The Role of NfuA Protein in \textit{Acinetobacter baumannii} Iron Metabolism

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ABSTRACT

THE ROLE OF NfuA PROTEIN IN ACINETOBACTER BAUMANNII IRON METABOLISM

by Thomas M. Park

*Acinetobacter baumannii* is a gram-negative bacterium that causes severe infections in immunocompromised patients, such as newborns, burn patients, and the elderly. Because the bacteria are strongly resistant to antibiotics, there is a dire need to develop new therapeutics to treat *A. baumannii* infections. Potential targets are proteins involved in bacterial iron metabolism, since iron is an essential micro-nutrient. Accordingly, random insertion mutagenesis analysis showed that the NfuA protein is needed when cells were cultured in the presence of 2,2'-dipyridyl, a synthetic iron chelator that generates iron-limiting conditions, and hydrogen peroxide and cumene hydroperoxide, which were used to mimic oxidative conditions. The role of NfuA was further confirmed by the observation that the genetic complementation of an *A. baumannii* ATCC 19606 T mutant with the parental allele was enough to restore the iron metabolism and oxidative stress phenotypes expressed by the wild-type strain. Electron paramagnetic resonance (EPR) analysis of overexpressed and purified NfuA demonstrated that this protein harbors an iron-sulfur cluster, which is a prosthetic group required in central metabolic processes. Interestingly, the inactivation of NfuA did not affect bacterial growth under non-oxidative and non-chelated conditions and did not impair the ability of the mutant to express the acinetobactin siderophore-mediated iron acquisition system. On the other hand, the ability of the *A. baumannii* ATCC 19606 T *nfuA* mutant to replicate inside human epithelial cells was significantly impaired when compared with the parental strain. Taken together, these observations suggest that NfuA plays a defined and important role in iron metabolism, resistance to oxidation, and intracellular replication without affecting bacterial iron acquisition processes. By understanding the function of NfuA and its importance to the *A. baumannii* virulence properties, we will come closer to understanding basic metabolic processes that could be alternative targets for new antibiotics designed to treat the infections this pathogen causes in humans.
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**Introduction**

*Acinetobacter baumannii* has been gaining attention as a notoriously difficult bacterium to treat, especially in hospital settings. *A. baumannii* was first described as a species in 1986 by Bouvet and Grimont (1986). The bacteria are gram-negative, aerobic, short, plump rods (Peleg et al., 2008). Though the bacteria are commonly found in the hospital environment, the natural environment for *A. baumannii* has not been fully established (Dijkshoorn et al., 2007; Peleg et al., 2008). *A. baumannii* usually infects patients with compromised immune systems, oftentimes with breaches in the skin and airway, and has been isolated from patients in burn units, intensive care units (ICUs), and from wounded soldiers (Peleg et al., 2008). It has been shown to cause pneumonia, bloodstream infections, skin/soft tissue infections, urinary tract infections, and meningitis among other manifestations (Peleg et al., 2008). Also, cases of necrotizing fasciitis have been reported where *A. baumannii* was isolated as the sole pathogen (Charnot-Katsikas et al., 2009).

*A. baumannii* has emerged as a clinically relevant bacterium mainly because of its ability to acquire resistance to antibiotics. Furthermore, it can survive for unusually long periods of time in the hospital environment (Peleg et al., 2008). It also resilient against desiccation and disinfectants and can be found on bed curtains, furniture, and equipment in hospitals (Dijkshoorn et al., 2007). Alarmingly, in hospitals, multidrug-resistant (MDR) and pandrug-resistant strains of *A. baumannii* have been isolated (Dijkshoorn et al., 2007). In ICUs across the United States, the resistance of *Acinetobacter* to nine antibiotics (ampicillin-sulbactam, ceftriaxone, cefepime, piperacillin, piperacillin-
tazobactam, imipenem, tobramycin, amikacin, and ciprofloxacin) increased significantly from a 12-year period from 1993-2004 (Lockhart et al., 2007). During this same period, 6.2% of all the organisms isolated were *Acinetobacter* (Lockhart et al., 2007). One study reported that extensively drug-resistant (XDR) *A. baumannii* infections of transplant recipients led to a 91% mortality rate (Shields et al., 2011).

A number of antibiotic resistance mechanisms exist for *A. baumannii*. The bacteria can modify antibiotic targets, inactivate antibiotics with enzymes such as β-lactamases, and encode efflux pumps to remove antibiotics from the cells (Dijkshoorn et al., 2007). One study shows that *A. baumannii* can regulate its antibiotic resistance by detecting sodium chloride in the environment (Hood et al., 2010). As more antibiotic-resistant strains of *A. baumannii* are increasingly being reported, it becomes more pressing to find new targets to treat infections caused by this species of bacteria.

One of the vulnerabilities of the bacteria is their dependence on iron. Nearly all bacteria require iron to survive. Iron plays an important role in cellular processes such as the oxidation-reduction reactions involved in metabolism and nucleotide synthesis (Neilands, 1991). Exploiting this vulnerability, the human body is an iron-limiting environment since compounds such as transferrin and lactoferrin sequester iron which creates conditions that prevent many species of bacteria from growing (Otto et al., 1992). However, when exposed to both natural and synthetic iron-chelating compounds, *A. baumannii* is still able to grow (Echenique et al., 1992). Many bacteria, including *A. baumannii*, have developed mechanisms to acquire iron in the human host by producing siderophores (Otto et al., 1992). Siderophores have an extremely high affinity for ferric
iron and thus can outcompete other organic complexes for iron (Wandersman & Delepelaire, 2004). Once bound to iron, the siderophores are then returned via specific outer membrane receptors of the bacteria (Wandersman & Delepelaire, 2004). There are three distinct siderophore biosynthesis gene clusters that have been identified within *A. baumannii* (Eijkelkamp *et al.*, 2011). *A. baumannii* ATCC 19606\(^T\) produces a siderophore called acinetobactin which has catecholate and hydroxamate functional groups (Yamamoto *et al.*, 1994). Furthermore, in response to an iron-deficient environment, the Fur (ferric uptake regulator) protein of *A. baumannii* modifies the level of transcription of genes, including some that are not directly related to iron acquisition (Eijkelkamp *et al.*, 2011).

Another vulnerability of the bacteria is their susceptibility to oxidative stress. The human host as well as the bacteria themselves produce reactive oxidative species (ROS) such as superoxide anion radicals (\(\text{O}_2^-\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and hydroxyl radicals (\(\cdot\text{OH}\)) (Toyokuni, 1999). These ROS can be by-products of aerobic respiration and metabolism (Soares *et al.*, 2010). ROS can attack biological macromolecules, particularly the cell membrane and cytoskeleton components (Sigler *et al.*, 1999). However, *A. baumannii*, along with other bacterial species that use oxygen as the final electron acceptor, have developed systems that prevent cellular damage from ROS. *A. baumannii* ATCC 19606\(^T\) harbors a plasmid that contains open reading frames that code for proteins that may relate to its observed resistance to organic peroxide-generating compounds (Dorsey *et al.*, 2006). It has also been shown that *A. baumannii* makes adaptive changes that induce oxidative stress responsive proteins when exposed to ROS.
(Soares et al., 2010). These changes may protect the bacteria from oxidative stress as well as from antibiotics (Soares et al., 2010).

Moreover, a relationship exists between the concentration of Fe$^{2+}$ in the cell and the level of ROS. When oxygen is present, iron may autoxidize in the following reaction (Sigler et al., 1999):

\[ 2 \text{Fe}^{2+} + \text{O}_2 + 2 \text{H}^+ \rightarrow 2 \text{Fe}^{3+} + \text{H}_2\text{O}_2 \]

This hydrogen peroxide can react with Fe$^{2+}$ to produce a hydroxyl radical in what is known as the Fenton reaction which can lead to DNA damage (Jang & Imlay, 2010):

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \]

Subsequently, Fe$^{3+}$ can react with a superoxide anion radical to yield Fe$^{2+}$ and oxygen (Sigler et al., 1999). Thus, bacteria control the intracellular concentration of Fe$^{2+}$ to prevent the metal-catalyzed generation of ROS (Sigler et al., 1999). Furthermore, bacteria have specific enzymes such as superoxide dismutase that reduce the levels of ROS (Cabiscol et al., 2000).

Bacteria have Fe-S cluster assembly systems which are important to survive under iron-deficient and oxidative stress conditions. These systems assemble and deliver Fe-S clusters to an apoprotein target (Py & Barras, 2010). Fe-S proteins are proteins that contain iron-sulfur clusters and are ubiquitous in living organisms (Py & Barras, 2010). These Fe-S proteins represent a major iron-containing protein pool within the cell. Fe-S clusters can have multiple redox states which enable them to participate in a number of essential cellular processes including respiration, metabolism, and gene regulation (Py & Barras, 2010). For example, enzymes in the electron transport chain, such as NADH
dehydrogenase and succinate dehydrogenase, contain Fe-S clusters that enable them to transfer electrons. Fe-S clusters also can sense oxidation and iron-related conditions and adaptively modulate gene expression (Py & Barras, 2010).

Three systems for Fe-S cluster assembly are known among a diversity of organisms: Nif, Isc, and Suf (Jin et al., 2008). Isc and Suf are the primary systems in assembling Fe-S clusters, most commonly [2Fe–2S] and [4Fe–4S] (Shepard et al., 2011). Isc is the housekeeping Fe-S cluster assembly system, and Suf is responsible for Fe-S cluster assembly under iron starvation and oxidative stress conditions (Outten et al., 2004; Shepard et al., 2011). The in vivo Fe-S cluster assembly process can be divided into three basic steps: formation of elemental sulfur, sulfur and iron cluster assembly, and cluster insertion into the apoprotein (Xu & Moller, 2008). All assembly pathways involve a cysteine desulfurase and a U-type and/or an A-type scaffold protein capable of assembling both [2Fe–2S] and [4Fe–4S] clusters (Bandyopadhyay et al., 2008). In Erwinia chrysanthemi, it is postulated that under iron-deficient and oxidative stress conditions, the Suf complex is required for a reductase to acquire a Fe-S cluster (Nachin et al., 2003). This reductase allows for the liberation of Fe^{2+} from siderophores and the repair of oxidatively damaged [4Fe-4S] clusters (Nachin et al., 2003). However, under oxidative stress conditions, the Isc system of Escherichia coli is inhibited by hydrogen peroxide (Jang & Imlay, 2010). All the currently available sequenced A. baumannii genomes are predicted to contain only an Isc system for Fe-S cluster biosynthesis.

In this study, we identified an NfuA protein in A. baumannii that might be involved in the maturation of Fe-S proteins. NfuA has been described in other species
such as *E. coli* (Angelini *et al.*, 2008), *Azotobacter vinelandii* (Bandyopadhyay *et al.*, 2008), and *Pseudomonas aeruginosa* (Daung-nkern *et al.*, 2010). The NfuA protein is essential for growth under oxidative stress and iron-limiting conditions in *E. coli* (Angelini *et al.*, 2008) and for aconitase maturation in *A. vinelandii* (Bandyopadhyay *et al.*, 2008). Thus, it has been proposed that NfuA is a Fe-S carrier protein that is involved in the maturation of apoproteins during iron starvation and oxidative stress (Py & Barras, 2010), processes that have not been investigated in *A. baumannii*. In this work we have identified NfuA as a Fe-S cluster biosynthesis protein that plays a role in the ability of this pathogen to grow under iron limitation, resist oxidation, and display virulence properties.
Materials and Methods

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this research project are listed in Table 1. The wild-type strain ATCC 19606<sup>T</sup>, the type strain isolated from urine (Bouvet & Grimont, 1986), was used in this experiment to generate the *nfuA* mutant and to serve as a control in the iron utilization, oxidation resistance, and cell invasion assays. To grow and maintain the bacteria, Luria-Bertani (LB) broth or agar were used (Sambrook & Russell, 2001). M9 minimal medium provided chemically defined conditions for bacteria grown during the iron starvation and oxidative stress experiments (Miller, 1972).

General DNA techniques.

Total DNA was isolated by ultracentrifugation in CsCl density gradients (Meade *et al.*, 1982) or by an adapted mini-scale method (Barcak *et al.*, 1991). Plasmid DNA was isolated using commercial kits (Qiagen). DNA digests were performed with restriction enzymes (New England Biolabs) and separated by size by agarose gel electrophoresis (Sambrook & Russell, 2001).

Transposition mutagenesis.

In an experiment described by Dorsey *et al.* in 2002, mutants were generated by using the EZ::TN<sup>R6K<sub>yori/KAN-2</sub></sup> Tnp transposome transposition mutagenesis system (Epicentre). The transposase complexes were introduced into *A. baumannii* ATCC 19606<sup>T</sup> cells by electroporation (Dorsey *et al.*, 2002). Kanamycin-resistant transformants
were screened on LB plates containing 40 μg kanamycin/mL. These transformants were subsequently toothpicked on LB plates with 100 μM 2,2’-dipyridyl (DIP). The colonies that displayed a phenotype of decreased growth under iron-limiting conditions were stored as glycerol stocks at -80°C for future experimentation.

**Screening for DIP growth-deficient mutants.**

Stock cultures of several ATCC 19606ᵀ insertion colonies that had previously demonstrated a growth-deficient phenotype under iron-limiting conditions were taken out of storage and plated on LB agar plates with 40 μg kanamycin/mL. These strains were then streaked on LB agar plates supplemented with increasing concentrations of DIP. The strains that demonstrated the greatest growth deficiency under iron-limiting conditions were analyzed to identify which gene was interrupted by the transposon. Only ATCC 19606ᵀ #1A, the nfuA mutant, will be discussed in this work.

**Rescue and identification of interrupted genes.**

Rescue of the genomic region with the insertion of the EZ::TN<R6Kγori/KAN-2> transposon was accomplished by performing an EcoRI-HF digestion of the genomic DNA followed by self-ligation using T4 DNA ligase (New England Biolabs). The resulting plasmid was transformed into *E. coli* EC100D pir+ cells using heat shock. Plasmid DNA was isolated from the colonies that grew on LB agar containing 40 μg kanamycin/mL. The plasmid DNA was used as a template to find the sequence of the genomic DNA flanking the transposon element by automated DNA sequencing. The
primers that were complementary to the inserted transposon were included in the mutagenesis kit. Primers 3642 and 3643 were designed and used for further extension of the nucleotide sequences (see Table 2). DNA sequencing was performed by using the BigDye sequencing protocol (Applied Biosystems) and the ABI 3100 automated DNA sequencer (Applied Biosystems). The DNA sequences were assembled using Sequencher (Gene Codes). DNA and amino acid sequences were analyzed using the DNASTAR software package (DNASTAR Inc., Wisconsin), BLAST (Basic Local Alignment Search Tool), and the tools provided by the ExPASy Molecular Biology Server (http://ww.expasy.ch).

**Growth curves for A. baumannii ATCC 19606\(^T\) and the nfuA mutant.**

Growth curves were performed with the *A. baumannii* ATCC 19606\(^T\) strain and the *nfuA* insertion mutant in LB broth and M9 minimal medium. 500 µL of starter cultures with an optical density at 600 nm (OD\(_{600}\)) of 0.5 were inoculated into 50 mL of LB broth or M9 minimal medium. After an initial time point reading, the OD\(_{600}\) of the LB cultures was read at 1, 2, 3, 4, 5, 6, 7, 8, and 24 hr. The M9 cultures were read at the same time points with an additional 13-hr time point. The cultures were incubated at 37°C with shaking at 200 rpm. The OD\(_{600}\) was read by a Beckman DU-640 UV-VIS spectrophotometer.
**Arnow’s colorimetric assay of A. baumannii ATCC 19606<sup>T</sup> and the nfuA mutant.**

The presence of extracellular catecholic compounds was detected using the Arnow’s colorimetric assay (Arnow, 1937) and supernatants of A. baumannii ATCC 19606<sup>T</sup> and nfuA M9 minimal medium iron-chelated cultures.

**Western blot analysis of A. baumannii ATCC 19606<sup>T</sup> and the nfuA mutant.**

Cells of the A. baumannii ATCC 19606<sup>T</sup> and the nfuA mutant were grown in LB broth under non-chelated and iron-limiting conditions using 100 µM DIP. Total cell lysates were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. Western blotting analysis was performed by using anti-BauA serum. The anti-BauA serum was produced in rabbits and was purified by SDS-PAGE and electroelution as described by Dorsey et al. (2004).

**Complementation of the nfuA mutant.**

Two complements were generated in this work, one with the A. baumannii ATCC 19606<sup>T</sup> nfuA gene and one with the E. coli MG1655 nfuA gene. The nfuA gene of both strains was amplified by the polymerase chain reaction (PCR). The ATCC 19606<sup>T</sup> nfuA gene was PCR-amplified from the genome using primers 3651 and 3652 that included BamHI restriction sites. The E. coli MG1655 nfuA gene was PCR-amplified from the genome using primers 3681 and 3682 that included BamHI restriction sites. Phusion DNA polymerase (New England Biolabs) was used for the PCR reaction. The blunt-ended amplicons were ligated into a pCR-Blunt II (Invitrogen) vector which was subsequently
transformed into *E. coli* TOP10 competent cells using heat shock. The cells were plated on LB agar with 40 µg kanamycin/mL. The plasmids of kanamycin-resistant colonies were sequenced to confirm that the insert had the same sequence as the parental DNA. The plasmid DNA (pMU937 and pMU949 harboring the *A. baumannii* and *E. coli nfuA* genes, respectively) was digested with *Bam*HI and ligated into the shuttle vector pWH1266. The vector was transformed into *E. coli* DH5α competent cells using heat shock. The cells were plated on LB agar with 100 µg ampicillin/mL and on LB agar with 20 µg tetracycline/mL. The plasmid (pMU944 and pMU952) isolated from a colony that was resistant to ampicillin and sensitive to tetracycline was electroporated into *A. baumannii* 19606 #1A *nfuA* mutant cells. The cells were cultured in LB broth with 100 µg/ml ampicillin. Restriction digestion and gel electrophoresis of the plasmid DNA isolated from the cells were performed to confirm the presence of pMU944 and pMU952 in the complemented *A. baumannii* 19606 #1A strains.

**Minimum inhibitory concentration assay of an iron-chelating agent.**

The minimum inhibitory concentration (MIC) assay of the iron chelator DIP was done in LB broth and M9 minimal medium. For the LB assay, 1 mL LB broth containing 0, 20, 100, 125, 150, 175, or 200 µM DIP was prepared. For the M9 assay, 1 mL M9 containing 0, 25, 50, 75, 100, 125, or 150 µM DIP was prepared. 10 µL of starter culture of ATCC 19606T, *nfuA::km*, *nfuA::km* harboring pMU944, and *nfuA::km* harboring pMU952 with an OD<sub>600</sub> of 0.5 were inoculated into the media. The cultures were
incubated overnight at 37°C with shaking at 200 rpm, and the OD$_{600}$ was read by a Beckman DU-640 UV-VIS spectrophotometer.

**MIC assay of oxidizing agents.**

The MIC assay of the oxidizing agents hydrogen peroxide and cumene hydroperoxide (CHP) was done in M9 minimal medium. For the hydrogen peroxide MIC assay, 1 mL media containing 0, 25, 50, 100, or 125 µM hydrogen peroxide was prepared. For the CHP MIC assay, 1 mL media containing 0, 10, 20, 25, or 40 µM CHP was prepared. 10 µL of starter culture of ATCC 19606$^T$, *nfuA::km*, *nfuA::km* harboring pMU944, and *nfuA::km* harboring pMU952 with an OD$_{600}$ of 0.5 were inoculated into the media. The cultures were incubated overnight at 37°C with shaking at 200 rpm, and the OD$_{600}$ was read by a Beckman DU-640 UV-VIS spectrophotometer.

**Overexpression and purification of the recombinant NfuA protein.**

The *nfuA* gene was amplified by PCR from ATCC 19606$^T$ total DNA using Phusion DNA polymerase (New England Biolabs) and primers 3673 and 3674. The amplicon was ligated into a pET-100D/TOPO (Invitrogen) vector which was subsequently transformed into *E. coli* TOP10 competent cells using heat shock. The cells were plated on LB agar containing 100 µg ampicillin/mL. The plasmid of an ampicillin-resistant colony was isolated and sequenced using T7 forward and reverse primers provided with the kit (Invitrogen) to confirm the proper orientation of the insert. The plasmid was then transformed into *E. coli* BL21(DE3) cells. The transformants overexpressed the ATCC
19606\textsuperscript{T} N-terminal His-tagged NfuA protein as described by Studier (2005). The cells were grown in ZYP media and auto-induced by adding lactose and incubating the culture overnight at 37°C with shaking at 200 rpm. The soluble protein was isolated by Ni\textsuperscript{2+} affinity column chromatography from total cell lysates as recommended by the manufacturer’s protocol (Pierce). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of the protein extracted from both a one-dimensional and two-dimensional protein gel confirmed that the overexpressed protein was indeed NfuA.

**Electron paramagnetic resonance of the NfuA protein.**

Electron paramagnetic resonance (EPR) was performed on the purified recombinant NfuA protein. The experiment was performed with a Bruker EMX X-band CW-EPR spectrometer composed of an ER 041XG microwave bridge, a TE\textsubscript{102} cavity, and a BVT 3000 nitrogen gas temperature controller (temperature stability of ±0.2°C). The EPR protocol was similarly performed as described in Breece et al. (2005). 250 mM of the NfuA protein was prepared in equilibrium buffer (50 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.3 M NaCl, 10 mM imidazole, 10 mM Tris pH 8.0) and glycerol was added to make a final concentration of 20% glycerol.

**Bacterial intracellular replication assay.**

A549 cells were cultured in Hanks’ balanced salt solution (HBSS) complete until they were over 90% confluent in a 24-well tissue culture plate with approximately 10\textsuperscript{5} cells
per well. Bacterial cells were grown in 24 hr LB cultures at 37°C with shaking at 200 rpm, collected by centrifugation at 15,000 rpm for 10 min, washed, resuspended, and diluted in HBSS. The A549 cells were infected with ATCC 19606\textsuperscript{T} cells, the nfuA mutant, and both the wild-type and mutant. Each well had a final number of approximately $10^3$ bacterial cells, estimated from the OD\textsubscript{600}. The cells were incubated overnight at 37°C in 5% CO\textsubscript{2}. The supernatant was discarded, and the A549 monolayer was lysed using dH\textsubscript{2}O. The resulting suspension was serially diluted and plated on nutrient agar. The co-infection of both ATCC 19606\textsuperscript{T} and the mutant was also plated on 50 μg kanamycin/mL. After overnight incubation of the cells at 37°C, the colony forming units (CFUs) were counted, and the CFUs/mL was calculated and recorded.
Results and Discussion

Screening for DIP growth-deficient mutants.

As previously mentioned, a mutant library of derivatives that had deficient growth under iron-limiting conditions was previously generated by our lab (Dorsey et al., 2002; Dorsey et al., 2004). These insertion derivatives were generated by the random interruption of genes by transposition using the EZ::TN<R6Kγori/KAN-2>Tnp Transposome system. The screening of the Tnp mutant library clones showed several strains that were unable to grow under iron-limiting conditions but were able to grow under non-chelated conditions. Out of all the strains tested, the *A. baumannii* ATCC 19606T #1A derivative, later found to harbor a single insertion within *nfuA* (ZP_05828133), was shown to have the most drastic growth decrease under iron-limiting conditions (Fig. 1). Thus, we sought to determine the role of the gene that was interrupted by the transposon that caused the resulting iron-utilization-deficient phenotype.

Nucleic acid sequence of *nfuA* and amino acid sequence of NfuA.

Through rescue cloning and DNA sequencing, it was found that the transposon of *A. baumannii* 19606 #1A interrupted the beginning of a gene encoding for a Fe-S cluster biogenesis protein. The gene is flanked by a gene encoding a rhomboid family protein upstream and a potential TonB-dependent receptor gene directly downstream (Fig. 2). However, based upon the large intergenic region, it is believed that this gene is monocistronic. It was found that the amino acid sequence of *A. baumannii* ATCC 19606T NfuA (ZP_05828133) shares 48% identity with the *E. coli* MG1655 NfuA
(NP_417873), 56% identity with the A. vinelandii NfuA (YP_002800022), and 59% identity with the P. aeruginosa PAO1 NfuA (NP_250538). The A. baumannii ATCC 19606 T NfuA protein has 212 amino acids and appears to be a modular protein with two functionally related domains (Fig. 3). The N-terminal region shares sequence homology with the protein IscA, a protein involved in iron-sulfur cluster biosynthesis. The C-terminal region shares sequence homology to NifU, another protein involved in iron-sulfur cluster biosynthesis. The amino acid sequence also has four conserved cysteine residues. Specifically, the NifU domain contains a CXXC motif that has been shown to be required for the function of NfuA in E. coli and A. vinelandii (Angelini et al., 2008; Bandyopadhyay et al., 2008). It has been demonstrated that both domains are important for the function of the E. coli NfuA in vivo (Angelini et al., 2008).

**Growth curves for A. baumannii ATCC 19606 T and the nfuA mutant.**

The growth curves for A. baumannii ATCC 19606 T and the nfuA mutant were determined to investigate whether the nfuA gene is related to the bacteria’s metabolism. In both LB and M9 minimal medium, the mutant strain had less growth than the wild-type between 3-7 hours after inoculation, but after seven hours, the mutant strain growth reached an equal optical density as the wild-type (Fig. 4). This result demonstrated that the decrease in growth of the mutant under iron-limiting conditions was not a result of a defect in general metabolism.
Arnow’s colorimetric assay of *A. baumannii* ATCC 19606<sup>T</sup> and the *nfuA* mutant.

Initially, it was hypothesized that a disrupted siderophore secretion and/or uptake system was responsible for the growth-deficient phenotype of the *nfuA* mutant under iron-limiting conditions. The Arnow’s colorimetric assay was performed to test for the presence of extracellular phenolic compounds, specifically catechols. The *nfuA* mutant was compared to the *A. baumannii* ATCC 19606<sup>T</sup> parental strain, used as a positive control, and an *A. baumannii* ATCC 19606<sup>T</sup> *entA* mutant, used as a negative control (Table 3). *EntA* is an early protein essential for acinetobactin synthesis. *A. baumannii* ATCC 19606<sup>T</sup> only produces one siderophore, acinetobactin, which is demonstrated by the observation that the *entA* mutant cannot grow under iron-limiting conditions (Penwell and Actis, manuscript in preparation). Thus, the possibility of a disrupted alternative siderophore system was eliminated. The detection of catechol in the culture supernatant of the ATCC 19606<sup>T</sup> #1A derivative demonstrated that the insertion inactivation of *nfuA* did not affect the acinetobactin-based iron utilization system.

Western blot analysis of *A. baumannii* ATCC 19606<sup>T</sup> and the *nfuA* mutant.

A western blot was performed on total cell lysates using polyclonal anti-BauA rabbit serum. BauA is the outer membrane receptor for acinetobactin, the siderophore produced by *A. baumannii* ATCC 19606<sup>T</sup> (Dorsey et al., 2004). The western blot showed that under non-chelated conditions, both *A. baumannii* ATCC 19606<sup>T</sup> and the *nfuA* mutant did not produce BauA (Fig. 5). Under iron-limiting conditions imposed by the addition of DIP, both the *A. baumannii* ATCC 19606<sup>T</sup> parental strain and the *nfuA* mutant
demonstrated approximately equal levels of BauA production. This indicates that the inactivation of \( nfuA \) does not affect the acinetobactin-mediated iron uptake mechanism and, therefore, the phenotype of the ATCC 19606\(^T \) #1A mutant is not due to a defect in iron uptake.

**Complementation of the \( nfuA \) mutant.**

To prove the role of \( nfuA \) in \( A. \) baumannii, two complemented derivatives were generated in this work, one with the parental \( A. \) baumannii ATCC 19606\(^T \) \( nfuA \) from plasmid pMU944 and another with the \( E. \) coli MG1655 \( nfuA \) from plasmid pMU952. The ATCC 19606\(^T \) #1A \( nfuA \) mutant was complemented with the \( E. \) coli NfuA protein because it shares 48% amino acid similarity with the \( A. \) baumannii NfuA and, therefore, we were interested if the function of these two proteins was also similar. The effect of these complementation experiments was examined by testing the iron starvation and oxidative stress response of the complemented derivatives as described in the following sections.

**MIC assay of an iron-chelating agent.**

For both the cultures with LB and M9 minimal medium, the \( nfuA \) mutant demonstrated less growth compared to \( A. \) baumannii ATCC 19606\(^T \) under iron-limiting conditions starting at a DIP concentration of 100 µM (Fig. 6). Interestingly, the complementation of the \( nfuA \) mutant with pMU944, which harbors the parental ATCC 19606\(^T \) allele, partially restored the phenotype of this mutant, whereas complementation of this mutant with pMU952, which harbors the \( E. \) coli \( nfuA \) gene, restored iron uptake to levels similar to
the wild-type. Apparently, the function of the *E. coli* NfuA resembled that of the *A. baumannii* NfuA and restored this activity in the ATCC 19606<sup>T</sup> #1A mutant better than the parental gene. These differences could be due to different levels of gene expression. It may be possible that the *nfuA* in pMU944 was overexpressing the *A. baumannii* NfuA because the *nfuA* gene is in the same orientation as the tetracycline promoter in the vector, and the excess NfuA may have had a toxic effect on the bacterial cells. Nevertheless, these results indicate that NfuA is important for *A. baumannii* ATCC 19606<sup>T</sup> to grow in an iron-limiting environment that it should encounter when it colonizes and infects the human host.

**MIC assay of oxidizing agents.**

Previous experiments in *E. coli* demonstrated that the NfuA protein, which was required during iron-limiting conditions, was also important for cell survival under oxidative stress conditions (Angelini *et al*., 2008). Accordingly, under oxidizing conditions from both hydrogen peroxide and CHP, which was used because it is a biologically relevant oxidizing agent in a host immune response (Dorsey *et al*., 2006), the ATCC 19606<sup>T</sup> #1A *nfuA* mutant demonstrated less growth compared to *A. baumannii* ATCC 19606<sup>T</sup> starting at a concentration of 100 µM hydrogen peroxide and 25 µM CHP (Fig. 7). Interestingly, the *nfuA* pMU944 complemented strain did not recover growth to wild-type levels, whereas the *nfuA* pMU952 complemented strain did, which is consistent with the results from the MIC of DIP. These results indicate that NfuA is important for *A. baumannii* to grow in an oxidizing environment.
**Electron paramagnetic resonance of the NfuA protein.**

The EPR spectrum of the ATCC 19606\(^T\) purified recombinant NfuA protein indicated that it contains a Fe-S cluster (Fig. 8) as predicted from its amino acid sequence similarity to other bacterial related proteins. In *E. coli* and *A. vinelandii*, NfuA has been shown to assemble a [4Fe-4S] cluster and transfer it to apoproteins (Fontecave & Ollagnier-de-Choudens, 2008). Thus, it is likely that the *A. baumannii* NfuA has a similar function related to intracellular iron metabolism and the assembly of iron-protein complexes.

**Bacterial intracellular replication assay.**

The bacterial intracellular replication assays demonstrated that NfuA was required for optimal replication within human lung epithelial cells (Fig. 9). It was found that 7.5 x 10\(^3\) CFUs/mL were recovered from A549 cells infected with the ATCC 19606\(^T\) parental strain, whereas significantly less bacteria, 1.2 x 10\(^3\) CFU/s/mL (p=0.01), were recovered from epithelial cells infected with the ATCC 19606\(^T\) #1A *nfuA* mutant. There was not a significant difference between the recovery of the co-cultured strains compared to that of solely the wild-type (p=0.23). When kanamycin was added to the wild-type and mutant combination, there was almost no recovery of bacteria. The wild-type was kanamycin sensitive and the *nfuA* mutant contained a kanamycin resistant cassette on its plasmid. Thus, this result indicated that co-infection with both *A. baumannii* ATCC 19606\(^T\) and the *nfuA* mutant caused the mutant to be competed out over the course of the infection. A549 lung epithelial cells were used because they have been shown to be a suitable
working model, and *A. baumannii* has been found to contribute to lower respiratory tract infections (Gaddy *et al.*, 2009).

**Conclusion**

The results collected during this study clearly demonstrate the importance of NfuA during iron starvation and oxidative stress in *A. baumannii*, independently of the bacteria’s capacity to acquire iron via the acinetobactin-mediated system. However, the precise function of NfuA still remains unclear. NfuA may be a scaffold for Fe-S cluster assembly or a Fe-S transporter that delivers the Fe-S cluster to apoprotein targets (Angelini *et al.*, 2008; Py & Barras, 2010). It is also possible that NfuA has a chaperone/repair function for damaged Fe-S proteins (Angelini *et al.*, 2008). *A. baumannii* ATCC 19606^T^ has the housekeeping Isc system for Fe-S cluster bioassembly but does not have the Suf system. As discussed earlier, the Suf system in *E. coli* is the primary Fe-S cluster assembly system under oxidative stress and iron starvation. Perhaps NfuA plays a similar role to the Suf system of *E. chrysanthemi* which is involved in the release of Fe^{2+} from siderophores and the repair of oxidatively damaged [4Fe-4S] clusters (Nachin *et al.*, 2003). However, more research needs to be done to deduce the specific role of NfuA in *A. baumannii*.

Since it has been reported that NfuA is important for aconitase maturation in *A. vinelandii* (Bandyopadhyay *et al.*, 2008), future enzyme kinetics assays should be done to see if the same is true for *A. baumannii*. The activity of aconitase, which contains a [4Fe-4S] cluster, was reduced in a strain of *A. vinelandii* where the *nfuA* gene was inactivated,
and this strain also could not grow under elevated oxygen concentrations (Bandyopadhyay et al., 2008). It may be possible that NfuA in *A. baumannii* targets other specific apoproteins, so therefore other Fe-S proteins that require NfuA activity will be examined.

It was demonstrated that NfuA is also important for the resistance of *P. aeruginosa* to fluoroquinolone antibiotics, such as ciprofloxacin and norfloxacin (Daung-nkern et al., 2010). These fluoroquinolones induce the formation of ROS which kill bacterial cells (Daung-nkern et al., 2010). Similar assays could be done with *A. baumannii*, using the Kirby–Bauer disc diffusion test with fluoroquinolone antibiotics to determine whether the *A. baumannii* ATCC 19606T #1A *nfuA* mutant is more susceptible than the wild-type (Bauer et al., 1966).

Eijkelkamp et al. (2011) recently reported that the *A. baumannii* ATCC 17978 *nfuA* gene, called *gntY* in their study, was upregulated 2.7-fold under iron-limiting conditions. Similarly, in *E. coli*, there is about a 2-fold increase in *nfuA* mRNA during oxidative stress and iron-limiting conditions (Angelini et al., 2008). Transcriptomic analyses demonstrated that *nfuA* is upregulated due to translational stress from misfolded proteins, heat shock, and kanamycin in *E. coli* (Grimm et al., 2010). Also, *nfuA* is upregulated during exposure to cobalt and is necessary for protection against this metal (Fantino et al., 2010).

To further explore how *A. baumannii* regulates the expression of *nfuA*, rabbit polyclonal antibodies prepared with purified His-tagged NfuA will be used to determine whether iron and oxidative stress conditions regulate the production of this protein in *A.*
baumannii. These observations will be further confirmed by testing for the presence of Fur-binding boxes in the promoter region controlling the transcription of *nfuA* using a Fur titration assay (FURTA) (Stojiljkovic *et al.*, 1994). All these studies will provide a more comprehensive picture of NfuA’s role in the physiology and pathobiology of *A. baumannii*.

In this work, we have begun to characterize a Fe-S biogenesis protein called NfuA in *A. baumannii* as an essential protein for the bacteria to grow and persist under iron starvation and oxidative stress conditions. Our data also supports the idea that NfuA is involved in Fe-S cluster assembly. Furthermore, this protein is required for optimal intracellular replication in certain host cells. By studying NfuA, we have come closer to understanding how *A. baumannii* can survive under stressful conditions that are analogous to those found in the human host and how it intracellularly utilizes iron. Hopefully, we will be able to find a way to exploit the vulnerabilities of *A. baumannii* so that new antibiotics can be developed to more effectively treat the threatening infections this pathogen causes in humans worldwide.
### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19606</td>
<td>Clinical isolate</td>
<td>ATCC</td>
</tr>
<tr>
<td>19606 #1A</td>
<td>*nfuA::EZ::TN &lt;R6Kγori/KAN-2&gt;; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>19606 #17</td>
<td>*entA::km; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Penwell <em>et al.</em></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>Used for DNA recombinant methods</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>EC100D&lt;sup&gt;+&lt;/sup&gt;</td>
<td>*pir&lt;sup&gt;r&lt;/sup&gt;, host for plasmid rescue maintenance</td>
<td>Epicentre</td>
</tr>
<tr>
<td>TOP10</td>
<td>Used for DNA recombinant methods</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>λDE3, T7 RNA polymerase</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>MG1655</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;λambda&lt;sup&gt;−&lt;/sup&gt;-<em>ilvG</em>-<em>rfb</em>-50<em>rph</em>-1</td>
<td>Labstrain</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pCR-Blunt II</td>
<td>PCR cloning vector; Km&lt;sup&gt;R&lt;/sup&gt;, Zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pWH1266</td>
<td>Shuttle vector; Ap&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hunger <em>et al.</em></td>
</tr>
<tr>
<td>pET100D</td>
<td>T7 expression vector; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pMU902</td>
<td>Plasmid rescue of self-ligation of EcoRI-digested DNA from mutant #1A; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pMU937</td>
<td>pBlunt harboring <em>nfuA</em> from 19606; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pMU941</td>
<td>pET100D harboring <em>nfuA</em> from 19606; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pMU944</td>
<td>pWH1266 harboring <em>nfuA</em> from 19606; Ap&lt;sup&gt;R&lt;/sup&gt; Tet&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pMU949</td>
<td>pBlunt harboring <em>nfuA</em> from MG1655; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pMU952</td>
<td>pWH1266 harboring <em>nfuA</em> from MG1655; Ap&lt;sup&gt;R&lt;/sup&gt; Tet&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Ap<sup>R</sup>, ampicillin resistance; Km<sup>R</sup>, kanamycin resistance; Tet<sup>R</sup>, tetracycline resistance; Zeo<sup>R</sup>, zeocin resistance
<table>
<thead>
<tr>
<th>Number</th>
<th>Nucleotide sequence</th>
</tr>
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<tbody>
<tr>
<td>3642</td>
<td>5’- GATCAGACGGTTCATGAC -3’</td>
</tr>
<tr>
<td>3643</td>
<td>5’- CACCACCTTCTTCTGGTGC -3’</td>
</tr>
<tr>
<td>3651</td>
<td>5’- <strong>GGATCC</strong>GATCAGACGGTTCATGAC -3’</td>
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<tr>
<td>3652</td>
<td>5’- <strong>GGATCC</strong>TCATGACGTCTTGCTCG -3’</td>
</tr>
<tr>
<td>3673</td>
<td>5’- CACCACCGATGACTGAGAACACC 3’</td>
</tr>
<tr>
<td>3674</td>
<td>5’- TTTGAAATAAGCACCCT -3’</td>
</tr>
<tr>
<td>3681</td>
<td>5’- <strong>GGATCC</strong>GTTATCAGCTGGTTGC -3’</td>
</tr>
<tr>
<td>3682</td>
<td>5’- <strong>GGATCC</strong>ACGACTGATACCCA -3’</td>
</tr>
</tbody>
</table>

Sequence underline indicates added restriction site.

**Table 3. Results of the Arnow’s assay.** The Arnow’s assay detected catechol compounds in culture supernatants. Cells were cultured in M9 minimal medium overnight at 37°C with shaking at 200 rpm.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arnow’s result</th>
</tr>
</thead>
<tbody>
<tr>
<td>19606&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>nfuA::km</em></td>
<td>+</td>
</tr>
<tr>
<td><em>entA::km</em></td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1.

![Diagram showing the effect of nfuA::km on protein expression from DIP 0 to DIP 200.]

Figure 2.

![Diagram showing the Rhomboid Family Protein and NfuA domains.]

Figure 3.

![Sequence alignment showing IscA-like and NifU-like domains.]

1-8: MSTENNTAVVAEIPLNLLITPSAQEYHLHLKQNTPGIGVRIFVHEHPGTRAECCMAYSAEEVVPDQKDYPDFPAYIDAPSIPYLLDA

IscA-like Domain

96-191: DYNKDFGGQITFRAPNSKVRPGDASEERITYVLAAlNEIpGLAGHGGNCLESVEQDDPHETLKFKGgCQgCgSALDVLKQGVE

NifU-like Domain

185-218: TRLKHEIPQRVQDQDHTQAEFGAYF

*
Figure 4.

a)

![LB Growth Curve](image1)

b)

![M9 Growth Curve](image2)
Figure 5.
Figure 6.

a)

![Graph LB](image)

b)

![Graph M9](image)
Figure 7.

a)

![Graph showing the effect of Hydrogen Peroxide on OD600.]

b)

![Graph showing the effect of Cumene Hydroperoxide on OD600.]

Legend:
- 1906
- nfuA::km
- nfuA::km pML844
- nfuA::km pML852
Figure 8.
Figure 9.

The diagram shows the comparison of (CFU/ml recovered)/(CFU/ml inoculum) for different strains. The strains compared are 19606, nfuA::km, nfuA::km/19606, and nfuA::km/19606 kan.
Fig. 1. Screening for DIP growth-deficient mutants. *A. baumannii* ATCC 19606<sup>T</sup> was plated with other strains including the *nfuA* mutant on increasing concentrations (µM) of DIP. The *nfuA* mutant showed less growth than the wild-type on DIP 100 µM and failed to grow on DIP 150 µM and DIP 200 µM, whereas the wild-type strain could grow under these concentrations.

Fig. 2. Visual representation of the genomic organization of *A. baumannii* ATCC 19606<sup>T</sup> DNA region harboring the *nfuA* gene. The vertical arrow indicates the transposon insertion site. A gene encoding a rhomboid family protein is upstream and a gene encoding a TonB-dependent receptor is downstream of the *nfuA* gene.

Fig. 3. Amino acid sequence and domain map of NfuA. Four conserved cysteine residues are emphasized. NfuA has an N-terminus IscA-like domain and a C-terminus NifU-like domain.

Fig. 4. Growth curves of *A. baumannii* ATCC 19606<sup>T</sup> and the *nfuA* mutant. The bacteria were grown in a) LB broth and b) M9 minimal medium. Absorbance was read at 600 nm to determine growth (n=3).

Fig. 5. Western blot of *A. baumannii* ATCC 19606<sup>T</sup> and the *nfuA* mutant total cell lysates with anti-BauA. Cells were cultured in LB in the presence of the iron chelator
DIP overnight at 37°C with shaking at 200 rpm. The immunocomplexes were detected with horseradish peroxidase labeled protein A and chemiluminescence.

Fig. 6. MIC assay of *A. baumannii* ATCC 19606^T^, the *nfuA* mutant, and cognate complemented derivatives in DIP. The bacteria were grown in a) LB broth and b) M9 minimal medium. Strains were grown overnight under increasing concentrations of iron chelator DIP at 37°C with shaking at 200 rpm. Absorbance was read at 600 nm to determine growth (n=3).

Fig. 7. MIC assay of *A. baumannii* ATCC 19606^T^, the *nfuA* mutant, and cognate complemented derivatives under oxidative conditions. Strains were grown overnight in increasing concentrations of the oxidizing agent a) hydrogen peroxide and the organic oxidizer b) CHP at 37°C with shaking at 200 rpm. Absorbance was read at 600 nm to determine growth (n=3).

Fig. 8. EPR spectrum of the purified NfuA protein. The peaks indicated by the black arrows suggest that the protein of interest harbors a Fe-S cluster. Recombinant NfuA protein was overexpressed and purified using metal chromatography.

Fig. 9. Bacterial replication in human epithelial cells. A549 lung epithelial cells were infected with cells of ATCC 19606^T^, the *nfuA* mutant, and a mix of both strains cultured
on plates grown with and without kanamycin. The cultures were plated, grown on nutrient agar, and the colony-forming unit (CFUs) were counted (n=12).
References


Bouvet, P. J., and P. A. Grimont. 1986. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov., and emended description of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. Int. J. Syst. Bacteriol. 36:228-240.


Acinetobacter baumannii ATCC 19606 and Vibrio anguillarum 775 are structurally and functionally related. Microbiology 150:3657–3667.


