A CONTINUED ANALYSIS OF IRON ACQUISITION SYSTEMS IN
ACTINOBACILLUS ACTINOMYCETEMCOMITANS

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Christopher John Shoemaker
Miami University
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ABSTRACT

A CONTINUED ANALYSIS OF IRON ACQUISITION SYSTEMS IN
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Actinobacillus actinomycetemcomitans (Aa) is a microaerophilic, gram-negative oral pathogen associated with localized aggressive periodontitis (LAP). Congo red and hemin binding assays showed that all tested strains were able to bind this stain and iron-containing compound, respectively. Inspection of the HK1651 genome indicated that this binding activity could be associated with the presence and expression of hms-like genes, which code for similar functions in other pathogens such as Yersinia pestis. The role of these genes was examined by testing the phenotype of a DF2200N derivative in which hmsF was disrupted by insertion mutagenesis. Such a derivative showed a large reduction in hemin and Congo red binding activity, which correlated with the production of a 71-kDA outer membrane protein. Iron-utilization assays showed no significant difference in the ability of the parental strain and the hmsF insertion derivative to use hemin under chelated conditions. These observations are in agreement with the presence of an hms-like locus in the genome of Aa, which seems to be involved in the interaction of cells with hemin but not the utilization of this compound as an iron source for reasons that remain to be elucidated. ExbBDTonB and TolQRA are energy transducing systems often used by bacteria for hemin acquisition. Both systems have been annotated in the Aa HK1651 genome. Single and double insertion derivatives of these systems were created using transposons coding for resistance to trimethoprim and kanamycin. DF2200N single and double mutants displayed no significant defect in hemin utilization when compared to the parental strain. These results suggest the expression of an alternative hemin acquisition system that does not require the energy transducing systems mentioned above. Analysis of the Aa HK1651 genome showed the presence of genes coding for a putative dipeptide transporter related to the Dpp system in Escherichia coli. Such a system was recently proposed as an alternative hemin acquisition factor. Further work will seek to analyze the role of this novel system during hemin acquisition in Aa.
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Christopher John Shoemaker

Approved by:

________________________, Advisor
(Luis A. Actis)

________________________, Reader
(Eric R. Rhodes)

________________________, Reader
(Sharon M. Menke)

Accepted by:

________________________, Director
University Honors Program

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Introduction:

Microbiology’s humble beginnings trace back to the 1680’s when Dutch glassmaker Anthony van Leeuwenhoek, using one of the earliest microscopes, first recorded the presence of observable microbes (Pennisi, 2005). On September 17, 1683, as part of his work, van Leeuwenhoek authored a letter to the Royal Society of London detailing the “little creatures” he observed in dental plaques removed from his mouth (Fred, 1933). Thus was the field of oral microbiology formed. It is now well-known that the dental plaques van Leeuwenhoek observed are actually complex biofilms composed of as many as 500 species of bacteria (Paster et al., 2001). The interactions of these bacteria, whether through microbe-microbe or microbe-host contacts, greatly impact the ability of the organisms to form a biofilm and, by consequence, evade antimicrobial agents and the host’s defenses to cause disease.

While most species of bacteria that reside in the oral cavity are commensal, a significant number are opportunistic pathogens capable of causing disease in immuno-compromised patients. One such disease is periodontitis, a chronic inflammatory disease implicated in tooth loss and cardiovascular disease (Office, 2000). In its most severe form periodontitis is known as localized aggressive periodontitis (LAP), recently renamed from localized juvenile periodontitis (LJP) (Armitage, 1999). By mechanisms still unknown, juvenile periodontitis targets only the incisors and premolars; however, adult periodontitis can affect all teeth. A report from the Surgeon General suggests that over 16% of all adults over 45 suffer from severe periodontal diseases (Office, 2000). Consequently, dental health care has blossomed into an over $60 billion-a-year industry.
Yet despite this, three out of every 10 people worldwide will lose all their teeth by age 65 (Pennisi, 2005).

Studies have shown that *Actinobacillus actinomycetemcomitans*, a gram-negative, microaerophillic coccobacillus, is a primary causative agent of LAP (Slots and Ting, 1999). *A. actinomycetemcomitans* stimulates rapid decomposition of the alveolar bone and periodontal ligament, ostensibly through the stimulation of bone resorption or osteoclast activity (Wilson and Henderson, 1995). Additionally, *A. actinomycetemcomitans* has been implicated in 0.6% of all endocarditis cases as well as in pneumonia, septicemia, bacteremia, osteomyelitis and urinary tract infections (Das et al., 1997; Van Winkelhoff and Slots, 1999). Amidst these allegations, *A. actinomycetemcomitans* is often detected in the oral cavity of otherwise healthy individuals, present in roughly 18% of all plaque samples examined (Haraszthy, 2000).

*A. actinomycetemcomitans in vitro* growth is optimized under 5% CO₂ at 37°C (Ohta et al., 1989). Colony morphology can be divided into two groups: rough clinical isolates that produce extensive fimbriae to aid in adhesion and smooth lab isolates characterized by a loss of fimbriae and the inability to adhere strongly to glass (Inouye, 1990). Similarly, it is noted that only *A. actinomycetemcomitans* strains which maintain the rough phenotype are pathogenic in an animal model (Schreiner et al., 2003).

The 2-megabase genome sequence of *A. actinomycetemcomitans* was recently made available by the efforts of David Dyer et al. at the University of Oklahoma and the Oralgen database (http://www.oralgen.lanl.gov/). Yet, much of the annotated genome remains listed as hypothetical, unclassified or unknown. Much work, therefore, remains
to be done in order to fully understand this important human pathogen. The focus of our lab group with this pathogen has been to characterize systems predicted to be involved in iron acquisition.

Iron is found in two naturally occurring ionic states: Fe(II) and Fe(III). Fe(II), or ferrous iron, is found under anaerobic (i.e. reduced) conditions and is soluble at a biological pH. In contrast, Fe(III), or ferric iron, is found under aerobic (i.e. oxidized) conditions and is largely insoluble (Wandersman and Delepelaire, 2004). This insoluble state greatly restricts iron’s availability to be directly assimilated by aerobic organisms. This is not to imply that iron is unreactive in aerobic environments. Interconversion of Fe(II) and Fe(III) under aerobic conditions occurs via the Fenton reaction (Wessling-Resnick, 1999).

\[
\begin{align*}
\text{Fe(III)} + \text{O}_2^- & \rightarrow \text{Fe(II)} + \text{O}_2 \\
\text{Fe(II)} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe(III)} + \cdot\text{OH} + \text{OH}^-
\end{align*}
\]

In the second step of the reaction, soluble Fe(II) reacts with hydrogen peroxide to form insoluble Fe(III) and a hydroxyl radical. Hydroxyl radicals resulting from this reaction can attack most major classes of biological molecules including proteins, DNA and lipids. Such damage often leads to cell necrosis or cancer (Taketani, 2005). Yet, despite its potential to cause harm, iron is necessary for cell proliferation, DNA synthesis, respiration, and protein synthesis. Out of all organisms, the only known exceptions to this are *Borrelia burgdorferi*, the causative agent of Lyme disease, and several microbes from the *Lactobacillae* family (Posey and Gherardini, 2000; Archibald, 1983). Iron’s critical biological function revolves around its versatile reduction capabilities. The redox potentials for ferrous and ferric iron vary greatly depending on the molecule to which
they are bound, making iron a critical component of many redox enzymes (Wandersman and Delepelaire, 2004). All organisms, consequently, must strictly control iron uptake to ensure that sub-toxic yet sufficient intracellular levels of iron are maintained. Host tissues restrict the availability of iron in the body through iron-binding compounds such as transferrin, lactoferrin, ferritin and hemin. As a result, successful bacterial pathogens must possess a high-affinity iron uptake system capable of out-competing the host system for iron (Wandersman and Delepelaire, 2004).

Hence, bacterial iron acquisition is an active area of microbial research. From previous work, it has been concluded that a majority of bacteria produce siderophores: compounds which, once secreted by the cell, chelate extracellular iron and are reabsorbed by the bacteria (Escolar et al., 1999). Other bacteria, such as *A. actinomycetemcomitans*, require physical interactions between iron-containing complexes and specific receptors located in the bacterial membrane. Currently, less is known about the alternative methods of inorganic iron acquisition used by organisms lacking a functional siderophore system. To date, our work with *A. actinomycetemcomitans* has led to an analysis of several gene systems with putative roles in iron acquisition, namely: *tolQRA, exbBDtonB*, and *hmsF*. By examining the functionality of the products of these genes we hope to acquire a better understanding of iron acquisition mechanisms in siderophore-independent pathogens.

Our attempt to elucidate the mechanism behind hemin utilization and transport in *A. actinomycetemcomitans* begins in the outer membrane. Transport of large, polar substances across the cytoplasmic membrane (CM) and outer membrane (OM) poses a
significant challenge to bacterial cells. Work by others (Wandersman et al., 2004; Wessling-Resnick, 1999) has implicated the ExbBDtonB system in energy-dependant transportation of siderophores and hemin across the outer membrane. The Tol and Exb systems represent energy-transducing systems by which the cell can drive high-affinity transport systems to accumulate substances present in the extracellular milieu.

While *A. actinomycetemcomitans* has no recognizable siderophore system, we decided to approach our investigation beginning with the ExbBDtonB system and the homologous TolQRA system. Previous work has demonstrated that the most likely source of biologically relevant iron for *A. actinomycetemcomitans* is hemin (Rhodes et al., 2007). Hemin is a complex porphyrin ring containing an atom of iron at its core and it is similar in structure to many siderophores, containing large planar regions and multiple polar side-chains (Fig 1). Thus, it was reasoned that the same energy-transducing systems involved in energizing siderophore transport in other organisms could also be implicated in driving hemin transport in *A. actinomycetemcomitans*.

TolQ and TolR are homologous to ExbB and ExbD, respectively (Fig 2). Similarly, TolA is homologous to TonB (Postle and Kadner, 2003). Both systems are present in the cytoplasmic membrane and operate via proton motive force (PMF) to activate TolA/TonB to an energized state from which solute transport can be driven through energy-dependent outer membrane transporters (Cascales et al., 2001). PMF is commonly utilized by bacteria to drive non-spontaneous reactions such as ATP synthesis, reverse electron transport, flagellar motors and, in this instance, solute transport (White,
Interestingly then, Tol and Exb transport proteins share structural homologies to another group of PMF-dependent proteins, MotA and MotB flagellar motors.

Collectively, the Tol and Exb systems have previously been implicated in the transport of siderophores, colicins and vitamin B12 in bacteria (Postle and Kadner, 2003). Previous work has also suggested that *tolQR* and *exbBD* are capable of cross-complementation *in vivo*. (Braun and Herrmann, 1993). Furthermore, it has been suggested that TonB can, at extremely low levels, form an energized conformation independently of ExbBD/TolQR function, however the implications of this assertion are not fully understood (Postle and Kadner, 2003).

An additional system of interest is the Hms system. This system consists of several components including HmsH, -F, -R, -S, and -T. HmsH and HmsF are predicted OM proteins while the remainder have shown an inner membrane function (Perry *et al.*, 2003). In *Yersinia pestis*, the Hms system has a described role in both hemin binding to the surface of the bacteria as well as in biofilm formation necessary for effective spread of the bacteria via its vector, the flea. Of particular interest is HmsF, which contains both a polysaccharide deacetylase and a demonstrated affinity for hemin and Congo red (Forman *et al.*, 2006). The Hms system of *A. actinomycetemcomitans* is thus of interest in regards to the mechanism and function of hemin binding in *A. actinomycetemcomitans* and the potential effects of this binding on biofilm formation during infection.

The ultimate goal of this work is multi-faceted. First, we seek to determine by what mechanism(s) *A. actinomycetemcomitans* can utilize hemin and how this affects the pathogenesis of the organism. Furthermore, we seek to find out if hemin utilization is
dependent on an energy transducing system and if so, what these systems are. In the long term, we hope to be able to use these answers to determine the role of hemin utilization on the virulence of this organism. This is done in the hopes of elucidating new methods for the development of antimicrobial agents which can be used in the treatment of infectious human diseases such as periodontitis.
Materials and Methods:

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. The strain DF2200N, a clinical isolate that preserved the aggregative and rough phenotypes observed after its initial isolation from a LAP patient (Fine et al., 1999), was used for further analysis because of its amenability to genetic manipulations, such as site-directed mutagenesis through allelic exchange mediated by homologous recombination (Rhodes et al., 2005). *A. actinomycetemcomitans* strains were cultured in *A. actinomycetemcomitans* growth medium (AAGM) broth or agar (Goncharoff et al., 1993) in 5% CO$_2$ at 37ºC. *Escherichia coli* strains were grown in Luria-Bertani broth or agar (Sambrook and Russell, 2001) at 37ºC. The iron content of AAGM medium was 1-3 µM as determined with a Variant Liberty 150 Inductive Coupled Plasma (ICP) atomic emission spectrometer, using standard conditions and a set of appropriate standards.

Cloning and analysis of the *tolQRA* system. A 2,988-basepair fragment containing the three open reading frames (ORFs) of *tolQRA* was PCR amplified from *A. actinomycetemcomitans* strain DF2200N using TAQ polymerase and the primers tolQR.entire.5’ (5’-TACAAGTGGAAACTGCTG,3’) and tolB.3’ (5’-CCTGTTCATGAGGAAGG-3’). The cloned fragment was ligated into pCR2.1 (Invitrogen) (pMU612) and sequenced on an ABI 310 Genetic Analyzer (Applied Biosystems) for confirmation.
**E. coli strain RA1051 complementation.** Cultures of *E. coli* strains W3110, RA1051, RA1051(pMU612) and RA1051(pMU634) were inoculated 1:100 into LB broth supplemented with increasing levels of 2,2’-dipyridyl, a synthetic iron chelator. Cultures were shaken at 37°C and measured after 18 h at OD<sub>600</sub> on a Beckman DU640 spectrophotometer. LB agar plates with varying concentrations of dipyridyl were inoculated with the strains listed above and observed for growth after 18 h at 37°C. Plate growth was inspected visually and recorded using a BioDoc-It gel documentation system (Bioscience Technology).

**Generation of tolQ, -R, -A isogenic derivatives.** An EcoRI digest was performed on pMU612, excising the *tolQRA* fragment from the plasmid and cleaving *tolQRA* in half. The product was separated using gel electrophoresis and excised from the gel using a QIAquick PCR purification kit (Qiagen). The two fragments, designated as *tolQR* and *tolA*, were ligated into pMB78, a pUC19 derivative containing a specific uptake signal sequence (USS). Random, *in vitro*, mutagenesis was performed on the pMU613 and pMU614 constructs using a Tn5<DHFR-1> transposon (Epicentre). The construct was then transformed into *E. coli* DH5α and screened for resistance to trimethoprim. Colony PCR of resistant colonies was utilized as a primary selection for desired insertions. Each insertion was later confirmed via DNA sequencing. Mutations in MU-3, MU-7 and MB1237, a competent form of DF2200N containing a plasmid expressing inducible competence factors, were obtained through homologous recombination between chromosomai DNA and the transformed suicide plasmid containing the desired insertion
mutation. MB1237 mutants were selected for using trimethoprim and confirmed via PCR and DNA sequencing. All *A. actinomycetemcomitans* mutant designations are listed in Table 2.

**Hemin complementation in exbBDtonB and tolQRA derivatives.** MB1237 derivatives containing either single or double insertions in the desired ORFs were streaked onto AAGM plates containing 370 µM dipyridyl. Filter paper disks were aseptically infused with 20µl hemin (10 mg/ml) and placed in the middle of the streaked quadrant. Plates were incubated in 5% CO₂ for 48 hours and photographed at 4x magnification using an Olympus SZX12 light microscope.

**Formation of an hmsF isogenic derivative.** The chromosomal region encompassing *hmsF* was PCR amplified using *Pfu* polymerase (Stratagene) with a forward (5'-TCTCAGCTGAAGCAAGTG-3') and reverse (5'-TATGCGCTGATTCTGACC-3') primer designed from the published sequence of *A. actinomycetemcomitans* strain HK1651 available online (http://www.oralgen.lanl.gov/). The DF2200N derived amplicon was cloned into pCR-BluntII-TOPO (Invitrogen) and the resultant construct was assigned as pMU606. The insert was confirmed via sequencing and subcloned into pMB78, resulting in pMU608. An EZ-Tn5<sup>™</sup><R6KγoriKan-2> (Epicentre) transposon kit was used to generate random insertions in pMU608. A desired *hmsF* insert (pMU620) was identified with colony PCR and mapped by DNA sequencing with primers provided in the EZ-Tn5<sup>™</sup><R6KγoriKan-2> kit. *A. actinomycetemcomitans*
MB1237 was transformed with the interrupted construct and allelic exchange between the assimilated plasmid and chromosomal DNA yielded isogenic insertion derivatives. Loss of the protein product associated with \( hmsF \) was confirmed by size-fractionating total cell lysates by SDS-PAGE on 12.5% polyacrylamide gels (Scopes, 1994).

**Congo red binding assay.** Desired *A. actinomycetemcomitans* strains (DF2200N, CU1000, CU1060, MB1237 and MU-14) were collected from an AAGM plate and resuspended in 0.5 ml of AAGM broth. Clumps were aseptically sheered using plastic pestles and a 1:100 inoculation was performed into glass tubes containing 3 ml AAGM broth and 30 µg/ml Congo red. Tubes were incubated in a 5% CO\(_2\) incubator at 37ºC. Cells were then collected via centrifugation and washed twice with Tris-buffered saline (TBS). Three 1 ml elutions of Congo red were performed using dimethyl sulfoxide (DMSO). The eluted DMSO was measured for Congo red at 488 nm in a Beckman DU640 spectrophotometer. A Bradford protein assay was used to standardize the recorded OD\(_{488}\) values to total protein levels (Bradford, 1976). This experiment was performed five times and the results were tabulated using Microsoft Excel.

**Electron microscopy of insertion derivatives.** For scanning electron microscopy (SEM) analysis, 5 ml of non-supplemented AAGM broth or broth containing 100 µM FeCl\(_3\), 50 µg/ml hemin or 100 µM dipyridyl were poured into 50 ml plastic conical tubes and inoculated with 0.05 ml of 2-day old cultures of the *A. actinomycetemcomitans* strains. A sterile plastic cover slip was added to each tube immediately after inoculation.
and incubated for 48 h at 37°C in 5% CO₂ without shaking. The cover slips were then removed and immediately submerged in a solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate and incubated at room temperature for 2 h. The fixative was removed and rinsed immediately with distilled water and then samples were dehydrated with increasing concentrations of ethanol, ranging from 25 to 100%. The dehydrated samples were then CO₂ critical point dried, gold-coated and visualized with a JEOL JSM-840A scanning electron microscope. These assays were done twice using fresh biological samples each time.

For transmission electron microscopy (TEM), cells were grown on AAGM agar plates. Cells were lifted from plates with a sterile loop and placed into 100 μl dH₂O in a 1.5 ml microcentrifuge tube and clumps were broken up by gently pipetting up and down. 10 μl of suspended cells were placed onto 300-mesh copper formvar-coated grids and allowed to dry for 10 to 15 minutes. Grids and cells were then stained with 0.1% ammonium molybdate for approximately 5 minutes. Excess stain was wicked away using a folded filter disk and the disks were set at room temperature for 5 minutes to allow the stain to dry. Grids were imaged at an accelerating voltage of 100 KeV on a Zeiss Supra TEM.

**Peroxide susceptibility assay.** DF2200N, CU1060 and MU-14 cells were streaked onto AAGM plates containing 50 μg/ml hemin. Filter-paper disks were placed on the streaked plates after being aseptically infused with 10μL of 5% hydrogen peroxide, t-butyl hydroxide or cumene hydroxide. Half of the streaked plates were immediately inoculated
with peroxide-infused disks while the other half were incubated for 8 hours before being inoculated. Plates were incubated at 37°C in 5% CO₂ for 48 hours following inoculation with peroxides and observed for zones of cell growth inhibition. This experiment was performed twice using fresh biological samples each time.
Results and Discussion:

*E. coli RA1051 complementation with exbBDtonB and tolQRA.*

Several putative energy transducing systems have been annotated in the genome of *A. actinomycetemcomitans*. In order to determine the functionality of these systems, we set up a complementation model in an iron transport-deficient *E. coli* derivative, RA1051. This strain is a derivative of *E. coli* W3110 and lacks *tolQ, exbB* and *exbD*, which are required for iron acquisition via the enterobactin system (Braun and Herrmann, 1993). This assay was used to confirm that the annotated Exb and Tol systems in *A. actinomycetemcomitans* are, in deed, present and functional. Parental *E. coli* strain W3110, a positive control containing the empty control vector pBR322, demonstrated only slight growth deficiencies when grown in LB broth containing iron-chelating dipyridyl concentrations ranging from 0 – 200 µM (Fig 3). In non-complemented RA1051, the negative control, a 90% reduction in growth was seen when dipyridyl concentrations in the media were increased from 0 uM to 200 uM. In contrast, RA1051 complemented with *tolQRA* showed growth similar to W3110 despite increasing dipyridyl concentrations. Similarly, *exbBD*-complemented RA1051 showed a mild restoration in growth. These results show that both energy-transducing systems, *tolQRA* and *exbBD*, present in *A. actinomycetemcomitans* are capable of rescuing growth and thus contain at least some functional activity. This confirms that these systems may have a role in transport functions in *A. actinomycetemcomitans*.

These complementation activities were further confirmed on agar plates. On plates, the rescue of RA1051 growth via complementation was more easily observed,
despite being less quantitative than growth in broth. On 0 µM dipyridyl plates, all four strains grew strongly, with parental strain W3110 and the tolQR-complemented strain growing more strongly than either the exbBD-complemented mutant or the RA1051 alone (Fig 4). At 100 µM dipyridyl, growth of non-complemented RA1051 was barely visible on the plate, while the other three strains grew without significant impairment. At 200 µM dipyridyl, W3110 and the tolQR-complemented mutant appeared unaffected, while the exbBD-complemented mutant appeared severely hampered. The RA1051 control presented no visible growth.

The incomplete restoration of growth in the exbBD-complemented mutant could be due to a weak interaction between the \textit{A. actinomycetemcomitans}-derived ExbBD and the endogenous \textit{E. coli} TonB. A complete complementation encoding for \textit{A. actinomycetemcomitans} ExbBDTonB was attempted, however was unsuccessful and abandoned in favor of the successful cloning of an alternative fragment encoding solely for ExbBD. Also of note, as mentioned previously, is the fact that RA1051 is deficient in both components (ExbB and ExbD) of the Exb system while only lacking one component (TolQ) of the Tol system. The double deficiency of the endogenous Exb system could result in the increased inefficiency seen when complementing the RA1051 mutation with \textit{A. actinomycetemcomitans}-derived Exb components. Regardless, both the Tol and Exb systems from \textit{A. actinomycetemcomitans} appear to have some, if not a strong, role in rescuing iron acquisition under iron-limiting conditions.
**exbBDtonB and tolQRA mutations in *A. actinomycetemcomitans.***

Having demonstrated the functionality of the *exbBDtonB* and *tolQRA* systems in *A. actinomycetemcomitans*, we sought to systematically disrupt each ORF in these two systems. As a result, a series of insertion mutations were made, as described earlier in this work, in the *exbBDtonB* system and in its homologous *tolQRA* system. PCR fragments of the mutated ORFs were larger than wildtype ORFs, corresponding to the ~1 kbp and ~2 kbp size of the EZ::Tn5<DHFR-1> and EZ::Tn5<KAN-2> inserts, respectively (Fig 5).

As mentioned above, previous work suggests the ability of *tolQRA* and *exbBDtonB* to cross-complement each other (Braun and Herrmann, 1993). To guard against this, double mutants, containing insertions in both systems were created to ensure against the possibility of a cross-complementation occurring. If cross-complementation were occurring, single mutations would have displayed no changes in phenotype as the deficiency would have been rescued by a functional alternative system.

Once created, each double mutant was tested for its ability to utilize hemin as a sole iron source. Results show that all mutants were capable of growth around the hemin-infused disk (Fig 6). Little to no variation was observed in the ability of any mutation to utilize hemin. These results, taken together with the RA1051 complementation, suggest that the Exb and Tol systems are required for *A. actinomycetemcomitans* transport functions, but are not essential in hemin utilization. Thus, these results for Exb and Tol in hemin acquisition suggests that additional hemin acquisition processes must be present that do not depend on energy transducing systems.
Alternatively, it is also possible that a novel energy-transducing system is present in *A. actinomycetemcomitans* which remains to be characterized.

**Future directions with the exbBD*ton*B and tolQRA system.**

Upon a review of the literature it was found that dipeptide transporters in *E. coli* have recently been proposed as an alternative hemin acquisition factor. An ATP-dependant dipeptide permease was discovered in *E. coli* to have the ability to transport hemin across the inner membrane (Letoffé *et al.*, 2006). The exact mechanisms of this interaction are unknown; however they open the door for an entirely new class of hemin transport systems. Upon analysis of the *A. actinomycetemcomitans* genome, a putative dipeptide transporter was found displaying homology to the hemin-related Dpp system in *E. coli*. This finding coincides with our work which demonstrates that the hemin transport functions of *A. actinomycetemcomitans* are independent of energy transducing systems. Further work should seek to analyze the role of this novel system in *A. actinomycetemcomitans* and the extent of its interaction with the ExbBD*ton*B and TolQRA systems during hemin binding and utilization.

**Hemin binding and hmsF.**

During preliminary experiments, it was discovered that *A. actinomycetemcomitans* is capable of binding hemin when grown on AAGM supplemented with this iron source. The obvious question we sought to address was: could this hemin binding function be related to its eventual uptake and utilization? Previous work with *Y. pestis* by Dr. Robert Perry at the University of Kentucky focused
on a cluster of genes known to encode the Hms proteins (Forman et al., 2006; Perry et al., 2003). These genes were found to play a role in iron utilization and biofilm formation in *Yersinia* and, most importantly, were found annotated in the *A. actinomycetemcomitans* genome. The ORFs encoding for HmsH and –F are co-transcribed in *A. actinomycetemcomitans* and are upstream of two hypothetical proteins, AA00492 and AA00493, homologous to HmsR and HmsS (data not shown). The *hmsF* mutant, designated as MU-14, was visually deficient at binding hemin and Congo red when grown on agar plates containing 50 µg/ml and 30 µg/ml of such compounds, respectively (Fig 7a and b). Congo red is a common stain which mimics hemin in its binding, but is superior in its ability to be eluted from cells with DMSO. A protein gel of total cell lysates from wildtype DF2200N and the mutant MU-14 show a visually distinct 71-kDa band in DF2200N representing the protein product of *hmsF*, which is lacking in the MU-14 insertion mutant (Fig 7c).

In an attempt to quantify the extent to which Congo red binding was impaired in the MU-14 derivative, a Congo red binding assay was developed. Congo red is a stain similar in structure and specificity to hemin with the unique property that it can be eluted off of the surface of cells (Daskaleros and Payne, 1987). Eluted Congo red levels were read with a spectrophotometer and standardized to total protein to take into account differential growth rates between strains. The MU-14 and smooth derivative CU1060 bound significantly less Congo red than did rough isolates DF2200N, CU1000 or MB1237 (Fig 8).
These initial results suggested that differences in Congo red binding could correlate with the presence of fimbriae. The rough strain controls (DF2200N, MB1237, and CU1000) all bound Congo red significantly while the smooth strain control (CU1060) did not. This led to the concern that the MU-14 mutation had simply created a smooth strain and had not specifically targeted a unique hemin-binding gene. To ensure that the phenotype remained rough, SEM of all samples was conducted to visualize gross changes in the morphology of colonies formed on plastic coverslips. MU-14 retained its ability to form large aggregates on plastic coverslips similar to DF2200N and CU1000. By contrast, smooth strain CU1060 was radically more dispersed than rough strains or MU-14 (Fig 9). Thus MU-14 retains its ability to form an aggregative biofilm typical of the rough phenotype. Further confirmation of maintained structural similarities between MU-14 and rough parental strains was sought using TEM. TEM was conducted to confirm that fimbriae were still present on MU-14 in order to assure that it was the loss of HmsF, not the loss of fimbriae, which was responsible for the drastic decrease in Congo red binding. Upon development of the TEM images, extensive fimbriae, similar to those on DF2200N or CU1000, were identified on MU-14 (Fig 10). Collectively, these microscopy results support our claim that hmsF encodes a uniquely hemin/Congo red binding protein which is not involved in the rough/smooth phenotype differences of A. actinomycetemcomitans.

Hemin complementation experiments were subsequently conducted to examine the effect of disrupted hemin binding on hemin utilization in A. actinomycetemcomitans. Despite the strong evidence (presented above) that hmsF is involved in hemin binding to
the outer surface of *A. actinomycetemcomitans*, these hemin complementation experiments demonstrated that MU-14 can still grow when hemin is provided as the only iron source (Fig 11). Thus one must ask what function hemin binding is fulfilling for the cell if such binding is not necessary for uptake and utilization.

**Hemin binding and peroxide susceptibility.**

One possibility was recently suggested by Dr. H. Fletcher at Loma Linda University (personal communication). Researchers there describe a role for hemin in bacterial pathogenesis and immune system evasion. Certain bacteria are capable of utilizing the reductive potential of hemin to combat oxidative damage induced by a host organism’s immune response during infection. This evidence suggests that hemin binding to the outer surface of bacteria increases, through unknown mechanisms, the organism’s resistance to a variety of reactive oxygen intermediates (ROIs). To test this hypothesis with *A. actinomycetemcomitans*, a peroxide susceptibility assay was adapted (Fig 12). Organic peroxides, such as cumene hydroperoxide and *t*-butyl hydroperoxide, were used in addition to hydrogen peroxide due to their biological relevance in a host immune response. However, none of the peroxide compounds tested displayed a large difference in toxicity between DF2200N and the MU-14 derivative. Incubation of DF2200N and MU-14 for 8 h before inoculating with peroxide disks did not result in any noticeable differences when compared to the samples that were immediately inoculated with peroxides after streaking (data not shown). Thus, hemin binding to the outer surface of *A. actinomycetemcomitans* does not appear to enhance the ability of this organism to survive challenge by ROIs. While the exact purpose of hemin binding to *A.*
*actinomycetemcomitans* has yet to be elucidated *in vitro*, the possibility exists that the significance of hemin binding will only be manifested in an *in vivo* infection model. It follows that such a model is the logical next step towards elucidating the function of *hmsF* in *A. actinomycetemcomitans*.

Conclusions and future directions with the *hms* system.

The quest to fully elucidate the mechanisms by which *A. actinomycetemcomitans* acquires hemin continues. An animal model for *A. actinomycetemcomitans* infection was developed by Schreiner *et al.* (2003) using Sprague-Dawley rats. Such a model will be ideal for testing the pathogenic capabilities of the various mutants created in this study. Although none of the mutations made in this work were capable of affecting hemin utilization, the potential effects of these mutations on the virulence of *A. actinomycetemcomitans* are large. Of particular interest is MU-14 and the effect that disrupted hemin binding will have on *A. actinomycetemcomitans*’ infection strategies and ability to evade the host immune response. Additional mutations in the remaining ORFs of the Hms system are currently being generated. The effect of these mutations on hemin utilization and/or biofilm formation in *A. actinomycetemcomitans* has yet to be speculated and, given the broad effects of these mutations in *Y. pestis*, could prove critical to our understanding of pathogenesis in *A. actinomycetemcomitans*. 

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Table 1. Bacterial strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>A. actinomycetemcomitans</em></td>
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<tr>
<td>DF2200N</td>
<td>Clinical isolate, rough phenotype</td>
<td>D. Fine</td>
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<tr>
<td>MB1237</td>
<td>Artificially inducible competent derivative of DF2200N; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>D. Figurski</td>
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<tr>
<td>CU1000</td>
<td>Clinical isolate, rough phenotype</td>
<td>D. Figurski</td>
</tr>
<tr>
<td>CU1060</td>
<td>Smooth derivative of CU1000</td>
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<td><strong>E. coli</strong></td>
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<td>DH5α</td>
<td>Used for recombinant DNA methods</td>
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<td>W3110</td>
<td>parental strain for RA1051</td>
<td>K. Postle</td>
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<td>RA1051</td>
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<td>Bolivar <em>al.</em></td>
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<td>pMB78</td>
<td>USS-containing suicide vector used for allelic exchange; Amp&lt;sup&gt;+&lt;/sup&gt;; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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<sup>a</sup>Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Tet, tetracycline; <sup>+</sup>, resistant

Table 2. Designations for mutagenized *Actinobacillus actinomycetemcomitans* strains.

<table>
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<tr>
<th>Strain</th>
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<th>Source or reference</th>
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<td><strong>MU-3</strong></td>
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<td><strong>MU-6</strong></td>
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<td><strong>MU-7</strong></td>
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<td>MB1237 with hms&lt;sup&gt;F&lt;/sup&gt;::EZ::TN&lt;sup&gt;KAN-2&lt;/sup&gt;; Cm&lt;sup&gt;-&lt;/sup&gt; Kan&lt;sup&gt;-&lt;/sup&gt;</td>
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<td><strong>MU-15</strong></td>
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<td><strong>MU-18</strong></td>
<td>MU-3 with tolA&lt;sup&gt;R&lt;/sup&gt;::EZ::TN&lt;sup&gt;DHFR-1&lt;/sup&gt;; Cm&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;-&lt;/sup&gt; Trim&lt;sup&gt;-&lt;/sup&gt;</td>
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<td><strong>MU-19</strong></td>
<td>MU-7 with tolA&lt;sup&gt;R&lt;/sup&gt;::EZ::TN&lt;sup&gt;DHFR-1&lt;/sup&gt;; Cm&lt;sup&gt;-&lt;/sup&gt; Kan&lt;sup&gt;-&lt;/sup&gt; Trim&lt;sup&gt;-&lt;/sup&gt;</td>
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<td><strong>MU-20</strong></td>
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<sup>a</sup>Trim, trimethoprim; Cm, chloramphenicol; Kan, kanamycin; <sup>+</sup>, resistant; <sup>-</sup>, sensitive.
Fig 1.

Hemin

Fig 2.

Tol–Pal  Exb–TonB

Outer Membrane

Pal

FepA or FhuA or FhuB

Peptidoglycan

Inner Membrane

TolQ  TolR  TolB  ExbD

TolA  TolN  TolC

ExbB
Fig. 3.
Fig. 4.
Fig 5.

(a) TolQ and ExbB

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(b) TolA and TonB

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Fig 6.
Fig 7.
Fig 8.
Fig 10.
Fig 11.

Fig 12.

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Hydrogen Peroxide  | 2-Butyl Peroxide  | Cumene Peroxide
Fig 1. **Structure of hemin and related siderophores.** The structure of hemin and several siderophores are shown for comparison. Similarities exist both in the large, planar shape of the structure and the polar side groups. Adapted from Fischbach *et al.*, 2006.

Fig 2. **A visual representation of the homology between ExbBDTonB and TolQRA**

Both are energy-transducing systems which rely on a proton motive force (PMF) to drive outer membrane transport of siderophores, colicins and vitamin B12. Similar homology can be seen between Tol/Exb and the Mot proteins which drive flagellar motion in certain bacteria. Adapted from Cascales *et al.*, 2001.

Fig 3. **E. coli RA1051 complementation in LB broth containing increasing levels of dipyridyl.** RA1051 was complemented with pMU612 and pMU634 fragments from *A. actinomycetemcomitans* and incubated in LB containing increasing concentrations of dipyridyl (DIP). Cell growth was measured at OD$_{600}$ after 18 h of incubation.


Fig 4. **E. coli RA1051 complementation on LB agar plates containing increasing levels of dipyridyl.** The same strains as seen in Figure 3 were plated on LB agar plates containing varying levels of dipyridyl (A, 0 µM; B, 100 µM; C, 200 µM) and incubated for 18 h at 37°C.
Fig 5. PCR confirmation of tolQRA and exbBDtonB single and double insertion mutations. FailSafe polymerase (Epicenter) was used to confirm insertion of the EZ::Tn5<KAN-2> transposon (~2 kbp) into the appropriate exb ORFs and the EZ::Tn5<DHFR-1> transposon (~1 kbp) into the appropriate tol system ORFs. DNA from A. actinomycetemcomitans strain DF2200N (DF) was used as a control. Lambda phage DNA digested with HindIII was used as a marker.

Fig 6. Hemin complementation of A. actinomycetemcomitans mutants on 100 μM dipyridyl plates. DF2200N, MB1237, MU-3, MU-6, MU-7, MU-15, MU-16, MU-20, MU-17, MU-18 and MU-19 were plated on AAGM agar plates containing 100 μM dipyridyl. A sterile filter-paper disk was aseptically infused with 20 μl hemin (10 mg/ml) and growth around the disk was documented. All pictures were taken at 4x magnification.

Fig 7. Comparative analysis of the DF2200N parental strain and the hmsF isogenic insertion derivative. DF2200N (1) and MU-14 (2) cells were streaked on AAGM agar containing either hemin (a) or Congo red (b). (c) SDS-PAGE of outer membrane proteins isolated from DF2200N (1) and MU-14 (2) cells. The arrow identifies a 71-kDa protein that is absent in the hmsF insertion mutant cells.

Fig 8. Congo red binding assays. MU-14, CU1060, MB1237, DF2200N, and CU1000 cells were grown for 24 h at 37°C in AAGM broth containing 30 μg/ml of Congo red.
Results were recorded as a ratio of Congo red eluted per µg of total protein (as determined via Bradford). All values are reported as percent relative to DF2200N values. N = 5; Error bars = 1 Standard deviation.

**Fig 9.** SEM analysis of the rough strains CU1000 (1), DF2200N (2), MB1237 (3), MU-14 (4) and smooth strain CU1060 (5) grown in AAGM broth on plastic slides. Slides were processed after 48 h incubation for SEM using ethanol dehydration, critical point drying and gold coating. All pictures were taken at 5000x magnification.

**Fig 10.** TEM analysis of MU-14 and control strains for fimbriae formation. Rough strains CU1000 (1) and DF2200N (2), smooth strain CU1060 (3), and mutant strain MU-14 (4) were visualized under TEM. Samples were lifted from AAGM agar plates onto formvar-coated grids and processed with 0.1% ammonium molybdate. Samples were visualized at 100 KeV on a Zeiss Supra TEM. Arrows indicate representative fimbriae.

**Fig 11.** Hemin complementation of MU-14 grown on AAGM agar containing inhibitory levels of dipyridyl. AAGM agar plates containing 370 µM dipyridyl were streaked with MU-14 using a sterile cotton swab. Filter paper disks were aseptically infused with 20 µL hemin (10 mg/ml) and placed appropriately. Plates were then CO₂ incubated for 2 days at 37°C and observed for growth. All pictures were taken at 4x magnification.
Fig 12. Peroxide sensitivity testing on an *hmsF* isogenic derivative using hydrogen, *t*-butyl and cumene peroxides. DF2200N and MU-14 cells were plated on AAGM agar with 50 µg/ml hemin and incubated for 48 h with filter paper disks infused with 10µl of 5% hydrogen, *t*-butyl and cumene peroxide. All pictures were taken at 4x magnification.
References


