 2000). In *A. actinomycetemcomitans*, several outer membrane proteins have been discovered, the best-described of which are the autotransporter adhesins.

7.1 Extracellular Matrix Protein Adhesin A (EmaA)

EmaA is an integral outer membrane protein of the autotransporter family. It is classified as a trimeric (type V_c) autotransporter and consists of three 202 kDa subunits that form a coiled-coil structure (Mintz 2004). Secretion depends on the presence of a unique extended 57 amino acid signal peptide (Jiang *et al.* 2011). This extended signal peptide ensures proper secretion at elevated temperatures such as those found at sites of inflammation (Jiang *et al.* 2012).

EmaA is important in the binding of *A. actinomycetemcomitans* to fibrillar collagen (type I, II, III, and V), an extracellular matrix protein found in abundance upon the desctruction of host cells during the pathogensis of periodontal disease (Mintz 2004). EmaA structures bind exposed collagen of damaged heart valves, as demonstrated using a rabbit model of infective endocarditis (Tang *et al.* 2008). Sequence analysis combined with the three-dimensonal structure, generated by electron tomography, indicates that this protein has multiple distinct domains including an ellipsoidal-shaped head and a stalk, which are connected by flexible neck regions (Yu *et al.* 2009). Collagen binding is associated with the head domain corresponding to the amino termini of the proteins (Ruiz *et al.* 2006, Yu *et al.* 2008). Multiple forms of the protein are expressed dependent on *A. actinomycetemcomitans* serotype. Serotypes b and c express a full-length form (202 kDa monomers), whereas serotypes a and d express a truncated version of the protein (175 kDa monomers) that also interacts with collagen (Tang *et al.* 2007). Protein function as well as stability depends on the presence of glycan moieties added by the LPS biosynthetic pathway (Tang and Mintz 2010, Tang *et al.* 2012b).

7.2 Outer Membrane Protein 100 (ApiA)

A. actinomycetemcomitans expresses a second trimeric autotransporter protein, which mediates binding to the extracellular matrix protein fibronectin, epithelial cells (Li *et al.* 2004) and also functions in invasion of eukaryotic cells (Asakawa *et al.* 2003). Interestingly, ApiA is only able to bind to epithelial cells derived from humans and Old World primates, mirroring the species tropism of *A. actinomycetemcomitans* (Yue *et al.*

2007). In addition to adhesion, this protein is involved in the activation of the innate immune response and in stimulation of host antimicrobial peptides (Ouhara *et al.* 2006).

7.3 *A. actinomycetemcomitans* Epithelial Cell Adhesin (Aae)

The monomeric (type V_a) autotransporter protein Aae also contributes to *A. actinomycetemcomitans* epithelial cell binding (Rose *et al.* 2003). In fact, a double mutant of *aae* and *apiA* shows an absence of binding to epithelial cells (Yue *et al.* 2007). Like ApiA, binding of Aae is also restricted to oral epithelial cells of humans and Old World primates, (Fine *et al.* 2005). Five alleles of *aae* have been identified in clinical isolates, each containing varying numbers of a 135-bp repeat (Pinheiro *et al.* 2011). The binding domain of this adhesin lies within the range of amino acids 201-240 as evidenced by competition experiments using synthetic peptides (Fine *et al.* 2010).

7.4 Outer Membrane Protein 34 (Omp34)

Omp34 is an abundant protein in the outer membrane of *A. actinomycetemcomitans*. It shares homology to the major porin OmpA of *E. coli* and cross-reacts with antibodies raised to this protein, indicating a conserved structure (Wilson 1991). The apparent molecular mass of Omp34 is 25-29 kDa, which corresponds to the predicted mass based on gene sequence (White *et al.* 1998). However, upon heating to 100°C the apparent molecular mass is increased to ~34 kDa. As such, this protein is referred to as a heat modifiable protein (Wilson 1991, Mintz and Fives-Taylor 1994b). Although the precise function of

Omp34 is unknown, it may play a role in immune evasion by binding to the Fc region of antibody molecules (Mintz and Fives-Taylor 1994b).

7.5 Bacterial Interleukin Receptor I (BilRI)

In addition to binding antibody molecules, *A. actinomycetemcomitans* outer membrane proteins can also modulate the host immune response by sensing host signaling molecules (Paino *et al.* 2013). The outer membrane protein BilRI binds specifically to the host cytokine interleukin-1β (IL-1β) (Paino *et al.* 2012). IL-1β is produced by periodontal ligament cells upon exposure to *A. actinomycetemcomitans* LPS and it appears that the bacterium transports the protein into the cytoplasm (Schytte Blix *et al.* 1999, Paino *et al.* 2012).

8. *A. actinomycetemcomitans* Inner Membrane Proteins

Integral membrane proteins are embedded via one to more than 15 alpha-helices owing to the hydrophobic nature of the phospholipid tails in the inner membrane (Silhavy *et al.* 2010). There is limited available information regarding the *A. actinomycetemcomitans* membrane proteome. Based on protein profiles of related organisms, the inner membrane is expected to be enriched in proteins that generate energy, transport macromolecules, and export toxic compounds. The inner membrane houses the m achinery required for oxidative phosphorylation and production of ATP (Silhavy *et al.* 2010). As this membrane is impermeable to hydrophilic or charged molecules, there are a wide array of transporters necessary to import nutrients and export toxic compounds (Silhavy *et al.* 2010). The

identity and substrate specificity of putative transporters in *A. actinomycetemcomitans* is unknown.

Proteins integral to the inner membrane also associate with freely soluble proteins called peripheral membrane proteins that form functional complexes. The most important of these complexes are the ABC transporters, integral membrane transporters that receive energy from association with a soluble ATPase. Ribosomes and the chromosome have also been shown to associate with inner membrane proteins in model Gram-negative organisms, making this the site of many important physiological processes (Silhavy *et al.* 2010). Despite the importance of the inner membrane in cellular physiology, only a small number of inner membrane proteins have been described in *A. actinomycetemcomitans*, the majority of which are components of the type I or type II secretion system. Morphogenesis protein C is the only inner membrane protein outside of the components of the secretion complexes that has been characterized in *A. actinomycetemcomitans*.

8.1 Morphogenesis Protein C

MorC is a relatively large (141 kDa) inner membrane protein that was first identified in *A. actinomycetemcomitans* (Gallant *et al.* 2008). Homologs of MorC are found among diverse families of Gammaproteobacteria including significant human pathogens such as *Haemophilus influenzae, Vibrio cholerae, E. coli*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* (Figure 1.5). *morC* is part of a three gene operon that is conserved in Gammaproteobacteria (Figure 1.6) including members of the Pasteurellaceae,

Enterobacteriaceae, and Vibrionaceae family. A two-gene operon structure consisting of

omp67 and *morC* is conserved in all seven families investigated. Omp67 encodes an outer

membrane protein paralogous to BamA, a principle component of the BAM complex

involved in integration of beta-barrel proteins into the outer membrane.
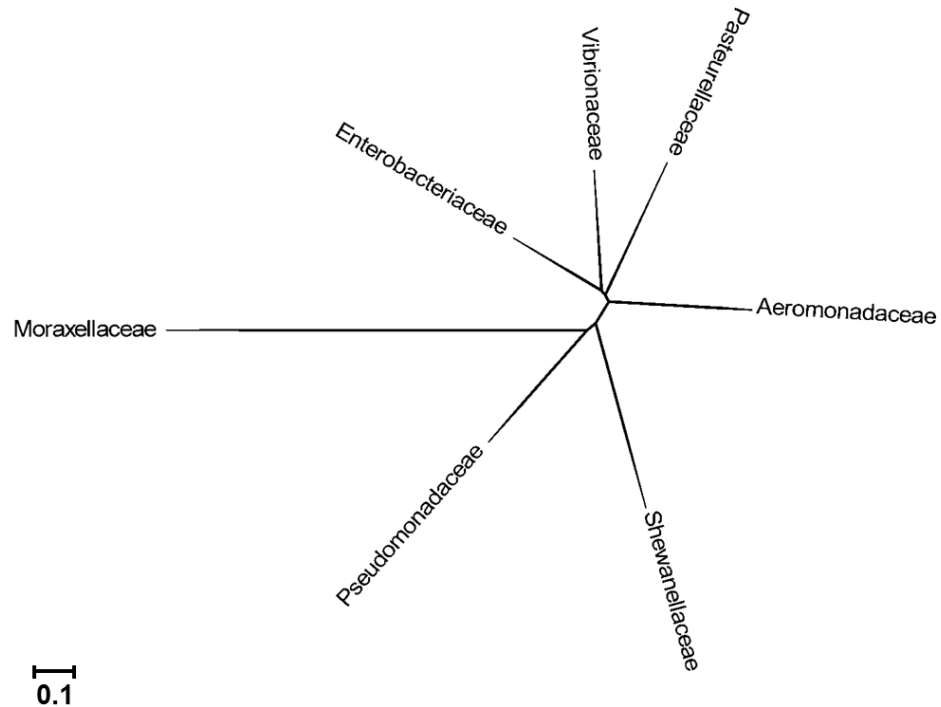
Figure 1.5 Phylogenetic tree of MorC sequences from related bacteria. The tree was generated using homologous MorC sequences from 435 bacterial genomes. Tree topology remained constant independent of methodology. Scale bar = number of amino acid substitutions per position.

Figure 1.6 Predicted operon organization of the *morC* operon in seven bacterial families.

All MorC homologs identified to date have a specific domain structure (Figure 2.1). The N-terminal domain belongs to COG2911 (Cluster of Orthologous Groups), a domain defined by sharing significant homology to sequences in other organisms (Tatusov *et al.* 1997). Although it is well-conserved, there is currently no known function ascribed to this domain. The N-terminal domain also contains a single membrane anchor domain consisting of 19 amino acids in *A. actinomycetemcomitans*. The presence of this transmembrane domain suggests an inner membrane localization and supports the localization determined by Gallant *et al.* (2008).

The C-terminal domain is a member of the DUF490 (Domain of Unknown Function) family and contains the highest sequence conservation. The DUF490 domain is part of the AsmA-like superfamily based on homology to the AsmA protein of *E. coli*. AsmA is responsible for the assembly of the outer membrane porin, OmpF (Deng and Misra 1996). This similarity implies that MorC may also be involved in the biogenesis of the outer membrane. The MorC sequence is conserved based on homology searches, however, no

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data exists regarding its functional conservation. Further research is required to investigate the functional conservation of this protein.

In *A*. actinomycetemcomitans, MorC is important in maintaining outer membrane structure (Gallant *et al.* 2008) and was named morphogenesis protein C for its effect on outer membrane morphology as visualized by transmission electron microscopy. Wild-type bacteria display an irregular or convoluted outer membrane, whereas inactivation of *morC* results in loss or significant reduction in convolutions (Gallant *et al.* 2008, Azari *et al.* 2013). The convolutions are restricted to the outer membrane as the contour of the inner membrane does not follow the irregular form of the outer membrane (Azari *et al.* 2013).

In addition to changes in the morphology of the outer membrane, inactivation of *morC* also results in a posttranslational reduction in leukotoxin secretion, which depends on a functional type I secretion complex (Gallant *et al.* 2008). Concomitant with reduction in leukotoxin is a reduction in cell size, an increase in autoaggregation, (Gallant *et al.* 2008) and an increased sensitivity to membrane destabilizing agents. These pleiotropic effects imply that multiple proteins may be affected by the loss of MorC in the membrane.

Taken together, the data suggest that MorC is a functionally conserved protein with important effects on membrane function. In this research, I propose to characterize the functional conservation of MorC using *A. actinomycetemcomitans* as a model organism. Homologous MorC proteins from various Gram-negative pathogens will be expressed in

the *morC* mutant and assayed for complementation of MorC phenotypes. In addition, truncation experiments of the endogenous *A. actinomycetemcomitans* MorC will be undertaken to define the functional domains of this protein. The effect of *morC* mutant on secretion systems of *A. actinomycetemcomitans* will also be investigated.

MorC is known to be involved in the type I secretion of leukotoxin, however, its effect on other secretion systems or proteins is unknown (Figure 1.7). The lack of a comprehensive membrane proteome of this organism further complicates the identification of specific proteins involved in the MorC phenotypes. Therefore, this research aims to identify the complete membrane proteome of *A. actinomycetemcomitans*. This proteome will be compared with the *morC* mutant using a quantitative mass spectrometry approach. Comparison of the two proteomes will identify putative proteins absent or modulated in abundance by the absence of MorC. The goal of this proposal is to identify additional proteins involved in the pleotropic phenotypes caused by inactivation of *morC*.
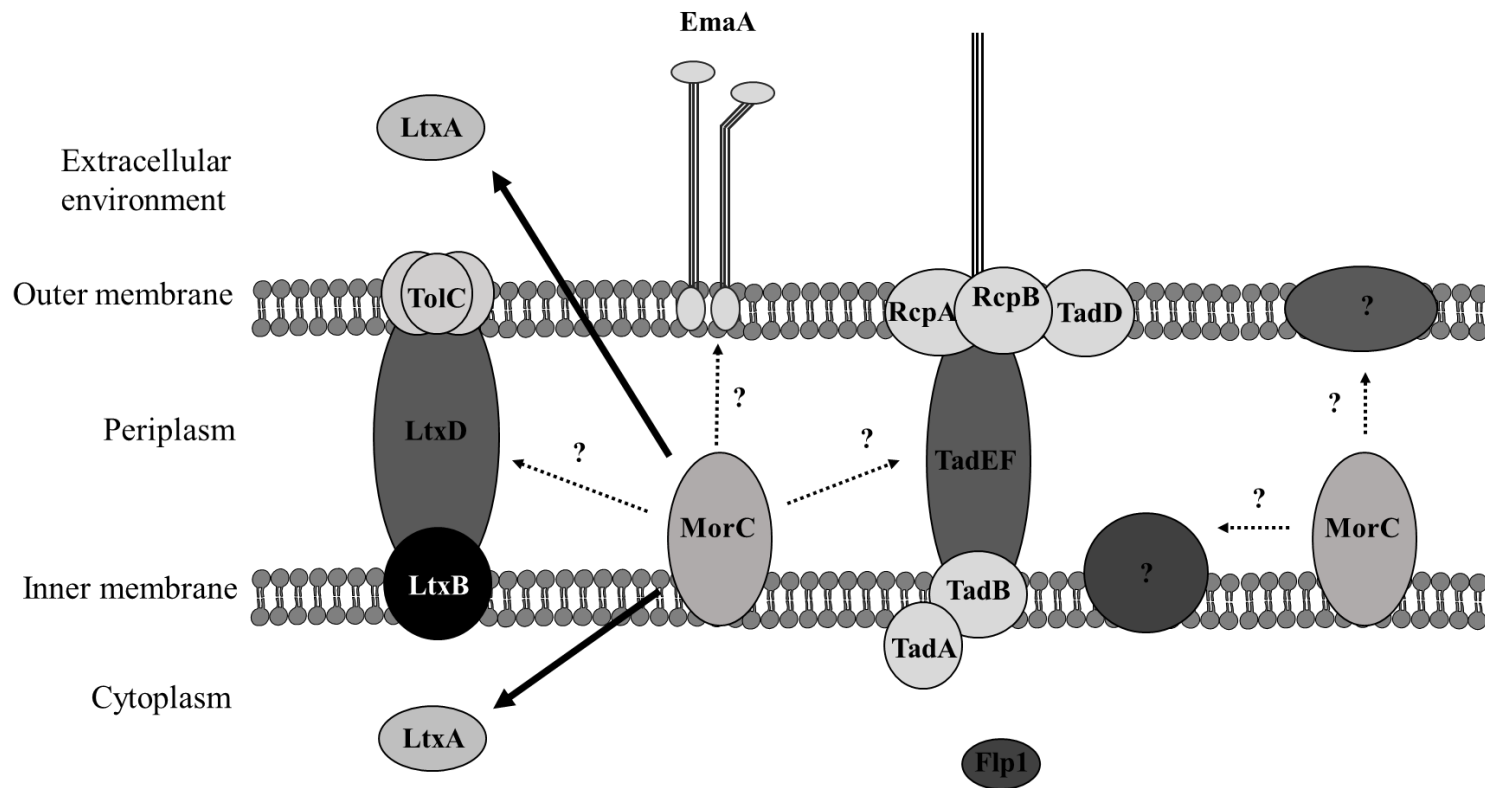
Figure 1.7 Model of the effect of *morC* inactivation on *A. actinomycetemcomitans* proteins. Solid arrows indicate proteins that are reduced in abundance in the *morC* mutant. Dotted arrows indicate hypothetical effects on secretion systems or other membrane proteins (labeled with question mark).

CHAPTER 2: The conserved carboxyl domain of MorC, an inner membrane protein of *Aggregatibacter actinomycetemcomitans*, is essential for membrane function

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Summary

Morphogenesis protein C (MorC) of *Aggregatibacter actinomycetemcomitans* is important for maintaining the membrane morphology and integrity of the cell envelope of this oral pathogen. The MorC sequence and operon organization was found to be conserved in Gammaproteobacteria, based on a bioinformatic analysis of 435 sequences from representative organisms. Functional conservation of MorC was investigated utilizing an *A. actinomycetemcomitans morC* mutant as a model system to express MorC homologs from four phylogenetically diverse representatives of the Gammaproteobacteria: *Haemophilus influenzae*; *Escherichia coli*; *Pseudomonas aeruginosa*; and *Moraxella catarrhalis*. The *A. actinomycetemcomitans* strains expressing the homologous proteins were assessed for sensitivity to bile salts, leukotoxin secretion, autoaggregation and membrane morphology. MorC from the most closely related organism (*H. influenzae*) was functionally identical to MorC from *A. actinomycetemcomitans*. However, the genes from more distantly related organisms restored some but not all *A. actinomycetemcomitans* mutant phenotypes. In addition, deletion mutagenesis indicated that the most conserved portion of the protein, the carboxyl terminus DUF490 domain, was necessary to maintain the integrity of the membrane. Deletion of the last ten amino acids of this domain of the *A. actinomycetemcomitans* MorC protein was sufficient to disrupt membrane stability and leukotoxin secretion. The data suggest that the MorC sequence is functionally conserved across Gammaproteobacteria and the carboxyl terminus of the protein is essential for maintaining membrane physiology.

Introduction

Aggregatibacter actinomycetemcomitans, a member of the Pasteurellaceae family, is a capnophilic, facultatively anaerobic bacterium implicated as the causative agent of human adult and localized aggressive periodontitis in adolescents (Socransky *et al.* 1998, Fine *et al.* 2007). In addition, *A. actinomycetemcomitans*, together with *Haemophilus* spp., *Cardiobacterium hominis, Eikenella corrodens*, and *Kingella* spp. are classified as HACEK organisms, which represent a group of oropharyngeal bacilli causing infective endocarditis (Paturel *et al.* 2004). *A. actinomycetemcomitans* is the most commonly isolated member of this group. This bacterium is also implicated in other systemic infections such as pneumonia and even brain infections (Scannapieco 1999, Rahamat-Langendoen *et al.* 2011)

The ability of this bacterium to survive within and colonize multiple tissues is highly dependent on the protein composition of the cell envelope. The protein/lipid composition of the envelope allows for the passage of specific molecules for growth and maintenance of homeostasis, while excluding environmental insults (Silhavy *et al.* 2010). *A. actinomycetemcomitans* expresses a novel membrane protein, morphogenesis protein C (MorC), that is essential for maintaining the distinct outer membrane morphology and membrane function of this organism. The deletion of this 141 kDa inner membrane protein in *A. actinomycetemcomitans* changes the membrane morphology from rugose to flat, reduces the secretion of leukotoxin posttranscriptionally, decreases cell size and increases autoaggregation (Gallant *et al.* 2008). Transformation with a replicating plasmid containing the endogenous *morC* gene restores all phenotypes and complemented strains

are identical to wild-type (Gallant *et al.* 2008). Although the absence of MorC results in the pleiotropic phenotypes, analysis of the *A. actinomycetemcomitans* cell envelope composition indicates that the protein is found in low quantities and absence of this protein only affects a specific subset of membrane proteins (Smith *et al.* 2015). Interestingly, the proteins of the leukotoxin secretion apparatus and characterized autotransporter proteins are unchanged in the mutant (Smith *et al.* 2015).

morC in *A. actinomycetemcomitans* is a member of a three gene operon including an outer membrane protein (*omp67*) and an exopolysaccharide phosphatase (*ppx*) (Gallant *et al.* 2008). Bioinformatic analysis indicates conservation of the MorC sequence and operon organization in multiple phylogenetically and physiologically diverse bacterial families (Gallant *et al.* 2008, Selkrig *et al.* 2012). Work in representative organisms of the Enterobacteriaceae family suggests an additional role for a MorC homolog (TamB/YftN) in protein translocation of the Flu autotransporter to the outer membrane (Selkrig et al. 2012). The membrane-related phenotypes of the *A. actinomycetemcomitans morC* mutant and the presence of homologous sequences in other organisms suggest that MorC function is conserved across diverse Gammaproteobacteria. Although MorC appears to be integral to the maintenance of cellular homeostasis, little is known about the protein domains and the functional conservation of this protein.

In the present study, a complementation strategy was used to determine the functional conservation of MorC using *A. actinomycetemcomitans* as a model organism. Homologous *morC* sequences were amplified, transformed into an *A. actinomycetemcomitans morC* mutant strain and assayed for complementation of *morC* phenotypes. MorC from the most

closely related organism was functionally identical to that from *A. actinomycetemcomitans*. Interestingly, MorC from more distantly related organisms restored some but not all mutant phenotypes. In addition, the carboxyl terminal DUF490 domain was necessary to maintain the integrity of the membrane. Deletion of the last 10 amino acids of this domain as well as conserved cysteine residues outside of the DUF490 domain were found to be required for proper protein function in *A. actinomycetemcomitans*. Taken together, these results suggest that MorC function is conserved across Gammaproteobacteria and the functional domain is associated with the carboxyl terminus of the protein.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *A. actinomycetemcomitans* strain VT1169 (wild-type) was grown statically at 37°C in a humidified 10% CO_2 atmosphere using TSBYE medium (3% trypticase soy broth, 0.6% yeast extract; Becton Dickinson, Franklin Lakes, NJ). *Escherichia coli*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* were grown using LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; Becton Dickinson) with agitation at 37°C. *H. influenzae* was grown statically at 37°C in a humidified 5% CO_2 atmosphere in BHI medium (3.7% brain heart infusion; Becton Dickinson) supplemented with 10 μg nicotinamide adenine dinucleotide ml^{-1} and hemin ml^{-1} (Sigma Aldrich, St. Louis, MO). Plasmids were maintained by addition to the medium of: 1 μg chloramphenicol ml^{-1} and 50 μg kanamycin ml^{-1} for *A. actinomycetemcomitans*; 50 μg

kanamycin ml^{-1} and 20 μg chloramphenicol ml^{-1} for *E. coli*. Where necessary, 0.3 mM diaminopimelic acid (DAP) was added to the medium to maintain *E. coli* strain β-2163.

Construction of *morC* deletion strain. Preliminary data indicated an increase in the propensity for homologous recombination in the insertion mutant (VT1650), when transformed with replicating plasmids carrying truncations of the *A. actinomycetemcomitans morC*. Therefore, it was necessary to develop a *morC* deletion strain of *A. actinomycetemcomitans* to eliminate homologous recombination of the truncated gene. An isogenic mutant of VT1169 with the *morC* gene deleted was generated by conjugation using a non-replicating broad host range plasmid (Mintz *et al.* 2002). The plasmid constructed for conjugation in *A. actinomycetemcomitans* is based on the mobilizable plasmid pGP704 (Miller and Mekalanos 1988). The kanamycin resistance gene from pUC-4k (Pharmacia, Kalamazoo, MI) was used as a selective marker in place of β-lactamase. The kanamycin cassette was restricted by BamHI followed by treatment with Klenow (New England Biolabs, Ipswich, MA). The cassette was ligated with pGP704 that had been digested with EcoRV and treated with shrimp alkaline phosphatase (Amersham Life Sciences, Buckinghamshire, UK). The ligation mixture was transformed into electrocompetent DH5α(λpir) *E. coli* cells. The resulting plasmid, pVT1460, was used as the template for inverse PCR to delete the β-lactamase gene as described previously (Mintz *et al.* 2002).

A. actinomycetemcomitans genomic DNA from VT1169 was purified using the Puregene genomic DNA isolation kit (Qiagen, Valencia, CA) and used as a template for PCR. A 1.5 kb fragment of the downstream *ppx* gene was amplified with a 5'XbaI

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restriction site (Table 2) and ligated with the spectinomycin adenyltransferase *aad9* gene, derived from pSL60 (Lukomski *et al.* 2000), amplified with primers containing 5'XhoI and 3'XbaI restriction sites (Table 2). A PCR product generated using the 5' *aad9* primer and the 3'*ppx* primer was ligated with the PCR2.1 TOPO vector (Invitrogen, Carlsbad, CA). The 5'XhoI/3'EcoRI PCR product was released and ligated with an amplified 1 kb fragment of the upstream *omp67* containing a 3'XhoI restriction site. The construct was introduced into the PCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and transformed into electrocompentent TOP10F- cells. Plasmids were purified from selected colonies (Qiagen, Valencia, CA) and characterized by restriction mapping and agarose gel electrophoresis. Digestion with EcoRI released the corresponding ~3.3 kb band, which was purified using the Qiagen gel purification kit (Qiagen, Valencia, CA, USA). The DNA was ligated with pVT1460 previously restricted with EcoRI and treated with shrimp alkaline phosphatase (USB, Santa Clara, CA), and the mixture was transformed into electrocompetent DH5α(λpir). Plasmids were isolated from this strain and transformed into β-2163 *E. coli* cells, auxotrophic for diaminopimelic acid (DAP) (Demarre *et al.* 2005).

Chromosomal deletion of *morC*. *E. coli* β-2163 containing pKM550 was used as a donor strain for conjugation with *A. actinomycetemcomitans* strain VT1169 as described by Gallant (2008) with modifications. Donor and recipient cells were grown to mid-logarithmic phase, centrifuged, washed and suspended at a 1:1 ratio in 100 µl TSBYE. The bacterial suspension was spotted onto a TSBYE plate containing DAP and air dried prior to incubation for 6 hours at 37°C in a humidified 10% CO_2 environment. After incubation, cells were plated onto TSBYE agar lacking DAP and containing 50 µg spectinomycin ml⁻

[1] for counter selection of the donor and recipient cells, respectively. Double homologous recombination events were identified by antibiotic resistance profiles and confirmed by PCR.

Modification of the *morC* sequence and amplification of *morC* homologs. Designated in-frame deletion constructs were generated by PCR using relevant primers (Table 2). Single nucleotide substitution mutants were generated using a commercially available mutagenesis kit following the manufacturer's instructions (Stratagene, La Jolla, CA). All plasmids were transformed into the *morC* deletion strain by electroporation (Gallant *et al.* 2008).

Genomic DNA from all organisms was prepared using the Puregene genomic DNA isolation kit (Qiagen, Valencia, CA) and used as a template for PCR with associated primers (Table 2) at an annealing temperature of 58°C. Products were introduced into the PCR2.1 TOPO vector (Invitrogen, Carlsbad, CA), transformed, and colonies were selected on LB agar containing kanamycin. Plasmids from selected transformants were isolated (Qiagen, Valencia, CA) and the amplicon was confirmed by DNA sequencing. *morC* was isolated and ligated into the appropriate restriction sites of a shuttle vector containing the *morC* promoter (Gallant *et al.* 2008). Transformants were screened by PCR and plasmids were transformed into the *morC*[-] strain of *A. actinomycetemcomitans* by electroporation (Gallant *et al.* 2008). All constructs were verified by nucleotide sequencing before and after transformation into *A. actinomycetemcomitans* at the Vermont Cancer Center DNA Analysis Facility, University of Vermont.

Detection of *A. actinomycetemcomitans* MorC.   Affinity purified polyclonal antiserum raised against the amino terminal half of the protein (Smith *et al.* 2015) was used to determine synthesis of MorC in strains expressing the modified forms of the protein. For whole cell lysate preparations, cells were grown to mid-logarithmic phase (OD_{495} = 0.2-0.3), collected by centrifugation and lysed by the addition of SDS. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Membrane isolation was performed based on the method previously described (Smith *et al.* 2014). Cells were grown to mid-logarithmic phase cells (OD_{495} = 0.3) in liquid culture (250 ml) and lysed using a French pressure cell (Thermo Scientific, Waltham, MA) at 18,000 psi. Cell debris was removed by centrifugation at 12 000 *g* for 20 min. Membranes were recovered by repeated centrifugation at 100 000 *g* for 30min with the intermediary pellets suspended in PBS (10mM sodium phosphate, 150 mM NaCl, pH7.4). Equivalent amounts of protein were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA)(Smith *et al.* 2015). Immunoblots were imaged using an Odyssey CLx infrared imaging system (LiCor Biosciences, Lincoln NE).

Autoaggregation assay. Autoaggregation was monitored using an assay modified from Ulett *et. al* (2007). A single colony of *A. actinomycetemcomitans* was inoculated into 5 ml TSBYE broth and grown overnight. The next day cells were suspended by gentle swirling for 10 s and the OD_{495} was recorded immediately using a Thermo Scientific Spectronic 20+ spectrophotometer with a culture tube adaptor (ThermoFisher Scientific, Waltham, MA) as time point 0. Cells were placed at 4 °C to prevent additional growth and OD_{495} readings were taken after 15 and 30 min. The optical density readings were

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standardized by setting the initial time point to 100%. Data for each strain was taken from a minimum of three trials and representative data was graphed.

Bile salt assay. Sensitivity to bile salts was measured using a broth microdilution assay based on the method of Floyd *et al.* with modifications (Floyd *et al.* 2010). Concentrations ranging from 20 mg bile salts ml^{-1} to 0.2 mg bile salts ml^{-1} (50% glycoholic acid, 45% taurocholic acid; ThermoFisher Scientific, Waltham, MA) were generated by two-fold serial dilution of a stock solution (20 mg ml^{-1}) in TSBYE broth in a sterile 96-well tissue culture plate (Nunc, Roskilde, Denmark). Overnight *A. actinomycetemcomitans* cultures were diluted to OD$_{495}$ = 0.1 and 20 µl (approximately 10^6 cells) were added to each well to a final volume of 200 µl. Plates were incubated for 24 hours and the minimal inhibitory concentration (MIC) was determined.

morC deletions and substitution mutants were tested for sensitivity to bile salt by relative growth in 2.5 mg bile salts ml^{-1}. Growth in bile salt was performed as above and measured at OD$_{495}$ using an ELx 800 plate reader (Biotek, Winooski, VT). The effect of bile salt on bacterial growth was expressed as a percentage (OD$_{495}$(bile salt)/ OD$_{495}$(no bile salt) x 100%). Strains were compared by ANOVA with Dunnett's post-test with significance defined as a p-value < 0.05 (GraphPad Prism 6.0, GraphPad Software, La Jolla, CA).

Leukotoxin detection. Leukotoxin was detected using the method of Tang and Mintz (2012a). Cell associated toxin was determined using mid-logarithmic phase cultures of *A. actinomycetemcomitans* (OD$_{495}$ = 0.3). Whole cell lysates were prepared, and aliquots removed for protein determination (BCA; Pierce, Rockford, IL). Secreted leukotoxin was

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prepared by filtration (0.22 µM filter; Corning Life Sciences, Tewksbury, MA) of 4 ml of growth medium concentrated 40-fold by ultrafiltration (50 kDa molecular weight cutoff; Millipore, Billerica, MA). Proteins, representing 20 µg of whole cell lysate or secreted proteins from 1×10^9 cells, were separated by electrophoresis on 4-15% gradient denaturing gels (Bio-Rad, Hercules, CA). For *morC* truncation and substitution mutants, secreted leukotoxin was normalized to whole cell lysate protein concentration (BCA assay, Pierce, Rockford, IL) as a measure of cell number. Proteins were separated by SDS-PAGE, transferred to PVDF or Immobilon-FL membranes (Millipore, Billerica, MA) and probed with an anti-leukotoxin antibody (provided by E. T. Lally, University of Pennsylvania). Immune complexes were bound by horseradish peroxidase or IRDye 800CW conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA and LiCor Biosciences, Lincoln NE, respectively). Detection was completed by addition of SuperSignal West Pico substrate (Pierce, Rockford, IL) followed by exposure to film (Eastman Kodak, Rochester, NY) or by secondary antibody fluorescent emissions measured using the Odyssey CLx system (LiCor Biosciences, Lincoln NE).

Ultrastructure analysis. Membrane ultrastructure was visualized by transmission electron microscopy (TEM) of negatively stained whole mount bacterial preparations as described previously (Azari *et al.* 2013). Bacteria were grown overnight in the appropriate broth medium. Cultures were diluted 1:10 with sterile media and allowed to grow to log phase ($OD_{495} = 0.2$). A 2 ml aliquot of cells was removed and centrifuged at $800 \times g$ for 5 minutes at 4°C. The supernatant was removed and the cell pellet was gently suspended in cold phosphate buffered saline (PBS, 10mM sodium phosphate, 150 mM NaCl, pH7.4). A

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5 µl aliquot of the cell suspension was added to carbon coated copper grids (400 mesh), rinsed with PBS, and stained with Nano-W (Nanoprobes, Yaphank, NY). The representative Gammaproteobacteria used for the *morC* homolog studies were visualized from colonies grown overnight on agar plates. Cells were transferred to carbon coated grids by first placing 5 µl of PBS on a group of colonies and then lowering a grid, carbon-side down, on top of the humidified colonies. 3 µl of buffer was added to the grid containing the transferred colonies to prevent drying and were further processed as described above prior to visualization. Grids were observed on a Tecnai 12 electron microscope (FEI, Portland, OR) operating at an acceleration voltage of 100 kV. Images were collected at nominal magnifications of 52 kx on a 2,048-by-2048-pixel charge-coupled camera with a pixel size of 14 µm (TVIPS, Gaunting, Germany).

Bioinformatic analysis. The BLASTP algorithm (e-value cutoff = 10^{-50}) (Altschul *et al.* 1990) was used to select homologous MorC sequences from the NCBI database using as query the *A. actinomycetemcomitans* sequence (Genbank Accession: AAY86707.1). Multiple phylogenetic trees were constructed using either neighbor joining, maximum likelihood or minimal evolution methods in the MEGA5 software package (Tamura *et al.* 2011). Transmembrane and signal anchor domains were predicted using Transmembrane Helix prediction based on hidden Markov Models (TMHMM) (Krogh *et al.* 2001). Conserved domains were identified using the NCBI Conserved Domain Search algorithm (Marchler-Bauer *et al.* 2011). Multiple sequence alignments were conducted using CLUSTALW or COBALT (Papadopoulos and Agarwala 2007, Sievers *et al.* 2011). Amino acid sequence of MorC was compared to similar sequences in solved 3-D strucutres using

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HHPred (Soding *et al.* 2005). Predicted organization of the *morC* operon was retrieved from the Prokaryotic Operon Database (Taboada *et al.* 2010, Taboada *et al.* 2012).

Results

Bioinformatic analysis of MorC. MorC plays an important role in the architecture of the outer membrane of *A. actinomycetemcomitans* (Gallant *et al.* 2008). A BLAST search revealed a total of 435 MorC homologs from a variety of Gram-negative bacteria. In this study, four MorC homologs from clinically relevant pathogens were used for further analysis. In order of phylogenetic relatedness to *A. actinomycetemcomitans*, the sequences chosen were: *H. influenzae* of the Pasteurellaceae (Genbank Accession: YP_248384.1); *E. coli* of the Enterobacteriaceae (NP_418642.1); *P. aeruginosa* of the Pseudomonadaceae (NP_251232.1); and *M. catarrhalis* of the Moraxellaceae (EKF83187.1). All the translated protein sequences have relatively the same size (1,200-1,300 amino acids) except for the *M. catarrhalis* protein, which contains 1696 amino acids. In relation to the *A. actinomycetemcomitans* MorC sequence, the sequence homology (identical and conserved residues) varied among the proteins: 69% (*H. influenzae*), 57% (*E. coli*), 46% (*P. aeruginosa*) and 42% (*M. catarrhalis*).

Analysis of the protein sequences revealed two conserved domain architectures despite primary sequence variability (Figure 1). An N-terminal domain belonging to the cluster of orthologous group, COG2911, is present in all sequences. A single transmembrane segment is predicted (TMHMM) in this domain, which supports the inner membrane localization previously reported (Gallant *et al.* 2008, Selkrig *et al.* 2012). In the *A.*

actinomycetemcomitans MorC sequence five cysteines are present: C26, C103, C110, C732 and C741. The C26 is present only in the most closely related homolog of *H. influenzae*. While the *Moraxella* sequence contains only the second pair of conserved cysteines, the rest of the sequences contain two pairs of conserved cysteines: C103, C110 and C732, C741. The second conserved domain is located at the C-terminus and is categorized as DUF490, a domain of unknown function. This region of the protein contains the greatest homology: 85% (*H. influenzae*), 53% (*E. coli*), 58% (*P. aeruginosa*) and 50% (*M. catarrhalis*).

Conservation of the primary amino acid sequence is also predicted to be maintained at the structural level. Protein structure prediction based on solved 3D structures (HHPred) indicated shared structural conformations between the five sequences. The *A. actinomycetemcomitans* and *H. influenzae* sequence predictions are similar in the transmembrane domain and amino acids 880-1012 (Table 4). The remaining proteins share these conformations and contain unique structural elements. The *E. coli* protein contains unique elements between amino acids 1059-1120. The *P. aeruginosa* protein contains elements between amino acids 1015-1062 and is the only sequence with a predicted structure at the carboxyl terminal end of the protein (1160-1221). The *M. catarrhalis* structure prediction is more closely related to *A. actinomycetemcomitans* and *H. influenzae* but contains additional elements between amino acids 180-265 (also present in the *P. aeruginosa* protein) and 468-716 (present in *P. aeruginosa* and *E. coli*).

Characterization of the heterologous expression of *morC* from different genera of

bacteria in the *A. actinomycetemcomitans morC* mutant strain.

Afimbriated *A. actinomycetemcomitans* grows relatively homogenously in broth. In

contrast, the *morC* mutant strain exhibits autoaggregation and grows in macroscopic

clumps (Gallant *et al.* 2008). This phenotype explains the difference in the maximum

optical density of the isogenic mutant ($OD_{495} = 0.45$) when compared with the parent strain

($OD_{495} = 0.65$)(Figure 2A). The growth phenotype, particularly the lag phase, was

differentially impacted by the introduction of the various *morC* homologs. The strain

containing the *H. influenzae* gene consistently displayed a higher maximum optical density

and a more rapid growth rate than the *A. actinomycetemcomitans* complemented strain.

Strains complemented with *P. aeruginosa* or *M. catarrhalis morC* displayed maximal

growth similar to the strain complemented with *A. actinomycetemcomitans morC*.

However, during the lag phase of growth (time points between 0 and 12 hours), these

strains consistently displayed optical densities less than that of the *A.*

actinomycetemcomitans complemented strain. The *E. coli morC* strain displayed growth

characteristics similar to the *A. actinomycetemcomitans* mutant. Based on these growth

curves, all subsequent experiments were performed using cells in early logarithmic phase

($OD_{495nm} = 0.2$-0.3).

Autoaggregation of each strain was assayed by monitoring the percentage of cells

remaining in the supernatant of a well-mixed culture over time using optical density

measurements (Figure 2B). Fewer cells in suspension indicates an increase in aggregation.

In the *morC* mutant, 30% of cells remain in suspension after half an hour compared to 80%

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of cells complemented with endogenous *morC*. Strains transformed with *morC* from *H. influenzae, E. coli,* or *M. catarrhalis*, exhibited autoaggregation similar to a strain complemented with the endogenous *morC* with 70% to 80% of cells remaining in the supernatant. Interestingly, mutant cells transformed with *Pseudomonas* homologs displayed no discernable autoaggregation and all cells remained in suspension during the time period tested.

Bile salt sensitivity was determined to examine the relative membrane stability of the different *morC* expressing strains. The minimal inhibitory concentration (MIC) of the wild-type *A. actinomycetemcomitans* was shown to be 10 mg bile salts ml^{-1}. The bile salt sensitivity of the other bacteria used in this study was determined to be > 20 mg bile salts ml^{-1}. Inactivation of the *A. actinomycetemcomitans morC* results in a four-fold reduction in the MIC to 2.5 mg bile salts ml^{-1}. Mutants transformed with the homologous *morC* sequences were tested for the restoration of the bile salt phenotype. The *H. influenzae* homolog rescued the bile salt sensitivity phenotype and restored the MIC to wild-type levels. This is in comparison to the remaining homologs, which did not restore bile salt sensitivity and the MIC remained at mutant levels (data not shown).

The loss of MorC in *A. actinomycetemcomitans* decreases the amount of both intracellular and secreted leukotoxin (Gallant *et al.* 2008). Intracellular and secreted toxin was detected by immunoblot using a leukotoxin-specific antibody (Figure 3). Leukotoxin was found to be reduced the *morC* mutant cells relative to a control producing *A. actinomycetemcomitans* MorC. Complementation with the heterologous gene from *H. influenzae* restored the secretion of the toxin in both compartments. Conversely,

transformation of the mutant with the *E. coli* homolog showed no detectable increase in leukotoxin abundance and the amount appeared to be reduced when compared with the *A. actinomycetemcomitans* mutant. The strains expressing the *P. aeruginosa* homolog were observed to contain increased amounts of toxin in both compartments compared with the mutant strain. In the *M. catarrhalis* expressing strain, leukotoxin was only slightly elevated above mutant levels. Interestingly, the magnitude of the increase was substantially greater in the medium.

The outer membrane of *A. actinomycetemcomitans* displays a rugose or convoluted phenotype when whole bacteria are observed by TEM. This membrane phenotype is shared by some but not all of the parent bacteria used in this investigation. *H. influenzae*, *M. catarrhalis*, and *E. coli* were all observed to have a rugose morphology. In contrast to these organisms, the outer membrane of *P. aeruginosa* displays a flat morphology (data not shown). Inactivation of the *A. actinomycetemcomitans morC* results in a change in the outer membrane from a rugose to a flat morphology (Figure 4). The wild type phenotype can be restored by transformation with the *A. actinomycetemcomitans morC* on a replicating plasmid containing the endogenous promoter. Transformation of the *A. actinomycetemcomitans* mutant with a plasmid encoding the homologs of MorC from *H. influenzae* and *E. coli* resulted in complementation of the membrane phenotype and displayed wild-type features. Homologous MorC from *M. catarrhalis* resulted in a subpopulation of cells with a wild-type outer membrane morphology, while the majority retained a flat outer membrane. In contrast, transformation with a similar plasmid encoding the *P. aeruginosa* homolog resulted in a flat outer membrane. In this strain, a number of

elongated and t-shaped cells were observed, suggestive of a defect in cell division. A summary of phenotypes for each strain is presented in Table 3.

Characterization of the DUF490 domain and conserved cysteine residues of *A. actinomycetemcomitans* MorC. The heterologous expression of *morC* from different genera of bacteria can substitute for the *A. actinomycetemcomitans* gene in the restoration of most of the mutant phenotypes. The DUF490 domain is the most highly conserved region of the protein. To determine if this domain is required for the physiological functions associated with this protein, amino acid substitution and deletion analysis were performed.

Truncated proteins corresponding to different regions of the DUF490 domain, based on secondary structure predictions, were generated by in-frame deletions of the corresponding nucleotide sequences and expressed in the *morC* mutant strain. The strain expressing MorC with the DUF490 domain (residues 1050-1292) deleted displayed phenotypes identical to the mutant strain for both leukotoxin secretion and bile salt sensitivity (Figure 5 A & 6, respectively). Similar phenotypes were also observed in a strain expressing the Δ1235-1292 construct. However, deletion of the last 10 amino acids (Δ1283-1292: AFDLLYQFEF) resulted in a strain displaying intermediate bile salt sensitivity, which is significantly different from both the strain expressing *A. actinomycetemcomitans morC* and mutant strains (Figure 5, ANOVA with Dunnett's post-test, $p < 0.05$). Interestingly, this strain did not restore leukotoxin secretion (Figure 6) and showed a mixed population of cells with rugose and flat, mutant-like, outer membranes (data not shown). The strain expressing a protein containing a deletion of the terminal

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phenylalanine (Δ1292) secretes more leukotoxin than the mutant and partially complements the leukotoxin secretion phenotype.

The role of the cysteine residues in determining the functionality of the *A. actinomycetemcomitans* protein was determined in complementation assays using substitution mutagenesis (conversion of cysteine to a serine residue) of the protein. Strains expressing proteins with substitutions at C26S (Figure 5B & 6), C103S, C110S (data not shown), or the C103S/C110S double mutant (Figure 5B &6) were indistinguishable from the wild type strain. However, expression of proteins with a substitution of either cysteine at 732 or 741 with serine (data not shown), or the double substitution construct (Figure 5 B & 6), failed to complement the mutant phenotypes.

MorC production was verified in the truncation strains by probing an immunoblot with affinity purified polyclonal anti-MorC serum. MorC of the expected size was found in the whole cell lysate preparations of all strains expressing a truncated protein (Figure 7A). However, we were unable to detect MorC production in the strains expressing MorC with cysteine substitutions C732S and C741S (Figure 7B). We tested for membrane localization of MorC in strains that produced the protein in whole cell lysate samples and displayed mutant phenotypes. Membrane-associated MorC of the expected molecular mass was detected in all of these samples (Figure 7C).

Discussion

The bacterial membrane protein composition is affected by physiological and environmental demands. Particular subsets of proteins are present or conserved to fulfill general membrane functions. Cellular homeostasis is maintained by the targeting and insertion of these proteins into the membrane. A variety of macromolecular complexes ensures proper protein integration and results in the generation of a functional membrane. We have previously identified a novel protein, MorC, in *A. actinomycetemcomitans* that impacts membrane morphology, barrier function and protein secretion (Gallant *et al.* 2008). Recently, MorC has been implicated in the modulation of fimbriae secretion and the architecture of the biofilm associated with fimbrial production (unpublished data). A homologous protein in the Enterobacteriaceae, TamB, has also been shown to be involved in protein secretion to the outer membrane (Selkrig *et al.* 2012). The MorC protein sequence is highly conserved across species and a bioinformatic analysis was used to select sequences present in Gammaproteobacteria to study the functional conservation of this protein.

MorC sequences from all families of Gammaproteobactera investigated display the same arrangement of two conserved domains. A single transmembrane helix consisting of ~20 amino acids (25-42 in *A. actinomycetemcomitans* and found in most of the species) is predicted at the N-terminal region of the protein within the COG2911 domain. This domain is found in hundreds of Gram-negative bacterial proteins, yet has no known function. The COG2911 is distinct from any other COGs and is not assigned to a protein family (Marchler-Bauer *et al.* 2011, Punta *et al.* 2012). A second conserved domain, DUF490, is

predicted to reside at the C-terminus of all investigated MorC homologs and exhibits the greatest sequence homology between MorC proteins. In contrast to COG2911, DUF490 is assigned to the AsmA (assembly suppressor mutations) protein family. AsmA is the prototypic protein of this family and is localized to the inner membrane of *E. coli* (Deng and Misra 1996). *E. coli* AsmA is involved in the insertion of proteins into the outer membrane (Deng and Misra 1996). Inactivation of the *morC* homolog in *Citrobacter rodentium* affects the display of a specific autotransporter on the cell surface (Selkrig *et al.* 2012). However, MorC does not appear to influence the integration of autotransporters in *A. actinomycetemcomitans* as the trimeric autotransporter EmaA was still observed attached to the outer membrane of the *morC* mutant (Gallant *et al.* 2008, Yu *et al.* 2009, Azari *et al.* 2013). A second autotransporter, Aae, was found to be equal in abundance between the wild-type and *morC* mutant (Smith *et al.* 2015). Interestingly, no AsmA homologs are predicted in the Pasteurellaceae as determined by BLAST analysis. Collectively, the available data implies that MorC is the only AsmA-like protein in *A. actinomycetemcomitans* and therefore could play a central role in membrane organization in this organism.

Resistance to chaotropic agents such as detergents or bile salts determines membrane stability (Nikaido 2003). Bile salt sensitivity is mediated by outer membrane proteins, which serve to stabilize the membrane by precluding intercalation of solubilizing agents or playing a role in active efflux (Begley *et al.* 2005). The ability of the *H. influenzae morC* gene product to restore the MIC in the *A. actinomycetemcomitans* mutant suggests a similar mechanism for bile salt resistance in the Pasteurellacae, which may be dependent on a

membrane protein composition that is not shared by distantly related organisms. The MorC derived from non-Pasteurellacae bacteria may not properly interact with and assemble the outer membrane proteins required for bile salt resistance found in *A. actinomycetemcomitans*.

Some secretion systems are maintained among diverse bacterial species. Hemolytic strains of *E. coli* secrete a repeats in toxin (RTX) or hemolysin that is dependent on a type I secretion system. Secretion requires the assembly of an ATPase (HlyB), a periplasmic channel (HlyD) and an outer membrane trimeric protein controlling secretion (TolC) (Delepelaire 2004). To the best of our knowledge, no accessory proteins associated with type I secretion systems have been identified in *E. coli*. The secretion of LtxA via an analogous type I secretion system in *A. actinomycetemcomitans* is dependent on the presence of MorC in the membrane, as indicated by the reduced levels of toxin secreted by the *morC* mutant. However, a similar role for MorC in a hemolytic *E. coli* strain is not evident as inactivation of *morC* in *E. coli* did not affect hemolysin secretion (data not shown). Therefore, the *A. actinomycetemcomitans* MorC appears to play a distinct role in the secretion of a type I effector molecule when compared with a homologous *E. coli* effector molecule secreted by an analogous system.

MorC homologs expressed in the *A. actinomycetemcomitans* mutant strain demonstrated the functional diversity of this protein. The expression of MorC derived from *P. aeruginosa*, an organism that secretes type I effector molecules (Guzzo *et al.* 1991, Duong *et al.* 2001, Wandersman and Delepelaire 2004) restored LtxA secretion. In addition, homologs derived from organisms expressing closely (*H. influenzae*) or more

distantly (*M. catarrhalis*) related MorC proteins, which do not have characterized type I secretion systems, also restored leukotoxin secretion in the *A. actinomycetemcomitans* mutant strain. In contrast, the bacteria expressing the *E. coli* MorC homolog failed to restore LtxA secretion. Disparity in the function of *E.coli* MorC is further illustrated by the inability of this homolog to restore LtxA secretion when expressed in the *A. actinomycetemcomitans* mutant strain. The inability of the *E. coli* MorC homolog to restore LtxA secretion may be attributed to the presence of a structural element that is absent in the other proteins. Based on structure predictions by HHPred, a distinct structural element composed of beta sheets and random coils is predicted between amino acids 1059-1120 of the *E. coli* sequence, which is not present in the other sequences (Table 4). This additional element may change the conformation of the *E. coli* MorC structure in such a manner that the protein does not interact either directly with a protein(s) of the secretion apparatus or indirectly with other proteins required for maintaining a membrane environment competent for LtxA secretion.

Electron microscopy images of *A. actinomycetemcomitans* reveal a rugose appearance of the bacterial membrane (Gallant *et al.* 2008, Azari *et al.* 2013). The rugose or convoluted surface of the bacteria is a trait shared with other members of the Pasteurellaceae and the Moraxellaceae families (data not shown). The molecules involved in the formation of membrane convolutions have not yet been identified. However, our data supports a role for MorC in the biogenesis of these structures. Inactivation of the *A. actinomycetemcomitans morC* renders the surface of the bacterium smooth, without obvious convolutions in 2D electron micrographs (Gallant *et al.* 2008, Azari *et al.* 2013). The smooth outer membrane

morphology of the mutant strain can be restored to the rugose or wild-type membrane phenotype by plasmids expressing *morC* derived from *A. actinomycetemcomitans*, *H. influenzae, E. coli* and *M. catarrhalis*. Expression of the *P. aeruginosa morC* in the mutant strain did not restore the rugose phenotype associated with the *A. actinomycetemcomitans* parent strain. The inability of the MorC from *P. aeruginosa* to restore the morphology of the outer membrane may be attributed to the formation of a structural element at the carboxyl terminus of the sequence (amino acids 1160-1221), which is absent in all other MorC sequences investigated. The change in the conformation of the protein may interfere with the binding of the *P. aeruginosa* MorC directly or indirectly to proteins that are involved in the formation of the convolutions associated with *A. actinomycetemcomitans* strains. Alternatively, the inability of the *P. aeruginosa* MorC to restore convolutions maybe related to the inherent lack of convolutions observed in *P. aeruginosa*.

Cells over-expressing *P. aeruginosa* or *M. catarrhalis* MorC exhibited aberrant cell division and altered cell shape. These observations may explain the difference in the growth curves of these strains when compared with the parent or mutant strains. These changes suggest the transcription and translation of the homologous genes in *A. actinomycetemcomitans*. Semi-quantitative RT-PCR indicated that all *morC* genes are equally transcribed (data not shown). However, we have not been successful in visualizing the synthesis of MorC homologs with N-terminal epitope tags (6xHis, FLAG, or HA) whereas the synthesis of *A. actinomycetemcomitans* MorC with the identical tags are easily detected (data not shown). Nevertheless, the ability of these constructs to complement

some or all of the mutant phenotypes implies the synthesis and membrane localization of MorC.

A signature sequence, present in the majority of outer membrane proteins (Struyve *et al.* 1991) necessary for the interaction with BamA/Omp85, is present in the *A. actinomycetemcomitans* MorC sequence. The sequence contains a terminal phenylalanine (phe) and a hydrophobic residue at positions -3,-5,-7 and -9 relative to the C-terminus of the protein. The terminal phe is important for the function of the prototypic protein (PhoE)(de Cock *et al.* 1997). The MorC sequence, AFDLLYQFEF, contains the requisite Phe (F) at the C-terminal and hydrophobic amino acids at positions -3 (F), -5 (Y),-7(L) and -9(F) relative to the C-terminus of the protein. Expression of a protein with this sequence deleted did not restore the mutant phenotypes to wild type, indicating the importance of this sequence for the function of MorC. Unlike the prototypic protein, deletion of the C-terminal Phe of the MorC sequence is not essential for restoration of bile salt resistance (Figure 6) but may play a role in leukotoxin secretion (Figure 5). Together, the data suggest that the DUF490 domain, in particular the terminal 10 amino acids, is essential for membrane function in *A. actinomycetemcomitans*.

In addition to the high degree of conservation found in the DUF490 domain, MorC also contains a pair of cysteines in the COG2911 domain (C103, C110) that are conserved among some species of Gammaproteobacteria. Expression of proteins containing mutation of these residues does not appear to be important in protein structure or function. Similarly, a cysteine (C26) found in *A. actinomycetemcomitans* and *H. influenzae* in the predicted signal anchor domain, was also shown not to be important for

the MorC phenotypes tested. However, in strains expressing the mutation of either C732 or C741, which are conserved in all of the homologs, do not complement the mutant phenotypes and we predict that these cysteine residues are important in maintaining the structure/function of the MorC protein. Therefore, we hypothesize that disulfide bonding between C732 and C741 is important for MorC function. Although these residues are highly conserved, they are not part of the DUF490 domain. This implies that the DUF490 domain is necessary but not sufficient for restoration of the *morC* phenotypes and that the COG2911 domain may play an indirect role in MorC function.

In this study, we have used *A. actinomycetemcomitans* as a model organism to investigate the function of MorC proteins derived from evolutionarily related and unrelated bacterial species. The proteins share varying levels of sequence similarity with the greatest homology located at the carboxyl region of the molecule. Though related by sequence, we have demonstrated a variation in the functional activity of the proteins, which may be attributed to the differences in secondary structures formed by specific sequences of amino acids in the DUF490 domain. In addition, we have determined that the DUF490 domain of the *A. actinomycetemcomitans* protein is required for function. Based on our data, we propose that MorC interacts with other membrane proteins to coordinate the biological functions associated with the *A. actinomycetemcomitans* membrane. Studies are ongoing to determine the interactions associated with MorC in the *A. actinomycetemcomitans* membrane.

Acknowledgements

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Tables

Table 2.1: Bacterial strains and plasmids.

Bacterial Strain or Plasmid	Description	Reference or Source
E. coli		
TOP10F-, DH10B	General cloning, *lac*⁻	Invitrogen
DH5α(λpir)	General cloning, λpir	
β-2163	DAP auxotroph, λpir	(Demarre *et al.* 2005)
A. actinomycetemcomitans		
VT1169	Spontaneous rifampin and nalidixic acid resistant mutant of SUNY465	(Mintz and Fives-Taylor 1994a)
VT1650	*morC* insertional inactivation strain. Spec^r	(Gallant *et al.* 2008)
KM555	*morC* deletion strain. Spec^r	This study
morC complement	*morC* mutant containing pKM303 - *A. actinomycetemcomitans morC*. Cm^r	(Gallant *et al.* 2008)
Plasmids		
pCR2.1-TOPO	TA cloning vector that replicates only in *E. coli*. Amp^r, Kan^r	Invitrogen
pKM1	*E. coli* and *A. actinomycetemcomitans* shuttle vector. Kan^r	(Ruiz *et al.* 2006)
pKM2	*E. coli* and *A. actinomycetemcomitans* shuttle vector. Cm^r	(Gallant *et al.* 2008)
pVT1460	Conjugative plasmid, replicates in λpir strains of *E. coli*, Km^r	(Mintz *et al.* 2002)
pKM550	pVT1460 containing *morC* deletion construct	This study

pKM303	pKM2 containing the 165bp *morC* promoter sequence. Cm^r	(Gallant *et al.* 2008)
pKM475	pKM1 containing the 165bp *morC* promoter sequence. Kan^r	This study
pKM557	pKM475 containing *A. actinomycetemcomitans morC*. Kan^r	This study
pKM408	pKM303 containing *A. actinomycetemcomitans morC*. Cm^r	(Gallant *et al.* 2008)
pKM303 – H.i.	pKM303 containing the *morC* homolog from *Haemophilus influenzae* 86-028NP. Cm^r	This study
pKM303 –E.c.	pKM303 containing the *morC* homolog from *Escherichia coli* K-12. Cm^r	This study
pKM303 – P.a.	pKM303 containing the *morC* homolog from *Pseudomonas aeruginosa* PAO1. Cm^r	This study
pKM303 – M.c.	pKM303 containing the *morC* homolog from *Moraxella catarrhalis* ATCC 43628. Cm^r	This study
C26S	Substitution of cysteine 26 to serine in pKM475	This Study
C103S, C110S	Substitution of cysteine 103 and 110 to serine in pKM475.	This Study
C732S, C742S	Substitution of cysteine 732 and 741 to serine in pKM475.	This Study
C732S	Substitution of cysteine 732 to serine in pKM475.	This Study
C741S	Substitution of cysteine 741 to serine in pKM475.	This Study
Δ1050-1292	In-frame deletion of bp 3148 to 3876 of *morC* in pKM475	This Study
Δ1235-1292	In-frame deletion of bp 3703 to 3876 of *morC* in pKM475	This Study
Δ1283-1292	In-frame deletion of bp 3847 to 3876 of *morC* in pKM475	This Study
Δ1292	In-frame deletion of bp 3874 to 3876 of *morC* in pKM475	This Study

Amp = Ampicillin, Cm = Chloramphenicol, Kan = Kanamycin, Spec = Spectinomycin.

Table 2.2: Oligonucleotide primers.

Oligonucleotide	Sequence (5'-3')
HimorCF	GCA<u>AAGCTT</u>ATGACTACAACCATCAG
HimorCR	GC<u>TCTAGA</u>CTAAATATAAAGTAAATC
EcmorCF	CCG<u>CTCGAG</u>ATGAATGGAAAAAAATC
EcmorCR	GC<u>TCTAGA</u>CTAAACTCGAACTGATAG
PamorCF	CCG<u>CTCGAG</u>GTGAAGGCGCTGAAG
PamorCR	CCG<u>GAATTC</u>TCAGTCGCGCTTCAAGA
McmorCF	CCG<u>CTCGAG</u>ATGACTCATCAGGATAATC
McmorCR	GC<u>TCTAGA</u>CTAAAACTTCCAACGATAA
Omp67F	TCTGGACGTATTGCTTTATCCGC
Omp67R	CTTC<u>CTCGAG</u>CTTATTATCCGTTCTTGTTGA
SpecF	TAAG<u>CTCGAG</u>TGACTAAATAGTGAGG
SpecR	CTTC<u>TCTAGA</u>CATGTGATTTTCCTCC
PpxF	CTTC<u>TCTAGA</u>TTATGAATAACGAAAATTTA
PpxR	TCAACGTGCCGACAGGCTTA
BamHI.P165.For	CGC<u>GGATCC</u>GCCCGTTTATTGAATCTACCTTCC
A.morC.C103S.For	GCAAATGCAATTAAGCAGCTTATGGAAATTAAAGGTTTGCG
A.morC.C110S.For	GCAAATGCAATTAAGCAGCTTATGGAAATTAAAGGTTTGCG
A.morC.C732S.For	GCCACTATTTCCGCGCACAGCTGGATACACAG
A.morC.C741S.For	CGGATTTAAGTTTCCCGCAAAGCTTTAAC
A.morC.C103S.Rev	CGCAAACCTTTAATTTCCATAAGCTGCTTAATTGCATTTGC
A.morC.C103S.Rev	CGCAAACCTTTAATTTCCATAAGCTGCTTAATTGCATTTGC
A.morC.C110S.Rev	CGCAAACCTTTAATTTCCATAAGCTGCTTAATTGCATTTGC
A.morC.C732S.Rev	CTGTGTATCCAGCTGTGCGCGGAAATAGTGGC
A.morC.C741S.Rev	GTTAAAGCTTTGCGGGAAACTTAAATCCG
MorC.SpHI.1049Rev	ACAT<u>GCATGC</u>TTATTCGCTCGGCAATGTTTTC

MorC.SpHI.1234Rev ....ACAT<u>GCATGC</u>TTATCTGTCGCCCACCCCG
MorC.SpHI.1282Rev ....................ACATGCATGCTTATTGATTAACACCCGAAACGGATTG
MorC.SpHI.1291Rev .... ACAT<u>GCATGC</u>TTATTCAAATTGATAGAGTAG
MorC.SpHI.TTA.Rev ....ACAT<u>GCATGC</u>TTAAAATTCAAATTGATAGAGTAG

Underlined sequences indicate restriction endonuclease sites.

Table 2.3: Summary of phenotypes.

Source of *morC*	Bile Salt Resistance	Leukotoxin	Membrane Morphology	Maximal Growth	Aggregation
morC mutant	-	-	-	-	-
A. actinomycetemcomitans	+	+	+	+	+
H. influenzae	+	+	+	+	+
E. coli	-	-	+	-	+
P. aeruginosa	-	+	-	+	+
M. catarrhalis	-	+	+	+	+

Table 2.4: MorC secondary structure predictions based on HHPred.

Aa	Hi	Ec	Pa	Mc	Reference Structure
*1-18	1-19	1-29	1-22	-	§2JPW (1-23), 2JPW (1-23), 1V54 (13-41), 1M56 (29-50)
21-43	24-46	-	-	-	1SPF (10-32), 1XRD (2-41)
-	-	-	-	34-63	3HD7 (79-108)
-	-	-	146-201	-	2NQN (66-123)
-	-	515-634	453-554	485-695	3PET (53-178), 3LYC (96-209), 3LYC (50-220)
880-1012	879-1011	857-988	830-961	1258-1389	3LYC (85-239)
-	-	-	1015-1062	-	4FZL (168-240)
-	-	1059-1120	-	-	3BB6 (34-97)
-	-	-	1160-1221	-	4FQE (56-115)
-	-	-	-	1323-1502	3SZE (537-514)

*Numbers represent the amino acid sequence from the MorC proteins.

§ PDB IDs for the reference structures are listed in order from left to right. Numbers in parentheses indicate the corresponding sequence of the reference structure.

"-"absence of predicted structural homologs

Figures
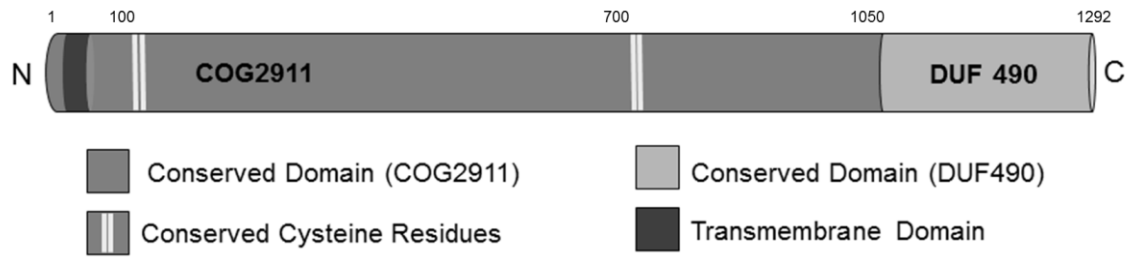
Figure 2.1 Diagram of a prototypic MorC. Domains common to all MorC proteins are shown to scale. Domain structure was predicted using the NCBI conserved domain search tool. Conserved cysteines were identified using CLUSTALW and transmembrane helices were predicted using the TMHMM algorithm.
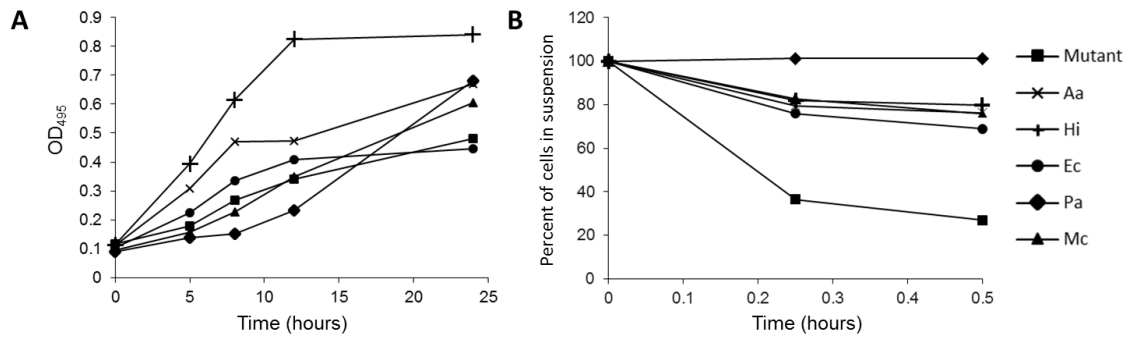
Figure 2.2 Representative growth curves and autoaggregation of transformed *A. actinomycetemcomitans* strains. A. Overnight cultures of *A. actinomycetemcomitans morC* mutant strains containing homologous *morC* genes on a replicating plasmid were diluted to $OD_{495} = 0.1$ and incubated at 37 °C in a 10% CO_2 atmosphere for 24 hours. Optical density was recorded at the time points indicated and data is representative of at least three independent experiments. **B.** Cells were grown overnight in broth and gently suspended. OD_{495} readings were taken at 0, 15, and 30 minutes and standardized to time point 0 as 100%. Percentages indicate the relative amount of cells in solution at the indicated time point. Data is representative of three independent experiments. Legend: *A. actinomycetemcomitans morC* mutant containing, empty vector (box), endogenous *morC* (x), *H. influenzae morC* (plus), *E. coli morC* (circle), *P. aeruginosa* (diamond), *M. catarrhalis* (triangle).

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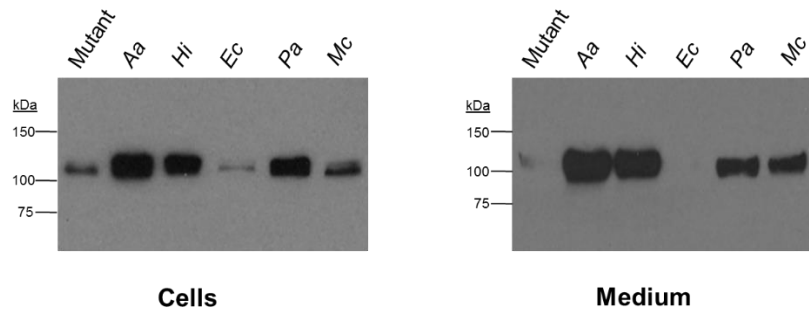
Figure 2.3 Immunoblot analysis of leukotoxin synthesis and secretion in *morC* homologs. Strains were grown to mid-log phase and 20 µg of protein (cell associated toxin) or secreted protein from 10^9 cells (medium) was probed with anti-leukotoxin antibody. Immunoblots are representative of three separate experiments. *A. actinomycetemcomitans morC* mutant (Mutant), *morC* mutant complemented (Aa), *H. influenzae morC* (Hi), *E. coli* (Ec), *P. aeruginosa* (Pa), and *M. catarrhalis* (Mc).
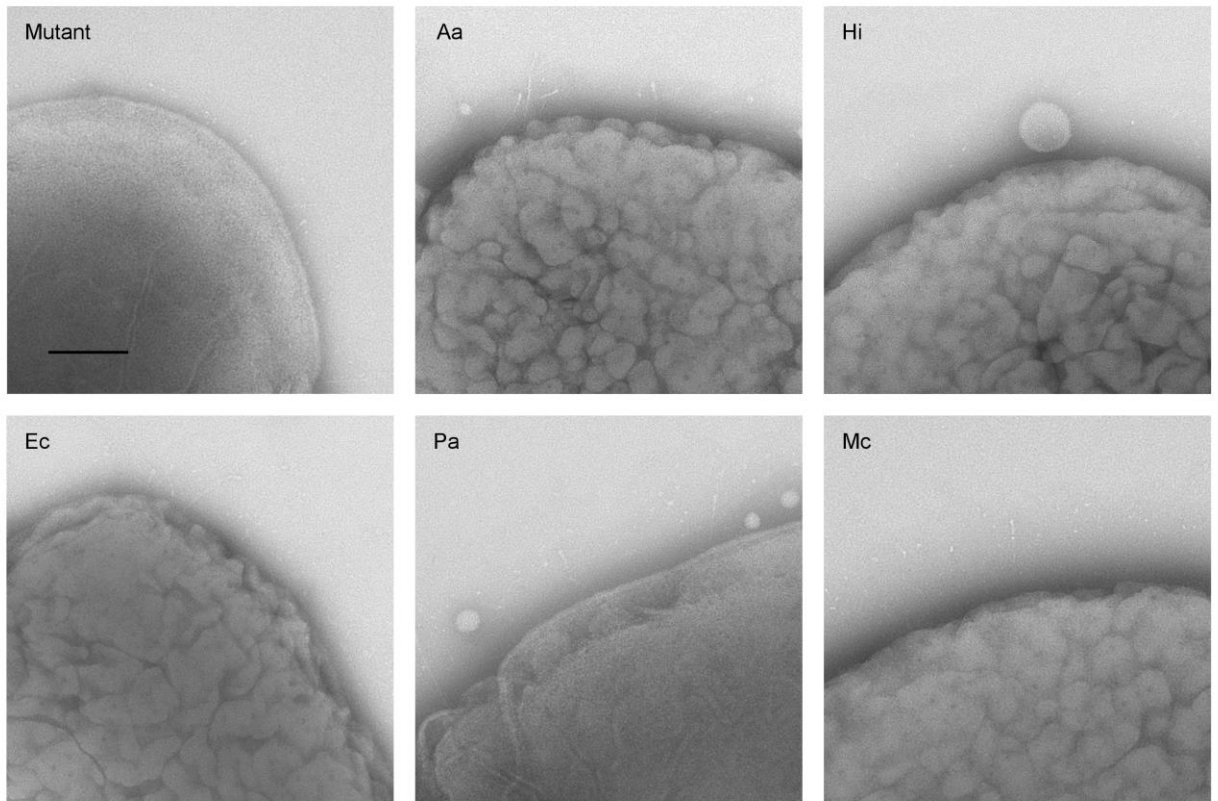
Figure 2.4 Electron micrographs of negatively stained whole mount preparations of *morC* **complementation strains.** Transmission electron micrographs of representative *A. actinomycetemcomitans* cells transformed with the *morC* homologs. *A. actinomycetemcomitans morC* mutant (Mutant), *morC* mutant transformed with *morC* from: *A. actinomycetemcomitans* (Aa), *H. influenzae* (Hi), *E. coli* (Ec), *P. aeruginosa* (Pa), and *M. catarrhalis* (Mc). Scale bar = 100 nm.
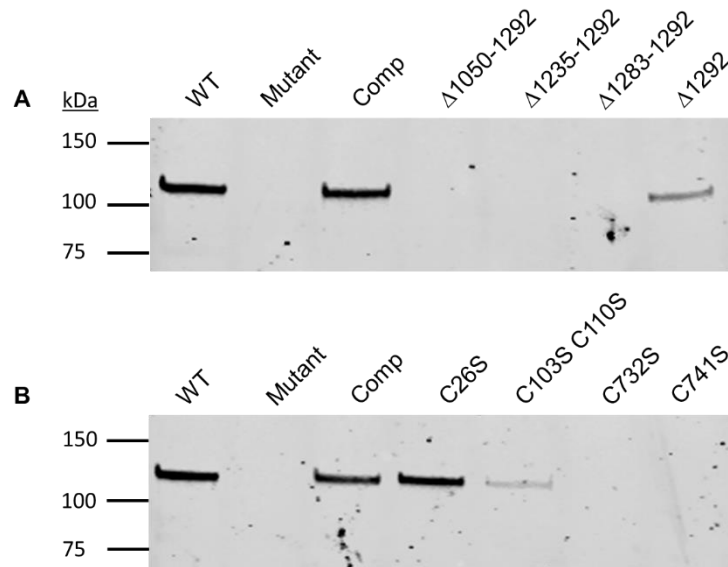
Figure 2.5 Immunoblot analysis of leukotoxin secretion in *A. actinomycetemcomitans* MorC truncation and substitution mutants. Secreted leukotoxin was prepared by filtration of 4 ml of growth medium from mid-log phase cells and concentrated 40-fold by ultrafiltration. Samples were normalized to whole cell lysate protein concentration as a measure of cell number and immunoblots were probed with anti-leukotoxin antibody. Immunoblots are representative of three separate experiments. **A.** *A. actinomycetemcomitans morC* mutant strains containing truncations in the DUF490 domain. **B.** *A. actinomycetemcomitans morC* mutant strains containing cysteine substitutes.
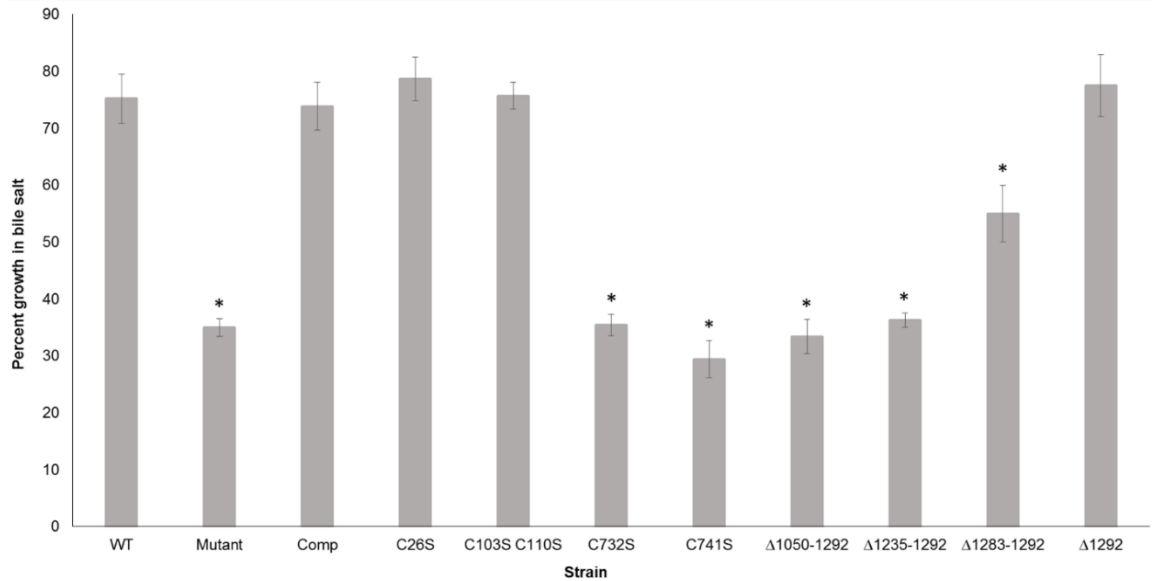
Figure 2.6 Bile salt sensitivity of *A. actinomycetemcomitans* MorC truncation and substitution mutants. Sensitivity to bile salts was measured in a 96 well plate growth assay. Mid-logarithmic phase cells were diluted into 2.5 mg bile salts ml^{-1} and growth for 24 hours. Optical density was measured at 495 nm. Growth of each strain in bile salt was compared to a control in TSBYE alone and expressed as a percentage. The (*) symbol indicates a significant difference from the wild-type strain (ANOVA, Dunnett's post-test, $p < 0.05$).
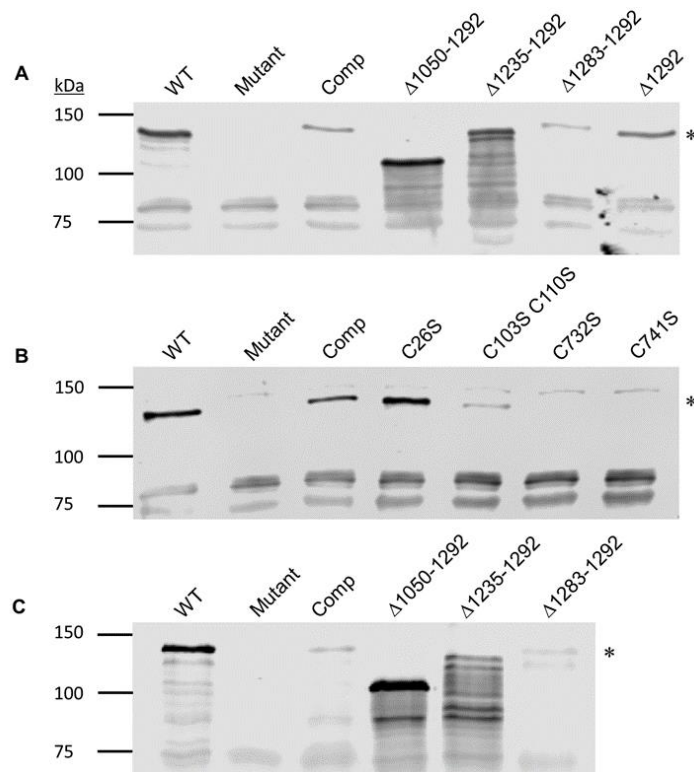
Figure 2.7 MorC expression in *A. actinomycetemcomitans* **truncation and substitution mutants.** MorC expression was determined by growing strains to mid-logarithmic phase and probing an immunoblot of 20 µg of whole cell lysate protein or 50 µg of whole membrane fraction with affinity purified polyclonal antiserum raised against the amino terminal half of the protein. Bacterial cell envelopes were prepared for strains that exhibited MorC expression in whole cell lysate preparations but were otherwise mutant for MorC phenotypes. The * indicates the predicted position of the 141 kDa wild-type MorC protein **A.** Whole cell lysates of carboxyl-terminal truncations of the DUF490 domain: Δ1050-1292 (115 kDa), Δ1235-1292 (134 kDa) Δ1283-1292 (139 kDa) and Δ1292 (141 kDa). **B.** Whole cell lysates of cysteine substitutes. **C.** Cell envelope preparations of carboxyl-terminal truncations.

CHAPTER 3: The cell envelope proteome of *Aggregatibacter actinomycetemcomitans*

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Key words: Periodontal disease, Secretion systems, Membrane proteins, Bioinformatics

Summary

The cell envelope of Gram-negative bacteria serves a critical role in maintenance of cellular homeostasis, resistance to external stress, and host-pathogen interactions. Envelope protein composition is influenced by the physiological and environmental demands placed on the bacterium. In this study, we report a comprehensive compilation of cell envelope proteins from the periodontal and systemic pathogen *Aggregatibacter actinomycetemcomitans* VT1169, an afimbriated serotype b strain. The urea-extracted membrane proteins were identified by mass spectrometry-based shotgun proteomics. The membrane proteome, isolated from actively growing bacteria under normal laboratory conditions, included 665 proteins representing 28% of the predicted ORFs in the genome. Bioinformatic analyses were used to annotate and predict the cellular location and function of the proteins. Surface adhesins, porins, lipoproteins, numerous influx and efflux pumps, multiple sugar, amino acid, and iron transporters, and components of the type I, II and V secretion systems were identified. Periplasmic space and cytoplasmic proteins with chaperone function were also identified. 107 proteins with unknown function were associated with the cell envelope. Orthologs of a subset of these uncharacterized proteins are present in other bacterial genomes, while others are found exclusively in *A. actinomycetemcomitans*. This knowledge will contribute to elucidating the role of cell envelope proteins in bacterial growth and survival in the oral cavity.

Introduction

Periodontal disease is a common inflammatory condition that affects the tissue surrounding the tooth resulting in reduced bone levels and ultimately tooth loss (Darveau 2010). *Aggregatibacter actinomycetemcomitans* is a pathogenic bacterium of the Pasteurellaceae family that colonizes the oral cavity of humans and old-world primates (Zambon *et al.* 1983). The presence of *A. actinomycetemcomitans* in the subgingival plaque of teeth is strongly implicated in the development of chronic adult periodontal disease and an acute form of the disease, localized aggressive periodontal disease (LAP), that mainly affects children and young adults (Zambon *et al.* 1983). *A. actinomycetemcomitans* is also capable of disseminating to distant tissues via the bloodstream and causes infective endocarditis, the inflammation of heart valves (Paturel *et al.* 2004, Tang *et al.* 2008). Respiratory, soft tissue, and brain infections are also associated with this bacterium (Kaplan *et al.* 1989, Scannapieco 1999, Rahamat-Langendoen *et al.* 2011).

The survival of *A. actinomycetemcomitans* in these disparate environments is dependent on the protein composition of the cell envelope. The cell envelope of this and other Gram-negative bacteria is comprised of three layers: an inner membrane adjacent to the cytoplasm, a periplasmic space containing the cell wall, and an outer membrane separating the periplasm from the extracellular environment. Both membranes are composed of a phospholipid bilayer containing peripheral and integral membrane proteins with the outer membrane forming an asymmetric bilayer due to the incorporation of lipopolysaccharide (LPS) in the outer leaflet (Silhavy *et al.* 2010).

The cell envelope, as a whole, is a critical structure involved in maintaining cellular homeostasis (Silhavy *et al.* 2010). This equilibrium is maintained by the dynamic interaction between the compartments of the cell. These interactions are mediated by individual proteins or protein complexes that allow for the transport of macromolecules between the cytoplasm and the external milieu or to be incorporated into the envelope itself. Therefore, the cell envelope can be viewed as a single unit inclusive of proteins found in both membranes, the periplasmic space, and peripherally associated with the inner membrane.

The nature of the proteins that comprise the cell envelope will be dependent on the cellular environment. However, a proportion of proteins or protein orthologs will be present in the envelope of most, if not all, Gram-negative bacteria. Despite the importance of these proteins, few envelope proteins have been characterized in *A. actinomycetemcomitans*. In this work, we used a gel-free mass spectrometry approach to detect proteins present in whole membrane fractions of VT1169, an afimbriated serotype b strain of *A. actinomycetemcomitans*. A total of 665 unique proteins were consistently identified. Bioinformatic analyses indicate that these proteins have diverse functions including virulence determinants, secretion, transport, metabolism, and energy generation. In addition, 107 proteins in the dataset were identified which have not been characterized.

Materials and Methods

Bacterial strains and growth conditions. VT1169, a well characterized laboratory-adapted strain generated by Mintz and Fives-Taylor (1994a), was used in this study. The genome of this afimbriated, serotype b strain of *A. actinomycetemcomitans* is homologous to the fimbriated, serotype b reference strain, HK1651. Bacteria were grown using TSBYE medium (3% tryptic soy broth, 0.6% YE; Becton Dickinson, Franklin Lakes, NJ) statically at 37°C in a humidified 10% CO_2 atmosphere. For membrane preparations bacteria were first grown on solid media (TSBYE containing 1.5% agar) and 2-3 colonies were inoculated into 6 mL TSBYE broth. 250 mL of pre-warmed TSBYE was inoculated with 5 ml of a 16 hour liquid culture and grown to mid-logarithmic phase ($OD_{495} = 0.3$).

Cell envelope preparation. Bacterial cell envelopes were prepared by differential centrifugation based on previously described methods (Tang *et al.* 2012a). Cells were harvested by centrifugation (8,000 x g for 10 minutes) and the resulting pellet was washed once with Dulbecco's phosphate buffered saline (PBS, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.46 mM KH_2PO_4, 0.46 mM $MgCl_2$, pH 7.4, Sigma Aldrich, St. Louis, MO). The washed cells were suspended in PBS containing protease inhibitors and 1 mM phenylmethylsulfonyl fluoride (Roche, Basel, Switzerland) and lysed using a French pressure cell press (Thermo Scientific, Waltham, MA) at 18,000 psi. Cell debris was removed by centrifugation at 10,000 x g for 30 min. Membrane and membrane-associated proteins were separated from cytoplasmic proteins by ultracentrifugation at 100,000 x g for 30 min (Optima TL Ultracentrifuge, Beckman, Brea, CA). The pellet was suspended

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in PBS and centrifuged as previously stated. The process was repeated and the final

membrane pellet was stored frozen at   -80°C.

Liquid chromatography/Mass spectrometry (LC/MS). Four biological replicates of *A.*

actinomycetemcomitans cell envelope preparations were analyzed. The protein

concentration of the preparations were quantified and 0.5 mg were suspended in 0.5 ml of

solublization buffer (6 M urea, 2 M thiourea, 20 mM HEPES, pH = 8.0). Suspensions were

sonicated on ice (3 bursts, 20 seconds each with 1 minute intervals) (Kontes 50W

Ultrasonicator, Kontes, Vineland, NJ). Extracted proteins were reduced by addition of

dithiothreitol (4.5 mM final concentration at 55°C for 30 min) and alkylated by addition of

iodoacetamide (10 mM final concentration at room temperature in the dark for 15 min).

The solution was diluted 4-fold with 20 mM HEPES, pH 8.0, and incubated with a 1:50

enzyme to substrate ratio with sequencing grade modified trypsin (Promega, Madison, WI)

at 37°C overnight. The reaction was terminated by the addition of trifluoroacetic acid

(TFA) to a final concentration of 1%. Resulting peptides were isolated (Sep-Pak tC18

cartridge; Waters, Milford, MA) and lyophilized. Peptides were reconstituted in 100 uL

1M HEPES, pH 7.5 and subjected to dimethyl labeling as previously described (Hsu *et al.*

2003, Boersema *et al.* 2009). Following labeling, TFA was added to a final concentration

of 7%, peptides isolated, and lyophilized. Peptides were suspended in 150 μL of 10 mM

potassium phosphate, pH 2.8, containing 25% CH_3CN (Buffer A). The peptide solution

(50μL) was fractionated using a strong cation exchange (SCX; PolySULFOETHYL A)

solid phase extraction (SPE) TopTip (10-200 μL, PolyLC, Columbia, MD) and step elution

(100 μL) with increasing concentrations of KCl added to Buffer A: 0 mM; 20-95 mM in 5

mM increments; 100 mM; 200 mM; and 350 mM. The fractions were dried under vacuum, desalted using Ziptips (Pierce, Rockford, IL) and dried again. Peptides were stored at -80°C prior to liquid chromatography/mass spectrometry (LC/MS) analysis.

Peptides from each of the 20 fractions were reconstituted in 2.5% CH_3CN containing 2.5% formic acid and analyzed by nano-scale LC/MS on an LTQ-Orbitrap Discovery coupled to a Surveyor MS Pump Plus (Thermo Fisher Scientific, Waltham, MA). Half of the digest was loaded directly onto a 100 μm x 120 mm capillary column packed with MAGIC C18 (5 μm particle size, 20 nm pore size, Michrom Bioresources, Auburn, CA) at a flow rate of 500 nL/min, and peptides were separated by a gradient of CH_3CN in 0.1 % formic acid (2.5-5% in 5 min, 5-35% in 100 min, 35-100% in 5 min, 100% in 10 min, followed by an immediate return to 2.5% and a hold at 2.5% for 15 min). Peptides were introduced into the linear ion trap via a nanospray ionization source and a laser pulled ~3 μm orifice with a spray voltage of 1.8 kV. Mass spectrometry data was acquired in a data-dependent "Top 5" acquisition mode with lock mass function activated (protonated polydimethylcyclosiloxane $(Si(CH_3)_2O)_6$; m/z 445.120011). An Orbitrap survey scan from m/z 360-1600 at 30,000 (FWHM) resolution was paralleled by 5 collision-induced dissociation (CID) MS/MS scans of the most abundant ions in the LTQ. MS/MS scans were acquired with the following parameters: isolation width: 2 m/z, normalized collision energy: 35%, Activation Q: 0.250 and activation time = 30 ms. Review mode for FTMS master scans was enabled. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 sec; exclusion list size: 180; exclusion duration: 60 sec). The minimum threshold was 500. Singly charged ions were excluded for MS/MS.

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Databases and data analysis. Product ion spectra were searched against the Los Alamos (Oralgen) annotation of the *A. actinomycetemcomitans* HK1651 genome. Sequences in forward and reverse orientations were analyzed using the SEQUEST search engine embedded in the Proteome Discoverer 1.4 software package (Thermo Fisher Scientific, Waltham, MA). Raw files from each fraction were processed as one contiguous input file and a single result file (.msf) was generated. Search parameters were as follows: full enzymatic activity and two missed cleavage sites allowed for trypsin; peptide MW of 350-5000 Da.; mass tolerance of 20 ppm and 0.8 Da for precursor and fragment ions, respectively; dynamic modifications on methionine (+15.9949 Da: oxidation) (4 maximum dynamic modifications allowed per peptide), static modification on cysteine (+57.0215 Da: carbamidomethylation) and on lysine and N-terminus (+28.0312984 Da). Cross-correlation (XCorr) values were applied to limit the false positive (FP) rates to less than 1% in each of the four data sets (with the Target/Decoy PSM Validator node). Proteins identified in three out of four biological replicates were included in the final analysis. All protein identification information from the result files (<1% FP; with protein grouping enabled) was exported to Excel spreadsheets, which are included as Supplementary Information.

Functional annotation of proteins. Protein sequences were given gene ontology terms (GO) (Ashburner *et al.* 2000) NCBI annotations, and KEGG pathway assignments via the BLAST tool (Altschul *et al.* 1990) using the Blast2GO software (http://www.blast2go.com/b2ghome) (Conesa *et al.* 2005). Sequences were also queried against the predicted proteomes of *E. coli* K-12 and *A. actinomycetemcomitans* ANH9381

with an e-value cutoff of 1^{-20} (www.ecogene.org, www.uniprot.org)(Jain *et al.* 2009, Zhou and Rudd 2013) to identify homologs of characterized proteins encoded in other genomes. Cluster of orthologous groups (COG) classes and protein family (PFAM) designations were assigned using the WebMGA server (http://weizhong-lab.ucsd.edu/metagenomic-analysis/)(Tatusov *et al.* 1997, Wu *et al.* 2011, Punta *et al.* 2012). COG classes that represented at least 10 sequences were manually divided into two broad categories (representing general cellular functions and transport) to construct pie charts. Transporters were identified by querying the transporter classification database (TCDB) using the BLAST algorithm with an e-value cutoff of 1^{-10} to identify significant hits (Saier *et al.* 2009). Secretion systems present in the dataset were manually identified by their annotation in Oralgen (www.oralgen.org), UniProt, the Human Oral Microbiome Database (HOMD) or BLAST searching against the HK1651 genome using known homologs (Chen *et al.* 2010). Curated protein annotations were assigned by manually synthesizing data from NCBI, Oralgen, EcoGene, HOMD, UniProt, and functional predictions from COG, PFAM, GO and TCDB.

Protein sequences without descriptive annotations were first analyzed for a predicted role in virulence or associated with other cellular processes using the VirulentPred server (http://203.92.44.117/virulent/index.html) (Garg and Gupta 2008) and querying the NCBI, HOMD, Oralgen, and PFAM databases. The PHMMER algorithm was utilized to predict the taxonomic distribution of these sequences (http://hmmer.janelia.org/)(Finn *et al.* 2011).

Protein localization prediction. Bioinformatic predictions were made based on a previously outlined approach (Emanuelsson *et al.* 2007). Localization to the cytoplasm, inner membrane, periplasm, outer membrane, or extracellular environment was predicted by combining data from the CELLO (http://cello.life.nctu.edu.tw/)(Yu *et al.* 2004) and PSORT algorithms (http://www.psort.org/psortb/index.html)(Yu *et al.* 2010). Predictions were refined by incorporating data from other bioinformatic tools: Sec signal peptides were predicted by SIGNALP (http://www.cbs.dtu.dk/services/SignalP/)(Petersen *et al.* 2011); lipoprotein signal peptides by LIPO (http://services.cbu.uib.no/tools/lipo)(Berven *et al.* 2006) and LIPOP (http://www.cbs.dtu.dk/services/LipoP/)(Juncker *et al.* 2003); twin arginine translocon signal peptides by TATP (http://www.cbs.dtu.dk/services/TatP/)(Bendtsen *et al.* 2005); transmembrane helices by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/)(Krogh *et al.* 2001); and outer membrane localization by BOMP (http://services.cbu.uib.no/tools/bomp)(Berven *et al.* 2004). Annotation and GO data was utilized to resolve ambiguous predictions and to assign proteins to the nucleoid, ribosome, or as membrane associated. When possible, localization of characterized proteins was manually assigned.

Results

The proteomics approach utilized in this study was carefully optimized regarding SCX fractionation, nanoscale chromatography and mass spectrometry acquisition, to maximize the coverage of the membrane proteome. With the optimized approach, 666, 737 828, 765 proteins were identified in replicate 1 to 4, respectively (Supplementary

Information). A total of 648 proteins were identified in 3 of the four biological replicates, 533 of which were found in all 4 replicates. The whole sequences of these proteins were queried against the *A. actinomycetemcomitans* HK1651 genome in the Oralgen and HOMD databases and separately against all genomes in NCBI to assign functional annotations. Homologs of all sequences were identified in the NCBI database, however >30% of these had ambiguous or conflicting annotation data. Annotations were improved by querying sequences against *A. actinomycetemcomitans* ANH9381 and *E. coli* K-12 predicted proteomes. This data was supplemented by manually incorporating information about conserved domains from the protein families (PFAM) and cluster of orthologous groups (COG) databases.

Sequences were queried against the PFAM database to identify function based on conserved domains. This database consists of defined groups of proteins organized into families based on related function or conservation among different organisms. PFAM data was available for 584 of the identified proteins. Clusters of Orthologous Groups (COG), which catalogs proteins based on conserved regions, was used as a complementary analysis to the PFAM data. The COG protein database compares predicted and known proteins in all completely sequenced microbial genomes to infer sets of orthologs. Each COG consists of any group of at least three proteins from distant genomes that are more similar to each other than they are to any other proteins from the same genomes (Tatusov *et al.* 1997). Each individual COG represents a specific motif and belongs to a larger group (COG class) with similar predicted function. The most common COG classes represented in our dataset were identified and are shown in Figure 1.

COG class data was available for 349 proteins of the cell envelope. A total of 203 proteins were predicted to be involved in general cell functions such as DNA transcription, replication, protein translation, and energy generation. Within this group, a subset of 27 proteins were classified to be involved in protein modification and 46 involved in cell envelope biogenesis (Figure 1A). Metabolic and transport functions were associated with 146 proteins (Figure 1B). Specific metabolic enzymes were identified by incorporating annotation data and predictions from the KEGG database. These included enzymes for the synthesis or breakdown of amino acids (alanine, cysteine, glycine, tryptophan, tyrosine, and others), carbohydrates (glucose, fructose, amino sugars), and nucleotides (purines and pyrimidines).

Specific protein secretion systems were identified by analysis of combined annotation data. Three types of secretion systems (Type I, II, and V) were identified in the dataset. In addition, we also identified the general secretory machinery (Sec) and twin-arginine translocon (TAT) complexes (Table 1). The Sec translocon transports unfolded polypeptides across the inner membrane or integrates membrane proteins. The TAT actively transports folded proteins across the inner membrane (Lee *et al.* 2006). All components of the Type I secretion required for leukotoxin secretion (LtxBD and TolC) as well as the Type V_a (Aae) and V_c autotransporter proteins (ApiA and EmaA) were present. Proteins encoded by the tight adherence (Tad) locus, classified as a subtype of the Type II secretion system, were identified. The fimbriae secretion apparatus (RcpC-TadG) was present in the preparations from this afimbriated strain. However, the structural subunit of fimbriae (Flp-1) and TadV, a processing enzyme of Flp-1, were not identified.

We identified multiple accessory complexes responsible for envelope biogenesis (Table 2). Outer membrane proteins contain repeating alternate hydrophobic amino acids at the carboxyl terminus forming structural elements termed β-barrels (Koebnik *et al.* 2000). The β-barrel assembly proteins (Bam), which form complexes to assemble these outer membrane proteins (Rigel and Silhavy 2012), were identified in the *A. actinomycetemcomitans* cell envelope. Homologs of the lipoprotein transport system (Lol) of *E. coli* were present in the *A. actinomycetemcomitans* preparation. Lipoproteins comprise a subset of membrane proteins with a lipid-modified cysteine residue at the amino termini, which are used to anchor the protein to the membrane. Lipoproteins are localized to either the inner or the outer membrane in Gram-negative bacteria (Tokuda and Matsuyama 2004). The LPS transport systems responsible for translocation of both the lipid and sugar components across the cell envelope were also identified in our preparations. LPS is composed of a lipid-anchored carbohydrate moiety and exists only in the outer leaflet of the outer membrane. Chaperones are an important subset of proteins required for maintaining protein structure and degrading unfolded proteins during secretion. Eleven proteins were annotated as chaperones (Table 3). Overall, our annotation scheme allowed the assignment of informative annotations to 84% of protein sequences. The curated annotations for each identified protein in this study can be found in the Supplemental data.

The remaining 16% or 102 proteins were uncharacterized. In an attempt at annotation, these proteins were analyzed using the VirulentPred algorithm, a method to predict putative novel virulence factors, and taxonomic conservation using PHMMER. A

combination of these programs identified 11 proteins that contained sequences similar to known virulence determinants and conserved across species (Table 4). The function of some of these proteins cannot be determined but are predicted to be associated with virulence. Taxonomic conservation of sequence is also considered to be of significance in terms of protein function. Analysis revealed conservation of these unknown proteins in many genera. Select proteins with interesting taxonomic distribution in terms of the diversity within families of prokaryotes are listed in Table 5.

Cellular localization of the annotated proteins was assigned using a combination of general prediction algorithms (PSORT and CELLO), and manual combination with more specialized localization programs such as TMHMM, LipoP, and SignalP (Figure 2A). Where possible, these predictions were manually curated based on functional annotation data and localization of known proteins. Approximately 66% of all identified proteins were predicted to be associated with the cell envelope. There were 57 predicted outer membrane proteins represented in the dataset. These proteins contain an amino terminal signal sequence for transport across the inner membrane. Both integral and peripheral membrane proteins, including lipoproteins, are included in this compartment. We identified 60 periplasmic proteins in the envelope preparations. These proteins are also translocated across the inner membrane, but are predicted to lack large domains of hydrophobic amino acids and are considered to be soluble and not associated with the membrane. Inner membrane proteins composed the largest subset (170) of the membrane proteins. These proteins were assigned to the inner membrane based on the prediction of the presence of at least one amphipathic helix that can span the membrane. The majority of the proteins (80)

are predicted to contain a single helical domain. However, diversity exists in the number of transmembrane domains and some of the proteins contain up to 16 transmembrane domains (Figure 2B). Many (38%) of these proteins had 5 or more transmembrane domains, indicating our ability to identify highly hydrophobic sequences with our mass spectrometry technique. Two proteins, leukotoxin and cytolethal distending toxin, which are considered extracellular, were identified but are expected to be present in the membrane during secretion to the extracellular milieu.

The localization of proteins that may interact directly with the polar head groups of lipids or directly with integral membrane proteins may not be correctly assigned using localization algorithms. Using the protein annotation data, proteins were designated membrane-associated if associated with cytoplasmic domains of transporters, ribosomal if associated with translation, or nucleoid if associated with DNA binding or replication. The remaining proteins were classified as cytoplasmic. These proteins are predicted to be soluble, contain no signal sequence, and are not predicted to be found as interacting partners with membrane proteins. Localization and annotation predictions for each protein can be found in the Supplemental data.

Discussion

In this study, we have adopted an alternative protocol for improved identification of the proteins associated with the cell envelope of *A. actinomycetemcomitans*. The protocol used in this study improves upon prior methodologies by eliminating limitations

associated with 2D gel electrophoresis (2D-GE). Techniques based on 2D-GE are restricted amount of protein that can be loaded, which affects the available protein for extraction and subsequent identification by MS, leading to false negatives. This phenomenon is especially significant when low abundance proteins are of interest (Gygi *et al.* 2000). Furthermore, specific nonionic or zwitterionic detergents are required for isoelectric focusing (IEF) and highly hydrophobic proteins are relatively insoluble in these detergents (Braun *et al.* 2007). There is also poor resolution of highly hydrophobic proteins by pH gradients and precipitation of these proteins at pH values close to their isoelectric points during IEF (Rabilloud 2009).

We have eliminated these potential pitfalls by using gel–free mass spectrometry-based shotgun sequencing (multidimensional protein identification technique, MudPIT)(Liu *et al.* 2002). SCX chromatography is routinely used as the first dimension of separation in MudPIT (Washburn 2004). In this study, SCX separation was performed on a SPE column, which in conjunction with LC/MS analysis serves as a robust method for characterizing complex proteomes, as previously reported (Dephoure and Gygi 2011). To yield the maximum proteome coverage, we carefully optimized the number of SCX fractions (20 fractions) as well as the elution concentrations so that peptides were equally distributed over the 20 fractions for subsequent LC/MS shotgun sequencing. Ion exchange chromatography of urea extracted membrane preparations and analysis of individual fractions by LC/MS increased the identification of envelope proteins ~4-fold in this study from previous studies of the *A. actinomycetemcomitans* proteome (Rylev *et al.* 2011, Zijnge *et al.* 2012).

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Functional annotation and cellular localization of the identified proteins were assigned using a combination of bioinformatic algorithms due to inherent weaknesses associated with the individual programs. To overcome this problem, we used programs in parallel and compared results looking for inconsistencies. Disagreements were resolved by incorporating results from other algorithms. For example: The localization assigned by PSORT is highly accurate. However, the program does not assign cellular localization to all queries. Assignments can be deduced using the CELLO program, which is less accurate but provides greater coverage (Gardy and Brinkman 2006). Disagreements between programs can then be mediated by the outcome of an independent tool, e.g. TMHMM, which is optimized for identification of specific motifs (Krogh *et al.* 2001). Using multiple algorithms takes advantage of the strengths while mitigating the weaknesses of the individual programs. This ultimately leads to more accurate bioinformatic predictions for the proteins associated with the cell envelope (Solis and Cordwell 2011).

The cell envelope of Gram-negative bacteria serve as the locus for many facets of bacterial physiology: transport of nutrients; metabolism; replication; and colonization. *A. actinomycetemcomitans* is saccharolytic and utilizes a multitude of sugars as the primary carbon source (Olsen *et al.* 1999). The media used in this study contains glucose as the major sugar. Therefore, the presence of a glucose transporter (AA01876) was expected. We also found transporters specific for other sugars such as mannose (AA01465-AA01467) and fructose (AA00332, AA00335). These sugars are not presumed to be present in the medium based on the manufacturer's stated composition. This indicates that these transporters are either upregulated, constitutively expressed, or contaminants are

present in the medium suggesting transporter regulation is exquisitely sensitive to the respective substrates. However, the observation of Brown and Whiteley (2007) suggests that transcripts corresponding to a subset of sugar transporters in *A. actinomycetemcomitans* are not changed in abundance by carbon source availability (Brown and Whiteley 2007).

Potential carbon sources for *A. actinomycetemcomitans* are free amino acids, which are found in abundance *in vivo* and in laboratory media (Syrjanen *et al.* 1990). However, except for the characterization of a single amino acid transporter (Jorth and Whiteley 2010), little is known about amino acid utilization in *A. actinomycetemcomitans*. Amino acid transporters are broadly categorized as being specific for polar or nonpolar amino acids, with individual transporters having high specificity for a single amino acid (Saier 2000). Six specific amino acid transporters were identified when grown under the culture conditions used in this study: arginine (AA00855, AA00858); cysteine/cystine (AA01509, AA01510, AA01524, AA01525) glutamic acid (AA00994); methionine (AA0415-AA0417) proline (AA00680); serine (AA00092). Transporters for oligopeptides (AA02893, AA02897-AA02799) and dipeptides (AA01568, AA01573) were identified, as well as for related amine containing compounds e.g. spermidine (AA02718-AA02721) and glycine betaine (AA02352). Interestingly, RNAs encoding amino acid transporters and metabolic proteins have been identified in an *in vivo* model of infection, implying a potential role for amino acid metabolism in disease (Jorth *et al.* 2013).

Cysteine is suggested to be an integral amino acid for the growth of *A. actinomycetemcomitans*. Substitution of yeast extract with cystine in TSBYE does not

alter the growth kinetics of the bacterium (Sreenivasan *et al.* 1993). Cysteine can be acquired by the bacterium from the degradation of polypeptides or *de novo* synthesis from serine. Notably, metabolic enzymes required for the synthesis of cysteine (AA02412, AA01502) were present. This suggests that even in the presence of a protein rich media, cysteine synthesis is still maintained. The biosynthesis of cysteine may be required *in vivo* due to the limitation of cysteine in gingival crevicular fluid of individuals with periodontal disease (Syrjanen *et al.* 1990). Taken together, the identification of multiple transport and metabolic systems imply that the metabolism of *A. actinomycetemcomitans* is more diverse than previously appreciated.

The composition of a subset of transporters include both membrane and membrane associated proteins. Both ATP binding cassette (ABC) and phosphoenolpyruvate transferase system (PTS) transporter families require integral membrane components that associate with soluble proteins on the inner leaflet of the inner membrane for function (Postma *et al.* 1993, Davidson and Chen 2004). The soluble components of these transporters are easily overlooked by *de novo* bioinformatic tools as they are indistinguishable from cytoplasmic proteins. Our annotation based approach allowed for manual curation of these proteins based on their predicted function as components of transporters. The majority of sugar transporters identified in this study belong to the PTS family. The remaining transporters are categorized as members of the major facilitator superfamily, which do not contain large cytoplasmic domains (Pao *et al.* 1998). The majority of amino acid transporters expressed in *A. actinomycetemcomitans* belong to the

ABC transporter family. Both of these transporter classes appear to be important in *A. actinomycetemcomitans* physiology.

The transport of proteins across cellular membranes is also important for cellular physiology. Conserved secretion systems were identified in the strain of *A. actinomycetemcomitans* utilized in this study. Type I, II, and V secretion systems were present in this bacterium. Proteomic and genomic analysis of other *A. actinomycetemcomitans* strains indicate the conservation of all these systems. Consistent with the study of Zijnge *et al*. (2012), we did not find type III or IV secretion systems in the envelope proteome consistent with the absence of these genes in the chromosome. Interestingly, a type VI secretion system is present in the genome of *A. aphrophilus*, a bacterium closely related to *A. actinomycetemcomitans* (Di Bonaventura *et al.* 2009). These secretion systems are typically involved in direct cell-to-cell interactions and delivery of effector molecules into the host (Tseng *et al.* 2009). We hypothesized a similar system may exist in *A. actinomycetemcomitans*, however, we found no evidence for a type VI secretion system in the genome based on BLAST searches against the NCBI database or annotation in the KEGG database.

The major virulence associated proteins of *A. actinomycetemcomitans* were identified in the studied strain. These included adhesins: EmaA, ApiA, and Aae (Asakawa *et al.* 2003, Rose *et al.* 2003, Mintz 2004); outer membrane proteins: Omp34, Omp18, Omp39, and Omp64 (Fives-Taylor *et al.* 1999, Komatsuzawa *et al.* 2002); and soluble toxins: leukotoxin and cytolethal distending toxin (Lally *et al.* 1989a, Mayer *et al.* 1999). In addition to these factors, several poorly annotated proteins shared characteristics of

virulence proteins based on bioinformatics analyses. These proteins may also be factors that contribute to the pathogenicity of this organism.

A. actinomycetemcomitans is typically isolated with fimbriae, which are considered an important virulence determinant. The fimbriae are composed of repeating subunits (Flp1) secreted through a subclass of the Type II secretion system consisting of 11 proteins encoded by the tight adherence (*tad*) locus (Tomich *et al.* 2007). The *tad* operon is suggested to be regulated by a single promoter (Kram *et al.* 2008). In the cell envelope of the afimbriated strain used in this study, the complete fimbrial secretion apparatus (RcpC-TadG) was present. However, the fimbriae subunit (Flp1) and the prepilin peptidase (TadV) were not detected. Typically, the loss of fimbriation is due to point mutations in the promoter region of the locus (Wang *et al.* 2005). However, studies in our laboratory indicate that the promoter is functional and nonsense mutations are present in *flp1* (data not shown) of the studied strain. The absence of Flp1 in the data set is explained by the presence of these stop codons in the gene. However, the absence of TadV and presence of RcpC-TadG suggests that a second promoter is present in the *tad* locus. Studies are underway to determine if the secretion apparatus is functional and if a second promoter exists in this locus in this strain of *A. actinomycetemcomitans*.

In addition to being a platform for membrane proteins, the cell envelope also interacts with structures traditionally considered to be purely cytoplasmic. Electron micrographs have shown an intimate association between the chromosome and bacterial membrane (Ryter 1968). Furthermore, regions of DNA transcribing genes coding for membrane proteins are found in close proximity to the membrane (Libby *et al.* 2012). This

is consistent with the observation that ribosomes directly interact with the Sec translocon through the signal recognition particle (SRP) while translating membrane proteins (Herskovits and Bibi 2000). As transcription and translation are linked, this provides an elegant means to protect membrane proteins from interacting with the cytoplasmic environment. These known interactions explain the presence of ribosome and nucleoid proteins in cell envelope preparations found in this and other proteomic studies (Huang *et al.* 2006, Marti *et al.* 2006).

Cell envelopes of Gram-negative bacteria are complex structures containing proteins critical for cell physiology and virulence. The proteome presented in this study includes both well characterized and hypothetical proteins predicted to exist based only on genomic sequencing. Some of these proteins share sequence identity with known proteins, whereas others remain functionally uncharacterized. This study may serve as a roadmap to study proteins required for colonization and survival in the oral cavity.

Acknowledgements

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Tables

Table 3.1. Classical secretion pathways identified in the *A. actinomycetemcomitans* cell envelope

Secretion system	Protein(s)	Function	Sequence ID(s)
Type I (Leukotoxin secretion)	LtxB	Leukotoxin secretion ATPase	AA02805
	LtxD	Leukotoxin secretion channel	AA02803
	TolC	Outer membrane pore	AA02077
Sec translocon	SecA	Sec translocon ATPase	AA01083
	SecB	Sec translocon chaperone	AA01506
	SecYG	SecYEG complex channel	AA01237, AA00786
	SecDFYajC	SecDFYajC inner membrane complex	AA02780-1, AA02779
	LepB	Leader peptidase	AA03004
Twin-arginine translocon	TatA	Twin arginine translocon protein A	AA01154
	TatB	Twin arginine translocon protein B	AA01153
Type II (Fimbrial secretion)	RcpC-B	Rough colony protein C-B	AA00870-2
	TadZ-G	Tight adherence protein Z-G	AA00873-80
Type V (Autotransporters)	EmaA	Collagen adhesin	AA00234
	Aae	Epithelial cell adhesin	AA02347
	ApiA	Epithelial cell adhesin	AA02485

Table 3.2. Outer membrane protein, LPS, and lipoprotein transport complexes

Transport system	Protein	Function	Sequence ID
Beta-barrel assembly module	BamA	Bam complex pore	AA00608
	BamCDE	Bam complex accessory lipoprotein	AA02350, AA01356, AA00998
LPS export system	MsbA	Transport of LPS/Lipid A across the inner membrane	AA01961
	LptBCFG	ABC transporter of LPS	AA02320, AA02323, AA01777, AA01776
	LptADE	Periplasmic LPS transporter	AA02321, AA00919, AA01088
Lipoprotein releasing system	LolCDE	ABC transporter of lipoproteins to the periplasm	AA01615-6, AA01618
	LolB	Outer membrane protein for lipoprotein insertion	AA02743

Table 3.3. Chaperone proteins

Chaperone	Sequence ID
ClpB	AA01211
ClpX	AA00120
DegQ	AA01869
DegS	AA01789
DnaJ	AA00659
DnaK	AA00657
GrpE	AA00766
GroEL	AA01284
Lon	AA02395
RseP	AA00606
YidC	AA03011

Table 3.4. Uncharacterized virulence-related proteins

Sequence ID	Predicted Localization	Predicted Function	Distribution	Conserved Domains	Domain Description
AA00179	Outer Membrane	Unknown	Alphaproteobacteria Betaproteobacteria Gammaproteobacteria	PF04575	Unknown function
AA00180	Outer Membrane	Transferrin binding	Pasteurellaceae Moraxellaceae Neisseriaceae	PF01298	Transferrin binding
AA00422	Inner Membrane	Virulence protein secretion	Bacteria	PF04393	Expression/localization of virulence factors
AA00702	Outer Membrane	Unknown	Pasteurellaceae	None	N/A
AA00781	Outer Membrane	Unknown	*A. actinomycetemcomitans*	None	N/A
AA01040	Outer Membrane	Lipoprotein	Gammaproteobacteria	PF03923	Lipoprotein
AA01688	Outer Membrane	Unknown	Bacteria	PF07703	Alpha-2-macroglobulin domain
AA02554	Periplasmic	Unknown	Gammaproteobacteria	PF09829	Unknown function
AA02614	Outer Membrane	Unknown	*Aggregatibacter Pasteurella Gallibacterium*	None	N/A
AA02748	Outer Membrane	Lipoprotein	*Aggregatibacter Haemophilus*	None	N/A
AA02811	Outer Membrane	Lipoprotein	Pasteurellaceae	None	N/A

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Table 3.5. Selected uncharacterized proteins with taxonomic conservation

Sequence ID	Predicted Localization	Predicted Function	Distribution	Conserved Domains	Domain Description
AA00300	Periplasmic	Unknown	Pasteurellaceae Pseudomonadaceae Campylobacteraceae Helicobacteraceae	PF07509	Unknown function
AA01162	Inner Membrane	Unknown	Proteobacteria Actinobacteria Bacteroidetes	PF04224	Quinol oxidase like
AA01577	Inner Membrane	Unknown	Proteobacteria Cyanobacteria	PF05128	Unknown function
AA02643	Inner Membrane	ABC Transporter	Bacteria	PF01061	ABC transporter

A **Predicted Localization of Proteins**

B **Predicted # of TM Helices**
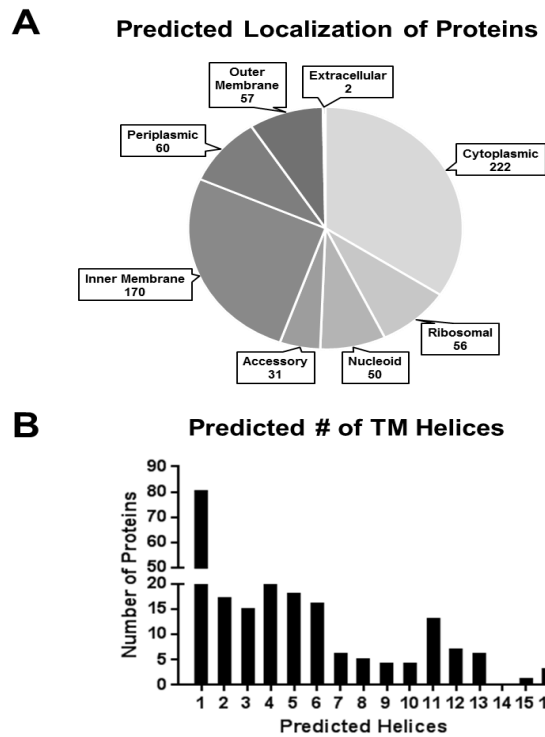
Figure 3.1. Functional predictions of identified proteins. Proteins were assigned to functional groups (COG classes) using the WebMGA server (http://weizhonglab.ucsd.edu/metagenomic-analysis/). Classes containing at least 10 sequences were utilized to generate pie charts of proteins involved in **(A)** general cellular functions and **(B)** transport and metabolic functions. COG class names were shortened for clarity. Classes represented: general cellular functions; Translation/ribosome: J, Envelope biogenesis: M, Energy production: C, DNA replication/repair: L, Protein modification/chaperone: O, Transcription: K, and for metabolism/transport of; Amino acids: E, Carbohydrates: G, Ions: P, Coenzymes: H, Lipids: I, Nucleotides: F, Proteins: U.
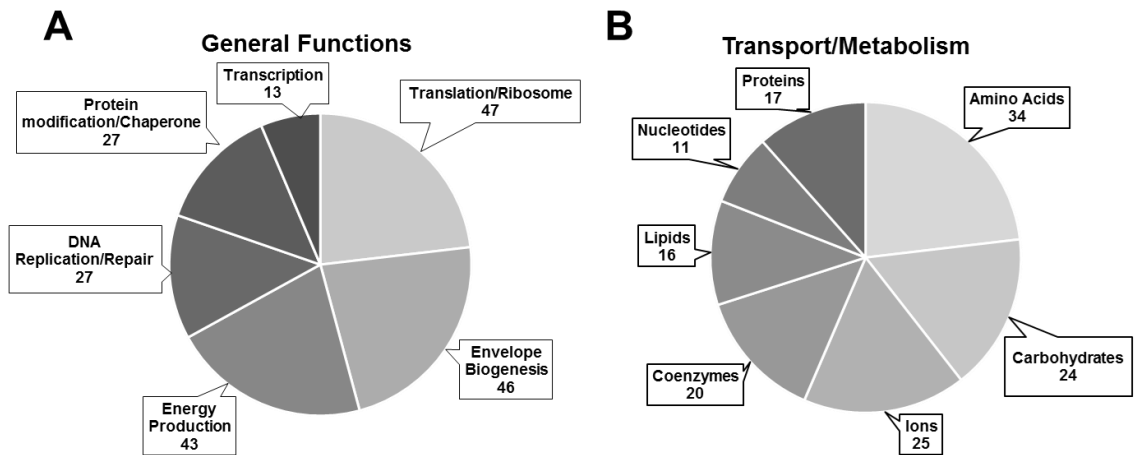
Figure 3.2. Predicted localization of identified proteins. (A) The 665 identified protein sequences were analyzed using the CELLO and PSORT algorithms. Data from SIGNALP, LIPO, LIPOP, TATP, TMHMM, BOMP, and functional annotations were incorporated into the analysis to refine the initial predictions. Proteins were then assigned to one of eight localizations based on bioinformatic predictions combined with manual curation. **(B)** The proteins containing at least one predicted transmembrane helix by TMHMM arranged by number of predicted helices.

CHAPTER 4: Alteration in abundance of specific membrane proteins of *Aggregatibacter actinomycetemcomitans* is attributed to deletion of the inner membrane protein MorC

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Running Title: Proteome changes dependent on MorC

Key words: Chaperone proteins, Leukotoxin secretion, Type I secretion

Abbreviations: WT: Wild-type, MT: Mutant

Abstract

Aggregatibacter actinomycetemcomitans is an important pathogen in the etiology of human periodontal and systemic diseases. Inactivation of the gene coding for the inner membrane protein, morphogenesis protein C (MorC), results is pleotropic effects pertaining to the membrane structure and function of this bacterium. The role of this protein in membrane biogenesis is unknown. To begin to understand the role of this conserved protein, stable isotope dimethyl labeling in conjunction with mass spectrometry was used to quantitatively analyze differences in the membrane proteomes of the isogenic mutant and wild-type strain. A total of 613 proteins were quantified and 601 of these proteins were found to be equal in abundance between the two strains. The remaining 12 proteins were found in lesser (10) or greater (2) abundance in the membrane preparation of the mutant strain compared with the wild-type strain. The 12 proteins were ascribed functions associated with protein quality control systems, oxidative stress responses, and protein secretion. The potential relationship between these proteins and the phenotypes of the *morC* mutant strain is discussed.

Introduction

The cell envelope of Gram-negative bacteria is an essential structure required for cellular homeostasis, protection from environmental stressors, and interactions with other microorganisms or eukaryotic tissues. Three physiologically distinct but contiguous compartments compose this structure: the inner membrane, a phospholipid bilayer adjacent to the cytosol; the periplasm, a viscous aqueous compartment containing the peptidoglycan cell wall; and the outer membrane, an asymmetric bilayer composed of lipids and lipopolysaccharide exposed to the extracellular environment (Silhavy *et al.* 2010). Each compartment contains conserved proteins with general cellular functions and proteins exclusively associated with the specific organism. Protein functions include roles in transport, which allows the envelope to serve as a barrier to exclude toxic compounds from the cell while simultaneously facilitating import of nutrients, energy generation, and secretion of proteins (Silhavy *et al.* 2010).

Canonical secretion systems have been well characterized in model organisms. These systems are composed of individual proteins or a complex of proteins spanning the lipid bilayer (Delepelaire 2004, Natale *et al.* 2008, Fronzes *et al.* 2009, Korotkov *et al.* 2012). A conserved type I secretion system is present in the periodontal pathogen *A. actinomycetemcomitans* and is associated with the secretion of a leukotoxin (LtxA) that destroys white blood cells (Kachlany *et al.* 2000a). This system is composed of an inner membrane ATPase (LtxB), a periplasmic channel protein (LtxD) and a pore protein (TolC, also known as TdeA in *A. actinomycetemcomitans*) that allows for toxin secretion across the outer membrane. The leukotoxin secretion system is homologous to that used for *E.*

coli hemolysin (HlyA)(Lally *et al.* 1989a). In *E. coli*, the secretion of HlyA does not appear to require accessory proteins. However, our laboratory has identified an *A. actinomycetemcomitans* mutant that displays a decrease in LtxA production through disruption of a gene not typically associated with the type I secretion system (Gallant *et al.* 2008). This mutation maps to a gene coding for a 141 kDa inner membrane protein, MorC (*Mor*phogenesis protein C).

Homologs of the MorC protein are suggested to be present in many other bacterial cells based on genomic sequences but relatively little is known about this protein's function in other systems (Gallant *et al.* 2008, Selkrig *et al.* 2012). In addition to the defect in LtxA secretion in *A. actinomycetemcomitans*, loss of MorC results in a change in outer membrane morphology from a rugose to smooth appearance, a decrease in size of the organism and an increase in autoaggregation (Gallant *et al.* 2008, Azari *et al.* 2013). All of these membrane-related phenotypes suggest a change in the membrane protein composition of the bacterium.

In this study, isotope dimethyl labeling quantitative proteomics was employed on cell envelope preparations to determine if the pleiotropic effects associated with the inactivation of *morC* are due to a generalized reduction in membrane proteins or quantitative changes in specific proteins in the mutant compared with the parent strain. 613 proteins were consistently quantified in whole membrane preparations of *A. actinomycetemcomitans*, >98% of which were found to be in equal abundance between the wild-type and mutant strains. The LtxA secretion apparatus was found to be among these proteins. A specific subset of 12 proteins was shown to be consistently changed. These

included leukotoxin, chaperone proteins, a fimbrial secretion system subunit, and oxidative stress response proteins. The potential function of these proteins in the modulation of leukotoxin secretion is discussed.

Materials and Methods

Bacterial strains and growth conditions. The wild-type strain (WT), VT1169, is an afimbriated serotype b strain of *A. actinomycetemcomitans* (Mintz and Fives-Taylor 1994a). The *morC* mutant (MT) strain is an isogenic mutant of VT1169 (Gallant *et al.* 2008). *A. actinomycetemcomitans* strains were routinely cultured in TSBYE medium (0.3% tryptic soy broth, 0.6% yeast extract; Beckton Dickinson, Franklin Lakes, NJ). Incubation was static at 37°C in a humidified 10% CO_2 atmosphere. Spectinomycin was added at a concentration of 50µg ml^{-1} for maintenance of the *morC* mutant.

Whole membrane isolation. Bacteria for each of the three biological replicates were streaked for isolation on fresh TSBYE plates from a stock frozen at -80°C. Several colonies were inoculated into liquid media and grown overnight. The overnight cultures were diluted in 250 ml broth and incubated until they reached mid-logarithmic phase (OD_{495} = 0.3). An aliquot of cells (~5 ml) was removed and subjected to testing for contamination by Gram-staining and inspection of growth characteristics by streaking to an agar plate. This aliquot was also used to verify the phenotypes of the wild-type and *morC* mutant cells. The remainder was utilized for membrane isolation based on the method of Smith *et al.* (Smith *et al.* 2014). Briefly, cells were lysed using a French pressure cell (Thermo

Scientific, Waltham, MA) and cell debris removed by centrifugation at 10,000 g for 30 minutes. Membranes were recovered by centrifugation at 100,000 g and the pellet suspended in PBS. The procedure was repeated three times and membrane pellets were stored dry at -80°C.

Dimethyl labeling, fractionation, nanoscale liquid chromatography-mass spectrometry (LC/MS). Whole envelope fractions were subjected to LC/MS analyses based on the methods of Smith *et al.* (Smith *et al.* 2014) with modifications. The WT and MT membrane pellets (~ 0.5 mg) were suspended in 0.5 mL of 6 M urea/2M thiourea/20 mM HEPES (pH 8) and sonicated on ice with 3 bursts of 20 seconds each, at 1-min intervals. Reduction and alkylation of disulfides was achieved by addition of dithiothreitol (final concentration of 4.5 mM) at 55°C for 30 min., followed by the addition of iodoacetamide (10 mM final concentration) and incubated in the dark at room temperature for 15 min. The solution was diluted 4-fold (20 mM HEPES, pH 8.0) followed by addition of sequencing grade trypsin (Promega, WI) with an enzyme to substrate ratio of 1:50. Following overnight incubation at 37°C, the reaction was terminated by the addition of trifluoracetic acid (TFA) to 1%. The acidified tryptic peptides were purified using Sep-Pak tC18 cartridge (WAT023590, 1cc, 100 mg; Waters, MA) and lyophilized. The lyophilized peptides were reconstituted in 100 uL 1M HEPES (pH 7.5) and subjected to dimethyl labeling as previously described (Hsu *et al.* 2003, Boersema *et al.* 2009). Briefly, 2 μL of 10% formaldehyde (Sigma, St. Louis, MO) and 4 μL of 500 mM sodium cyanoborohydride ($NaCNBH_3$) (Sigma St. Louis, MO) were added to the WT peptides. 2 μL of 10% *d2*-formaldehyde (diluted from a 20% solution, 98% D2; Cambridge Isotope

Laboratories, MA) and 4 μL of 500 mM sodium cyanoborodeuteride NaCNBD$_3$ in 1 M

NaOH (98.7% D2; CDN Isotopes, Canada) were added to the MT peptides. The peptide

mixtures were incubated at room temperature for 1 h. The labeling and incubation steps

were repeated and it was confirmed that > 99% of the peptides were labeled. The resulting

peptides were acidified by addition of TFA to 7%, combined, purified by Sep-Pak tC18,

and lyophilized. The lyophilized peptides were suspended in 150 μL of buffer A (10 mM

potassium phosphate (pH 2.8) and 25% acetonitrile (CH$_3$CN)). Fifty μL of the peptide

solution was fractionated on a PolySULFOETHYL A (silica SCX) cation exchange TopTip

(0-200 μL sized, PolyLC, MD) by step elution (in 100 μL aliquots) with solutions of 0, 20,

25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, and 350 mM of KCl in

buffer A. The eluates were lyophilized, ziptipped (Pierce, Rockford, IL) and dried again.

The peptides were stored at -80°C until liquid chromatography/mass spectrometry

(LC/MS) analysis.

Protein identification and data analysis. Database searches for protein identification

were conducted following the methods of Smith *et al.* (Smith *et al.* 2014). Product ion

spectra were searched against the OralGen annotation of the *A. actinomycetemcomitans*

HK1651 genome (www.oralgen.org) containing sequences in forward and reverse

orientations using the SEQUEST search engines in Proteome Discoverer 1.4 (Thermo

Fisher Scientific, MA). The 20 raw files from each experiment were processed as one

contiguous input file and a single result file (.msf) was generated. Search parameters were

as follows: fully enzymatic activity and two missed cleavage sites allowed for trypsin;

peptide MW of 350-5000.; mass tolerance of 20 ppm and 0.8 Da for precursor and fragment

ions, respectively; dynamic modifications on methionine (+15.9949 Da: oxidation) (4 maximum dynamic modifications allowed per peptide), static modification on cysteine (+57.0215 Da: carbamidomethylation) as well as static isomeric dimethyl modifications on lysine and N-terminus (+28.0312984 Da: for WT and +34.068961 Da for MT). The raw files were searched separately with "light" or "heavy" labels in the same workflow. Cross-correlation (XCorr) values were applied to limit the false positive (FP) rates to less than 1% in the data sets (with the Target/Decoy PSM Validator node). The relative abundances of peptides between WT and MT (isotopologues) were quantitated by integrating the intensities of peptide ion elution profiles with the Precursor Ions Quantifier node in the Proteome Discoverer (Parameters: Mass precision: 2 ppm, Protein quantification using all peptides, and Single-peak Quan channels were used). The XCorr cut-off values for doubly (2+) and triply (3+) charged peptides are as follows: replicate 1: 2.115 (2+); 2.25 (3+); replicate 2: 2.035 (2+); 2.245 (3+); replicate 3: 2.1 (2+); 2.205 (3+). All the protein identification and quantification information (<1% FP; with protein grouping enabled) was exported from the .msf result files to Excel spreadsheets, which are included as Supplementary Information 1-3.

Statistical analyses. Quantitative and statistical metrics were used to identify proteins with differential abundance between WT and MT. All MT:WT ratios were $\log_2$ transformed to normalize data. Proteins present in all three biological replicates with $\log_2$ ratios consistently less than -1 or greater than 1 (corresponding to a two-fold change) were considered for analysis. For each replicate, z-scores were calculated for individual protein ratios based on the mean and standard deviation from that experiment. Average z-scores

across all replicates were calculated and proteins with an average z-score of ±2 (corresponding to a p-value of less than 0.05) were classified as increased or decreased. Exact p-values were calculated based on the normal distribution. All analyses were conducted in Excel 2013 (Microsoft, Redmond, WA) and JMP 11.0 (SAS Institute, Cary, NC).

Bioinformatic analyses. Prediction of subcellular localization of changed proteins was based on multiple bioinformatic tools according to the method of Smith *et al*. (Smith *et al.* 2014). Functional annotations were assigned to each protein using the WebMGA server to identify cluster of orthologous groups (COG) assignments, gene ontology (GO) terms, and enzyme commission (EC) numbers (Bairoch 1999, Ashburner *et al.* 2000, Tatusov *et al.* 2000). The BLAST tool was used to identify similar *Escherichia coli* K-12 or *A. actinomycetemcomitans* ANH9381 sequences using the EcoGene and Uniprot databases respectively (e-value cutoff = 1e^{-20}, www.ecogene.org, www.uniprot.org). Transporters were identified by querying the transporter classification database with an e-value cutoff of 1e^{-10} (Saier *et al.* 2009). All available information for each protein was compiled and used to manually synthesize a descriptive annotation and biological function.

Generation and purification of MorC antibodies. Polyclonal antibodies were generated in rabbits (Cocalico Biologicals, Reamstown, PA) using a 63 kDa expressed fragment of MorC, corresponding to amino acids 46-620. The protein was expressed in *E. coli* BL21(DE3) cells as a maltose-binding protein (MBP) fusion protein from the pMAL-c2x plasmid (New England Biolabs, Ipswich, MA). The 1725 bp fragment was amplified using primers containing engineered restriction sites for directional cloning into the BamHI and

HindIII sites of the plasmid with high fidelity polymerase (Roche, Basel, Switzerland). Maximal protein expression was achieved by induction of exponential-phase *E. coli* cells with 0.3 mM IPTG for 2 h at 37°C. The MBP-fusion protein, obtained following lysis and centrifugation (Mintz 2004), was purified by amylose affinity chromatography (New England Biolabs, Ipswich, MA) using a batch/column method. The immobilized MBP fusion protein was eluted by the addition of 10 mM maltose to the column buffer. The recombinant protein was collected and concentrated by ultrafiltration (Millipore, Billerica, MA) with a 10,000 kDa cut off and used as the immunogen.

Serum antibodies were concentrated by the addition of 50% ammonium sulfate, dialyzed overnight with phosphate buffered saline (PBS) and incubated with a glutathione s-transferase (GST) fusion protein of MorC immobilized to cyanogen bromide-activated-Sepharose 4B (Sigma Aldrich, St. Louis, MO). The GST-63 kDa protein was generated by expression of the *morC* fragment, described above, using the pGEX-6P-1 expression plasmid (GE Healthcare Bio-Sciences, Pittsburgh, PA). Purified antibodies were eluted by 0.2M glycine, pH 2.8 and neutralized with 3M Tris, pH 8.8. Antibodies were stored as a 50% glycerol stock at -20°C.

Generation of 6x Histidine Tagged Leukotoxin B (LtxB). The *ltxB* gene was amplified by PCR from genomic DNA using primers corresponding to the 5' (AAA<u>GTCGAC</u>ATG*CACCACCACCACCACCAC*GACTCACAGAAAAATACT AATC) and 3' (AA<u>TCTAGA</u>TTAATTTACTTGTAATTGGTG) termini of the gene with engineered SalI and XbaI (underlined) endonuclease restriction sites, respectively, for directional cloning into an *A. actinomycetemcomitans* shuttle plasmid (Gallant *et al.* 2008).

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A 6x His sequence was engineered into the 5' primer following the endonuclease restriction site (italics). The PCR product was cloned and the 6xHis*ltxB* construct containing plasmid was introduced into the wild-type and *morC* mutant cells by electroporation.

Immunoblotting of *A. actinomycetemcomitans* proteins. Immunoblots were performed to validate the proteomics results. Protein concentrations in whole cell lysates were calculated by the BCA method (Thermo Scientific, Waltham, MA). Secreted leukotoxin was prepared based on the method of Tang *et al.* (Tang *et al.* 2012a). Spent liquid media from *A. actinomycetemcomitans* strains was concentrated by ultrafiltration using a 50,000 MW cutoff (Millipore, Billerica, MA). Equal amounts of protein were separated by SDS-PAGE on 4-15% gradient gels (Bio-Rad, Hercules, CA) and transferred to an Immobilon-FL PVDF membrane (Millipore, Billerica, MA). Membranes were incubated with Odyssey blocking buffer (LiCor Biosciences, Lincoln, NE), washed three times with PBS-T (Dulbecco's phosphate buffered saline pH, 7.4 + 0.1% tween-20) and probed with either anti-Aae [18], anti-MorC, anti-LtxA (Lally *et al.* 1989b, Rose *et al.* 2003) or antibodies specific to the 6xHis epitope tag (Abcam, Cambridge, UK). Binding of primary antibodies was detected by species-specific secondary antibodies conjugated with fluorescent tags according to the manufacturer's instructions (Li-Cor Biosciences, Lincoln, NE). Fluorescence was detected on an Odyssey Clx infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Lipid analysis. Lipid analysis was performed based on the method of Whittaker *et al.* using cells grown in liquid culture (Whittaker *et al.* 2007). Mid-logrithmic phase *A. actinomycetemcomitans* was grown in 1L broth, collected by centrifugation and washed

once with PBS. Fatty acids were saponified, methylated and extracted with methyl tert-butyl ether (MTBE) followed by a second extraction of the organic phase with 0.3N NaOH. Analysis was performed using a gas chromatograph (GC2010, Shimadzu, Kyoto, Japan) equipped with a split injector (temperature: 260°C, injection volume: 1 μL, split ratio: 1:20) and a mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) using a Rtx-2330 column (30 m length × 0.25 mm diameter × 0.20 μm film thickness; Restek Bellefonte, PA). FAME were detected in full scan mode (m/z 45 to 500) and identification was achieved using authentic standard mixtures (BAME mix, Supelco Inc., Bellefonte, PA; GLC-463 and GLC-603 from Nu-Chek Prep. Elysian, MN). Mass spectra were based on the web-accessible mass spectra database at American Oil Chemists' Society (AOCS) lipid library (http://lipidlibrary.aocs.org/).

Results and Discussion

Heterogeneity in individual protein abundance exists in bacterial cells (Taniguchi *et al.* 2010). Some structural proteins, enzymes, and ribosomal subunits are found in high abundance while other proteins are found at much lower levels (Ishihama *et al.* 2008). In this study, we used a dimethyl labeling strategy (outlined in Figure 1) to quantitatively assess the differences in the membrane proteomes of the isogenic mutant and wild-type strain. To detect peptides derived from the 141 kDa MorC, hypothesized to be a low abundance protein the fractionation procedure was carefully optimized to detect MorC as well as to maximize the proteome coverage. Following trypsin digestion and dimethyl

labeling, peptides were separated into 20 fractions on a strong cationic exchanger (Smith *et al.* 2014). Only after this extensive fractionation of the membrane extract were peptides from MorC detected, suggesting that MorC is either present in low abundance in the membrane or is not easily extracted under the conditions used here.

We identified 751, 923, and 817 proteins (< 1% FP) in biological replicates 1, 2, and 3 respectively, with MT:WT abundance ratios determined for around 95% of the identified proteins (replicate 1: 715 ratios; replicate 2: 888 ratios; replicate 3: 771 ratios). Proteins that were identified and quantified in all three replicates are listed with their associated MT:WT ratios in Supplementary Information 4. In this study, 613 proteins were identified with quantifiable ratios in all replicates (Figure 2A). Subsequent statistical analyses were performed on the dataset including these 613 proteins. MT:WT ratios were subject to $\log_2$ transformation to normalize the ratios. Values of ±1 indicate a two-fold decrease or increase in abundance in the *morC* mutant. Average values were plotted for each protein to visualize the entire dataset and the distribution was found to be clustered around a $\log_2$ ratio of zero (Figure 2B). The average $\log_2$ ratio across all experiments was -0.175 (SD = 0.79) with 88% of protein ratios falling within one and >95% within two SD of the mean. These data demonstrate that the majority of proteins identified are equal in abundance between wild-type and *morC* mutant cells, implying that the phenotypes of the *morC* mutant are due to specific protein changes rather than a general decrease in membrane proteins.

Biological and technical variation between replicates was controlled for by stringent statistical criteria for identification of differentially abundant proteins. Proteins

were initially filtered based on MT:WT ratios and z-scores were calculated if their ratios were consistently changed by at least two-fold between all three experiments. Those which were differentially expressed in MT vs. WT were defined as having average ratios >2 standard deviations from the mean (corresponding to a p-value of < 0.05). Based on these criteria, we deduced that 601 out of 613 proteins (>98%) were unchanged in abundance between WT and MT and 12 proteins were significantly increased or decreased in the *morC* mutant cells (Table 1, Figure 2B).

Changes in the membrane protein profiles were confirmed using available antibodies to known *A. actinomycetemcomitans* proteins. In all immunoblots, protein concentrations from both strains were normalized before separation by gel electrophoresis to account for the increased aggregation phenotype of the mutant strain. The abundance of most of the proteins identified were shown to be unchanged between the two strains by quantitative proteomics and subsequent statistical analyses. The epithelial cell adhesin Aae (AA02347), an outer membrane protein secreted via the type V autotransporter pathway (Rose *et al.* 2003), was included in this cohort of proteins. No difference in abundance between the strains was seen using antibodies specific for Aae (Figure 3A). MorC (AA00961) was not detected in the envelope preparation of the mutant strain compared with the wild-type strain. The absence of MorC in the mutant strain was confirmed by immunoblotting (Figure 3B). A reduction in leukotoxin secretion has been described as a phenotype for the *morC* mutant strain (Gallant *et al.* 2008). Quantification of immunoblots detecting leukotoxin from spent medium demonstrated an 82% reduction in the secretion of the toxin from the mutant compared with the wild-type strain (Figure 3C). A similar

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decrease (77%) in leukotoxin secretion was observed across all replicates as detected by our statistical analysis of the MS data.

The defect in leukotoxin secretion is hypothesized to result from reduction in abundance of one or more proteins directly associated with the type I secretion apparatus in the membrane. The secretion system required to transport leukotoxin from the cytoplasm to the environment consists of three components. An ATPase (LtxB, AA2805) that transports the toxin across the inner membrane to the periplasmic channel (LtxD, AA2803) and an outer membrane pore (TolC, AA02077) to release the toxin to the environment (Lally *et al.* 1991, Kachlany *et al.* 2000a). The average abundance of each of these three proteins was found to be equal in both strains (Supplementary material). The abundance of LtxB was verified to be equal between wild-type and *morC* mutant cells as determined by immunoblotting (Figure 3D).

As previously mentioned, TolC forms the outer membrane pore for LtxA secretion. This protein is also associated with drug efflux systems and is required for export of specific antimicrobials (Crosby and Kachlany 2007). We have tested the *morC* mutant strain for differences in susceptibility to these antimicrobial agents and have not found any differences between the wild-type and mutant strains (data not shown). This finding suggests that TolC is still functional in the *morC* mutant and implies that the defect in LtxA secretion is attributed to the loss of functionality associated with either LtxB, D or both proteins.

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The decrease of leukotoxin secretion from the *morC* mutant is not attributed to a decrease in the proteins composing the secretion apparatus. This finding raises the possibility of a change in the 3-dimensional structure of one or more of the protein components. Lipids are known to be important for the correct folding of membrane proteins (Mitchell 2012). Therefore, analysis of the fatty acid composition of the two strains was conducted by GC-MS to identify potential changes. The types and percentages of fatty acids identified in this study were in close agreement with previously published data from a different strain of *A. actinomycetemcomitans* (Braunthal *et al.* 1980). However, no obvious differences in fatty acid composition were detected between the wild-type and mutant strains (Supplementary Table 1). The absence of a difference in fatty acids implies that the lipid composition of the membrane is not affected by the absence of MorC in the membrane.

Proteins differentially expressed between WT and MT across replicates are visualized by a heat map in Figure 3E. Bioinformatic analysis (Table 2) of these sequences provided information about the cellular location, conserved domains, enzyme activity and predicted functions (Smith *et al.* 2014). Four of the proteins are predicted to be chaperones, which are critical to the maintenance of cellular homeostasis. Chaperones interact with other proteins to maintain the proper tertiary structure for secretion and also serve to degrade misfolded or aggregated proteins (Kim *et al.* 2013). Three chaperones were found to be reduced (HtpX, SlyD, GroES) in the *morC* mutant strain. An increase in misfolded or aggregated proteins in *E. coli* (Pallen and Wren 1997) results in a membrane stress response and the up-regulation of specific chaperones including DegP. Although the *A.*

actinomycetemcomitans genome does not appear to encode DegP, a protein with overlapping function, DegQ, was found to be increased in the mutant strain (Table 2). The overall decrease in abundance of known chaperones may lead to an increase in the incorporation of misfolded proteins into the membrane of the mutant strain resulting in the phenotypes associated with this mutant.

Two other proteins that were demonstrated to be reduced in abundance in the mutant strain are involved in an oxidative stress response: SodA, an enzyme that detoxifies superoxide radicals (Touati 1988) and MsrB, responsible for reducing oxidized methionines (Weissbach *et al.* 2005). Interestingly, a mutation in quinol peroxidase (*qpo*), another oxidative stress response protein reduces leukotoxin secretion in *A. actinomycetemcomitans* (Takashima and Konishi 2008). Leukotoxin is also inactivated by reactive oxygen species and interacts directly with a superoxide dismutase in *A. actinomycetemcomitans* (Balashova *et al.* 2007). Our findings support a link between oxidative stress and secretion of LtxA, however the precise mechanism is not known.

Three protein secretion systems have been identified in *A. actinomycetemcomitans*: Type I, II, and V (Zijnge *et al.* 2012, Smith *et al.* 2014). Our analysis suggests the absence of MorC in the membrane affects the secretion of proteins involved with two types of secretion systems: leukotoxin (LtxA) secreted via the type I secretion pathway and TadB, is a structural component of a subtype of the type II secretion pathway. Proteins secreted via the type V systems are not affected by the absence of MorC in the membrane. This class of proteins includes the monomeric (Aae) and trimeric (EmaA, ApiA) autotransporter proteins (Asakawa *et al.* 2003, Rose *et al.* 2003, Mintz 2004). This finding is in contrast

to the observation that secretion of the monomeric autotransporter Flu (Ag43) is impacted in a strain of *E. coli* in which the *morC* homolog (*tamB* or *ytfN*) was inactivated (Selkrig *et al.* 2012).

A. actinomycetemcomitans expresses pili (fimbriae) that are essential for biofilm formation, adhesion and pathogenesis (Tomich *et al.* 2007). An 11 protein complex (RcpCAB–TadZABCDEFG) is associated with secretion of the fimbriae subunit (Flp1). We have previously identified this secretion system in the strain used in this study, which does not possess fimbriae (Smith *et al.* 2014). In this study, we observed a consistent decrease in the abundance of ten of these proteins in the *morC* mutant, of which only TadB (AA00875) achieved statistical significance (see Table 1 for TadB and supplemental material for RcpCAB-TadZACDEFG, AA00870-874 and AA00876-880). TadB is predicted to localize to the inner membrane with six transmembrane domains (Kachlany *et al.* 2000b). The rationale for the decrease of these proteins in an afimbriated strain is not apparent. This observation does raise the possibility that MorC is required for fimbrial subunit secretion in strains that express fimbriae.

Conclusion

In this study, we have presented quantitative changes in the abundance of membrane proteins in an *A. actinomycetemcomitans morC* mutant strain. The majority of membrane proteins remained unchanged in mutant cells as compared with the parent strain. This suggests that the phenotypes associated with the mutant are not related to a generalized decrease in the abundance of membrane proteins. Proteins composing the type I secretion apparatus for leukotoxin were included in this group, which indicates that the defect in toxin secretion is not due to the change in abundance of these proteins nor the fatty acid composition of the membrane. The findings of this study support the hypothesis that the pleotropic phenotypes of the *morC* mutant are associated with the decrease in the abundance of specific proteins. These proteins are associated with functions involving cellular homeostasis and oxidative stress response. The role of these proteins in relationship to the phenotypes displayed by the *morC* mutant strain are under investigation.

Acknowledgements

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Tables

Table 4.1. Statistical analysis of differentially abundant proteins.

Oralgen ID	Average Ratio (log$_2$)	SD (log$_2$)	Average z-score	p-value
AA01213	-4.07	1.21	-6.07	<0.0001
AA00086	-2.74	1.27	-4.06	<0.0001
AA00561	-2.62	0.69	-3.88	<0.001
AA00470	-2.44	0.92	-3.6	<0.001
AA02012	-2.35	0.12	-3.47	<0.001
AA00068	-2.31	1.08	-3.4	<0.001
AA02806	-2.23	0.69	-3.29	<0.01
AA00875	-1.84	0.41	-2.69	<0.01
AA00401	-1.76	0.62	-2.57	<0.05
AA01280	-1.53	0.26	-2.22	<0.05
AA02212	1.48	0.47	2.33	<0.05
AA01869	1.79	0.33	2.8	<0.01

Table 4.2. Bioinformatic analysis of proteins significantly changed in abundance.

Oralgen ID	Annotation	Biological Function	Protein Name	COG	GO	EC	TCDB	Predicted Localization
AA01213	Competence protein	Transport of DNA	ComEA	COG2931	0005509		3.A.11.1.4	OM
AA00086	SEPHCHC synthase	Vitamin K biosynthesis	MenD	COG0225	0030976			IM
AA00561	Transcription factor	Competence gene expression	TfoX	COG0208				C
AA00470	Chaperone protein (metalloprotease)	Protein folding and transport	HtpX	COG0234	0004222	3.4.24		P
AA02012	Peptide methionine sulfoxide reductase	Resistance to oxidative stress	MsrB	COG0265	0016671	1.8.4		P
AA00068	Superoxide dismutase	Resistance to oxidative stress	SodA	COG4965	0004784	1.15.1.1		P
AA02806	Leukotoxin A	Toxin	LtxA	COG1555			1.C.11.1.3	E
AA00875	Tight adherence protein B	Fimbrial secretion system subunit	TadB	COG3070			3.A.7.15.1	IM
AA00401	Peptidyl-prolyl cis-trans isomerase	Protein folding and transport	SlyD	COG0501	0006457			P
AA01280	Chaperone protein	Protein folding and transport	GroES	COG1047	0006457			C

| AA02212 | Ribonucleoside diphosphate reductase | Ribonucleotide biosynthesis | NrdF | COG1165 | 0004748 | 1.17.4.1 | C |
| AA01869 | Chaperone protein | Protein folding and transport | DegQ | COG0605 | | 9.B.160. 1.10 | P |

COG: Cluster of orthologous groups, GO: Gene ontology, EC: Enzyme commission, TCDB: Transporter classification database, C: cytoplasmic, E: extracellular, IM: inner membrane, OM: outer membrane, P: periplasmic
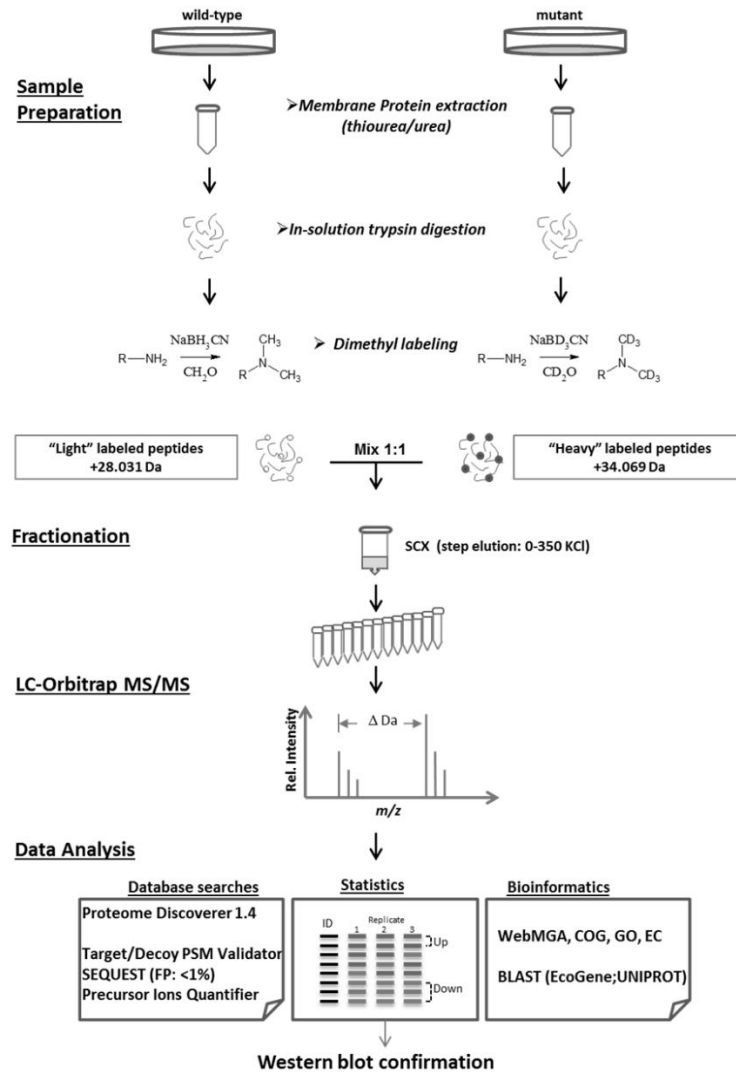
Figure 4.1. Quantitative proteomics workflow. Bacterial cells were grown to mid-logarithmic phase and whole envelopes were isolated by differential ultracentrifugation. Envelope-associated proteins were solubilized in a urea/thiourea buffer, incubated with trypsin, isotope labeled and fractionated by cation exchange chromatography. Fractions were subject to LC/MS analysis and data analyzed using various bioinformatics techniques.

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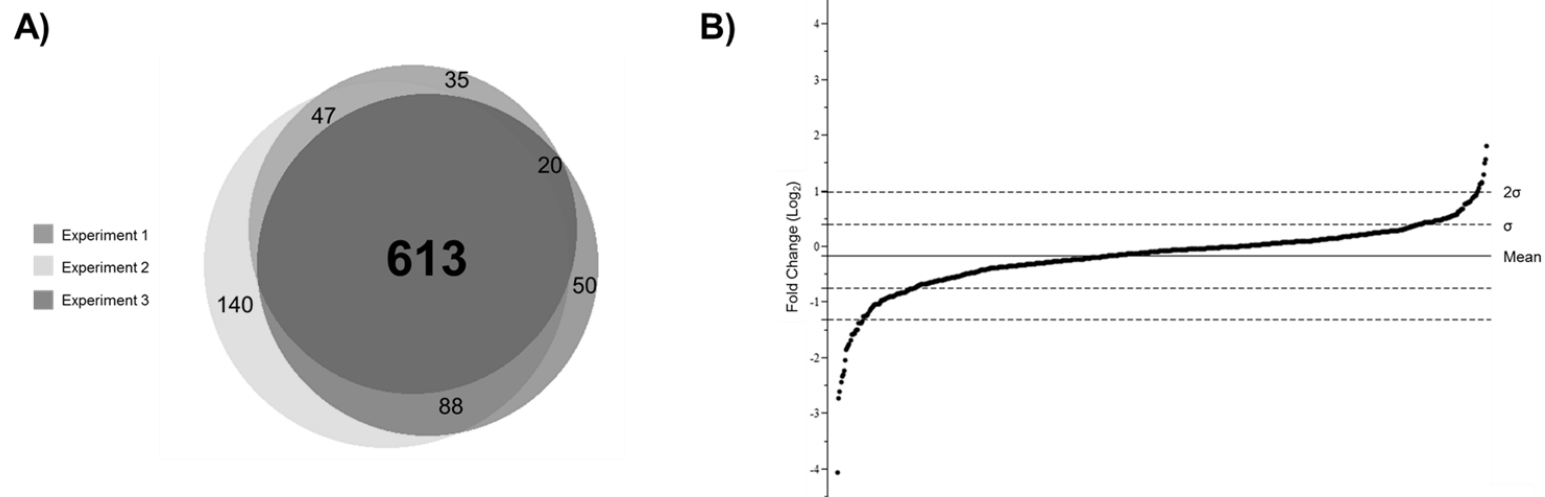
Figure 4.2. Characterization of the dataset. A) A Venn diagram was constructed by inputting identified proteins into the BioVenn website (http://www.cmbi.ru.nl/cdd/biovenn/). Each circle represents a single biological replicate and numbers indicate how many proteins are present in each section. B) Ratios for each consistently identified protein were $\log_2$ transformed and plotted in ascending order using JMP 11.0. Each point in the graph represents a single protein.
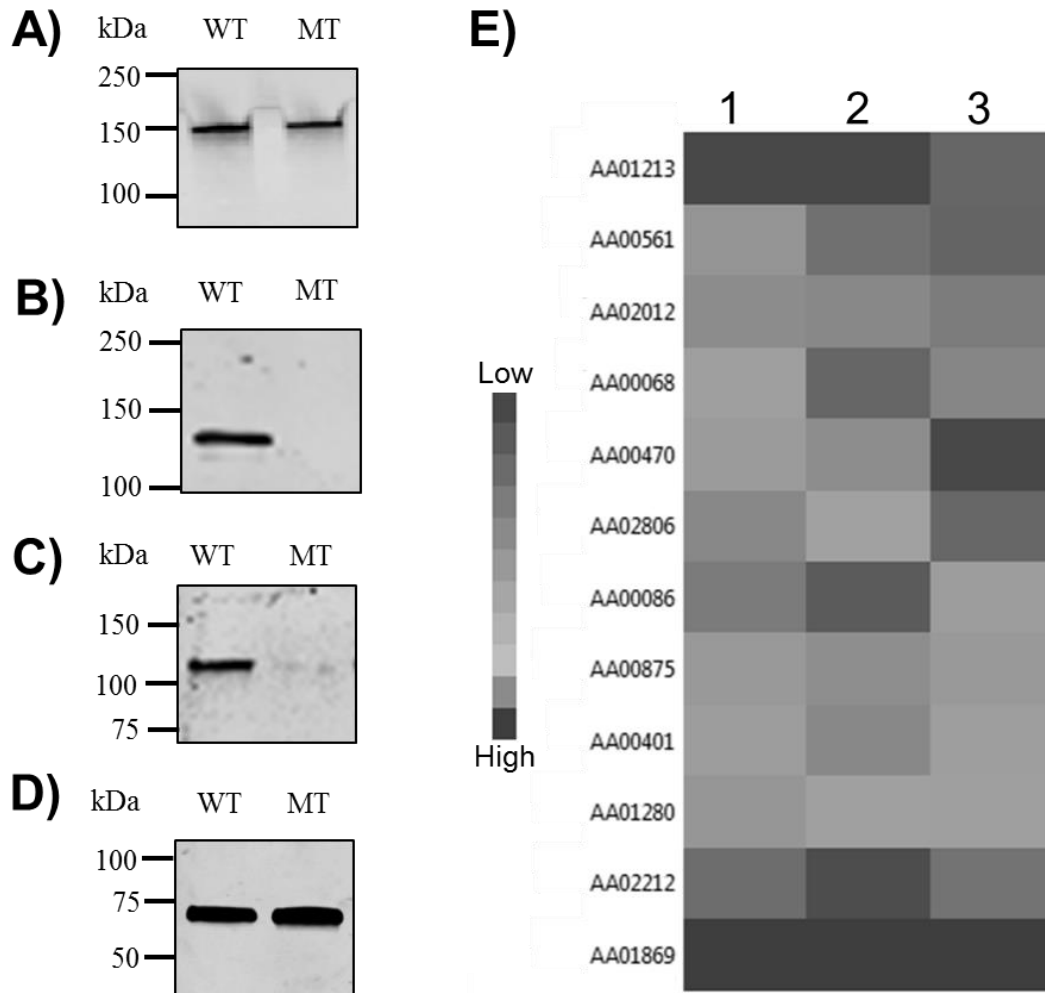
Figure 4.3. Western blotting and Bioinformatic analyses of significantly changed proteins. A-C) Immunoblots of membrane protein using antibodies specific to: A) Aae; B) MorC; C) leukotoxin (LtxA); and D) 6xHis tagged leukotoxin B (LtxB). E) Heat map of the $\log_2$ transformed ratios for each differentially abundant protein using JMP11.0. Each column is a single replicate and each row represents a single protein. Light gray indicates proteins reduced in the mutant whereas dark gray indicates an increase.

CHAPTER 5: The inner membrane protein, MorC, is involved in fimbriae production and biofilm formation in *A. actinomycetemcomitans*

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Key words: Secretion systems, microcolony formation, adhesion

Abstract

Fimbrial subunit synthesis and assembly on the surface of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* is essential for biofilm formation on abiotic and biotic surfaces. A recent quantitative proteomics study employing an afimbriated strain has revealed that the abundance of the fimbrial secretion apparatus proteins in the membrane is dependent on the inner membrane protein MorC (Smith *et al.*, 2015). In this study, we have identified and complemented the defect in fimbriae production to generate a strain that displayed all of the hallmarks of a fimbriated bacterium. These hallmarks include distinct star-like colony morphology, robust biofilm formation, biofilm architecture, and presence of fimbriae as detected by electron microscopy. An isogenic *morC* mutant strain transformed with the identical plasmid did not display any of the phenotypes of fimbriated strains. Deletion of *morC* in a naturally fimbriated clinical strain maintained the characteristic colony morphology and produced biofilms with a mass similar to the parent strain. However, the biofilm formed by the *morC* mutant strain displayed a significantly altered microcolony architecture as observed by confocal microscopy. These results suggest that MorC influences fimbrial secretion and biofilm formation in *A. actinomycetemcomitans*.

Importance

Aggregatibacter actinomycetemcomitans is a fastidious Gram-negative human pathogen associated with periodontal diseases and infective endocarditis in humans. The inner membrane protein MorC (morphogenesis protein C) is important for membrane physiology and has not been associated with any canonical protein secretion systems. In this work, we have determined that MorC is associated with fimbrial secretion and biofilm architecture. The presence of protein homologs in the membrane of other Gammaproteobacteria suggests a conserved function of this protein in this order. To our knowledge, this is the first report of a protein not associated with a canonical secretion system that affects production of fimbriae in *A. actinomycetemcomitans*.

Introduction

Aggregatibacter actinomycetemcomitans, a facultatively anaerobic, coccobacillus shaped bacterium of the Pasteurellaceae family, which colonizes the oral cavity of humans and Old World primates, is typically associated with periodontal diseases (1). Accumulation of this bacterium results in tissue inflammation, expression of extracellular matrix remodeling enzymes, and bone reduction leading to loss of tooth attachment to the jawbone (2-4). This bacterium is also a member of the HACEK (*Haemophilus* spp., *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Kingella kingae*) family of pathogens that causes culture-negative infective endocarditis (5). Clinical isolates of *A. actinomycetemcomitans* have a distinct colony morphology containing a characteristic star or cigar shaped internal structure (6). This unique morphology is associated with the expression of long bundled fimbriae or pili extending from the surface of the bacterium (7). These fimbriae are associated with "non-specific" adherence of the organism to abiotic and biotic surfaces and favor formation of a tenacious biofilm (8).

The structural fimbrial subunit and the secretion machinery are encoded by the well-conserved 14 gene tight adherence (*tad*) locus (9). The fimbriae structural protein (Flp1) is expressed as a pre-protein, which is cleaved by the TadV peptidase to generate the mature form of the protein (10). The processed subunits are secreted via an 11 protein complex (RcpCAB, TadZA-G) analogous to a canonical type II secretion system (9). Continuous planktonic growth of fimbriated strains leads to the generation of afimbriated cells. This conversion typically arises from point mutations in the promoter region of the *tad* locus (11). Loss of fimbriae is accompanied by an increase in the size of the colonies

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and loss of the star-shaped colony morphology (6). Afimbriated strains also display reduced adherence and do not colonize host tissues as effectively as the fimbriated form (12). However, afimbriated strains of *A. actinomycetemcomitans* show reduced auto-aggregation and are easier to manipulate in the laboratory.

A. actinomycetemcomitans and other members of the Pasteuracellae family display a rugose outer membrane morphology compared with the smooth membrane morphology of other bacteria (13). The presence of an inner membrane protein of *A. actinomycetemcomitans*, morphogenesis protein C (MorC), has been shown to be necessary for the expression of this membrane morphology (14). Loss of MorC in the membrane results in the transformation of the membrane morphology from rugose to flat (13, 14). In addition to this dramatic morphological change, the absence of this protein results in pleotropic membrane-related effects including a decrease in cell size, an increase in autoaggregation, an impaired secretion of leukotoxin (14) and an increase in sensitivity to membrane destabilizing agents,. A recent comparative quantitative membrane proteomic study of the parent strain and the *morC* isogenic mutant revealed a change in the abundance of 12 proteins between these two strains, including proteins associated with fimbriae secretion (15).

In this study, we have identified the defect for fimbriae production in a laboratory strain of *A. actinomycetemcomitans* and have complemented fimbriation by introducing a plasmid expressing the fimbrial structural subunit (Flp-1) and the prepilin peptidase (TadV). The transformed strain expressed all of the hallmarks of a fimbriated strain. When an identical plasmid was introduced into the *morC* mutant strain, afimbriated phenotypes

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were still observed. Inactivation of *morC* from a clinical, naturally fimbriated isolate resulted in an altered biofilm architecture when compared to the parent strain. Microcolonies of the mutant strain were significantly smaller in volume and more rounded than the wild-type. The experimental data suggest that MorC is necessary for maintenance of proper biofilm architecture in *A. actinomycetemcomitans*. This represents the first report of a protein independent of the canonical fimbrial secretion complex modulating secretion.

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *A. actinomycetemcomitans* strains were routinely cultured in TSBYE medium (0.3% tryptic soy broth, 0.6% yeast extract, 1.5% agar; Beckton Dickinson, Franklin Lakes, NJ). Clinical strains of *A. actinomycetemcomitans* were grown exclusively on solid media to avoid the potential loss of fimbriation that results from growth in liquid media (11). All strains were grown at 37°C in a humidified 10% CO_2 atmosphere, without agitation for planktonic growth. Where required, chloramphenicol was incorporated into the medium at a concentration of 1 µg ml^{-1} and spectinomycin at 50 µg ml^{-1}. *Escherichia coli* was propagated on LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; Beckton Dickinson) at 37°C in ambient air with agitation. For plasmid selection and maintenance, ampicillin was used at 100 µg ml^{-1} and chloramphenicol at 20 µg ml^{-1}.

***flp1-tadV* plasmid construction.** Primers were designed to generate the amplicon encompassing the *flp-1-tadV* region of the tad locus using the annotation of the *A. actinomycetemcomitans* strain HK1651 genome in the Human Oral Microbiome Database (HOMD, www.homd.org). The region of interest was amplified using the primers listed in Table 2 (annealing temperature: 58°C, extension time: 45 seconds), ligated into the pGEM TA cloning vector (Promega, Madison, WI), and transformed into *E. coli* DH10B (Invitrogen, Carlsbad, CA). Fidelity of the cloning process was verified by DNA sequencing at the Advanced Genome Technology Core at the University of Vermont. The insert was released from the plasmid, purified using a commercial kit (Qiagen, Valencia, CA), ligated into the shuttle vector pKM2 and transformed into DH10B cells (14). The resulting plasmid was used to transform *A. actinomycetemcomitans* wild-type or *morC* mutant cells. PCR-positive transformants were selected, the plasmid purified and sequenced.

Construction of a *morC* deletion mutant strain. Inactivation of the *morC* gene in the clinical isolate VT1257 was performed according to the method of Gallant *et al*. with modification (14). DNA fragments upstream and downstream of the *morC* gene were fused to a spectinomycin resistance cassette designed to allow for translational readthrough to mitigate potential polar effects (16) using primers shown in Table 2. The construct was introduced into the conjugative plasmid pVT1460. A strain of *E. coli*, auxotrophic for diaminopimelic acid (DAP), was used as the donor strain to transfer the plasmid to *A. actinomycetemcomitans* (17). Conjugation mixtures were plated on media containing

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spectinomycin to select for transconjugates. Selected transconjugates were screened genotypically and phenotypically to verify that the *morC* gene was inactivated.

Crystal violet biofilm assay. Biofilm assays were based on the method of Merritt *et al.* (18). *A. actinomycetemcomitans* strains were grown on solid media and cells were collected by scraping with a sterile glass slide, followed by suspension in sterile TSBYE. Cell number was standardized based on protein concentration due to the autoaggreation of the fimbriated strains. Equal cell equivalents were inoculated into a sterile 96-well microtiter plates (Nunc, Roskilde, Denmark) and grown to stationary phase. Following growth, supernatants were aspirated and remaining non-adherent cells removed by three consecutive washes with PBS (136.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.46 mM KH_2PO_4, 0.46 mM $MgCl_2$, pH 7.4; Sigma Aldrich, St. Louis, MO). Biofilms were stained with 0.1% crystal violet for 20 minutes, washed, and solubilized using a 2:1 solution of water:glacial acetic acid. Biomass was quantified by absorbance at 630 nm on an ELx800 plate reader (Biotek, Winooski, VT). A two-tailed Student's t-test was used to identify significant differences ($p < 0.05$) between the biomass of each strain.

Confocal microscopy. Cells were grown in glass-bottom dishes (MatTek, Ashland, MA). Following growth, supernatants were removed by aspiration and non-adherent cells were removed by three washes with Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.4; Sigma, St. Louis, MO). Biofilms were stained with SYTO 9 (Invitrogen, Carlsbad, CA) in TBS at a concentration of 5 μM for 30 minutes. The staining solution was decanted and unbound stain was removed by four washes with TBS. Images were recorded at the University of Vermont Microscopy Imaging Center using a Zeiss LSM 510 META

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confocal microscope (Zeiss, Oberkochen, Germany) with a plan-apochromat 63x objective and an excitation wavelength of 488 nm. Random fields were selected, Z-slices were acquired at increments of 0.37 µm and Z-stacks were generated. Stack sizes depended on biofilm depth and ranged from 5.2 to 40 µm. Surface coverage of the biofilm was determined using the particle analysis function of the ImageJ program (19). Shape, volume and surface area of individual microcolonies were quantified using the Volocity software package (PerkinElmer, Waltham, MA). All experiments were performed in triplicate. For comparisons between strains, a Student's t-test was used with significance defined as p-value <0.05.

Transmission electron microscopy. *A. actinomycetemcomitans* cells were visualized by transmission electron microscopy (TEM) based on the method described by Azari *et al.* (13). Briefly, appropriate strains were streaked for isolation on solid media. Colonies were directly transferred to carbon coated grids and stained using Nano-W (Nanoprobes, Yaphank, NY). Images were collected using a Tecnai 12 electron microscope (operating at 100,000V; FEI, Portland, OR) equipped with a 2,048-by-2048-pixel charge-coupled camera with a pixel size of 14 µm (TVIPS, Gaunting, Germany) at 52,000 nominal magnification.

Results

A recent quantitative membrane proteomic study revealed a consistent decrease in the abundance of the fimbrial secretion apparatus in the *morC* mutant strain compared to the parent strain (15). The relevance of this observation was obscured by the afimbriated nature of these strains. To fully comprehend the importance of these results, it was necessary to determine the molecular basis of the defect that caused the afimbriation for complementation and restoration of fimbriae production. The presence of the components of the fimbrial secretion apparatus in the membrane eliminated the presence of point mutations in the promoter region of the locus as a potential mechanism. Sequencing of the structural subunit gene revealed changes in the nucleotide sequence, which introduced stop codons, predicted to result in the truncation of the expressed protein. No additional changes were found in the non-funcional Flp2 subunit or in the TadV peptidase sequence. However, based on the proteomic data, TadV was not identified in the membrane of either strain (15, 20). Therefore, the wild type and *morC* mutant strains were transformed with a replicating plasmid expressing the *flp1-tadV* region of the locus, under the control of an endogenous *A. actinomycetemcomitans* promoter, and investigated for production of fimbriae.

Colonies of the afimbriated strains are large (>1mm) and translucent with a regular edge and lack internal structures (Figure 1 A). Transformation of the wild type strain *in trans* resulted in small (<1 mm), opaque colonies with irregular edges and internal structures (Figure 1 C), a colony morphology typical of a clinical isolate (Figure 1 E). Colonies of the isogenic *morC* mutant were identical to the wild-type afimbriated strain (Figure 1 B). In contrast, the isogenic mutant transformed with the *flp1-tadV* plasmid

formed colonies indistinguishable from the parent strain (Figure 1 E). Plasmids isolated from these strains were sequenced and found to be identical.

The internal structures associated with the transformed wild type and clinical strain colony morphologies are typically related to the expression of fimbriae. Visualization of these cells by transmission electron microscopy revealed fimbriae associated with the clinical strain (Figure 2 A). Fimbriae were not detected in preparations of the afimbriated wild-type or the isogenic *morC* mutant strains (Figure 2 B and C, respectively). After transformation with the *flp1-tadV* plasmid, fimbriae were observed in the wild-type afimbriated strain (Figure 2 D). However, there were noticeable differences in the characteristics of the fimbriae between the clinical and the complemented strain. The fimbriae associated with the clinical isolate appeared longer and more abundant than the fimbriae of the transformed strain. In addition, not all of the cells selected for maintenance of the plasmid expressed fimbriae as detected by TEM (data not shown). The *morC* afimbriated isogenic mutant transformed with the identical *flp1-tadV* plasmid did not express fimbriae as revealed by the absence of fimbriation in all mutant cells imaged by TEM (Figure 2 E). Visualization of acellular regions of the grids did not show the presence of fimbrial fragments or bundles of fimbriae detached from the cell envelope as observed with clinical strains, thus, further confirming the lack of fimbriae of the mutant cells.

Biofilm formation is dependent on the fimbriation state of *A. actinomycetemcomitans*. The naturally fimbriated, clinical strain formed a relatively robust biofilm with a greater biomass relative to the afimbriated wild type and isogenic mutant strains, as determined by the standard static biofilm assay based on crystal violet retention

(Figure 3). The biofilm formed by the wild type strain transformed with the *flp1-tadV* plasmid was equivalent to the clinical strain. However, the biomass of the isogenic mutant expressing the same construct was unchanged when compared with the parent strain (Figure 3).

Biofilms are spatially well-ordered structures with a specific architecture that can be visualized by confocal microscopy. For investigating the biofilm architecture of the wild-type and *morC* mutant strains transformed with the *flp1-tadV* plasmid, cells were seeded and grown on a glass surfaces and imaged by confocal microscopy. The wild-type afimbriated strain formed a relatively poor biofilm, lacking obvious microcolony formation, with numerous individual cells and minimal cell coverage of the glass surface (Figure 4 A). These biofilm attributes were unaffected by inactivation of *morC* (Figure 4 B). After transformation of the wild-type afimbriated strain with the *flp1-tadV* plasmid, the biofilm morphology displayed different characteristics compared to the original afimbriated strain. In addition to the numerous individual cells, microcolonies with irregular edges were observed (Figure 4 C). In contrast, the identical plasmid introduced into the *morC* mutant displayed biofilm features similar to the parent strain (Figure 4 D).

The architecture of the biofilm formed by the wild-type afimbriated strain expressing the *flp1-tadV* construct (Figure 4 C) was compared with the biofilm produced by a clinical, fimbriated strain (Figure 5 A). The naturally fimbriated strain formed large, discrete microcolonies, with few free cells in between. In contrast the wild-type transformed strain formed diffuse microcolonies with individual cells in between the microcolonies (Figure 4 C). The surface area covered by both strains was equivalent and

represented 8042 μm^2 per field for the afimbriated strain transformed with *flp1-tadV* and 8962 μm^2 per field for the naturally fimbriated strain. However, the average volume of the biofilm was significantly lower for the afimbriated strain transformed with *flp1-tadV* than for the naturally fimbriated strain (32715 μm^3 and 114134 μm^3 per field, respectively).

The data thus far suggested that MorC is required for the surface expression of fimbriae and biofilm formation in the laboratory strain. To determine the role of *morC* in biofilm formation of a naturally fimbriated clinical strain, a *morC* deletion mutant was constructed. The *morC* mutant strain showed all the phenotypes associated with the afimbriated *morC* mutant strain including: lack of toxin secretion, sensitivity to bile salt and altered membrane morphology. The mass of the biofilm formed by the fimbriated mutant was similar to the parent strain as determined by the crystal violet assay (data not shown). However, there were significant differences in the microcolony architecture of the parent and mutant naturally fimbriated strains (Figure 5 A and B, respectively). Large, amorphous microcolonies were commonly observed in in the biofilm formed by the wild-type strain, while smaller and rounder microcolonies were observed in the mutant strain. The average volume of a microcolony formed by the wild-type strain is 2219 μm^3 and 1309 μm^3 for the *morC* mutant strain, which represents a significant, almost two-fold reduction in microcolony size.

Discussion

The protein composition of the *A. actinomcyetemcomitans* membrane envelope evolved to fit the specific environmental niche that the bacterium colonizes in the oral cavity. A membrane proteome study revealed the presence of specific secretion systems responsible for protein integration and secretion across the membranes (20). The macromolecular composition of the secretion complexes has been well characterized in other bacterial systems and functional accessory proteins have not been identified (21). In *A. actinomycetemcomitans*, the absence of MorC, a large (141 kDa) inner membrane protein not associated with any known secretion system, impairs membrane function (14). Furthermore, a decrease in the abundance of leukotoxin, an effector molecule of a type I secretion system, and of a subunit of a modified type II secretion system associated with fimbriae secretion were observed (14, 15). However, the inherent absence of fimbriae in the studied strain obfuscated the functional significance of this finding.

Proteomic and DNA sequence analysis of the genetic locus associated with fimbrial production provided a basis for the complementation of fimbriae expression in the afimbriated strain under study. The effect on fimbriation appears to occur posttranscriptionally since both the wild-type and morC mutant afimbriated strains show equal amounts of TadZ (15), the product of the seventh gene of the 14 gene *tad* operon (22). In *trans* expression of a prototypic *flp1-tadV* sequence promoted the production of fimbriae in the afimbriated wild type strain. However, microscopic examination of the transformed wild type strain revealed the presence two different colony morphologies. The majority of colonies (>95%) were small, rough in appearance, and adherent to agar but

lacked the internal star shaped structure. Colonies displaying these phenotypes have been observed previously and are described as an intermediate form between fimbriated and afimbriated strains (6). The remaining colonies displayed a star-shaped internal structure characteristic of fimbriated strains and were indistinguishable from a strain isolated directly from the oral cavity. Heterogeneity in colony morphology was reproduced upon re-plating individual colonies, implying that the fimbriation phenotype in this laboratory strain exhibited incomplete penetrance. This suggests that the intermediate colony morphology represents a population of cells containing different fimbriation states.

Fimbriae are associated with biofilm formation in *A. actinomycetemcomitans* (9) and differences in the mass of the formed biofilm are apparent between the fimbriated and afimbriated forms. The biofilm forming ability of the afimbriated strain transformed with the *flp1-tadV* plasmid was enhanced compared to the parent strain and was equivalent to the naturally fimbriated strain. Interestingly, only a small percentage of the transformed cells expressed fimbriae. This observation suggests that fimbriation of all cells is not required for the tenacious biofilm formation associated with this bacterium *in vitro*.

Clinical strains of *A. actinomycetemcomitans* form biofilms composed of well defined, discrete microcolonies with few free cells in between the microcolonies (23)(Figure 5 A). When compared to the clinical strain, the wild-type transformed strain displayed a different phenotype with less well defined microcolonies and a greater abundance of free cells. In addition to altered biofilm architecture, this strain displayed fimbriae that appeared more fragile when compared to those present on the naturally fimbriated strain. These observation suggest that maximum fimbriae production and

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integrity is critical for the biofilm architecture formed by the *A. actinomycetemcomitans* strains used in this study.

In similar fimbrial secretion systems, subunits are secreted via a piston like mechanism mediated by inner membrane proteins of the complex, which ensure the proper spatial organization of the polymerizing structure (24). The abnormal fimbriae observed in the transformed strain may be due to defects in the export mechanism of the fimbrial subunit. The laboratory adapted strain used in this study may have accumulated mutations in one or more of the fimbrial secretion proteins resulting in a defect in subunit export. The reduced fimbriae export may explain the absence of fimbriae production in the *morC* mutant strain. Since wild-type cells are impaired in fimbrial secretion, the further reduction of the abundance of the export apparatus in the mutant (15) would result in a rate of subunit export not compatible with formation of fimbriae.

The data associated with the afimbriated strain suggested that inactivation of *morC* would lead to loss of fimbriae in a naturally fimbriated strain. This was not observed and may be attributed to the extensive genomic variation between strains as reported for *A. actinomycetemcomitans* (25). However, altered microcolony architecture was evident in the isogenic *morC* mutant of the clinical strain. The fimbriae did not appear obviously different between the two strains (data not shown). Therefore, the change in the microcolony phenotype may result from a decrease in the abundance of the secretion apparatus in the membrane of the mutant strain leading to fewer functional fimbriae expressed on the surface of the bacteria.

To date, genes outside the *tad* locus are not known to have an effect on fimbriation of *A. actinomycetemcomitans*. In this work, we have demonstrated that the novel inner membrane protein MorC is associated with fimbrial secretion and microcolony formation in *A. actinomycetemcomitans*. This finding builds on our previous observations that MorC plays an important role in the membrane physiology of this pathogen. Interestingly, proteins homologous to MorC are present in Gammaproteobacteria and may contribute to the fimbriae secretion and biofilm architecture of these bacteria.

Acknowledgements

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Tables

Table 5.1: Bacterial strains and plasmids.

Bacterial Strain or Plasmid	Description	Reference or Source
E. coli		
DH10B	Laboratory strain for general cloning, *lac*[-]	Invitrogen
A. actinomycetemcomitans		
HK1651	Sequenced serotype b fimbriated strain	ATCC 700685
VT1257	Fimbriated clinical isolate	Maria Saarela, IDH, Finland
KM700	Isogenic *morC* mutant of VT1257. Spec[r]	This study
VT1169	Afimbriated strain derived from SUNY465.	(Mintz and Fives-Taylor 1994a)
VT1650	Isogenic *morC* mutant of VT1169. Spec[r]	(Gallant *et al.* 2008)
KM278	*morC* mutant containing pKM2	(Gallant *et al.* 2008)
KM409	*morC* mutant containing pKM2/*morC*	(Gallant *et al.* 2008)
KM611	VT1169 containing pKM586	This study
KM612	VT1650 containing pKM586	This study
KM609	VT1257 containing pKM687	This study
KM699	KM700 containing pKM687	This study
Plasmids		
pGEM	TA cloning vector. Replicates in *E. coli*. Amp[r]	Promega
pKM2	*E. coli* and *A. actinomycetemcomitans* shuttle vector. Cm[r]	(Gallant *et al.* 2008)
pVT1642	pKM2 containing JP2 leukotoxin promoter	(Tang and Mintz 2010)
pKM586	pVT1642 containing the *flp1-tadV* region of *A. actinomycetemcomitans* strain HK1651	This study

Amp = Ampicillin, Cm = Chloramphenicol, Spec = Spectinomycin

Table 5.2: Oligonucleotide primers used in this study (restriction sites are underlined)

Primer Name	Sequence (5'-3')	Description
Flp1Xho1F	CCG<u>CTCGAG</u>TTGAATTTTATTTTCTATAAAC	5' Primer, *flp1*
TadVXho1R	AAT<u>CTCGAG</u>ATTTGATAGAGCCATGTTTATCATAAAGCC	3' Primer, *tadV*
Omp67F	TCTGGACGTATTGCTTTATCCGC	5' Primer *morC* upstream
Omp67R	CTTC<u>CTCGAG</u>CTTATTATCCGTTCTTGTTGA	3' Primer *morC* upstream
PpxF	CTTC<u>TCTAGA</u>TTATGAATAACGAAAATTTA	5' Primer *morC* downstream
PpxR	TCAACGTGCCGACAGGCTTA	3' Primer *morC* downstream
SpecF	TAAG<u>CTCGAG</u>TGACTAAATAGTGAGG	5' Primer pSL60 cassette
SpecR	CTTC<u>TCTAGA</u>CATGTGATTTTCCTCC	3' Primer pSL60 cassette
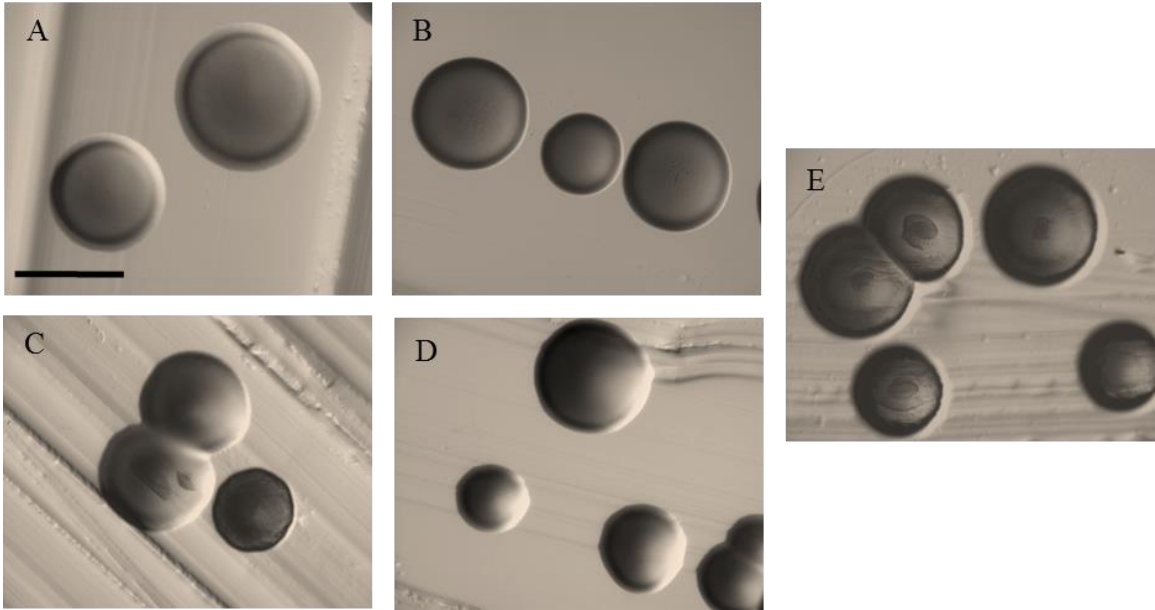
Figure 5.1. *A.* *actinomycetemcomitans* **Colony morphologies.** Bacteria were grown on solid TSBYE media for 3 days at 37°C in a humidified 10% CO_2 atmosphere. Images were taken using a Leica MZ16F Stereo Microscope (Leica, Buffalo Grove, IL). A; Wild-type B; *morC* mutant C; Wild-type/pKM2/*flp1-tadV* D; Fimbriated clinical isolate E; *morC* mutant/pKM2/*flp1-tadV*. Scale bar: 1 mm.

Figure 5.2 Transmission electron microscopy of strains of *A. actinomycetemcomitans*

A. actinomycetemcomitans strains grown on solid media were transferred directly to carbon coated grids and subject to transmission electron microscopy. A; Fimbriated clinical isolate. B; Wild-type C; *morC* mutant D; Wild-type/pKM2/*flp1-tadV* E; *morC* mutant/pKM2/*flp1-tadV*. Scale bar: 100 nm.
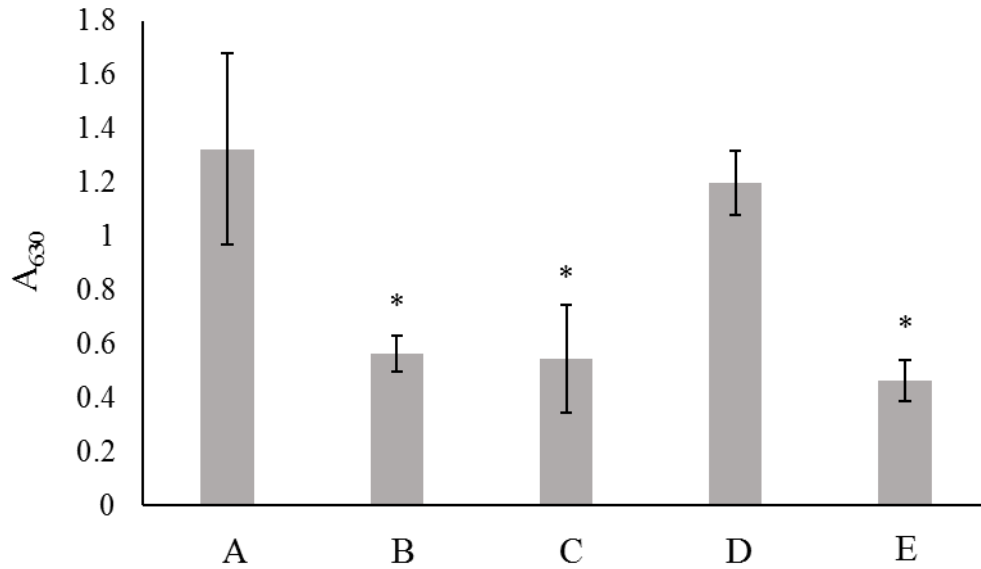
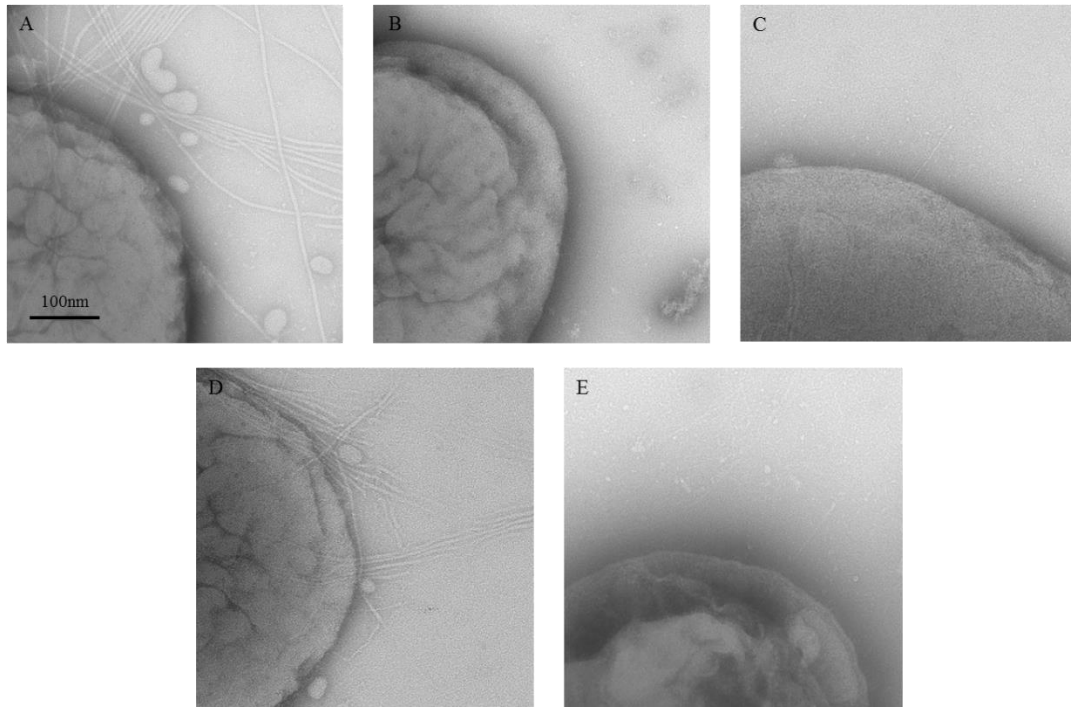**Figure 5.3 Biofilm assay.** Equal amounts of bacteria were inoculated into 96-well plates and grown to stationary phase to allow for biofilm formation. Media was aspirated and biofilms were washed and stained with crystal violet. Amount of stain retained (quantified by absorbance at 630 nm) corresponds to the relative biomass of the biofilm. Error bars indicate standard deviations from at least three experiments. A; Fimbriated clinical isolate. B; Wild-type C; *morC* mutant D; Wild-type/pKM2/*flp1-tadV* E; *morC* mutant/pKM2/*flp1-tadV*. (*) indicates a significant difference from the wild-type fimbriated strain (ANOVA with Dunnett's post-test, $p < 0.05$).
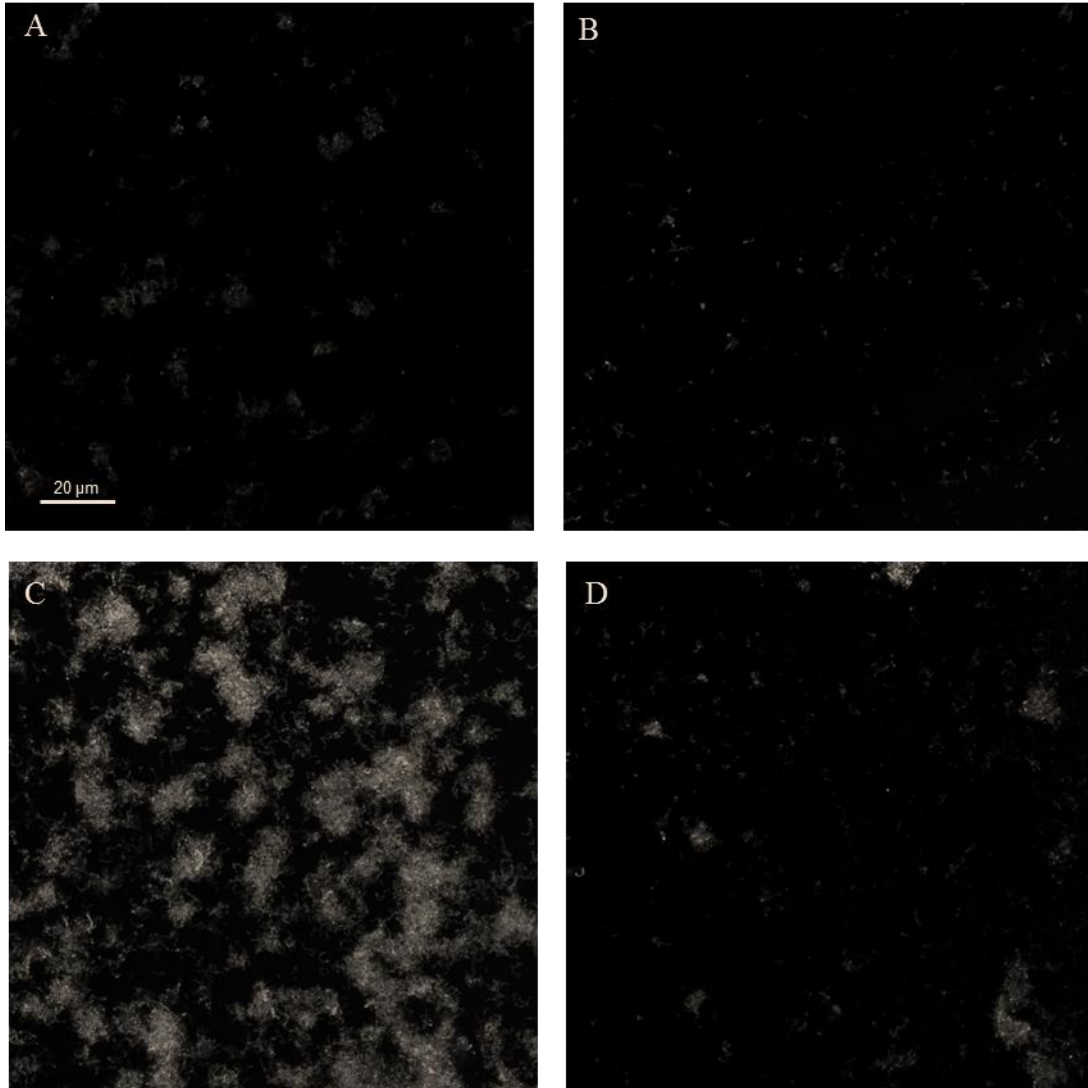
Figure 5.4 Confocal microscopy of biofilms generated by laboraory strains of *A. actinomycetemcomitans.* *A. actinomycetemcomitans* strains were grown as biofilms in glass-bottomed dishes and stained with SYTO 9. A series of z-stack images was generated with a Zeiss LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany). A-D are representative fields from the following strains: A; Wild-type B; *morC* mutant C; Wild-type/pKM2/*flp1-tadV* E; *morC* mutant/pKM2/*flp1-tadV*
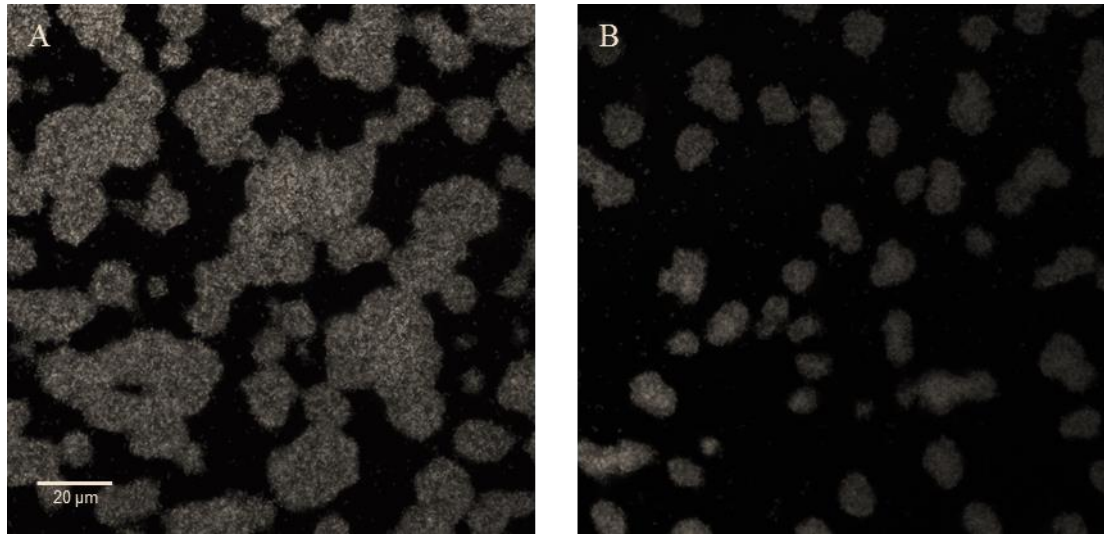
Figure 5.5 Confocal microscopy of biofilms generated by clinical strains of *A. actinomycetemcomitans*. Biofilms were prepared, stained, and imaged as in Figure 4. A; Fimbriated clinical isolate VT1257 B; Isogenic *morC* mutant of VT1257. Images are representative of three separate experiments.

CHAPTER 6: Discussion and future directions

1. Discussion

A. actinomycetemcomitans, a Gram-negative rod shaped bacterium of the Pasteurellaceae family, is recognized as a significant human pathogen implicated in the etiology of oral and non-oral infections (Zambon *et al.* 1983, Paturel *et al.* 2004, Tang *et al.* 2008). As a Gram-negative bacterium, it contains a double membrane system termed the cell envelope consisting of an inner and outer membrane separated by a periplasmic space (Silhavy *et al.* 2010). Each compartment of this envelope contains a specific subset of proteins critical for maintenance of cellular homeostasis (Silhavy *et al.* 2010). Loss or inactivation of any of these proteins will have dramatic impact on the physiology of the cell. Accordingly, the absence of MorC in the cell envelope of *A. actinomycetemcomitans* results in multiple pleiotropic effects. The conserved nature of the *morC* sequence and operon organization implies that this protein may be important for the maintenance of cellular physiology in other Gammaproteobacteria.

Much significance is associated with conservation of protein sequence and structure. However, establishing conservation of protein function, outside of substituting heterologous enzymes conserved in well-defined and essential metabolic functions, has not been extensively studied. The functionally conservation of MorC among diverse families of Gammaproteobacteria was tested using *A. actinomycetemcomitans* as the model organism. MorC from all organisms tested (*H. influenzae*, *E. coli*, *P. aeruginosa*, and *M. catarrhalis*) was found to rescue at least some of the phenotypes in *A.*

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actinomycetemcomitans. This implies that there is some functional conservation of this protein among these divergent bacteria.

MorC is found in low abundance in the inner membrane, based on our analysis. This fact, along with the extensive pleiotropic effects observed in the mutant, implies that MorC alone does not generate all of the observed phenotypes and may interact with other proteins. If MorC acts as a scaffolding protein in a complex, each interacting partner may mediate a MorC phenotype. Correct interaction between MorC and specific membrane-associated proteins may be required to complement each of the observed phenotypes. Therefore, complementation depends on the protein composition of the cell membrane which in turn is influenced by the organism and its environmental niche. In addition, heterologous MorC may interact with a subset of proteins resulting in partial complementation.

The MorC homologs used in this study evolved alongside a different complement of proteins than exist in the membrane of *A. actinomycetemcomitans*. As the proteome of *A. actinomycetemcomitans* is necessarily unique, it is possible that the interactive partners may not be present in *A. actinomycetemcomitans* or that homologous proteins may be unable to interact with MorC from divergent organisms due to structural differences. A similar species-specific interaction has been shown in for a protein-interacting domain of the BamA protein of *E. coli*. This domain interacts only with outer membrane proteins from the same organism (Robert *et al.* 2006). A conceptual diagram of this hypothesis shown in Figure 6.1. Aberrant interactions with other membrane proteins potentially

explain the differential complementation observed with the MorC homologs expressed in

A. actinomycetemcomitans.
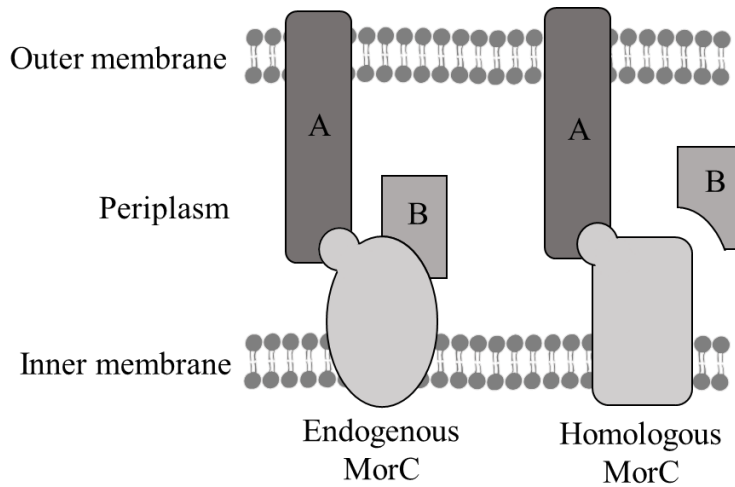
Figure 6.1 Potential model for differential complementation among homologous MorC proteins in the *A. actinomycetemcomitans* membrane. The endogenous MorC from *A. actinomycetemcomitans* can interact properly with protein A and B resulting in restoration of all wild-type phenotypes. Due to differences in structure, the homologous protein can interact with A but not B, restoring only phenotype A to wild-type while phenotype B remains mutant.

Protein-protein interactions have been shown between the MorC homolog (TamB) and the

Omp67 homolog (TamA) in a member of the *Enterobacteriaceae* family (Selkrig *et al.*

2012). Structural data has revealed that the *E. coli* TamA/TamB complex spans the entire

periplasmic space and may exert an inward force on the outer membrane (Shen *et al.* 2014).

Based on sequence conservation of MorC and Omp67, we hypothesize that a similar

periplasm-spanning complex is formed in *A. actinomycetemcomitans*. This connection may

exert forces on the outer membrane, effectively pulling it towards the inner membrane,

serving as the anchor point for the observed convolutions of *A. actinomycetemcomitans*.

Links between the outer membrane and components of the periplasm are common and

essential for cell viability as demonstrated by the interaction between Braun's lipoprotein and the peptidoglycan cell wall (Vollmer *et al.* 2008). Further experiments will be required to test this hypothesis.

In *E. coli*, the DUF490 domain of the MorC otholog, TamB, is important for mediating protein-protein interaction with Omp67 (Selkrig *et al.* 2012). This observation is consistent with results of our truncation experiments showing that the DUF490 domain is required for MorC function in *A. actinomycetemcomitans*. In particular, the last 10 amino acids are essential, implying that MorC function in *A. actinomycetemcomitans* may rely on interaction with Omp67 via the DUF490 domain. However, the phenotypes of a mutant in *omp67* do not mirror the phenotypes of the *morC* mutant (Danforth and Mintz, unpublished data), suggesting that other proteins are involved in the MorC phenotypes. Some of these partners may interact specifically with MorC or the MorC/Omp67 complex but not Omp67 alone (Danforth and Mintz, unpublished data).

If MorC does indeed interact with proteins in the membrane of *A. actinomycetemcomitans*, it follows that abundance of other proteins may be modulated in the *morC* mutant. Therefore, a proteomics approach was taken to characterize the membrane-associated proteins of *A. actinomycetemcomitans*. Historically, large-scale identification of proteins was accomplished using 2-dimensional gel electrophoresis (2D-GE). This technology allows for the separation of proteins by charge in the first dimension followed by size in the second dimension. However, a limitation of this technique is that membrane proteins

are notoriously difficult to detect due to bias against the hydrophobic transmembrane domains in the isoelectric focusing step (Rabilloud 2009). To overcome this limitation, a gel-free method (Dephoure and Gygi 2011) was utilized that allowed for the detection of over 665 proteins.

Over 20% of the membrane proteome consisted of proteins with no close homologs (e-value $> 10^{-20}$) in *Escherichia coli*, which supports the observation that not all MorC homologs complement the *A. actinomycetemcomitans* phenotypes. The majority of proteins found in this study existed at a 1:1 ratio between the wild-type and mutant cells. In contrast, depletion of a secretion apparatus in *E. coli* responsible for the biosynthesis of LPS and maintenance of outer membrane integrity, results in a general reduction of over 100 proteins (Martorana *et al.* 2014). Since a general decrease in protein abundance has not been shown for the *morC* mutant, it is likely that non-protein components of the membrane are not affected. This was confirmed by fatty acid analysis data indicating the lack of change in fatty acid composition between wild-type and mutant cells.

The observed decrease in leukotoxin secretion in the *morC* mutant was originally hypothesized to be due to a decrease in the abundance of the proteins involved in type I secretion apparatus. However, based on the quantitative proteomic analysis, the abundance of the proteins associated with this complex were not found to be changed (Figure 6.2). The functionality of the inner membrane (LtxB) and periplasmic components (LtxD) of this secretion system are difficult to assess and are beyond the scope of this study.

However, TolC, the outer membrane, pore forming component can be functionally assayed. In *A. actinomycetemcomitans*, TolC is necessary for resistance to the antibiotic erythromycin through association with an efflux pump (Crosby and Kachlany 2007). To investigate the functionality of TolC, the minimal inhibitory concentration (MIC) for erythromycin was compared between the wild-type and mutant strain. The MIC was found to be equal between the two strains, suggesting that TolC is active in the *morC* mutant strain.
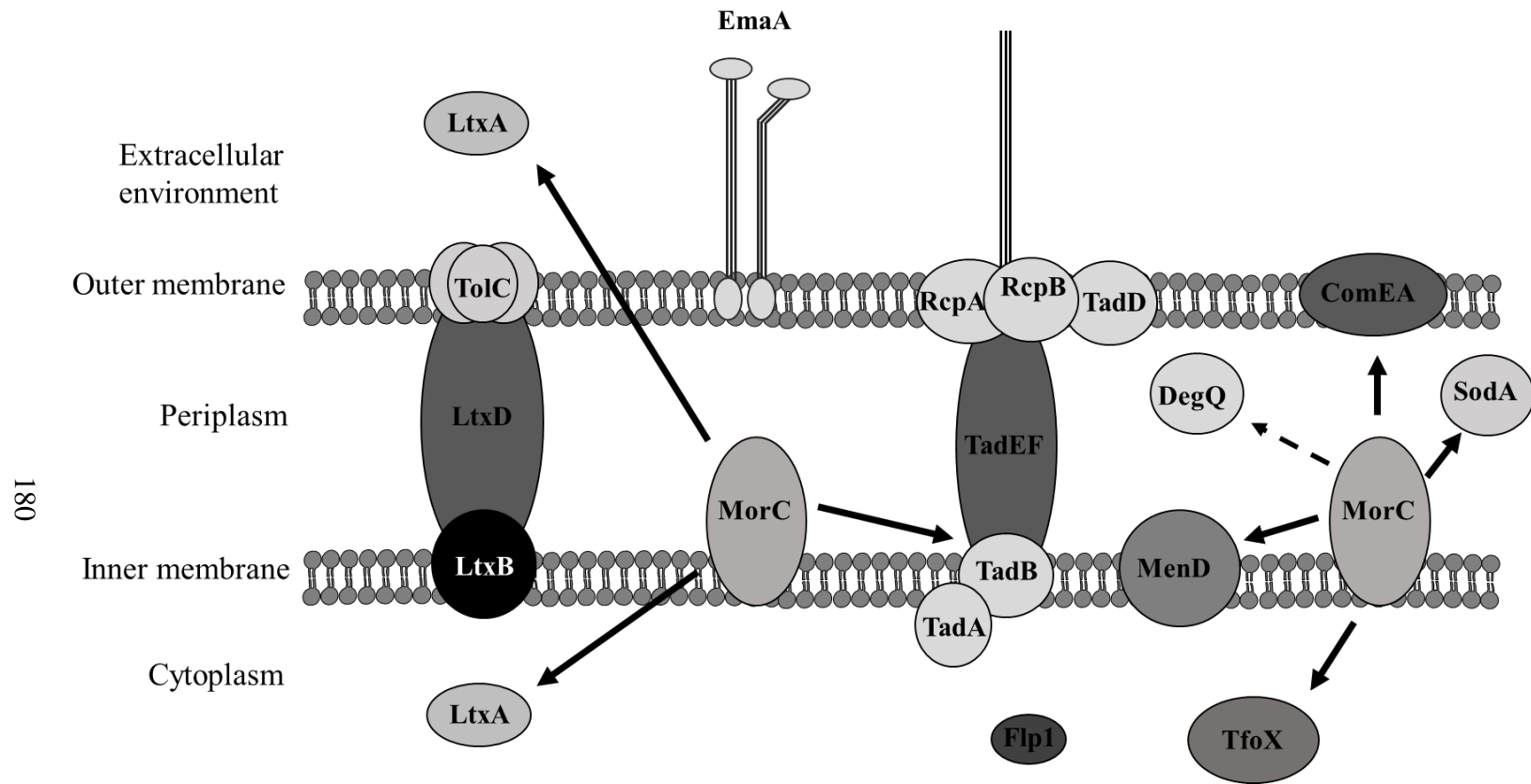
Figure 6.2 Current model of the effect of *morC* inactivation on *A. actinomycetemcomitans* proteins. Solid arrows indicate proteins that are reduced in abundance in the *morC* mutant. Dashed arrow indicates increase in abundance in the mutant. Some proteins have been omitted for clarity.

Leukotoxin production has been linked to the presence of reactive oxygen species, and a direct interaction between the toxin and a superoxide dismutase has been proposed (Balashova *et al.* 2007, Takashima and Konishi 2008). Reduction in abundance of a superoxide dismutase (SodA) and a peptide methionine sulfoxide reductase (MsrB) was observed in the *morC* mutant, implying increased oxidative stress. An increase in oxidative stress is hypothesized to destabilize the leukotoxin protein, resulting in misfolding of the toxin (Balashova *et al.* 2007, Takashima and Konishi 2008). The observed decrease in folding-associated chaperones and concomitant increase in a degradation-associated chaperone protein may also contribute to the degradation of leukotoxin (Kim *et al.* 2013).

Reduction in chaperone proteins suggests an overall increase in misfolded proteins. Improperly folded proteins are more likely to form aggregates, especially in the periplasm (Kim *et al.* 2013) and may not be inserted into the membrane. Protein aggregation activates the membrane stress response and results in an increase in specific periplasmic chaperones such as DegP, responsible for degrading misfolded proteins (Pallen and Wren 1997). The *A. actinomycetemcomitans* genome does not encode DegP but does contain DegQ, a periplasmic chaperone with overlapping functionality with DegP (Kolmar *et al.* 1996, Waller and Sauer 1996). In the *morC* mutant, DegQ was increased in abundance, indicating that this bacterium may be under membrane-related stress. DegQ may play a compensatory role by preventing the majority of outer membrane and periplasmic proteins from misfolding, as evidenced by the limited changes in abundance found between the wild-type and mutant strains.

In addition to reduction of chaperone proteins and oxidative stress response proteins, decreases in the the fimbrial secretion system complex proteins were observed in the *morC* mutant. This complex (Tad) is composed of 14 proteins, all but one were found to be consistently reduced in the mutant strain. Only TadZ was found to be equal in abundance between the wild-type and mutant. TadZ is encoded by a gene in the middle of the *tad* locus and is the only protein of the locus that can be found independently of the secretion complex (Perez-Cheeks *et al.* 2012). This effectively rules out transcriptional regulation as a mechanism for the reduced Tad abundance. No observed differences in transcription of several of the genes of the operon were observed by RT-PCR, suggesting a posttranslational mechanism for the reduction of fimbrial secretion proteins.

Consistent with the finding that the Tad complex is reduced in the afimbriated *morC* mutant, inactivation of *morC* in a naturally fimbriated strain of *A. actinomycetemcomitans* also affected fimbriation. The mutant in a clinical strain formed significantly altered microcolony structure implying a defect in secretion or structure of fimbriae. Complete loss of a single complex protein leads to afimbriation (Clock *et al.* 2008). In the *morC* mutant fimbriae are still present but may be impaired due to reduction of the abundance of the number of complexes formed. These observations, combined with the fact that TadZ is not affected seems to imply that the secretion complex is not properly assembled in a *morC* mutant of a naturally fimbriated strain.

Taken together, our evidence suggests that MorC has a specific effect on multiple proteins associated with the cell envelope. We hypothesize that these effects are mediated by a complex comprised of MorC and Omp67 and that this interaction is necessary for proper localization of membrane proteins. Interactions between MorC and Omp67 have been observed in *E.coli* and are important for MorC function (Selkrig *et al.* 2012). Further, bioinformatics analyses indicate that Omp67 is a paralog of BamA, a key component of the BAM complex involved in the integration of outer membrane proteins. In *E. coli*, Omp67/MorC appears to play a similar role to the BAM complex for a specific autotransporter (Shen *et al.* 2014). However, unlike proteins of the BAM complex, MorC and Omp67 are not essential genes (Gallant *et al.* 2008, Rossiter *et al.* 2011). Therefore, it is unlikely that the Omp67/MorC system shares overlapping functionality with the BAM complex. We hypothesize that MorC in *A. actinomycetemcomitans* interacts with Omp67 and acts as a scaffold for a unique transport complex for a subset of outer membrane proteins summarized in Figure 6.3. It is clear that loss of MorC effects at least one outer membrane protein (ComEA, involved in DNA binding and natural competence) and may possibly affect others involved in fimbrial secretion.
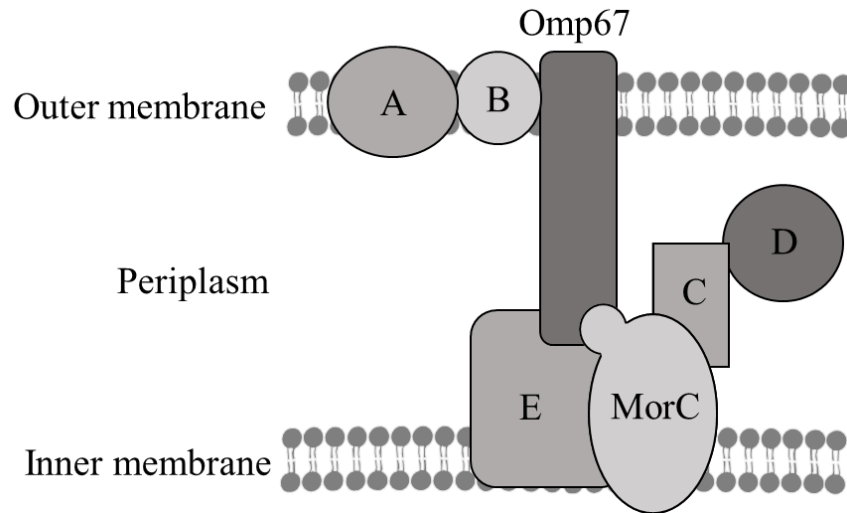
Figure 6.3 Model for MorC interactions with other membrane-associated proteins. MorC is known to interact with Omp67 in a related organism via the DUF490 domain (represented by small circle protruding from MorC). As Omp67 is a relative of BamA, we predict that it will also form a similar complex in the outer membrane with proteins (A and B). Given that the *omp67* mutant does not mirror the phenotypes of the *morC* mutant strain we also hypothesize there is a complex of proteins associated with MorC (C and D). Finally, some proteins may interact with both Omp67 and MorC (E).

In conclusion, this work has demonstrated that MorC plays a critical role in maintaining secretion of major virulence determinants of *A. actinomycetemcomitans*. Specific changes in the protein composition of the cell envelope were identified and indicate a direct or compensatory role of these proteins in maintaining membrane physiology. The functional conservation of MorC also implies an important role for this protein in other Gram-negative bacteria. This work suggests a role of MorC as an accessory or a scaffold protein involved in secretion.

2. Future Directions

1. Characterization of proteins affected by loss of MorC. Our proteomics analyses identified 12 proteins that are modulated in abundance in the *morC* mutant. Given the multiple membrane-related effects on the mutant, it stands to reason that the affected proteins may play a direct or indirect role in one or all of these phenotypes. Identification of proteins directly responsible for each MorC phenotype will give a better understanding of the function or MorC as well as insight into membrane physiology of *A. actinomycetemcomitans*.

Functionality of these proteins will be evaluated by insertional inactivation or deletion of each gene coding for these proteins in *A. actinomycetemcomitans* by homologous recombination (Gallant *et al.* 2008). Assays for bile salt resistance, membrane morphology, and leukotoxin secretion will be carried out to identify which, if any, phenotype the protein in question is involved in. Quantitative mass spectrometry of the mutants when compared with the wild-type cells will allow for identification and verification of other proteins involved in the observed phenotype. If no phenotype is observed, it can be concluded that the observed reduction in the affected protein is a secondary effect of *morC* inactivation rather than a driver of the MorC phenotypes.

2. Identification of proteins interacting with MorC in the membrane of *A. actinomycetemcomitans*. We hypothesize that pleiotropic effects related to membrane physiology depend on protein-protein interactions with MorC in the membrane. This

hypothesis is supported by our observation that multiple proteins are modulated in abundance and this alteration is not a result of changes in membrane lipid composition. Therefore, it seems likely that MorC influences the abundance of other membrane-associated proteins by direct interaction. Further evidence for this is found in *E. coli* where the MorC homolog, TamA, directly interacts with the Omp67 protein (Selkrig *et al.* 2012).

Currently, there are no known interacting partners of *A. actinomycetemcomitans* MorC. We will identify protein-protein interactions via immunoprecipitation experiments using a MorC antibody. Whole membranes from *A. actinomycetemcomitans* will be isolated and treated with a crosslinking agent. This mixture will be incubated with the anti-MorC antibody conjugated to agarose beads. The beads will be isolated and immune complexes released, run on SDS-PAGE gels, and bands analyzed by mass-spectrometry. The *morC* mutant will serve as a negative control. Alternatively, FLAG, HA, or 6xHis tagged MorC proteins that exist in our laboratory could be used in the pulldown experiments. This would allow for use of a high-affinity monoclonal antibody to the appropriate epitope tag in the event that the polyclonal anti-MorC antibodies are ineffective. We expect to find Omp67 among the MorC interacting partners. We also expect to find some of the proteins identified in the quantitative proteomics studies. If MorC is shown to directly interact with other proteins in the membrane, this will support our hypothesis that MorC and Omp67 form a novel secretion system.

3. Inactivation of Omp67 and comparison of phenotype to the *morC* mutant. Omp67 interacts with the MorC homolog in *E. coli* and is also hypothesized to interact with MorC in *A. actinomycetemcomitans*. We have created an *omp67* mutation in *A. actinomycetemcomitans* and are in the process of characterizing the phenotypes. If interactions between the two proteins were required for all observed phenotypes, an *omp67* mutant should have identical phenotypes to the *morC* mutant strain. However, preliminary data suggest that the phenotypes of this mutant are not identical to that of the MorC mutant.

This implies that there are MorC specific phenotypes that do not depend on Omp67 (see Figure 6.3 for model). The MorC phenotypes will be tested in the *omp67* mutant to evaluate the relative contribution of this protein to the observed phenotypes. In addition, we plan to perform quantitative mass spectrometry on this mutant and compare the proteome to that of the *morC* mutant. This will allow us to identify proteins and phenotypes specific to each mutation and those that depend on the MorC-Omp67 interaction. Ultimately, this will give us more information about the function of each protein as well as the hypothesized complex.

4. Quantification of gene expression in the *morC* mutant. Results from our proteomics study indicated that a transcription factor (TfoX) was reduced in abundance. Given that our study was limited to investigating membrane-associated proteins and did not take into account mRNA levels, the functional significance of this change is unclear. Therefore, we

propose to use RNA-Seq technology to quantify gene expression in the wild-type and *morC* mutant strains of *A. actinomycetemcomitans*.

We will utilize cells grown to mid-logrithmic phase in broth culture as in the proteomics experiments. RNA will then be extracted, isolated, and converted into cDNA for next generation sequencing. Using the bioinformatic techniques used for the quantitative proteomics study, we quantify differences in gene expression between the two strains. This will allow us to investigate expression levels of cytoplasmic proteins and potentially identify new MorC phenotypes.

5. Investigation of the membrane morphology phenotype. Our phenotypic data, combined with structural information of the *E. coli* MorC homolog indicates that this protein participates in a cell envelope spanning complex. This complex is predicted to be anchored to the inner and outer membrane. *A. actinomycetemcomitans* has been shown to have a rugose outer membrane with discrete connection points in which the outer membrane comes in contact with the inner membrane (Azari *et al.* 2013). This raises the possibility that MorC may mediate these connections. To investigate this possibility, we will use transmission electron microscopy to detect immunogold labeled antibodies specific to MorC in ultrathin sections of bacteria. We expect that these antibodies will localize to the connection points between the inner and outer membranes if our hypothesis about the nature of the MorC/Omp67 complex is correct.

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