



Communication

# Phenotypic Heterogeneity in Resistance and Biofilm Formation Among Clinical *Acinetobacter baumannii* Isolates in Northwestern Mexico

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## Abstract

*Acinetobacter baumannii* is a major nosocomial pathogen characterized by multidrug resistance and persistence in hospital environments. This study evaluated antimicrobial susceptibility and biofilm-forming capacity in ten clinical *A. baumannii* isolates obtained from a tertiary care hospital in Northwestern Mexico to explore phenotypic traits associated with persistence. Minimum inhibitory and bactericidal concentrations were determined using broth microdilution according to Clinical and Laboratory Standards Institute guidelines. All isolates were resistant to ciprofloxacin, whereas variable resistance was observed for tobramycin (60%) and ceftazidime (20%); all strains remained susceptible to colistin. Bactericidal assays revealed heterogeneous responses across antibiotics. Biofilm formation, assessed by crystal violet staining, showed that most isolates produced weak to moderate biofilms, while three strains were non-producers. Significant variability in biofilm biomass was observed ( $p < 0.001$ ). Notably, no direct association was found between antimicrobial resistance profiles and biofilm-forming capacity, indicating phenotypic heterogeneity among isolates. These findings suggest that persistence-related traits in *A. baumannii* may not be solely predicted by resistance patterns, highlighting the complexity of adaptive responses under antimicrobial stress.

**Keywords:** *Acinetobacter baumannii*; antimicrobial resistance; biofilm formation; phenotypic heterogeneity; persistence



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## 1. Introduction

*Acinetobacter baumannii*, a Gram-negative opportunistic pathogen and a key member of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), is recognized by the World Health Organization as a priority for the development of novel therapies due to its notable persistence and multidrug resistance [1,2]. The pathogen can cause a range of infections, including sepsis, pneumonia, meningitis, urinary tract infections, and skin and soft tissue infections [3]. Rapid acquisition of resistance, environmental persistence, and high associated morbidity and mortality make *A. baumannii* a serious threat, particularly for critically ill patients in intensive care units [4,5].

With a highly adaptable genome, *A. baumannii* rapidly acquires multidrug resistance, including resistance to carbapenems and colistin [6], which enables survival under harsh conditions and promotes extensive colonization in hospitals [7]. Several virulence-associated traits contribute to its pathogenicity, including membrane-associated proteins, secretion systems, and iron acquisition mechanisms [8]. In addition, biofilm formation, mediated by pili, collagen adhesion, and the biofilm-associated protein encoded by the *bap* gene, enhances persistence, chronic infection, and antibiotic resistance, rendering many treatments ineffective and supporting survival and spread in healthcare settings. These features collectively reflect complex adaptive responses to environmental and antimicrobial stress conditions that favor long-term persistence [6,9,10].

Building on these mechanisms of persistence and virulence, the widespread formation of *A. baumannii* biofilms on medical devices has become a pressing international issue, fueling infection rates and antibiotic resistance [11]. In Mexico, previous studies have demonstrated that clinical *A. baumannii* isolates exhibit high levels of antimicrobial resistance, particularly to carbapenems, associated with the dissemination of OXA-type carbapenemases and successful international clonal lineages. Bocanegra-Ibarias et al. [12] reported the presence of carbapenem-resistant clones harboring *bla*OXA-58 and *bla*OXA-72 genes in a tertiary-care hospital in Monterrey, with persistent clones associated with biofilm production and resistance profiles. Similarly, genomic and epidemiological analyses have identified the circulation of international clones such as IC2, IC5, and IC7 in Mexican hospitals, carrying characteristic resistance determinants including *bla*OXA-66, *bla*OXA-65, and *bla*OXA-239 [13]. Although biofilm formation has been recognized as an important factor contributing to persistence, previous Mexican studies have reported variable associations between biofilm production and antimicrobial resistance. Bocanegra-Ibarias et al. [12] found that biofilm formation was associated with specific resistant clones, whereas other studies have shown that this relationship is not always consistent, suggesting that biofilm capacity and antimicrobial resistance may represent independent adaptive traits. Rosales-Reyes et al. [14] also reported the presence of multidrug-resistant *A. baumannii* isolates in Mexico and highlighted the relevance of resistance mechanisms and virulence-associated characteristics in hospital persistence.

Understanding the relationship between antimicrobial resistance and biofilm formation is essential to better define the mechanisms underlying persistence, therapeutic failure, and dissemination in hospital environments [15]. Despite the well-established roles of antimicrobial resistance and biofilm formation in *A. baumannii* persistence, the relationship between these phenotypic traits remains poorly understood [9]. In particular, it is unclear whether highly resistant strains exhibit enhanced biofilm-forming capacity as part of a coordinated adaptive strategy or whether these traits vary independently. Therefore, this study aimed to evaluate antimicrobial resistance and biofilm-forming capacity in clinical *A. baumannii* isolates to explore potential associations between these phenotypic traits and their contribution to bacterial persistence.

## 2. Results

### 2.1. Minimum Inhibitory Concentrations Revealed Multidrug Resistance in *A. baumannii*

To assess antibiotic susceptibility, broth microdilution assays were conducted. These assays determined the minimum inhibitory concentrations (MICs) of selected antibiotics (Table 1) according to CLSI guidelines, providing a precise characterization of resistance profiles and allowing the identification of strains with intermediate susceptibility. The MIC results confirmed resistance to ciprofloxacin in all ten *A. baumannii* strains and revealed subtle variations in aminoglycoside susceptibility. Notably, strain AbAK02 exhibited intermediate susceptibility to tobramycin (6.4 µg/mL), while strains AbAK03, AbAK04,

AbAK06, AbAK07, AbAK08, and AbAK09 showed MIC values of 16 µg/mL, indicating resistance to this aminoglycoside. In contrast, all strains remained susceptible to colistin, with MICs ranging from 0.125 to 0.25 µg/mL among those tested. Additionally, ceftazidime resistance (MIC > 32 µg/mL) was previously confirmed in strains AbAK01 and AbAK03 [16]. Overall, the MIC-based findings supported the classification of AbAK03, AbAK04, AbAK07, AbAK08, and AbAK09 as multidrug-resistant strains, consistent with disk diffusion results [17] and reinforcing the phenotypic confirmation of genomic resistance predictions.

**Table 1.** Minimum inhibitory concentrations of ten *A. baumannii* isolates from a public tertiary hospital.

| <i>Acinetobacter baumannii</i> Strain | Antibiotic MIC (µg/mL) <sup>a</sup> |                   |                   |               |                            |                               |                 |                     |
|---------------------------------------|-------------------------------------|-------------------|-------------------|---------------|----------------------------|-------------------------------|-----------------|---------------------|
|                                       | Colistin (CN)                       | Ceftriaxone (CRO) | Ceftazidime (CFZ) | Amikacin (AN) | Ampicillin-Sulbactam (SAM) | Piperacillin-Tazobactam (TZP) | Tobramycin (NN) | Ciprofloxacin (CIP) |
| AbAK01                                | - (S)                               | 6.4 (S)           | >32 (R)           | 6.4 (S)       | 4/0.4 (S)                  | 8/3.2 (S)                     | 0.1 (S)         | 4 (R)               |
| AbAK02                                | - (S)                               | 1.6 (S)           | 0.2 (S)           | 6.4 (S)       | 4/1.6 (S)                  | 4/0.8 (S)                     | 6.4 (I)         | 6.4 (R)             |
| AbAK03                                | 0.125 (S)                           | 6.4 (S)           | >32 (R)           | 64 (R)        | 4/1.6 (S)                  | 8/3.2 (S)                     | 16 (R)          | 4 (R)               |
| AbAK04                                | 0.125 (S)                           | 1.6 (S)           | 0.2 (S)           | 64 (R)        | 4/0.4 (S)                  | 8/4 (S)                       | 16 (R)          | 4 (R)               |
| AbAK05                                | - (S)                               | 3.2 (S)           | 4 (S)             | 0.1 (S)       | 4/1.6 (S)                  | 16/4 (S)                      | 0.125 (S)       | 6.4 (R)             |
| AbAK06                                | - (S)                               | 3.2 (S)           | 4 (S)             | 64 (R)        | 4/1.6 (S)                  | 16/4 (S)                      | 16 (R)          | 6.4 (R)             |
| AbAK07                                | 0.125 (S)                           | 8 (S)             | 4 (S)             | 64 (R)        | 4/1.6 (S)                  | 16/4 (S)                      | 16 (R)          | 6.4 (R)             |
| AbAK08                                | 0.125 (S)                           | 3.2 (S)           | 4 (S)             | 64 (R)        | 4/1.6 (S)                  | 4/1.6 (S)                     | 16 (R)          | 6.4 (R)             |
| AbAK09                                | 0.25 (S)                            | 6.4 (S)           | 1.6 (S)           | 64 (R)        | 8/6.4 (S)                  | 4/1.6 (S)                     | 16 (R)          | 6.4 (R)             |
| AbAK10                                | - (S)                               | 8 (S)             | 4 (S)             | 0.5 (S)       | 4/1.6 (S)                  | 4/1.6 (S)                     | 0.2 (S)         | 4 (R)               |
| References                            | This study                          | This study        | [16]              | This study    | This study                 | [16]                          | This study      | This study          |

<sup>a</sup> MIC, Minimum Inhibitory Concentration; I, intermediate susceptibility; R, resistant; S, susceptible.

## 2.2. Minimum Bactericidal Concentrations Revealed Heterogeneous Resistance in *A. baumannii*

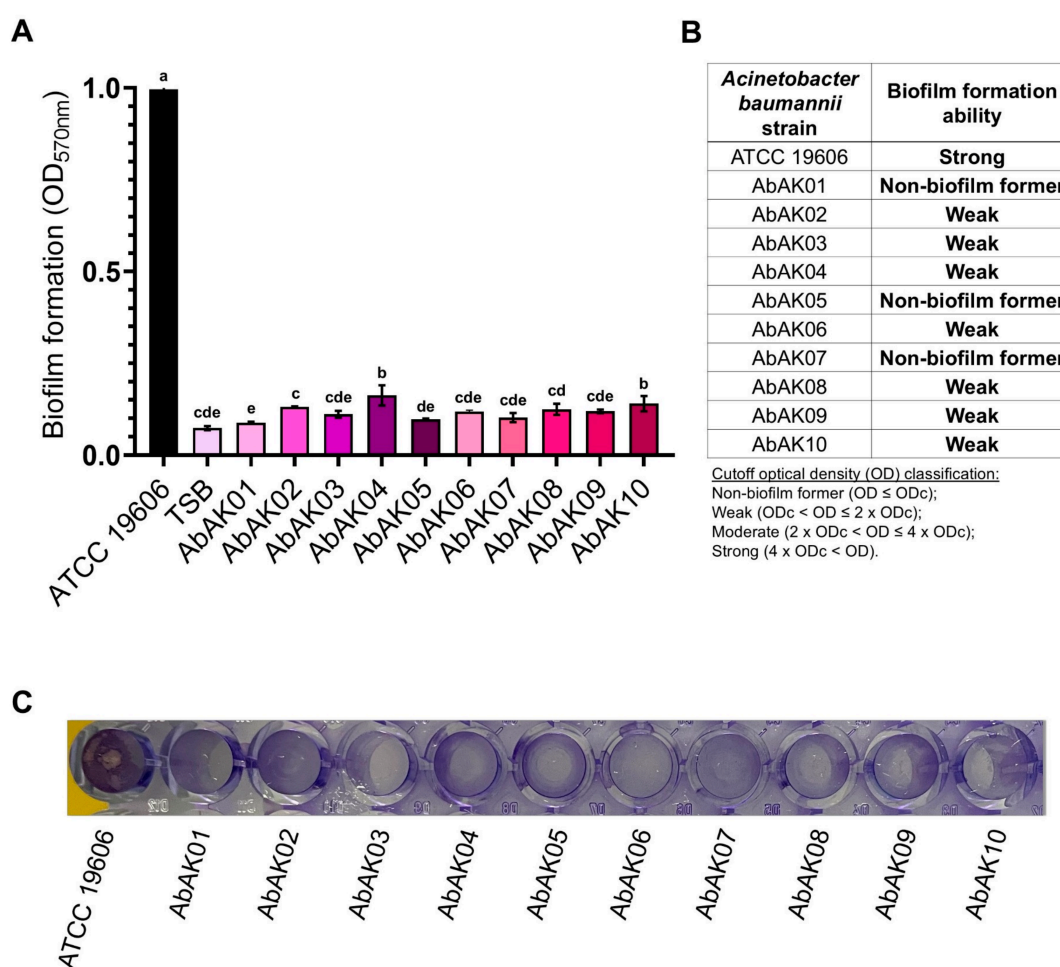
The minimum bactericidal concentrations (MBCs) of eight antibiotics were determined for ten clinical *A. baumannii* strains (Table 2). Colistin was active against a subset of strains, whereas ceftazidime showed limited bactericidal activity, with several strains exhibiting high-level resistance (MBC > 32 µg/mL). Aminoglycosides and β-lactam/β-lactamase inhibitor combinations displayed variable activity across strains, with some isolates showing intermediate susceptibility. Ciprofloxacin MBCs were consistently in the range of 4–6.4 µg/mL. These results revealed heterogeneous resistance profiles among the clinical isolates and highlighted the therapeutic challenges posed by multidrug-resistant *A. baumannii*.

## 2.3. Biofilm Formation by *A. baumannii* Clinical Isolates on an Abiotic Surface

The crystal violet assay used to measure biofilm biomass of the *A. baumannii* strains revealed that most isolates were capable of producing biofilms on an abiotic surface, while three strains did not form detectable biofilms (Figure 1A). A statistically significant difference was observed ( $p < 0.001$ ; Tables S1–S3). Strains AbAK04 and AbAK10 produced significantly higher biofilm levels than AbAK01 and AbAK05. The remaining strains, AbAK02, AbAK03, AbAK06, AbAK07, AbAK08, and AbAK09, displayed moderate biofilm formation, with differences among them not reaching statistical significance. Figure 1B illustrates the biofilm-forming ability of each strain, while Figure 1C shows the biofilm formed in the 96-well microtiter plate, where the intensity of crystal violet staining corresponded to the degree of adherence measured by optical density at 570 nm.

**Table 2.** Minimum bactericidal concentrations of ten *A. baumannii* isolates from a public tertiary hospital.

| <i>Acinetobacter baumannii</i> Strain | Antibiotic MBC (μg/mL) <sup>a</sup> |                   |                   |               |                            |                               |                 |                     |
|---------------------------------------|-------------------------------------|-------------------|-------------------|---------------|----------------------------|-------------------------------|-----------------|---------------------|
|                                       | Colistin (CN)                       | Ceftriaxone (CRO) | Ceftazidime (CFZ) | Amikacin (AN) | Ampicillin-Sulbactam (SAM) | Piperacillin-Tazobactam (TZP) | Tobramycin (NN) | Ciprofloxacin (CIP) |
| AbAK01                                | -                                   | 6.4               | >32               | 6.4           | 1.6/0.4                    | 4/1.6                         | 0.1             | 4                   |
| AbAK02                                | -                                   | 0.4               | 0.1               | 6.4           | 1.6/0.4                    | 3.2/0.4                       | 6.4             | 6.4                 |
| AbAK03                                | 0.125                               | 6.4               | >32               | 64            | 3.2/1.6                    | 4/1.6                         | 16              | 4                   |
| AbAK04                                | 0.125                               | 0.8               | 0.1               | 64            | 1.6/0.4                    | 4/1.6                         | 16              | 4                   |
| AbAK05                                | -                                   | 3.2               | 3.2               | 0.1           | 3.2/1.6                    | 16/4                          | 0.125           | 6.4                 |
| AbAK06                                | -                                   | 3.2               | 3.2               | 64            | 3.2/1.6                    | 16/4                          | 16              | 6.4                 |
| AbAK07                                | 0.125                               | 6.4               | 3.2               | 64            | 1.6/0.4                    | 16/4                          | 16              | 6.4                 |
| AbAK08                                | 0.125                               | 3.2               | 4                 | 64            | 1.6/0.4                    | 4/1.6                         | 16              | 6.4                 |
| AbAK09                                | 0.25                                | 6.4               | 1.6               | 64            | 6.4/1.6                    | 4/1.6                         | 16              | 6.4                 |
| AbAK10                                | -                                   | 6.4               | 3.2               | 0.5           | 3.2/1.6                    | 4/1.6                         | 0.1             | 4                   |
| References                            | This study                          | This study        | This study        | This study    | This study                 | This study                    | This study      | This study          |

<sup>a</sup> MBC, Minimum Bactericidal Concentration.**Figure 1.** Biofilm production by ten clinical *A. baumannii* strains. (A) Quantitative assessment of biofilm formation. Data represent the mean of three independent experiments ( $n = 3$ )  $\pm$  standard deviation (SD); error bars represent the SD. Different letters indicate statistically significant differences ( $p < 0.001$ ). Biofilm formation is expressed as a biofilm index relative to the reference strain *A. baumannii* ATCC 19606 (positive control). (B) Summary of biofilm-forming capacity among the clinical strains. (C) Representative crystal violet staining of biofilm formed in 96-well polystyrene microtiter plates.

### 3. Discussion

Antimicrobial resistance has become one of the most alarming global health threats, and *Acinetobacter baumannii* plays a major role in this crisis due to its remarkable ability to acquire and disseminate resistance determinants, especially in healthcare settings [1,18]. In this study, antibiotic resistance and biofilm-forming capacity were evaluated in ten clinical multidrug-resistant *A. baumannii* strains isolated from a tertiary care hospital in Northwestern Mexico [16], to provide relevant information that could guide clinical management and inform infection control strategies. Phenotypic antimicrobial resistance surveillance revealed a high prevalence of resistance to commonly used antibiotics, with all strains resistant to ciprofloxacin (100%), 60% resistant to tobramycin, and 20% to ceftazidime, while all remained susceptible to colistin. These resistance patterns are consistent with previous reports, including global analyses conducted between 2000 and 2016 that documented the widespread multidrug resistance of *A. baumannii* and its capacity to develop resistance to nearly all available antimicrobials [19].

Previous investigations conducted in Mexico have also demonstrated the circulation of multidrug-resistant *A. baumannii* in healthcare settings and have reported variations in antimicrobial susceptibility profiles among clinical isolates. Bocanegra-Ibarias et al. [12] characterized drug resistance patterns and clonal dynamics of *A. baumannii* isolates from a Mexican hospital, highlighting the dissemination of resistant clones and the contribution of antimicrobial resistance mechanisms to their persistence in clinical environments. Similarly, Rosales-Reyes et al. [14] reported resistance profiles and phenotypic characteristics of Mexican clinical isolates, emphasizing the variability of resistance and virulence-associated traits among circulating strains. These previous studies provide an important epidemiological context for the resistance patterns observed in the present study; however, differences in susceptibility profiles may reflect variations in hospital settings, antimicrobial selective pressure, geographic distribution, and the genetic background of the analyzed isolates.

The newly characterized strains (AbAK01–AbAK10) exhibited amikacin resistance patterns similar to those reported for nosocomial isolates in Mexico [20], including five pandrug-resistant and extensively drug-resistant strains (Aba/76 ISSSTE 2015, Aba/75 ISSSTE 2015, Aba/78 ISSSTE 2015, Aba/79 HCG 2016, and Aba/80 HGM 2016) collected from multiple hospitals across different regions [21]. Likewise, the colistin susceptibility observed in these isolates aligns with that previously described for *A. baumannii* strains 10324, 7835, and 9201 [22], highlighting the urgent need for continuous phenotypic and genomic monitoring to better understand emerging resistance trends.

The epidemiological diversity of *A. baumannii* circulating in Mexico has also been supported by genomic surveillance studies. Barrios-Camacho et al. [13] analyzed Mexican *A. baumannii* genomes and identified the predominance of international clonal lineages, including IC2 (CC208), IC5 (CC205), and IC7 (CC229), carrying characteristic resistance determinants such as OXA-type  $\beta$ -lactamases and additional antimicrobial resistance-associated genes. The identification of these successful lineages suggests that antimicrobial resistance dissemination in Mexico is not only driven by acquisition of individual resistance determinants but also by expansion of highly adapted clones capable of persistence and transmission in healthcare environments.

Consistent with these phenotypic resistance profiles, previous genomic analyses of these isolates identified several resistance determinants, including  $\beta$ -lactamase genes and aminoglycoside-modifying enzymes [23]. The resistance observed to ceftazidime and amikacin in several isolates was consistent with the presence of these genomic determinants, providing additional evidence of a genotype–phenotype association in the studied strains. Nevertheless, variability in susceptibility levels among isolates carrying similar resistance

determinants suggests that phenotypic resistance may also be influenced by regulatory, physiological, or gene-expression factors beyond gene presence alone.

Given the critical role of biofilm formation in infection persistence and therapeutic failure, the virulence traits contributing to hospital survival were assessed through the ability of *A. baumannii* to form biofilms on abiotic surfaces. While previous studies have reported that 60–90% of *A. baumannii* isolates are strong biofilm producers [24] and that approximately 98.5% of clinical isolates reported by Alcantar-Curiel et al., 2017 [20] formed biofilms on polystyrene, the degree of biofilm-forming capacity was not specified. In this analysis of ten clinical isolates, 70% exhibited only weak biofilm-forming ability, contributing to a better understanding of the phenotypic variability of this trait in clinical strains.

The differences observed between previous reports from Mexico and the present study highlight the heterogeneous nature of biofilm formation among clinical *A. baumannii* populations. Bocanegra-Ibarias et al. [12] evaluated Mexican clinical isolates and reported variability in biofilm production, including differences among strains with distinct antimicrobial resistance profiles. Similarly, Rosales-Reyes et al. [14] described biofilm-associated characteristics among Mexican *A. baumannii* isolates, supporting the concept that biofilm formation is a variable phenotype influenced by strain-specific factors. Therefore, the predominance of weak biofilm formation observed among the AbAK01–AbAK10 isolates does not necessarily indicate reduced persistence potential, but rather reflects the diversity of adaptive strategies present among circulating *A. baumannii* strains.

In a previous genomic study [23], several determinants associated with biofilm formation and persistence were identified in these isolates, including *bap*, *ompA*, *omp38*, the *pgaA-D* and *csuA/BABCDE* operons, and regulatory systems such as *bfmRS* and *abaR/abaI*. These determinants have been implicated in adhesion, biofilm development, and environmental persistence [1,25]. In particular, the *bap* gene, commonly linked to this process [25], was detected in nine of the ten genomes analyzed (AbAK01–AbAK09), while it was absent in strain AbAK10. However, it has been reported that the presence of the *bap* gene does not always correspond to robust biofilm formation, suggesting that other genetic elements, such as *ompA*, which acts as a porin and partially contributes to biofilm formation [9,26], may influence this trait independently or in coordination with *bap*. Additionally, differences between genomic predictions and experimentally measured biofilm formation may be influenced by methodological factors, including incubation conditions, nutrient availability, surface characteristics, and the criteria used for biofilm classification. These variables can affect the expression of biofilm-associated pathways and the amount of biomass detected in vitro.

This observation is consistent with previous findings from Mexican clinical isolates, in which the presence of biofilm-associated determinants did not always predict the intensity of biofilm formation. Such discrepancies between genotype and phenotype have also been described for other persistence-associated traits in *A. baumannii*, indicating that gene regulation, expression levels, and interactions among multiple pathways may determine the final biofilm phenotype rather than the presence of individual genes alone.

Beyond *bap* and *ompA*, the previous genomic analysis also identified additional determinants associated with biofilm development, surface colonization, and persistence, including type IV pilus-associated genes such as *pilA* and other pilus assembly components, genes involved in exopolysaccharide production, including the *pgaA-D* operon, and efflux-related determinants such as *adeC* [23]. Type IV pili contribute to initial attachment, surface motility, and biofilm maturation in *A. baumannii*, while the *pgaABCD* operon is involved in the synthesis of poly- $\beta$ -1,6-N-acetylglucosamine, an important component of the extracellular matrix [27,28]. Efflux systems, including AdeABC-related components, have also been associated with bacterial persistence and adaptation under antimicrobial

and environmental stress conditions. Although these determinants were detected among the analyzed genomes [13], their presence alone did not consistently predict the biofilm phenotype observed in this study. The variability observed among isolates carrying multiple biofilm-associated genes indicates that gene regulation, expression levels, and interactions among different pathways may play a critical role in determining biofilm formation capacity [29].

The observation that strains carrying several biofilm-associated determinants nevertheless formed weak or negligible biofilms highlights the complexity of the genotype–phenotype relationship underlying biofilm formation. This variability is consistent with the fact that biofilm formation is a multifactorial process governed by well-orchestrated mechanisms responding to various intrinsic and extrinsic factors, such as the physicochemical properties of surfaces, bacterial cell characteristics, and environmental conditions [30]. Furthermore, variation in experimental approaches, including biofilm incubation periods, growth media, and threshold values applied to categorize weak, moderate, or strong biofilm producers, may contribute to differences among studies and should be considered when comparing phenotypic results.

These observations underscore the need for further research to understand the mechanisms regulating biofilm formation in *A. baumannii*. The crystal violet assay used in this study is a widely applied quantitative approach for evaluating biofilm formation by measuring the total biomass attached to abiotic surfaces. However, this method does not differentiate between viable and non-viable cells and does not provide information regarding biofilm architecture, extracellular matrix organization, or structural heterogeneity [31,32]. Furthermore, the crystal violet assay does not allow the characterization of additional physicochemical properties that may influence biofilm development, such as cell surface hydrophobicity, which can be evaluated through approaches based on bacterial adhesion to hydrocarbons or surface wettability measurements, or extracellular polymeric substance (EPS) composition, including polysaccharide content, which can be assessed through biochemical quantification methods or specific staining approaches [33,34]. Therefore, complementary microscopy-based approaches, such as fluorescence microscopy or field-emission scanning electron microscopy (FESEM), would be valuable in future studies to further characterize the organization of biofilms formed by clinical *A. baumannii* isolates and to better understand the mechanisms contributing to the phenotypic variability observed in this study. Together with analyses of surface properties and EPS composition, these approaches could provide a more comprehensive understanding of the mechanisms contributing to the phenotypic variability observed among isolates.

Additionally, some limitations of this study should be considered. The analysis was performed using a limited number of clinical isolates ( $n = 10$ ) obtained from a single tertiary-care hospital, which may restrict the extrapolation of these findings to other healthcare settings or geographic regions. Although these isolates provide relevant information regarding antimicrobial resistance and biofilm-associated phenotypes in *A. baumannii*, future studies including a larger collection of isolates from multiple institutions will be necessary to further evaluate the diversity of these traits.

Collectively, these findings demonstrate that antimicrobial resistance and biofilm formation exhibit substantial phenotypic variability among clinical *A. baumannii* isolates. Furthermore, the discordance observed between the presence of resistance- and biofilm-associated determinants and the corresponding phenotypes highlights the complexity of genotype–phenotype relationships and suggests that persistence-related traits are influenced by factors beyond gene content alone.

## 4. Materials and Methods

### 4.1. Bacterial Strain and Growth Conditions

The ten *A. baumannii* strains AbAK01, AbAK02, AbAK03, AbAK04, AbAK05, AbAK06, AbAK07, AbAK08, AbAK09, and AbAK10 were generously provided by a tertiary care public hospital [Instituto Mexicano del Seguro Social (IMSS)] in Culiacán, Sinaloa, Mexico. The isolates were obtained in 2024 from hospitalized patients and were provided in anonymized form for research purposes. Due to institutional confidentiality policies, detailed clinical metadata, including the clinical specimen source, hospital admission information, and patient-related data, were not available to the investigators. Therefore, this study did not involve human subjects.

These isolates were previously subjected to whole-genome sequencing and comparative genomic analysis by Orozco-Ochoa et al. [23]. The genomic characterization identified antimicrobial resistance determinants, including intrinsic and acquired  $\beta$ -lactamase genes such as *bla*OXA-51-like and *bla*ADC, as well as the carbapenemase gene *bla*OXA-398 in selected isolates. It also revealed virulence-associated genes and biofilm-related determinants, including *bap*, *ompA*, the *csuA/BABCDE* operon, and *pgaABCD*. The genomic information generated in the previous study was used to contextualize the phenotypic antimicrobial susceptibility and biofilm formation analyses performed in the present study.

The strains were routinely incubated on tryptone soy agar (TSA) plates (Oxoid Ltd., Hants, UK) at 37 °C for 18–24 h.

### 4.2. Antimicrobial Susceptibility Profile

The broth microdilution method was used in triplicate to determine the minimum inhibitory concentrations (MICs) of the ten clinical *A. baumannii* strains against eight antibiotics, grouped into five classes: colistin (CT) [Lipopeptides]; ceftriaxone (CRO) and ceftazidime (CFZ) [Cephems]; ampicillin-sulbactam (SAM); piperacillin-tazobactam (TZP) [ $\beta$ -Lactams]; amikacin (AN) and tobramycin (NN) [Aminoglycosides]; and ciprofloxacin (CIP) [Quinolones and fluoroquinolones]. The procedure followed the Clinical and Laboratory Standards Institute (CLSI) guidelines [35], using *Escherichia coli* ATCC 25922 as the positive control strain. Bacterial counts were carefully adjusted so that each well contained  $5 \times 10^5$  CFU/mL for the MIC testing. CLSI breakpoints were applied to interpret MIC values as follows: colistin (resistant,  $\geq 4$   $\mu$ g/mL); ampicillin-sulbactam (susceptible:  $\leq 8/4$   $\mu$ g/mL; resistant,  $\geq 32/16$   $\mu$ g/mL); piperacillin-tazobactam (susceptible:  $\leq 16/4$   $\mu$ g/mL; resistant,  $\geq 128/4$   $\mu$ g/mL); ceftriaxone (susceptible:  $\leq 8$   $\mu$ g/mL; resistant,  $\geq 64$   $\mu$ g/mL); amikacin (susceptible:  $\leq 16$   $\mu$ g/mL; resistant,  $\geq 64$   $\mu$ g/mL); ceftazidime (susceptible:  $\leq 8$   $\mu$ g/mL; resistant,  $\geq 32$   $\mu$ g/mL); tobramycin (susceptible:  $\leq 4$   $\mu$ g/mL; resistant,  $\geq 16$   $\mu$ g/mL); and ciprofloxacin (susceptible:  $\leq 1$   $\mu$ g/mL; resistant,  $\geq 4$   $\mu$ g/mL).

The broth dilution method was used to determine the minimum bactericidal concentrations (MBCs) of the antimicrobials [35]; 100  $\mu$ L from wells showing no visible growth after 24 h incubation at 37 °C was plated onto TSA plates and incubated for 48 h at 37 °C. The MBC was defined as the lowest antimicrobial concentration at which the inoculum viability was reduced by  $\geq 99.9\%$  or no apparent growth was observed [36,37]. MBC assays were performed in triplicate for each strain and antimicrobial compound.

### 4.3. Phenotypic Assay for Biofilm Formation

Biofilm formation assays were performed as described by [38] with minor modifications. For inoculum preparation, the selected strains were seeded in 5 mL of TSB (Oxoid Ltd., Hants, UK) and incubated at 37 °C for 24 h. Subsequently,  $10^1$  and  $10^2$  dilutions were prepared in TSB. From the  $10^2$  dilution, 200  $\mu$ L was inoculated in triplicate into the wells of a 96-well flat-bottom polystyrene plate. The negative control wells contained 200  $\mu$ L of TSB,

and the positive control wells contained 200  $\mu\text{L}$  of *A. baumannii* ATCC 19606. The plate was incubated at 37 °C for 24 h to allow biofilm maturation. After incubation, the contents of the wells were discarded by inverting the plate, and the wells were washed three times with 300  $\mu\text{L}$  of sterile phosphate-buffered saline (PBS; pH 7.2). After washing, the plate was air-dried in an inverted position, and bacterial fixation was performed using 50  $\mu\text{L}$  of 95% ethanol per well for 30 min. The microtiter plate was then emptied by simple flicking and left to air-dry overnight in an inverted position at room temperature. The adherent biofilm layer formed in each microtiter plate well was stained with 150  $\mu\text{L}$  of 1% crystal violet (Sigma-Aldrich, Naucalpan de Juárez, Mexico) for 15 min. After staining, the stain was aspirated with a pipette. The dye bound to the cells was resolubilized by eluting from the adhered cells using 150  $\mu\text{L}$  of 33% glacial acetic acid (*v/v*) per well for 10 min at room temperature. The optical density (OD) was measured at 570 nm using a Synergy HT microplate reader (BioTek, Inc., Winooski, VT, USA). The results obtained were interpreted by establishing a cut-off value that distinguished biofilm-producing strains from non-biofilm-producing strains. The OD cut-off value (OD<sub>c</sub>) was defined as the mean OD of the blank plus three standard deviations (SDs). Based on this, the strains were classified as non-adherent ( $\text{OD} \leq \text{OD}_c$ ), weakly adherent ( $\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$ ), moderately adherent ( $2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$ ), and strongly adherent ( $4 \times \text{OD}_c < \text{OD}$ ).

#### 4.4. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, Inc., CA, USA) and Minitab® 19, Minitab Statistical Software (Minitab, LLC.). In the biofilm formation assay, quantitative values are reported as the mean  $\pm$  standard deviation. Significant differences were assessed through one-way analysis of variance (ANOVA) followed by Tukey's test, with statistical significance defined at  $p < 0.001$ .

## 5. Conclusions

The clinical *Acinetobacter baumannii* isolates analyzed in this study revealed substantial phenotypic variability in antimicrobial resistance and biofilm formation. The lack of correlation between these traits demonstrates that resistance profiles alone are insufficient to predict persistence-related behaviors in this pathogen. These findings highlight the importance of combining phenotypic characterization with genomic surveillance to better understand the adaptive strategies of clinical *A. baumannii* isolates and improve infection-control approaches.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jgbg1020015/s1>, Table S1: One-way ANOVA of biofilm formation among *A. baumannii* strains; Table S2: One-way ANOVA model summary for biofilm formation; Table S3: Multiple comparisons of biofilm formation among *A. baumannii* strains using Tukey's method (95% confidence).

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