Acinetobacter baumannii has emerged as a notorious multidrug-resistant pathogen, and development of novel control measures is of the utmost importance. Understanding the factors that play a role in drug resistance may contribute to the identification of novel therapeutic targets. Pili are essential for A. baumannii adherence to and biofilm formation on abiotic surfaces as well as virulence. In the present study, we found that biofilm formation was significantly induced in an imipenem-resistant (Imp\(^r\)) strain treated with a subinhibitory concentration of antibiotic compared to that in an untreated control and an imipenem-susceptible (Imp\(^s\)) isolate. Using microarray and quantitative PCR analyses, we observed that several genes responsible for the synthesis of type IV pili were significantly upregulated in the Imp\(^r\) but not in the Imp\(^s\) isolate. Notably, this finding is corroborated by an increase in the motility of the Imp\(^r\) strain. Our results suggest that the ability to overproduce colonization factors in response to imipenem treatment confers biological advantage to A. baumannii and may contribute to clinical success.

**MATERIALS AND METHODS**

Bacterial strains and MIC determination. AB08-5110S is an A. baumannii clinical isolate susceptible to imipenem (Imp\(^s\)). The imipenem-resistant strain AB08-5110R (Imp\(^r\)) was generated by exposing the Imp\(^s\) strain to 16 successive *in vitro* passages (1:1 dilution in equal proportions with fresh medium at 37°C and 180 rpm agitation) in Mueller-Hinton (MH) broth under subinhibitory concentrations of imipenem (16 mg/liter). The Imp\(^r\) strain was streaked onto heart infusion (HI) agar (Oxoid) after the 16th passage, and a single colony from each plate was subcultured at 37°C and 180 rpm agitation and was used to determine the MIC of imipenem using the broth dilution method. MICs were confirmed by two independent replicates of the Etest (Oxoid), and *Escherichia coli* ATCC 25922 was used as the control strain. The MIC results were interpreted according to 2010 CLSI guidelines. Finally, pulsed-field gel electrophoresis (PFGE) was employed for genetic profiling and for the confirmation of the two strains as noncontaminants, and bacterial strains were stored at −70°C in a brain heart infusion (BHI) medium containing 20% glycerol.

**Growth curves.** The growth kinetics of our strains were determined in the absence and in the presence of sub-MIC (16 and 1 mg/liter), MIC (32 and 2 mg/liter), and 2× MIC (64 and 4 mg/liter) of imipenem for Imp\(^r\) and Imp\(^s\) strains, respectively. Briefly, cultures in the stationary phase were used to inoculate 5-ml aliquots of MH broth to an initial optical density at 600 nm (OD\(_{600}\)) of 0.01. These cultures were then incubated at 37°C and 180 rpm agitation. When bacterial growth reached an OD\(_{600}\) of 0.35 (early log phase, chosen based on the literature), the cultures were...
supplemented with sub-MIC, MIC, and 2 × MIC of imipenem (Merck). Cultures were then incubated at 37°C under constant shaking, and their turbidity values (OD_{600}) were estimated every 30 min. This assay was repeated at least three times for each strain. GraphPad Prism was used for the determination of growth curves and statistical significance (t test).

**TEM analysis.** A. baumannii Impr^+ and Imp^+ strains were streaked onto HI agar and were grown overnight at 37°C. Three colonies from each plate were then inoculated separately into 10 ml of MH broth, and the cultures were incubated overnight at 37°C and 150 rpm agitation. Overnight cultures were used for a 1:100 inoculation (OD_{600} 0.05) and were incubated at 37°C and 180 rpm agitation until an OD_{600} of 0.35 was reached, and then impenem was added as previously mentioned. Bacterial cultures were further incubated for 3 to 4 h and were harvested for preparation prior to transmission electron microscopy (TEM). Untreated bacterial cultures were used as controls. For pilus observation, whole-cell mounts were prepared using noncentrifuged bacterial cells suspended in growth medium. One drop of the bacterial suspension was placed onto a 50-nm-thick Formvar film that was previously placed onto a copper index TEM grid (Marivac). The cell suspension was allowed to air dry, and after 5 to 10 min, the remaining solution was removed using filter paper. The samples were rinsed with 2 ml of HEPES buffer (pH 6.8) followed by 1 ml of saline (PBS) and then allowed to air dry. Biofilms were stained with crystal violet (1%, wt/vol), and optical density (OD_{590}) was measured using a microplate reader. Each assay was performed in triplicate. The following conditions were used as controls. For gene expression analyses, RNA was extracted using the TRI reagent (Molecular Research Center) according to the manufacturer’s protocol and was purified twice using an RNeasy Mini RNA isolation kit (Qiagen). RNA samples were evaluated for their purity and concentration using the Implen NanoPhotometer (Implen GmbH), and integrity was evaluated using microfluidic capillary electrophoresis (Agilent 2100 Bioanalyzer). Samples with an RNA integrity number (RIN) of ≥8 were used for microarray and quantitative reverse transcription-PCR (qRT-PCR) analyses. Subsequently, 8 × 15K custom genomic microarrays, representing each A. baumannii AYE coding sequence, were developed using the Agilent eArray package (Agilent Technologies). At least four 60-mer DNA oligonucleotides were incorporated into the chip design, and pilus gene expression was determined as part of the A. baumannii transcriptome. For cDNA synthesis and microarray hybridization, approximately 200 ng of each total RNA was polyadenylated and reverse transcribed using the T7 oligonucleotide (deoxyribosylthymine [dT]) primer. Following cDNA synthesis, the cRNA was transcribed and labeled using the Quick-Amp Labeling kit (Agilent Technologies) according to the manufacturer’s protocol. The labeled cRNA was then purified using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions and was fragmented and hybridized to the custom-designed 8 × 15K one-color gene expression microarray slide for 16 h at 65°C. After washing, slides were scanned using an Agilent G2565CA microarray scanner (Agilent Technologies) at a 5-μm scanning resolution. The microarray data were extracted using Feature Extraction software v10.1 and were normalized and analyzed using GeneSpring GX 10.0 analysis platform software (Agilent Technologies). Signal intensity values were adjusted to the minimal intensity (0.05), and data were normalized to the 50th percentile per chip. Differential gene expression was assessed using a volcano plot and was determined as a ≥2-fold change with a false discovery rate of 0.05. Unpaired t test analysis was used to determine statistical significance. Finally, quantitative RT-PCR was employed to confirm the expression levels of the type IV pilus (TFP) genes pilN, pilV, pilT, pilQ, pilM, pilW, pilP, pilI, pilO, pilL, and pilY and a type IV pilin protein gene (Table 1; see also Table S1 in the supplemental material). Bacterial cDNA was synthesized using 200 ng of total RNA, which was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Gene sequences were retrieved from GenBank, and

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**Table 1. A. baumannii Imp^+ gene dysregulation determined by microarray and confirmed by qPCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>ORF no. (ATCC 17978)</th>
<th>Fold change*</th>
<th>Biological role</th>
</tr>
</thead>
<tbody>
<tr>
<td>pilM</td>
<td>A1S_3195</td>
<td>2.9</td>
<td>Required for cell surface expression of type IV pili and associated with twitching motility</td>
</tr>
<tr>
<td>pilN</td>
<td>A1S_3194</td>
<td>3.9</td>
<td>Required for type IV pili adherence by promoting the functional display of PilC (the adhesion protein)</td>
</tr>
<tr>
<td>pilO</td>
<td>A1S_3193</td>
<td>2.6</td>
<td>Type IV pilin assembly protein required for twitching motility</td>
</tr>
<tr>
<td>pilV</td>
<td>AS1_3882</td>
<td>3.8</td>
<td>Type IV pilin assembly proteins required for twitching motility; plays an essential role in PilO stability</td>
</tr>
<tr>
<td>pilT</td>
<td>A1S_0897</td>
<td>3.8</td>
<td>Type IV pilus hybrid sense kinase/receptor regulator</td>
</tr>
<tr>
<td>pilQ</td>
<td>A1S_3191</td>
<td>3.2</td>
<td>Type IV fimbriae biogenesis protein tip-associated adhesin</td>
</tr>
<tr>
<td>pilW</td>
<td>A1S_3168</td>
<td>2.9</td>
<td>Type IV pilin structural subunit</td>
</tr>
<tr>
<td>pilP</td>
<td>A1S_3192</td>
<td>2.8</td>
<td>Regulator of twitching motility by controlling pilus extension and retraction</td>
</tr>
<tr>
<td>pilI</td>
<td>A1S_2812</td>
<td>2.7</td>
<td>Type IV fimbriae biogenesis protein tip-associated adhesin</td>
</tr>
<tr>
<td>pilL</td>
<td>A1S_2811</td>
<td>2.8</td>
<td>Type IV fimbriae biogenesis protein tip-associated adhesin</td>
</tr>
<tr>
<td>pilY</td>
<td>A1S_3167</td>
<td>2.1</td>
<td>Type IV pilin assembly proteins required for twitching motility; plays an essential role in PilO stability</td>
</tr>
<tr>
<td>pilO</td>
<td>A1S_3194</td>
<td>3.9</td>
<td>Type IV pilin structural subunit</td>
</tr>
<tr>
<td>pilN</td>
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<td>11.1</td>
<td>Type IV pilin structural subunit</td>
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<tr>
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<td>A1S_0897</td>
<td>5.6</td>
<td>Type IV pilus hybrid sense kinase/receptor regulator</td>
</tr>
<tr>
<td>pilQ</td>
<td>A1S_3191</td>
<td>5.7</td>
<td>Type IV fimbriae biogenesis protein tip-associated adhesin</td>
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<td>pilP</td>
<td>A1S_3192</td>
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<tr>
<td>Type IV pili</td>
<td>AS1_3883</td>
<td>2.7</td>
<td>Required for cell surface expression of type IV pili and associated with twitching motility</td>
</tr>
</tbody>
</table>

*Induced by a sub-MIC of imipenem (16 mg/liter).
Gene-specific primers were designed using the Primer Express software v3.0 (Applied Biosystems), which produces 50- to 150-bp amplicons. Primer pair efficiency was determined by carrying out RT-PCR on serial dilutions of cDNA, and valid pairs presented efficiency of amplification between 90% and 100% with a minimum $R^2$ of 0.98. A SYBR green PCR master mix was used following the manufacturer’s protocol (Applied Biosystems), and experiments were performed in triplicate using the StepOnePlus real-time PCR system (Applied Biosystems). A final volume of 20 μl was adopted for each reaction mixture, which contained 10 μl Power SYBR green master mix (250 nM for each primer), 7 μl nuclease-free water (NFW), and 5-ng cDNA template (0.05-ng template for 16S rRNA). The quantitative PCR (qPCR) thermal cycling parameters were as follows: 10 min at 95°C for polymerase activation followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of the amplicons was verified by melting curve analysis (60°C to 95°C) with a heating rate of 0.3°C per 15 s. The experiments were performed in triplicate. No-template and no-reverse transcription controls were included. The relative quantitation was determined by the $\Delta \Delta C_T$ method after normalizing it to the endogenous gene. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). A $P$ value of <0.05 was considered significant.

Motility assay. Twitching motility was investigated as previously described (25). Briefly, Imp$^R$ and Imp$^S$ overnight cultures were stab inoculated through 1% Mueller-Hinton (MH) agar plates and were incubated at 37°C overnight. Of note, cultures and plates were employed with and without a sub-MIC of imipenem, and twitching motility was measured at 10 mm around the site of inoculation, and assays were performed in triplicate. Motile strains were defined as those exhibiting a zone of >10 mm around the site of inoculation, and assays were performed in triplicate. Statistical analyses were performed using GraphPad Prism 6.

RESULTS

An imipenem-resistant Acinetobacter baumannii strain survives during antibiotic treatment. The MIC values of imipenem for Acinetobacter baumannii Imp$^R$ and Imp$^S$ strains were found to be 32 mg/liter and 2 mg/liter, respectively. In addition, in vitro subculturing of the two strains under different concentrations of the antibiotic (sub-MIC, MIC, and 2× MIC) revealed various growth rates, whereby the Acinetobacter baumannii Imp$^R$ strain sustained normal growth at a sub-MIC (0.5-fold) (Fig. 1A). Furthermore, transmission electron microscopy (TEM) demonstrated that only minor morphological changes occurred in Imp$^R$ under that concentration, which is contrary to the cell damage observed when the Imp$^S$ strain was exposed to the same condition (see Fig. S1 in the supplemental material).

Biofilms are complex structures, often polymicrobial communities constituted by proteins, polysaccharides, and other molecules. One of the main factors influencing biofilm formation is the synthesis of bacterial surface components and cell appendages (i.e., pili, flagella, adhesins) (26, 27). Different Acinetobacter pili were found to be essential for (i) biofilm formation on medically relevant abiotic surfaces, such as polystyrene (11), (ii) adherence to biotic and abiotic surfaces (28), and (iii) twitching motility (29). Therefore, considering the critical role of pilin in Acinetobacter biofilm formation and the fact that Imp$^R$ pilus synthesis is not affected by a subinhibitory concentration of imipenem, we decided to assess the impact of the antibiotic treatment on Imp$^R$ biofilm production. Interestingly, we found that its exposure to a sub-MIC of imipenem significantly induced biofilm synthesis compared to that in Imp$^S$ and uninduced Imp$^R$ isolates (Fig. 1B).

Pili gene expression is altered in response to a subinhibitory concentration of imipenem. The results of our biofilm formation assay and TEM analysis suggest that the Imp$^R$ strain is resistant to the cell-damaging effects caused by a subinhibitory concentration of imipenem, as would be expected from a resistant isolate. Accordingly, biofilms have been reported to prevent damage that directly kills bacterial cells (30). This was shown to be due, in part, to drug diffusion issues caused by altered biofilm architecture, as observed in resistant mutants and persister cells (31). Since biofilms are known to be dependent on pilus synthesis, we employed microarrays to investigate whether the expression of pilin-related genes would contribute to an explanation of our results. Microarray experiments were performed separately for each sub-MIC-treated Acinetobacter baumannii strain and were compared to those of the respective untreated controls. In the Imp$^R$ strain, 91 open reading frames (ORFs) were downregulated and 182 were upregulated, including 12 pilus-associated genes, one biofilm synthesis protein sequence (ABAYE1394, 2.77-fold), and an alginate biosynthesis regulatory protein gene (algR, ABAYE3509, 2.25-fold) (Table 1; see also Table S2 in the supplemental material). Of note, the AlgR response regulator is also required for the production of
type IV pili in _Pseudomonas aeruginosa_, and biofilms in alginate-overproducing isolates present a highly structured architecture that responds for resistance to antibiotics and to the immune response of cystic fibrosis patients (32). Among the 12 pilus genes upregulated by >2-fold in the Imp* strain are pilN, pilV, pilT, pilQ, pilM, pilW, pilP, pilJ, pilO, pilL, pilY, and a type IV pilin protein gene (Table 1), whereas the same sequences were unaffected in the Imp* strain (see Table S3 in the supplemental material). In addition, the overexpression of the pil genes by 3-fold or more was confirmed by quantitative RT-PCR (Fig. 2A). The gene expression results are in agreement with our microscopy data and also contribute to an explanation of the increased biofilm formation exhibited by the Imp* strain when treated with a sub-MIC of imipenem (Fig. 1B and 2A). Finally, a twitching motility assay was performed to confirm the influence of the treatment on the expression of the Imp* pilus-related genes. Note that twitching motility was chosen as it is a well-characterized trait of _A. baumannii_, which is powered by the extension and retraction of type IV pili. As expected, Imp* cells showed increased twitching motility at the agar-plate interface (above 10 mm), while it was inhibited in Imp strains (see also Fig. S2 in the supplemental material). These findings are corroborated by the reduced cell viability exhibited by the Imp* strain when exposed to a sub-MIC of imipenem (Fig. 1A). In addition, the elevated motility of the Imp* strain appears to be a consequence of the strain’s ability to survive treatment with a sub-MIC of imipenem (Fig. 1A and 2B), which correlates to the upregulation of the pilus-related genes (Fig. 2A) and other phenotypes (Fig. 1B; see also Fig. S1 in the supplemental material). Of note, motility was not affected among untreated strains grown on MH medium. In summary, the data presented herein demonstrate that our Imp* strain survives treatment with a sub-MIC of imipenem, a phenotype that correlates with increased biofilm synthesis, motility, and expression of pilus-associated genes.

**DISCUSSION**

_A. baumannii_ is recognized as a multidrug-resistant organism (1, 2), and an understanding of how environmental stress factors influence the expression of its virulence genes is expected to contribute to the development of novel directed therapies (5). Despite recent efforts, more studies are necessary to elucidate the correlation between _A. baumannii_ virulence and antibiotic resistance (33). In _A. baumannii_, pili are essential for attachment to and colonization of biotic and abiotic surfaces, biofilm formation, and infection (11–13, 29). Bacteria in biofilms may exhibit increased antibiotic resistance due to cooperation among multiple microbial species, which confers numerous advantages, such as passive resistance, enlarged gene pool with more efficient DNA sharing, quorum-sensing systems, and other synergies (27). Imipenem is one of the drugs most commonly used against _A. baumannii_ infections worldwide (1, 2, 34). Bearing this in mind, we attempted to contribute to the elucidation of the relationship between the synthesis of _A. baumannii_ colonization factors and resistance to imipenem. To achieve this, we assessed phenotype alteration and differential gene expression in imipenem-resistant and imipenem-susceptible strains that were induced by their exposure to a subinhibitory concentration of the antibiotic.

Growth kinetics shows that the _A. baumannii_ Imp* strain exhibited sustained growth when exposed to a sub-MIC (Fig. 1A) and a significantly extended lag phase in relation to the susceptible cells when treated with the MIC (Fig. 1A). Notably, a recent study found that exposure of uropathogenic _E. coli_ (UPEC) strains to sub-MICs of ciprofloxacin increased the expression of adhesive determinants, including peripherally located pili, compared to those of unexposed strains (35). Using TEM analysis, we observed that our _A. baumannii_ Imp* strain underwent significantly fewer morphological changes and retained most pili in response to treatment with a value of less than or equal to the MIC of imipenem. In contrast, Imp* cells grown under all treatments were severely damaged (see Fig. S1 in the supplemental material). In addition, it is known that exposure of MDR _A. baumannii_ to sub-MICs of imipenem increases biofilm formation and stimulates other virulence mechanisms (36). Likewise, sub-MICs of this antibiotic were found to boost the production of _P. aeruginosa_ biofilm and alginate (37). In fact, our microscopy and biofilm quantification results demonstrate a strong correlation between the maintenance of pili and increased biofilm formation when the Imp* strain is exposed to a subinhibitory concentration of imipenem. Taken together, our findings agree with the literature, suggesting that (i)
biofilm-producing organisms present an altered growth rate upon exposure to antibiotics (38) and (ii) pilus synthesis has a direct impact on biofilm formation (13, 39, 40), although Impt mutant strains remain to be assessed regarding their phenotype during imipenem treatment.

Different types of pili, including type IV pili (TFP), have been found in Gram-negative bacteria such as Pseudomonas aeruginosa, Neisseria gonorrhoeae, and A. baumannii (7, 8, 29, 41–43). In these pathogens, pili are known to mediate a range of functions, from binding to abiotic and biotic surfaces to participation in biofilm formation, twitching motility, virulence, and persistence in the environment. Furthermore, pili have been found to facilitate DNA uptake and transfer between bacterial cells (29, 44–46).

However, the role of pili in antibiotic resistance needs to be further investigated. Therefore, using transcriptomic approaches, we assessed the expression of pili genes in both Impt and Imp0 strains in response to supplementation of the cultures with a sub-MIC of imipenem. Microarray analysis showed significant upregulation of the TFP biogenesis and function (29, 41, 47–51). In fact, PilM, PilN, PilO, and PilP are required for assembly of the pilus subunits (PilA) into the pilus and constitute the core mechanism necessary for mechanical functioning (41). In the present study, their corresponding genes were upregulated between 2.6- and 3.9-fold upon treatment and were corroborated by quantitative RT-PCR (Table 1 and Fig. 2A). Therefore, we concluded that exposure of A. baumannii Impt to a sub-MIC of imipenem influenced its biology by enhancing the expression of genes critical for pilus synthesis and biofilm formation. Although the experiments performed do not allow us to directly confirm that A. baumannii colonization factors play a direct role in drug resistance, this statement may be true, as biofilms can act as physical barriers to antibiotic molecules (30, 31). Notably, A. baumannii comparative genomics showed that drug-resistant strains possess a higher catalytic capacity than sensitive ones due to the presence of virulence factors known to play a key role in infection, biofilm formation, iron uptake, quorum sensing, and gene expression (52). Furthermore, pilin-encoding genes (i.e., pil family) were found in antibiotic-resistant A. baumannii but are absent from the sensitive strain A. baumannii SDF (52, 53). To substantiate our molecular data, we performed twitching motility assays and observed that Impt strain motility was increased under exposure to a sub-MIC of imipenem. Our findings are in agreement with previous reports, whereby growth of bacterial pathogens under sub-MICs of tobramycin, tetracycline, norfloxacin, or cefodizime led to an increase in pilus synthesis, biofilm formation, motility, type III secretion system, and toxin production as a result of gene upregulation (54–56).

In addition, it is plausible that a subinhibitory concentration of imipenem also affects alternative stress response pathways, leading to the gene dysregulation that influenced biofilm formation and other noninvestigated mechanisms that contributed to the in situ survival of the Impt strain. This second hypothesis is supported by the upregulation of several transcriptional regulators (TetR, LysR, MerR, AlgR; 2.25- to 36.36-fold) and the downregulation of the ClpXP protease (2.7-fold), which is necessary for the stabilization of the Spx repressor and the transcriptional activator by protoxysis (32) (see Table S2 in the supplemental material). Finally, three genes corresponding to efflux systems were upregulated up to 3-fold when the Impt strain was grown under a sub-MIC of imipenem and may have participated in its survival.

Taken together, our findings help to explain the biology of an imipenem-resistant A. baumannii strain and suggest the possibility that multiple attributes may play a role in its survival during suboptimal chemotherapy. In addition, the molecular mechanism responsible for the overexpression of the aforementioned pilin genes is under investigation and is expected to shed light on the rational design of directed therapies against this troublesome pathogen. In summary, our results suggest that the pilus biosynthetic machinery may be an amenable target for the control of A. baumannii.

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G. N. Dhabaan, S. AbuBakar, and H. Hassan designed the experiments, and H. Hassan supervised the research. G. N. Dhabaan and G. M. Cerqueira wrote the paper. G. N. Dhabaan, S. AbuBakar, H. Hassan, M. Al-Haroni, and G. M. Cerqueira revised the paper. G. N. Dhabaan and S. P. Pang performed the experiments. G. N. Dhabaan did the data analysis. All authors read and approved the final manuscript.

The authors declare that they have no competing interest.

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