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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.

Keywords

Membrane Morphology; Toxin secretion; TamB; YtfN; DUF490

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 (Fine *et al.* 2007; Socrasky *et al.*

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1998). 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 (Rahamat-Langendoen *et al.* 2011; Scannapieco 1999)

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; Selkig *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 (Selkig *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% CO₂ 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% CO₂ 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 restriction site (Table 2) and ligated with the spectinomycin adenyltransferase *aad9* gene, derived from pSL60 (Lukowski *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 electrocompetent 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 DH5 α (λ pir). 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 μ l 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% CO₂ environment. After incubation, cells were plated onto TSBYE agar lacking DAP and containing 50 μ g 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 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% glycocholic 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 ($OD_{495}(\text{bile salt}) / OD_{495}(\text{no bile salt}) \times 100\%$). 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 (2012). 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 prepared by filtration (0.22 μ M 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 μ g 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 5 μ l 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 μ l of PBS on a group of colonies and then lowering a grid, carbon-side down, on top of the humidified colonies. 3 μ l 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 μ m (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 structures using HHPred (Soding *et al.* 2005). Predicted organization of the *morC* operon was retrieved from the Prokaryotic Operon Database (Taboada *et al.* 2012; Taboada *et al.* 2010).

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; Selkirk *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% 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 in 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 1235–1292 construct. However, deletion of the last 10 amino acids (1283–1292: 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 phenylalanine (1292) 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 (Selkig *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 Gammaproteobacteria 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 (Azari *et al.* 2013; Gallant *et al.* 2008; Yu *et al.* 2009). 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 Pasteurellaceae, which may be dependent on a membrane protein composition that is not shared by distantly related organisms. The MorC derived from non-Pasteurellaceae 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 (Duong *et al.* 2001; Guzzo *et al.* 1991; 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 (Azari *et al.* 2013; Gallant *et al.* 2008). 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 (Azari *et al.* 2013; Gallant *et al.* 2008). 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.

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Figure 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. 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 (square), endogenous *morC* (cross), *H. influenzae* *morC* (plus), *E. coli* *morC* (circle), *P. aeruginosa* *morC* (diamond), *M. catarrhalis* *morC* (triangle).

Figure 3. Immunoblot analysis of leukotoxin synthesis and secretion in *morC* homologs

Strains were grown to mid-log phase and 20 μ g of protein (cell associated toxin) or secreted protein from 10^9 cells (secreted toxin) 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 morC* (Ec), *P. aeruginosa morC* (Pa), and *M. catarrhalis morC* (Mc).

Figure 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 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 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 strain expressing endogenous *morC* (ANOVA, Dunnett's post-test, $p < 0.05$).

Figure 7. MorC expression in *A. actinomycetemcomitans* truncation and substitution mutants
 Strains were grown to mid-logarithmic phase and 20 μ g of whole cell lysate or 50 μ g of whole membrane proteins were probed with affinity purified polyclonal antiserum. 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 MorC protein **A**. Whole cell lysates of carboxyl-terminal truncations of the DUF490 domain: 1050–1292 (115 kDa), 1235–1292 (134 kDa) 1283–1292 (139

kDa) and 1292 (141 kDa). **B.** Whole cell lysates of cysteine substitutes. **C.** Cell envelope preparations of carboxyl-terminal truncations.

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Table 1

Bacterial strains and plasmids.

Bacterial Strain or Plasmid	Description	Reference or Source
<i>E. coli</i>		
TOP10F-, DH10B	General cloning, <i>lac</i> ⁻	Invitrogen
DH5α(λpir)	General cloning, λpir	
β-2163	DAP auxotroph, λpir	(Demarre <i>et al.</i> 2005)
<i>A. actinomycetemcomitans</i>		
VT1169	Spontaneous rifampin and nalidixic acid resistant mutant of SUNY465	(Mintz and Fives-Taylor 1994)
VT1650	<i>morC</i> insertional inactivation strain. Spec ^r	(Gallant <i>et al.</i> 2008)
KM555	<i>morC</i> deletion strain. Spec ^r	This study
<i>morC</i> complement	<i>morC</i> mutant containing pKM303-A.a. Cm ^r	(Gallant <i>et al.</i> 2008)
Plasmids		
pCR2.1-TOPO	TA cloning vector that replicates only in <i>E. coli</i> . Amp ^r , Kan ^r	Invitrogen
pKM1	<i>E. coli</i> and <i>A. actinomycetemcomitans</i> shuttle vector. Kan ^r	(Ruiz <i>et al.</i> 2006)
pKM2	<i>E. coli</i> and <i>A. actinomycetemcomitans</i> shuttle vector. Cm ^r	(Gallant <i>et al.</i> 2008)
pVT1460	Conjugative plasmid, replicates in λpir strains of <i>E. coli</i> , Km ^r	(Mintz <i>et al.</i> 2002)
pKM550	pVT1460 containing <i>morC</i> deletion construct	This study
pKM303	pKM2 containing the 165bp <i>morC</i> promoter sequence. Cm ^r	(Gallant <i>et al.</i> 2008)
pKM475	pKM1 containing the 165bp <i>morC</i> promoter sequence. Kan ^r	This study
pKM557	pKM475 containing <i>A. actinomycetemcomitans morC</i> . Kan ^r	This study
pKM408	pKM303 containing <i>A. actinomycetemcomitans morC</i> . Cm ^r	(Gallant <i>et al.</i> 2008)
pKM303 – H.i.	pKM303 containing the <i>morC</i> homolog from <i>Haemophilus influenzae</i> 86-028NP. Cm ^r	This study
pKM303 – E.c.	pKM303 containing the <i>morC</i> homolog from <i>Escherichia coli</i> K-12. Cm ^r	This study
pKM303 – P.a.	pKM303 containing the <i>morC</i> homolog from <i>Pseudomonas aeruginosa</i> PAO1. Cm ^r	This study
pKM303 – M.c.	pKM303 containing the <i>morC</i> homolog from <i>Moraxella catarrhalis</i> 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
1050–1292	In-frame deletion of bp 3148 to 3876 of <i>morC</i> in pKM475	This Study
1235–1292	In-frame deletion of bp 3703 to 3876 of <i>morC</i> in pKM475	This Study
1283–1292	In-frame deletion of bp 3847 to 3876 of <i>morC</i> in pKM475	This Study
1292	In-frame deletion of bp 3874 to 3876 of <i>morC</i> in pKM475	This Study

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

Table 2

Oligonucleotide primers.

Oligonucleotide	Sequence (5' -3')
HimorCF	GCAA <u>AGCTT</u> ATGACTACAACCATCAG
HimorCR	GCTCTAGACTAAATATAAAGTAAATC
EcmorCF	CCGCTCGAGATGAATGGAAAAAATC
EcmorCR	GCTCTAGACTAAACTCGAACTGATAG
PamorCF	CCGCTCGAGGTGAAGGCGCTGAAG
PamorCR	CCGGAATTCTCAGTCGCGCTTCAAGA
McmorCF	CCGCTCGAGATGACTCATCAGGATAATTC
McmorCR	GCTCTAGACTAAAACCTCCAACGATAA
Omp67F	TCTGGACGTATTGCTTTATCCGC
Omp67R	CTTCCTCGAGCTTATTATCCGTTCTTGTTGA
SpecF	TAAGCTCGAGTGACTAAATAGTGAGG
SpecR	CTTCTCTAGACATGTGATTTTCCTCC
PpxF	CTTCTCTAGATTATGAATAACGAAAAATTA
PpxR	TCAACGTGCCGACAGGCTTA
BamHI.PI65.For	CGCGGATCCGCCCGTTTATTGAATCTACCTTCC
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	GTAAAGCTTTGCGGGAACTTAAATCCG
MorC.SpHI.1049Rev	ACATGCATGCTTATTCGCTCGGCAATGTTTTC
MorC.SpHI.1234Rev	ACATGCATGCTTATCTGTCGCCCACCCCG
MorC.SpHI.1282Rev	ACATGCATGCTTATTGATTAACACCCGAAACGGATTG
MorC.SpHI.1291Rev	ACATGCATGCTTATTCAAATTGATAGAGTAG
MorC.SpHI.TTA.Rev	ACATGCATGCTTAAATTCAAATTGATAGAGTAG

Underlined sequences indicate restriction endonuclease sites.

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Table 3

Summary of phenotypes.

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

MorC three-dimensional structure predictions based on HHPred.

Aa	Hi	Ec	Pa	Mc	Reference Structure
*1-18	1-19	1-29	1-22	-	δ_2 JPW (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”