Application of a Unique Insertion Mutagenesis Method to Characterize Genes Responsible for Biofilm Formation by *Acinetobacter baumannii*

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by

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Abstract

Acinetobacter baumannii is a bacterium capable of causing severe upper respiratory infections in patients whose immune systems have been suppressed. A. baumannii forms biofilms on a variety of surfaces. Biofilms are bacterial communities present as layers of cells that can form on devices such as catheters and endotracheal tubes, which can be very problematic because they enhance the ability of bacteria to resist the host responses and antimicrobial compounds. The purpose of this study was to use a unique DNA insertion system to identify some of the genes responsible for biofilm formation by this organism. After isolating DNA from selected insertion derivates of A. baumannii 19606, we were able to determine that one of the interrupted genes that abolished biofilm formation had a high degree of similarity to the csuE gene from Vibrio parahaemolyticus. This gene is part of the csu chaperone-usher operon and could potentially play a role in the assembly of pili at the cell surface. This study was significant because it helped further describe one of the potential virulence factors of A. baumannii, few of which have been well characterized.
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Introduction

*Acinetobacter baumannii* is recognized as one of the many species of bacteria that is capable of causing nosocomial infections. We have demonstrated in our laboratory that this organism is able to form biofilms on a variety of surfaces, such as glass, polystyrene, and polyethylene. While biofilm formation by *A. baumannii* has not been thoroughly studied as a virulence factor, it is certainly reasonable to believe that this organism is very capable of adhering to medical devices. The first section of this work, then, provides the reader with a description of nosocomial infections and their impacts in society.

Before reading about the actual study, it is also important to provide a background that describes some of the unique characteristics of *A. baumannii*. This organism, because it is opportunistic and only causes problems within a host whose immune system has been suppressed, is not as well known by the general public. This background will give the reader new insight as to why it is important that microbiologists continue to gain a better understanding of this organism’s virulence factors.

Biofilms will be described in detail. These bacterial communities are ubiquitous, and while most are beneficial, those that form on medical devices, such as catheters and endotracheal tubes, can be devastating. This work attempted to identify some of the genes responsible for biofilm formation by *A. baumannii*, and this section reveals some of the unique features of biofilms. Following this section, the actual study and its significance will be explained.
Description of Nosocomial Infections

Overview and significance

Nosocomial infections continue to cause much concern across the world (5), for these infections can occur in a wide range of hospitalized patients and can be very problematic and devastating. Nosocomial infections are infections that develop within a hospital or are produced by microorganisms acquired during hospitalization (10). While our understanding of these infections continues to improve, there is still much to learn about hospital-acquired infections if we are to ameliorate the current situation. One of the most important reasons why nosocomial infections continue to occur is because of patient susceptibility (5). For example, many elderly citizens often make up a large proportion of the patients in a hospital, and their immune systems are often not as effective as a healthy adult’s in ridding the body of harmful pathogens; therefore, it is the elderly who are often victims of these nosocomial infections. In addition to patient susceptibility, the other, possibly more important, reason why nosocomial infections can be so devastating is attributed to the rise in bacterial resistance to a wide range of antibiotics. If the bacteria that establish a biofilm on a medical device are able to evade the action of many different antimicrobial compounds, then the biofilm will persist for a longer period of time, potentially creating long-lasting problems for the host.

Unfortunately, the medical devices that are used to aid patients often serve as a site of bacterial colonization and can greatly increase the risk of acquiring a nosocomial infection (39). In one study (39) that looked at multiple sites of nosocomial infections in a coronary care unit (CCU), it was revealed that urinary tract infections, pneumonia, and blood stream infections were almost always associated with an invasive device (93% with
urinary catheters, 82% with ventilators, and 82% with central lines). While this study also revealed that these data differ from nosocomial infection data from other critical care areas, it certainly brings attention to the fact that nosocomial infections are still prevalent.

*Description of Acinetobacter baumannii*

**Significance in the clinical setting**

Like other organisms that are most problematic in causing nosocomial infections, *A. baumannii* is resistant to many different antibiotics. A ten-year study in a U.S. hospital (27) found that *Acinetobacter* accounted for 1.4% of all nosocomial infections. Even though this percentage may seem small at first glance, 1.4% represents a large number of patients considering the thousands who acquire a nosocomial infection each year. This bacterium certainly has been shown to play a role in the nosocomial-infection problem, but there is still much that needs to be known about *A. baumannii* if we hope to prevent the problem from growing worse.

Bacteria from the genus *Acinetobacter* are gram-negative, catalase-positive, strict aerobes. Recently, acinetobacters have gained much attention, as they are being recognized as important opportunistic pathogens in hospital settings (7). Among the different *Acinetobacter* species, *A. baumannii* is the most important human pathogen that causes pneumonia, bacteremia, secondary meningitis, and urinary tract infections (8, 18, 24, 33). Unfortunately, these infections are often very difficult to treat because *A. baumannii* is very resistant to a wide range of antibiotics, including, but not limited to, β-lactams, aminoglycosides, quinolones, and cephalosporins (6). The resistance of some strains is due to the expression of molecules and mechanisms, such as β-lactamases and
aminoglycoside-modifying enzymes (6), and low permeability of the outer membrane to certain antibiotics (9), that allow them to evade the effects of certain antimicrobial compounds. Even imipenem, regarded as the most effective antimicrobial compound to treat *Acinetobacter* infections (6), no longer has activity against 100% of *Acinetobacter* strains.

Because *Acinetobacter* spp. are opportunistic pathogens, the bacteria generally do not cause problems in a healthy host. Severe problems can arise, however, when patients are immunocompromised or are subjected to open wounds or burns. In one study (50), it was observed that 60 out of 79 major burn patients acquired *A. baumannii*, a statistic that is consistent with the fact that *A. baumannii* is the most common flora on most burn units (29).

**Virulence factors**

Unfortunately, as mentioned before, very little is known about the mechanisms of the virulence factors of *A. baumannii*. One essential factor for a pathogen is the ability to acquire iron from its external environment, and this virulence factor is currently being investigated (25). Because bacteria experience very iron-limited conditions when inside a human host, they must have a mechanism for obtaining this essential element, and *A. baumannii* is no exception.

In one study (16), *A. baumannii* survived under iron-limiting conditions through the production of siderophores. Siderophores are important compounds that scavenge iron from human hosts, and catechol compounds have been measured quantitatively in strains of *A. baumannii* (16). Because iron is such an important nutrient to the cell, it is
essential that bacteria be able to obtain this element very quickly. It is no surprise that, in
vitro, A. baumannii is able to obtain maximum cellular concentrations of iron within 5
minutes of being in an iron-limiting environment (16). One group of researchers (51)
also characterized the acinetobactin molecule from A. baumannii 19606. This
siderophore is closely related to the plasmid-encoded anguibactin, a siderophore
produced by Vibrio anguillarum (51).

While slime production enhances virulence in some strains of Acinetobacter (36),
some studies have revealed that there is no difference in virulence between slime-
producing and non-slime-producing strains (as measured by the 50%
lethal dose in mice) (35). Even though slime may play a role in virulence, it is probably
not an essential virulence factor (36), as one study reported that only 14 out of 100 strains
produced any slime, and no direct correlation could be drawn between the degree of
virulence and the amount of slime produced (35).

As microbiologists continue to study and understand some of these virulence
factors of A. baumannii, it is important that the ability of this organism to form a biofilm
and contribute to virulence be fully understood. Although it has been shown in our
laboratory that A. baumannii is fully capable of adhering to plastic surfaces, the
environment within a human host is very different from that of a plastic test tube. The
more information that can be gathered about the genes and mechanisms responsible for
biofilm formation, the greater the chance is that medical personnel can effectively treat
problematic infections caused by these bacterial communities.
Biofilms

General characteristics and steps in formation

A very important topic that has received much attention in recent years is that of biofilms. Because my research involved isolating and genetically characterizing transposon-harboring cells that were unable to form a biofilm, it is necessary that the important components and interactions involved in a biofilm are described. A biofilm can generally be thought of as a microbial community forming at a phase boundary, generally, but not always, at a liquid:solid interface (48). It is important to realize that biofilms are often very dynamic and heterogeneous in nature, as different species of bacteria interact and sometimes communicate with one another (14).

There are several necessary steps that are required for biofilm formation. Because almost all biofilms occurring in the environment are heterogeneous, one important step is that, after the primary colonizers adhere to a surface, coadhesion occurs (11). During coadhesion, bacteria begin to release substances such as polymers and make fibrils that anchor the bacteria to the substratum. This step is a very crucial one in the formation of the biofilm, for if the bacteria are not able to withstand the shear forces of the environment, then the entire biofilm will detach (11).

One interesting feature of certain biofilms is the ability of the bacteria to release a biosurfactant that discourages the adhesion of other strains and species of bacteria (31). While this feature has not been well studied, these biosurfactants could play an important role in biofilms that form on medical and prosthetic devices, as these medical biofilms can be heterogeneous in nature but could prevent certain species of bacteria from adhering to the biofilm once it has been established. Clearly, while the general steps of
biofilm formation appear to be straightforward, each one involves complex interactions and events that are essential if the biofilm is to persist within its environment.

**EPS**

One important feature of all studied biofilms seems to be that they possess extracellular polymeric substances (EPS) (17). These EPS often distinguish biofilms from bacteria that are simply attached to a solid surface, because the EPS matrix is frequently responsible for providing the binding substance that allows cells to form a bacterial community. The EPS consist mostly of polysaccharides and proteins (17), but other macromolecules such as DNA and lipids may also be important to maintain the integrity of the EPS (32). *Pseudomonas aeruginosa*, an important model organism for studying biofilms, is dependent on extracellular DNA for biofilm formation (47), so clearly the polysaccharides are not always the essential molecules. The importance of the EPS is evident from the fact these extracellular substances make up between 50 and 90% of the total organic matter in a biofilm (13, 31, 34).

As mentioned above, protein also can be an important component of the EPS and the biofilm community. Many proteins in the EPS matrix are enzymes (19) that degrade macromolecules to provide nutrients available for microbial metabolism. It was also shown in one study (49) that the EPS can interact and provide protection for enzymes such as lipase, making the enzyme less sensitive to temperature changes. While there is still much to learn about the role of EPS in biofilm formation and maintenance, it is clear that this glycocalyx-like substance is responsible for many of the observed characteristics of biofilms.
Role of outer membrane molecules, pili, and flagella

The roles that flagella, pili, and outer membrane proteins play in biofilm formation are often very important ones. Studies showed that biofilm formation could be abolished by creating mutant organisms that lacked the ability to produce one of these molecules and surface-exposed structures. In one study (37), researchers discovered that \textit{P. aeruginosa} mutants that were unable to express their distinguishing type IV pili were also unable to form biofilms on a polyvinyl chloride abiotic surface. This same study also revealed that non-motile \textit{P. aeruginosa} organisms, characterized by the mutation in the \textit{flgK} gene, were unable to form the microcolonies that the wild-type organisms were able to form. Hence this study, while it certainly cannot be applied to all organisms, lends a possible explanation as to the important role that pili and flagella play in biofilm formation.

In another study (15), researchers characterized an outer membrane protein (OMP), Ag43, in a strain of \textit{Escherichia coli}. Interestingly, they found that this OMP affected biofilm formation in minimal medium but not in Luria-Bertani broth. This study revealed that bacteria may be capable of up- or down-regulating biofilm formation depending on their surrounding environment. Because the human host is a source for many different nutrients, some bacteria may be able to establish a biofilm more effectively on implanted devices such as catheters than on Petri dishes in the laboratory because of the contrast of the nutrients available in their environment.

Although the host may provide nutrients needed by the bacteria, the bacterial cells must also be able to evade many of defense mechanisms expressed by the host. Many biofilms are often composed of the host’s own flora (3), so many of these bacteria have
already established relationships with the host that prevent them from being recognized by the host’s immune system as foreign. But for other organisms, including *A. baumannii*, the regulation of the expression of extracellular molecules is important if the bacteria are to survive and establish a biofilm.

**Treatment techniques for biofilms**

While many biofilms in nature go unnoticed and under appreciated on a day-to-day basis, there is certainly an urgency to study and understand biofilms in the clinical setting. In addition to the concerns of the transfer of resistance genes by bacteria, it is easy to understand why biofilms cause much concern within the medical community when the problem is viewed from an economic standpoint. While the rate of infection due to biofilms in hip replacements, for example, is only about 1.5-5%, over 90,000 of these replacement surgeries are performed in the UK alone, and the cost of treating a biofilm-infected patient is anywhere from seven to ten times higher than the cost of the surgery itself (3). The cause behind these problems stem from the fact that, as discussed earlier, more and more strains of bacteria are resistant to a myriad of antibiotics, and this resistance leads to enhanced colonization and eventual biofilm formation. Also, even if the bacterial cells are susceptible to a certain antibiotic, those same cells present as a biofilm are often able to then confer resistance to that antibiotic, revealing the unique feature that biofilms are capable of displaying traits that individual cells within that biofilm are unable to express. While it is certainly important to understand how to effectively destroy these biofilms, perhaps more of a focus should be placed on training physicians so they will use powerful antibiotics only when absolutely necessary.
Because it is often impossible to completely prevent biofilm formation within a human host, health professionals are taking as many steps as possible to limit the morbidity and mortality caused by these bacterial communities. For instance, antibiotics are administered to patients after virtually all surgical implantations (3). It is often a patient’s own flora that can be responsible for a biofilm found on a prosthetic device, and the antibiotics serve to prevent any problems before they start. In addition to giving antibiotics, hospitals are careful to follow rigid aseptic techniques during surgical procedures (30). These practices have helped reduce the incidence of infection following prosthetic joint surgery.

Another technique, though used more frequently in people who already have infected artificial prostheses, is to mix antibiotic powder with the material that is used to make the actual prosthesis. While this idea could possibly be applied to all implant procedures, it is controversial, most likely as a result of the fact that biofilms are capable of acquiring resistance to many antibiotics much more easily than normal organisms. If a medical staff is unable to prevent biofilm formation, then the infection must be promptly treated. The most common treatment is removal of the device (3, 4); this method is the most reliable in making sure that the biofilm has the least chance of persisting in the patient.

While not used on a regular basis, some studies have demonstrated that passing a small DC current in vitro through a biofilm in the presence of an antibiotic eradicated the biofilm (26). The antibiotic showed little effect on the biofilm without the current. Because it is very difficult to treat biofilms, it is essential that prevention methods continue to be studied. One of the goals of my project was to characterize the genes and
functions that are responsible for biofilm formation by *A. baumannii*. If these genes can be isolated and characterized, it is possible that antimicrobial compounds that target the genetic sequences and/or their products can be made, leading to a successful prevention of biofilm formation.

**Biofilms and their resistance to antibiotics**

One unique feature of biofilms is that the cells integrated into the biofilm display traits they would not normally be able to express as single-celled organisms (1). One of the traits, a high degree of resistance to many antimicrobial compounds, is the biggest problem facing the medical community. One reason why bacteria in a biofilm can display such resistance is that the bacteria at the substratum layer receive much less oxygen and nutrients than the cells at the upper layers (1). Consequently, these organisms often slow down or completely stop their growth, making antibiotics such as penicillin ineffective, since many antimicrobials only kill growing cells.

The chemical makeup and thickness of the EPS itself physically prevents antimicrobials from reaching cells’ surfaces (42). Highly charged antimicrobials, such as tobramycin, or chemically reactive molecules, such as halogens, will be quenched within the EPS and never be able to penetrate the cells (1). In addition to the EPS, the physical location of the bacterial cells is important in understanding the increased resistance displayed by cells within a biofilm. Because biofilms are three-dimensional and are composed of many layers of cells, those cells at the bottom layer are may be exposed to subinhibitory concentrations of antibiotics for extended period of time. All of these factors are important to study as the medical community and microbiologists try to
prevent these biofilms that result in serious and infectious diseases from forming and persisting in infected patients.

*A. baumannii* and biofilms

*A. baumannii* certainly plays an important role in the biofilm crisis. Not only has it been shown that certain strains of this bacterium can effectively form a biofilm (45), but the added feature of already being resistant to many antibiotics even before biofilm formation complicates matters severely. Because *A. baumannii* is mostly associated with infections of the respiratory tract (41), this organism would be likely to form biofilms on ventilators and tracheal tubes. In one study (23) researchers found over 50 mg of dry weight bacteria in 30 of 40 tracheal tubes. While this experiment analyzed other gram-negative bacteria, *A. baumannii* should not be overlooked as an important bacterium of these infections that form on tracheal tubes and other implanted devices.

**Purpose of my Project**

The significance of my study was that I characterized some of the biofilm-related genes in *A. baumannii*, which will aid in the understanding of the biofilm formation process and its effects on the virulence of this important nosocomial pathogen. This project employed a technique called insertion mutagenesis to help characterize and describe *A. baumannii* genes involved in biofilm formation. It was based on the utilization of the EZ::TN™ R6K<sup>γ</sup>ori <KAN-2> Tnp Transposome™ System, a commercially available (Epicentre) DNA-protein complex (Fig. 1) that was electroporated into electrocompetent *A. baumannii* cells.
Transposomes are unique because they contain both the DNA transposon element and the transposase needed to mobilize a particular fragment of DNA (22). In the description that follows, insertion mutagenesis proved to be an effective method of characterizing genes responsible for biofilm formation by *A. baumannii*. Because the exact nucleotide sequence of the DNA transposon element was known, we were able to directly sequence out from the ends of the transposon to identify the gene whose interruption resulted in biofilm defects.

**Fig. 1. The EZ::TN™ R6K*yori* <KAN-2> Tnp Transposome™ System.** The transposase proteins (Tnpase) are the enzymes necessary to integrate the DNA transposon into the *A. baumannii* chromosome. The triangles represent inverted repeats that play a role in the integration of the transposon. The Km<sup>R</sup> region of the DNA confers resistance to kanamycin and was complementary to the *aph* probe used in this study. The R6Kγ origin of replication is unique because it is recognized by the Pi protein that was expressed by the *pir* gene present in the genome of the *E. coli* EC100D cells.

Transposition mutagenesis for *Acinetobacter* was first described in *Acinetobacter calcoaceticus* RAG-1 (28). This species is very similar to *A. baumannii*, and an “*A. calcoaceticus*-*A. baumannii* complex” is sometimes described in the literature (20). Even though *A. baumannii* is able to acquire DNA via conjugation (12), we found that this method was unsuccessful in introducing the transposon into the *A. baumannii* genome. Electroporation yielded much better results and was used throughout this work to
introduce the transposon into *A. baumannii*. A self-ligated plasmid obtained from the total DNA of *A. baumannii* insertion derivatives was then introduced into *E. coli* cells via transformation or electroporation.

**Materials and Methods**

**Bacterial strains and growth conditions.** The *A. baumannii* 19606 prototype strain was purchased from ATCC. The *E. coli* TransforMax EC100D (*pir*+) strain was obtained from Epicentre. Both *A. baumannii* and *E. coli* strains were maintained on either Luria-Bertani (LB) agar or broth (40), and kanamycin at the concentration of 40 µg/mL was added when appropriate. Immediately following electroporation, the *A. baumannii* and *E. coli* cells were suspended in SOC medium (40) and incubated in a rotary shaker at 200 rpm. *A. baumannii* cells that grew on LB agar with kanamycin were inoculated into LB broth contained in the wells of microtiter plates made of polystyrene.

**General DNA procedures.** Total DNA was isolated from the parental strain and biofilm-defective mutants using the Qiagen DNeasy kit or a mini chromosomal method (2). The DNA was digested with either *Eco*RI or *Nde*I using the manufacturer’s recommendations (New England Biolabs). The DNA was loaded into wells on an agarose gel, and the DNA was size-fractioned by electroporation (40), using *Hind*III-digested λ DNA as a size marker. Rescued DNA from insertion derivatives were sequenced using the ABI Prism 310 or 3100 instruments and the primers provided with the insertion mutagenesis kit as well as custom-designed primers. Nucleotide and amino acid sequences were analyzed with BLAST (http://www.ncbi.nlm.nih.gov). The custom-
designed primer pairs (5’-TACTGGTTTGGCCTATCC-3’) and (5’-CGTAAGGCTACTCATGTC-3’) were used for PCR amplification of the internal region of the csuE-like gene.

**Insertion mutagenesis, mutant screening and rescue of interrupted sequences.** The EZ::TN™ <R6Kγ/ori/KAN-2> Tnp Transposome™ was used during the electroporation, as suggested by the manufacturer (Epicentre). A 2510 Eppendorf electroporator at 2500 V and 2-mm-wide cuvettes were used during this study. Electrocompetent cells (100 µL) were electroporated using 1 µL of the EZ::TN™ <R6Kγ/ori/KAN-2> Tnp Transposome™, diluted with 1 mL SOC medium (40) immediately after the electroporation, and placed into a 5 mL polystyrene tube. After allowing the cells to shake for 1 h at 37°C in a 200-rpm rotary shaker, transposome-harboring clones were isolated on LB agar plates containing 40 µg/mL kanamycin. DNA from cells that did not form biofilm similar to the parental 19606 strain was rescued by self ligation of EcoRI- or Ndel-digested genomic DNA followed by electroporation of *E. coli* EC100D (pir⁺) electrocompetent cells.

**Biofilm assay.** Biofilm formation by *A. baumannii* was quantified using the microtiter plate assay described by Vuong et al. (46). Each microtiter well was filled with 200 µL of LB, inoculated with cells, and incubated at 37°C for approximately 18 h. After incubation, the culture medium was aspirated and the wells were rinsed with water. The wells were filled with 200 µL of a 1:4 (V:V) mixture of a 0.1% crystal violet:water stain and incubated for 5 minutes. After this time, the wells were rinsed with tap water,
dried, and analyzed for the absence of a purple band near the top of the well. The dark purple band indicated that the cells were successful in attaching to and forming a biofilm on the plastic surface. Possible biofilm-defective colonies were incubated in both polystyrene tubes and Petri dishes to confirm the deficient phenotype. After incubation, an 80%-20% ethanol-acetone solution was added to the polystyrene tubes containing the parental strain and potential mutant. This step allowed for the solubilization of the crystal violet retained by the cells attached to the sides of the tubes. Biofilm formation was quantified using a Beckman spectrophotometer. A reading to measure only the growth of the bacteria was taken at 600 nm and biofilm absorbance (following solubilization) was measured at 580 nm. An OD_{580} vs. OD_{600} ratio was constructed on a graph for the parental strain and each potential mutant. This graph was to demonstrate that the lack of biofilm formation was not due to the cells’ inability to grow in LB broth.

**Genetic complementation experiments.** The csuE gene from the parental strain was amplified using PCR techniques and was cloned into the shuttle vector pWH1266 to restore the biofilm phenotype of the parental strain. The complementing plasmid was electroporated into an insertion derivative that was unable to form biofilm on polystyrene. Following a stagnant incubation in LB broth at 37°C in a polystyrene tube, biofilm restoration was determined by the presence of a purple band around the edge of the tube.
Results and Discussion

After finding that a transposition mutagenesis system based on the delivery vector pLOFKm was unsuccessful in effectively generating colonies that were unable to form biofilms, we used the Epicentre transposome to investigate some of the genes responsible for biofilm formation by A. baumannii. Using replica-plating experiments, we found that, after electroporation of the transposome, about 2% of the A. baumannii 19606 colonies were able to grow on both LB agar plates without antibiotic pressure and LB agar plates containing 40 µg/mL of kanamycin.

Southern blot analysis using the aph probe revealed that, from 18 selected kanamycin-resistant derivatives, all contained the kanamycin resistance gene (present in the transposome), which could not be detected in the genome of the parental strain (Fig. 2). Most of the derivatives appeared to contain a single transposome insertion and some of them were mapped in different regions of the same gene (Table 1). These results indicate that, most of the time, the transposome inserted in different regions of the A. baumannii genome. Although the Bio144 insertion derivative was exclusively studied in this work, Table 1 shows that the generated insertion derivatives could be used to study genes responsible for functions in A. baumannii other than biofilm formation.

To identify insertion derivatives that were unable to form a biofilm, cells were first grown on LB agar with kanamycin after electroporation. The cells that grew were plated on a large grid and then inoculated into the wells of microtiter plates. Only one colony out of the 3,000 that were inoculated into the wells did not adhere to the plastic well in a way similar to the parental strain. The biofilm-defective phenotype of this derivative, which was named Bio144, was confirmed using Petri dishes (Fig. 3).
Table 1. Characterization of *A. baumannii* 19606 insertion derivatives

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Target site</th>
<th>Gene - function disrupted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GCCCTAAAA</td>
<td><em>dapA</em> - dihydrodipicolinate synthase</td>
</tr>
<tr>
<td>9</td>
<td>CGCGGATAC</td>
<td><em>trpD</em> - anthranilate phosphoribosyltransferase</td>
</tr>
<tr>
<td>10</td>
<td>GTTTATTCA</td>
<td><em>argF</em> - ornithine carbamoyltransferase</td>
</tr>
<tr>
<td>12</td>
<td>GGGCCATAC</td>
<td><em>argG</em> - argininosuccinate synthase</td>
</tr>
<tr>
<td>13</td>
<td>ATAGAATGG</td>
<td><em>aceE/aceA</em> - pyruvate dehydrogenase E1</td>
</tr>
<tr>
<td>15</td>
<td>AATGGAAAC</td>
<td><em>proA</em> - γ-glutamyl phosphate reductase</td>
</tr>
<tr>
<td>16</td>
<td>AATATACGT</td>
<td><em>cysL/nirA/sir</em> - sulfite reductase</td>
</tr>
<tr>
<td>19</td>
<td>CTACGATGC</td>
<td><em>trpE</em> - anthranilate synthase</td>
</tr>
<tr>
<td>23</td>
<td>CTATTCACA</td>
<td><em>trpE</em> - anthranilate synthase</td>
</tr>
<tr>
<td>26</td>
<td>CATGTGAAT</td>
<td><em>cysL/nirA/sir</em> - sulfite reductase</td>
</tr>
<tr>
<td>28</td>
<td>CATGTGAAT</td>
<td><em>cysL/nirA/sir</em> - sulfite reductase</td>
</tr>
<tr>
<td>31</td>
<td>CTGCAAACC</td>
<td><em>hisH</em> - glutamine amidotransferase</td>
</tr>
<tr>
<td>44</td>
<td>GTTTTACGT</td>
<td><em>cysL/nirA/sir</em> - sulfite reductase</td>
</tr>
<tr>
<td>144</td>
<td>GTCACAAAC</td>
<td><em>csuE</em> - chaperone/usher secretion system</td>
</tr>
</tbody>
</table>

*Potential genes and functions were predicted by BLASTp and BLASTx searches.*
Fig. 2. Southern blot analysis of the *A. baumannii* 19606 parental strain and EZ::TN <R6K*γori*/KAN-2> insertion derivatives. *Hind*III-digested λ DNA (lanes 1 and 22), *Eco*RI-digested total DNA isolated from the parental strain (lane 2) and insertion derivatives (lanes 3-20), and the *aph* amplicon (lane 21), were size-fractionated by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose and probed with λ DNA and the *aph* amplicon labeled with ³²P-dCTP.

In addition to qualitatively comparing the lack of biofilm formation of Bio144 to that of the parental strain, we wanted to confirm that the inability of Bio144 to establish a biofilm was not caused by its inability to grow in LB. Using a ratio that compared amount of biofilm the bacteria formed to the amount of bacterial growth, we observed that the 19606 parental strain had a much higher OD₅₈₀/OD₆₀₀ ratio than Bio144 (Fig. 4). These data were important because they demonstrated that Bio144’s inability to form a biofilm was not caused by a lack of or impaired growth in LB broth.
Fig. 3. Picture showing the difference between the biofilm phenotype of the parental strain 19606 and the Bio144 mutant. Each plate, containing LB broth, was inoculated with the appropriate colony and grown overnight at 37°C in stagnant conditions. The following day, each plate was stained with crystal violet for 5 minutes and then rinsed with water. The purple band indicated a biofilm had been established.

After sequencing out from the ends of the transposome into the interrupted gene and comparing the sequence data with the GenBank database using BLASTx as a tool, we determined that the interrupted gene encoded a protein that had a high degree of similarity to the CsuE protein of *Vibrio parahaemolyticus*. The *csuE* gene from *V. parahaemolyticus* is the last in the *csu* operon that is composed of the predicted polycistronic locus *csuABCDE*. The operon potentially codes for functions required for the secretion and assembly of bacterial pili responsible for adhesion in other gram-negative bacteria (43). Although CsuE most likely does not directly adhere to extracellular surfaces, its interruption could prevent molecules such as pili from being
assembled at the extracellular surfaces of cells. This claim was supported by the results obtained by electron microscopy analysis, which revealed many pili were clearly seen radiating from the surface a parental-strain cell, but no pili were seen at the surface of the Bio144 isogenic derivative (Fig. 5, Fig. 6).

These figures establish the importance of pili and their role in biofilm formation by *A. baumannii*. In order to form a biofilm, the bacteria must be able to adhere to some solid surface, and these pili, even if they are being synthesized within the cytoplasm, are not effective in attachment unless they are able to transverse the plasma membranes and radiate toward the extracellular environment after assembly.
Fig. 5. Electron micrograph of the 19606 parental strain of *A. baumannii*. The parental strain 19606 cells were grown in Tris M9 agar. Ammonium molibdate and was used to stain the cells.

Fig. 6. Electron micrograph of the Bio144 insertion derivative. The Bio144 insertion derivative cells were grown in Tris M9 agar. Ammonium molibdate and was used to stain the cells.
The important role of the *csuE*-like gene in biofilm formation was confirmed by complementation experiments using a derivative of the pWH1266 shuttle cloning vector. Using the sequence data from the Bio144 insertion derivative, we PCR amplified the *csuE* gene from the genome of the parental strain and cloned it into pWH1266. While electroporation of the plasmid by itself did not alter the phenotype of the Bio144 insertion derivative, electroporation of the pWH1266 derivative harboring the *csuE* gene restored the parental phenotype: the biofilm mutant designated as Bio144 was again able to fully adhere to the plastic polystyrene surface (Fig. 7).

In one study (44) that is discussed here to further stress the potential importance of pili in biofilm formation, researchers identified and characterized a protein in the chaperone-usher pathway of *P. aeruginosa* that was necessary for biofilm formation. This gene was, like the *csuE* gene described in *V. parahaemolyticus*, also part of a gene cluster, and the researchers designated it as *cupA* (with genes *cupA1*-*cupA5*). After establishing that the *P. aeruginosa* flagella and type IV pili played important roles in the biofilm formation of this organism, they introduced a mutation in the *cupA3* gene. This gene encodes the usher component, and it was discovered that fewer of the mutant bacteria were able to adhere to the well of a microtiter plate when compared with the parental strain (44). While *A. baumannii* is certainly a different organism from *P. aeruginosa*, these two bacteria have sometimes been compared with one another, and this study could offer new insight to the role of the chaperone-usher system in *A. baumannii*.

One gene of particular interest that we discovered within the *A. baumannii* genome was *gacS*. The GacS protein has been described in several organisms and acts as a sensor histidine kinase in a two-component regulatory system (21). While we found
Fig. 7. Genetic complementation of the \textit{A. baumannii} biofilm-deficient mutant \textbf{Bio144}. The 19606 parental strain (sample 2) and the Bio144 insertion derivative harboring no plasmid (sample 3), the shuttle vector pWH1266 (sample 4), or a derivative of the latter containing the parental \textit{csuE} gene (lane 5) were tested for attachment/biofilm formation after stagnant culture in LB broth. A tube containing sterile LB broth (sample 1) was the negative control. Tubes were stained with crystal violet to detect bacterial cells.

that a mutation in the \textit{gacS} gene did not prevent biofilm formation by \textit{A. baumannii} (data not shown), one study (38) reported that GacA, the response regulator of this two-component system, was essential for biofilm formation in \textit{P. aeruginosa}. As we continue to study \textit{A. baumannii} and its potential virulence factors, it will be very helpful if we are able to isolate and characterize the second component of this two-component regulatory system.
In conclusion, as we continue to attempt to identify more genes that are responsible for biofilm formation by *A. baumannii*, it is clear that the expression of several genes is necessary if the bacteria are able to bind to a surface and consequently create a bacterial community. While pili seem to be essential for biofilm formation by *A. baumannii*, it will also be important to understand if global regulatory molecules, such as GacA and GacS, play a role in the ability of *A. baumannii* to establish a biofilm. This study has shown that insertion mutagenesis is an effective method for generating phenotypic mutants and then identifying the gene(s) that are responsible for that phenotype. This study was significant because it helped characterize one of the potential virulence factors that is not as well understood in *A. baumannii* as in other bacterial pathogens.
References


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