Hindawi
International Journal of Microbiology
Volume 2024, Article ID 6959403, 14 pages
https://doi.org/10.1155/2024/6959403

Research Article

Pseudomonas aeruginosa Isolates from Water Samples of the Gulf of Mexico Show Similar Virulence Properties but Different Antibiotic Susceptibility Profiles than Clinical Isolates

Luis E. Romero-González,[1] **Luis F. Montelongo-Martínez**,[2] **Abigail González-Valdez**,[3] **Sara E. Quiroz-Morales**,[3] **Miguel Cocotl-Yañez**,[2] **Rafael Franco-Cendejas**,[4] **Gloria Soberón-Chávez**,[3] **Liliana Pardo-López**,[1] **and Víctor H. Bustamante**[1]

[1]*Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico*
[2]*Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México, Coyoacán, Mexico*
[3]*Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México, Coyoacán, Mexico*
[4]*Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra Ibarra," Ciudad de México, Mexico*

Correspondence should be addressed to Víctor H. Bustamante; victor.bustamante@ibt.unam.mx

Received 25 January 2024; Revised 14 March 2024; Accepted 27 April 2024; Published 16 May 2024

Academic Editor: Karl Drlica

Pseudomonas aeruginosa is an opportunistic pathogen found in a wide variety of environments, including soil, water, and habitats associated with animals, humans, and plants. From a One Health perspective, which recognizes the interconnectedness of human, animal, and environmental health, it is important to study the virulence characteristics and antibiotic susceptibility of environmental bacteria. In this study, we compared the virulence properties and the antibiotic resistance profiles of seven isolates collected from the Gulf of Mexico with those of seven clinical strains of *P. aeruginosa*. Our results indicate that the marine and clinical isolates tested exhibit similar virulence properties; they expressed different virulence factors and were able to kill *Galleria mellonella* larvae, an animal model commonly used to analyze the pathogenicity of many bacteria, including *P. aeruginosa*. In contrast, the clinical strains showed higher antibiotic resistance than the marine isolates. Consistently, the clinical strains exhibited a higher prevalence of class 1 integron, an indicator of anthropogenic impact, compared with the marine isolates. Thus, our results indicate that the *P. aeruginosa* marine strains analyzed in this study, isolated from the Gulf of Mexico, have similar virulence properties, but lower antibiotic resistance, than those from hospitals.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium ubiquitously distributed in the environment. It is an opportunistic pathogen that exhibits high genomic plasticity and metabolic versatility, enabling its survival in a wide range of habitats including soil and different aquatic ecosystems such as wastewater, freshwater, and the sea [1, 2]. *P. aeruginosa* is a major cause of hospital-acquired infections, is recognized as one of the most life-threatening bacteria, and has been designated as a priority pathogen by the World Health Organization (WHO) for the research and development of new antibiotics [3, 4].

P. aeruginosa infections have been reported in various organisms, including plants, insects, fishes, mammals, and humans [5–8]. This bacterium has the ability to induce both acute and chronic infections in humans, including hospital-acquired pneumonia, cystic fibrosis-related respiratory

infections, abdominal and urinary tract infections, and skin conditions such as folliculitis and external otitis [9]. Additionally, it is frequently associated with bacteremia, particularly in patients with severe burns or those with compromised immune system, such as patients with cancer or with acquired immune deficiency syndrome [10].

Numerous factors have been associated with the ability of *P. aeruginosa* to cause infections in hosts [11]. *P. aeruginosa* expresses various protein secretion systems, including a type three secretion system (T3SS), which is a syringe-like protein complex that translocates effector proteins/toxins from bacteria into the cytoplasm of host cells [12]. This T3SS secretes at least four effector proteins: ExoU, ExoS, ExoT, and ExoY; ExoS and ExoU are the most studied due to their clinical relevance [13]. ExoS-secreting strains induce cell apoptosis and actin cytoskeleton disruption [14], while ExoU secretion causes rapid cell integrity loss and lysis, promoting bacterial persistence and dissemination [15]. The production of ExoS and ExoU varies among *P. aeruginosa* strains and is mostly mutually exclusive, with strains expressing one of them, but not both [16]. Furthermore, the presence of these effectors correlates with the phylogenetic group of the *P. aeruginosa* strains; those from phylogroup 1 secrete ExoS, while those from phylogroup 2 secrete ExoU [17]. *P. aeruginosa* also expresses cell-associated components such as flagella, type IV pili, and lipopolysaccharide, as well as extracellular factors such as exopolysaccharides, rhamnolipids (RLs), pyocyanin, siderophores, and proteases [18, 19].

An additional virulence trait of *P. aeruginosa* is the ability to form biofilm. Biofilm formation depends on the production of a matrix of extracellular polymeric substances that maintain bacteria together [20]. Biofilm provides bacteria a survival strategy against temperature fluctuations, lack of nutrients, and the effect of antibiotics, thereby enhancing bacterial persistence on both living and nonliving surfaces [21, 22]. Additionally, quorum sensing (QS) is a pivotal factor in the virulence of *P. aeruginosa* [23]. QS is a bacterial regulatory system that coordinates the expression of multiple genes necessary for communal bacterial behavior; additionally, it controls virulence factors expression while promoting biofilm formation [24, 25].

The emergence of drug-resistant *P. aeruginosa* strains represents a global threat to human health. *P. aeruginosa* strains exhibit intrinsic resistance to several antibiotics, primarily due to the low permeability of the outer membrane and the expression of antibiotic efflux pumps [26]. Additionally, *P. aeruginosa* strains present acquired resistance to many antibiotics, which is mediated by the acquisition of genes encoding enzymes that inactivate antibiotics, as well as by mutations leading to overexpression of efflux pumps or to the modification of antibiotic target sites [3, 27].

P. aeruginosa strains present genomic plasticity, which reflects their adaptive nature [28]. However, a conservation of virulence-associated traits among clinical and environmental isolates of *P. aeruginosa* has been reported [29, 30]. A whole-genome comparison allows for the classification of *P. aeruginosa* strains into three major clades or groups [31]. Strains from group 1, including *P. aeruginosa* PAO1, are more common in both clinical and environmental niches than strains from groups 2 and 3, represented by *P. aeruginosa* PA14 and *P. aeruginosa* PA7, respectively [17, 32].

Most studies on the virulence and antibiotic resistance of *P. aeruginosa* have focused on clinical isolates. In the context of One Health, which emphasizes addressing health threats at the human-animal-environment interface, the study of environmental *P. aeruginosa* strains is essential [33]. Despite that *P. aeruginosa* strains have been isolated from the sea [34–38], there is limited information regarding their virulence and antibiotic resistance properties.

In this study, we analyzed different virulence phenotypes and the susceptibility to distinct antibiotics of *P. aeruginosa* strains isolated from the Gulf of Mexico (GoM), specifically comparing them to strains from hospital patients and clinical environments. As expected, we detected strains belonging to phylogroups 1 and 2 among the environmental and clinical isolates analyzed, and we found that the marine and clinical strains tested possess similar virulence traits. In contrast, the marine strains were more susceptible to antibiotics than the clinical strains, which correlates with the anthropogenic impact evaluated on these strains. Our study provides additional evidence showing the pathogenicity of environmental *P. aeruginosa* strains from the GoM. Furthermore, it illustrates the anthropogenic impact on the selection of antibiotic-resistant *P. aeruginosa* strains.

2. Materials and Methods

2.1. Bacterial Strains. Bacteria strains used in this study are listed in Table 1. *P. aeruginosa* marine isolates were previously recovered from seawater samples from the GoM and identified by the analysis of their 16S rRNA gene sequence [38, 39]. The *P. aeruginosa* isolates P1165, P1483, P1503, P1546, and P1547 were obtained from clinical samples in the Instituto Nacional de Rehabilitación (National Institute of Rehabilitation) Luis Guillermo Ibarra Ibarra in Mexico City and identified by a semiautomated system Vitek 2 Compact® (bioMereux Marcy l'Etoile, France). *P. aeruginosa* isolates P6103 and P3536 were obtained from clinical environments [40, 41]. Clinical isolates were confirmed as *P. aeruginosa* by phylogenetic analysis of their 16S rRNA gene using the EZBioCloud database [43] (Table 2). Additionally, all *P. aeruginosa* strains were phenotypically characterized by growth on cetrimide agar (Merck, Germany), a selective medium for where the production of pyocyanin and fluorescein pigments is observed.

2.2. Biofilm Formation. The biofilm-forming ability of *P. aeruginosa* strains was assessed using the crystal violet assay in polystyrene microtiter plates (Costar®, Corning Incorporated), as previously described [44]. Bacterial strains were cultured in lysogeny broth (LB) for 24 h at 37°C. Absorbance at 570 nm (OD_{570}) was measured using a microtiter plate reader (BioTek™ Epoch2™, San Diego, CA,

Table 1: Bacterial strains used in this study.

Strains	Relevant features[a]	Reference or source
P. aeruginosa GOM1	Isolated from seawater (55 m depth)	[38]
P. aeruginosa LP17	Isolated from seawater (55 m depth)	[39]
P. aeruginosa LP21	Isolated from seawater (55 m depth)	[39]
P. aeruginosa LP34	Isolated from seawater (1000 m depth)	[39]
P. aeruginosa LP35	Isolated from seawater (1000 m depth)	[39]
P. aeruginosa LP36	Isolated from seawater (1000 m depth)	[39]
P. aeruginosa LP89	Isolated from seawater (30 m depth)	[39]
P. aeruginosa P6103	Nosocomial isolate	[40]
P. aeruginosa P3536	Environmental hospital isolate	[41]
P. aeruginosa P1165	Isolated from blood culture	This study
P. aeruginosa P1483	Isolated from endotracheal aspirate	This study
P. aeruginosa P1503	Isolated from tendon tissue	This study
P. aeruginosa P1546	Isolated from infected wound	This study
P. aeruginosa P1547	Isolated from urine culture	This study
P. aeruginosa PAO1 (ATCC15692)	Reference strain	American-type culture collection (ATCC)
P. aeruginosa 27853	Reference strain	American-type culture collection (ATCC)
P. aeruginosa 9027	Reference strain	American-type culture collection (ATCC)
P. aeruginosa PA14	Reference strain	[42]
Escherichia coli 25922	Reference strain	American-type culture collection (ATCC)
Escherichia coli DH5α	Laboratory strain	Invitrogen

[a]Marine strains from the same depth were isolated from different water samples taken in the same station of the GoM.

Table 2: Identification of *P. aeruginosa* isolates by 16S rRNA gene sequence analysis.

Isolate	Bacteria with best match of 16S rRNA gene[a]	Query cover (%)	Identity (%)
P6103	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.37
P3536	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.87
P1165	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.93
P1483	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.65
P1503	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.01
P1546	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	97	99.29
P1547	*P. aeruginosa* strain JCM 5962 (BAMA01000316.1)	98	99.16

[a]Accesion number is indicated between parentheses.

USA). Experiments were conducted in triplicate. A cut-off OD value (ODc) was calculated as three standard deviations (SDs) above the mean value of absorbance at OD_{570} of the negative control: ODc = mean value of $OD5_{570}$ for negative control + (3 × SD of negative control). The final OD value (OD_f) was calculated as the mean value of absorbance at OD_{570} of the respective strain minus the ODc value: OD_f = mean value of OD_{570}–ODc value. Then, the bacterial strains were classified as previously described [44]. Strains with $OD_f \leq$ ODc were classified as no biofilm producer; ODc < $OD_f \leq 2 \times$ ODc were classified as weak biofilm producers; $2 \times$ ODc < $OD_f \leq 4 \times$ ODc were classified as moderate biofilm producers, while $OD_f > 4 \times$ ODc were classified as strong biofilm producers.

2.3. Pyocyanin and Elastase Production. Pyocyanin and elastase production was assessed in *P. aeruginosa* cultures grown in LB. The elastase activity was measured using the previously described Elastin-Congo Red assay [37]. Pyocyanin production was quantified from bacterial culture supernatants as previously described [45]. The pyocyanin concentration in $\mu g\,ml^{-1}$ was calculated by multiplying the absorbance value at 520 nm with pyocyanin-specific molar extinction coefficient (17.072).

2.4. Antibiotic Susceptibility. The antibiotic susceptibility of *P. aeruginosa* strains was assessed using the microdilution method in cation-adjusted Mueller–Hinton broth, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [46]. The tested antibiotics were ceftazidime (CAZ), piperacillin (PIP), meropenem (MER), norfloxacin (NOR), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IMI), and amikacin (AMI). Multidrug resistance (MDR) was defined as nonsusceptibility to at least one antibiotic from three or more antibiotic categories, as described before [47]. *P. aeruginosa* ATCC 27853 was utilized as control strain for antimicrobial susceptibility testing. Antibiotics were purchased from Sigma-Aldrich.

2.5. Pathogenicity Assays. The virulence of *P. aeruginosa* isolates was analyzed using the *Galleria mellonella* (wax moth) infection model, following the established protocols

[48]. Groups of ten *G. mellonella* larvae were inoculated with 10 µl of a bacterial suspension (1×10^2 CFU in 1X PBS) through the lower left proleg using an insulin syringe. The infected larvae were then incubated at 30°C without food and monitored at different times postinjection to record mortality. *P. aeruginosa* PAO1, a human clinical isolate, and the nonpathogenic *Escherichia coli* DH5*α* strain were used as positive and negative controls, respectively.

2.6. Rhamnolipids Production. Bacterial strains were first cultured overnight in LB and then subcultured in a PPGAS medium with aeration at 37°C for 24 h [49]. Supernatants were obtained by centrifugation at 14,000 rpm for 10 min. Rhamnolipids were detected in the supernatants using thin-layer chromatography (TLC) following the method described by Matsuyama [50].

2.7. Detection of Secreted ExoS and ExoU Toxins. *P. aeruginosa* cultures grown in LB supplemented with 20 mM Mg_2Cl + 5 mM EGTA, up to an OD_{600} 1.5, were used to detect ExoS and ExoU. Proteins from supernatants were precipitated with trichloroacetic acid and separated by 15% SDS-PAGE electrophoresis, transferred onto a nitrocellulose membrane, and probed by Western blot assays using anti-ExoS or anti-ExoU polyclonal antibodies, as reported previously [51]. Anti-GroEL polyclonal antibodies (Sigma) were used to detect the GroEL protein as a load control. Blots from membranes were developed with the HRP luminol Super Signal chemiluminescent substrate (Thermo Scientific).

2.8. Class 1 Integron Identification. Detection of the *intI1* gene was performed as previously described [52]. Purified genomic DNA was used as the template to amplify by PCR a fragment of *intI1* using the HS463a and HS464 primers (Table 3). PCRs were performed using GoTaq Flexi DNA Polymerase (Promega, Wisconsin, USA) according to the manufacturer's instructions. Amplification conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, 90 s extension at 72°C, and a final extension step at 72°C for 5 min.

2.9. Phylogroup Identification. PCR amplification of *exoS* and *PA14300* genes was used to identify *P. aeruginosa* phylogroups as proposed before [53]. PCRs were performed using GoTaq Flexi DNA Polymerase (Promega, Wisconsin, USA). Briefly, a 25 µl PCR mixture was prepared, containing 2 µl of purified genomic DNA, 5 µl of 5X Green GoTaq buffer, 1 µl of 4% DMSO, 2.5 µl of 25 mM MgCl2, 2 µl each oligonucleotide (1.0 µM), 0.4 µl of GoTaq enzyme (1.25 U), 1 µl of a dNTPs mix (10 µM each), and sterile water to reach the final volume. Amplification conditions included an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 75 s, with a final elongation step at 72°C for 5 min. The oligonucleotides used are shown in Table 3.

2.10. Statistical Analysis. Data are shown as mean ± standard deviation. Statistical analysis was carried out by using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA), with one-way ANOVA combined with Dunnett's multiple comparison test, or the log-rank test, as required. A P value of <0.05 was considered statistically significant.

3. Results

3.1. The Marine and Clinical P. aeruginosa Isolates Tested Show Similar Virulence in G. mellonella. The *G. mellonella* larvae are highly susceptible to *P. aeruginosa* infection, making them a suitable model for assessing the pathogenicity of this bacterium [54]. Thus, we evaluated the virulence of seven *P. aeruginosa* isolates from the GoM and, for comparison, seven clinical isolates of *P. aeruginosa* in *G. mellonella* larvae. All clinical isolates and five out of the seven marine isolates caused death of the 100% of the larvae at 44 h postinfection, a phenotype similar to that of the *P. aeruginosa* PAO1 strain used as a positive virulence control (Figures 1(a) and 1(b)). The virulence of the *P. aeruginosa* PAO1 strain has been previously demonstrated in *G. mellonella* and mice [55, 56]. The other two marine strains killed 100% of the larvae at 68 h postinfection (Figure 1(a)). As expected, injection with the nonvirulent strain *E. coli* DH5*α* did not induce larval mortality (Figure 1). These results indicate that, in overall terms, the marine and clinical isolates of *P. aeruginosa* tested show similar virulence in the *G. mellonella* larvae.

3.2. The Marine and Clinical P. aeruginosa Isolates Tested Form Biofilm. Biofilm formation has been reported as an important factor contributing to the virulence of *P. aeruginosa* [57]. We compared the capacity of the marine and clinical *P. aeruginosa* isolates to form biofilm in plastic microplates. Both the marine and the clinical isolates exhibited different levels of biofilm formation (strong, moderate, or weak); the seven marine isolates formed biofilm, whereas two out of the seven clinical isolates did not form biofilm (Figure 2). The *P. aeruginosa* PAO1 and *E. coli* DH5*α* strains, used as positive and negative controls of biofilm formation, respectively, presented the expected phenotype (Figure 2). These results show that the tested marine isolates can form biofilm like clinical isolates.

3.3. The Marine and Clinical P. aeruginosa Isolates Tested Express Factors Associated with Virulence. Several factors have been associated with the virulence of *P. aeruginosa*, including the ExoS and ExoU toxins, pyocyanin, elastase, and rhamnolipids [58, 59]. We determined the production of these factors in the marine and clinical *P. aeruginosa* strains assessed in our study. *P. aeruginosa* secretes the toxins ExoS and ExoU through a T3SS; ExoS is a dual function protein with GTPase-activating proteins and ADP-ribosyltransferase domains that disrupt the host actin cytoskeleton and impair cell-to-cell adhesion, whereas ExoU, found in cytotoxic *P. aeruginosa* strains, has a phospholipase activity and significantly contributes to infection severity [16, 60]. To evaluate

Table 3: Primers used in this work.

Primers	Sequence (5′ to 3′)	Target gene	Reference or source
For class 1 integron identification			
HS463a	CTGGATTTCGATCACGGCACG	*intI1*	[52]
HS464	ACATGCGTGTAAATCATCGTCG	*intI1*	[52]
For phylogroup classification			
exoS-Fwd	CAATCGCTTCAGCAGAGTC	*exoS*	This work
exoS-Rv	CAACTGGTCGGTGATTTCG	*exoS*	This work
PA14300-Fwd	GCGTTGATACTCAAGGCGTTTG	*PA14300*	This work
PA14300-Rv	GAGGGGGATGTCGGCAAG	*PA14300*	This work

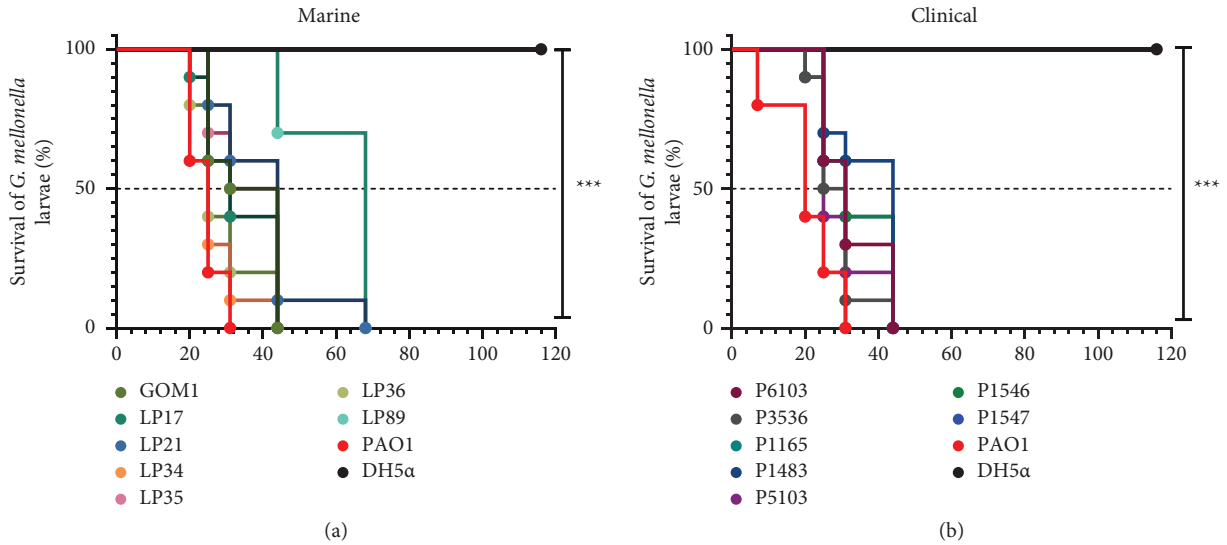

Figure 1: Marine and clinical *P. aeruginosa* isolates show similar virulence in *G. mellonella*. *G. mellonella* larvae were infected with 1×10^2 CFU of marine or clinical *P. aeruginosa* strains, and their survival was monitored for 5 days. Kaplan–Meier survival curves of infected larvae with marine (a) or clinical (b) strains were generated. Control groups included *P. aeruginosa* PAO1 (pathogenic) and *E. coli* DH5α (nonpathogenic). Larvae not injected or injected with 1X PBS did not kill any larvae (data not shown). *P* values were calculated using the log-rank test. ***, $P < 0.001$.

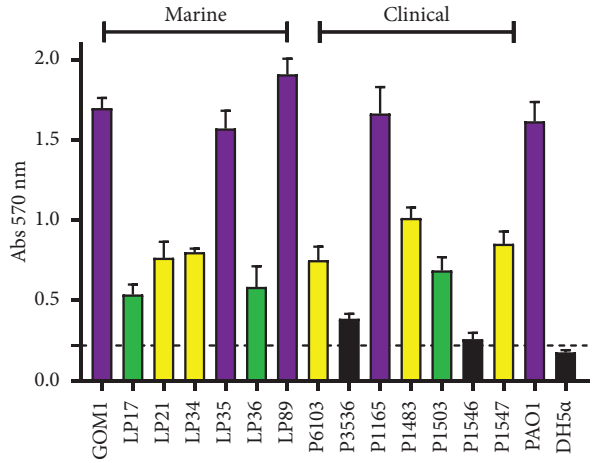

Figure 2: Marine and clinical *P. aeruginosa* isolates form biofilm. Biofilm formation was quantified using the crystal violet staining method. The strains were classified based on their biofilm-producing capacity: strong (purple), moderate (yellow), weak (green), or not biofilm forming (black), following the criteria established by Stepanović [44]. The *P. aeruginosa* PAO1 and *E. coli* DH5α strains were used as positive and negative controls of biofilm formation, respectively. The dotted line shows the cut-off value used for the classification of biofilm formation. The bars represent the average of three independent experiments ± SD.

the expression and secretion of ExoS and ExoU, we detected the presence of these proteins in the supernatant of bacterial cultures. Our findings demonstrated that six out of the seven marine isolates and four out of the seven clinical isolates secreted ExoS, a phenotype displayed by the *P. aeruginosa* PAO1 strain (Figure 3). Meanwhile, one marine strain and three clinical isolates secretes ExoU, a phenotype displayed by the *P. aeruginosa* PA14 strain (Figure 3). As expected, ExoS and ExoU toxins were not detected in the *P. aeruginosa* ATCC 9027 strain, which was included as a negative control (Figure 3). These results demonstrate that the marine and clinical isolates assessed can produce and secrete ExoS or ExoU toxins.

The genomic analysis of *P. aeruginosa* has revealed the presence of two main groups within its population structure [31]. These groups exhibit distinct production patterns of the effector proteins ExoS and ExoU [17]. To determine the phylogroup for the marine and clinical *P. aeruginosa* isolates tested in our study, we conducted a PCR analysis focused on the presence of the *exoS* and *PA14300* genes, which have been found to be significant in delineating the major groups 1 and 2 of *P. aeruginosa*, respectively [53]. The *exoS* gene was detected in six marine isolates and four clinical isolates, while the *PA14300* gene was detected in one marine isolate and three clinical isolates (Figure 4). As expected, the detection of the *exoS* or *PA14300* genes by PCR correlated with the detection of the ExoS or ExoU proteins by the Western blot (Figures 3 and 4). No amplification of the *exoS* or *PA14300* genes was obtained for the *P. chlororaphis* 9446 strain that was used as a negative control (Figure 4). Together, our analysis supports that, among the *P. aeruginosa* isolates tested, six marine and four clinical isolates belong to group 1, whereas one marine and three clinical isolates belong to group 2. These results are in agreement with previous studies showing the presence of phylogroups 1 and 2 among clinical and environmental isolates [17, 32].

Pyocyanin and elastase are additional virulence-associated factors synthesized by *P. aeruginosa* [61]. Pyocyanin, a phenazine molecule, is associated with the generation of reactive oxygen species, while elastase is a protease that facilitates bacterial colonization and leads to tissue damage in hosts [62, 63]. The production of these factors was detected and compared between the analyzed marine and clinical *P. aeruginosa* isolates. The *P. aeruginosa* PAO1 and *E. coli* DH5α strains were assessed as positive and negative controls, respectively. The marine and clinical isolates exhibited different levels of pyocyanin production; in general terms, the marine isolates showed higher levels of pyocyanin than the clinical isolates (Figure 5(a)). Five out of the seven marine isolates presented pyocyanin levels equal to or higher than the *P. aeruginosa* PAO1 strain, while only one clinical isolate showed pyocyanin levels higher than those shown by the *P. aeruginosa* PAO1; this last clinical isolate exhibited the highest pyocyanin production among all strains tested (Figure 5(a)). On the other hand, the marine isolates showed a higher elastase production than most of the clinical isolates; except for one marine isolate, the rest of the isolates tested presented lower levels of elastase than the *P. aeruginosa* PAO1 strain (Figure 5(b)). As expected, pyocyanin or elastase production was not observed in the *E. coli* DH5α strain (Figures 5(a) and 5(b)). Collectively, these results indicate that the analyzed marine isolates exhibit pyocyanin and elastase production levels comparable to or even higher than those shown by clinical isolates.

Extracellular virulence factors, including RLs, play a role in the virulence of *P. aeruginosa* [64]. RLs are amphiphilic glycolipids with detergent and solubilizing properties; *P. aeruginosa* produces both mono-RLs (containing 1 molecule of rhamnose) and di-RLs (containing 2 molecules of rhamnose) [65]. RL production was detected by TLC in the culture supernatants of the marine and clinical *P. aeruginosa* isolates studied. Purified mono- and di-RLs, and the *P. aeruginosa* PAO1 strain that produces both RL types, were assessed as positive controls. All the marine and clinical isolates, and the *P. aeruginosa* PAO1 strain, produced both mono- and di-RLs (Figure 6). These results indicate that the marine and clinical isolates tested produce mono- and di-RLs like the *P. aeruginosa* PAO1 strain.

3.4. The Marine Isolates Tested Show Low Antibiotic Resistance Compared to Clinical Isolates. Antibiotic resistance is a widespread feature in clinical bacteria, but it can also be present in environmental bacteria [66]. We analyzed the susceptibility of the marine and clinical isolates studied to antibiotics commonly used to treat *P. aeruginosa* infections (ceftazidime, piperacillin, meropenem, imipenem, norfloxacin, ciprofloxacin, gentamicin, and amikacin). As could be expected, most of the clinical isolates displayed resistance or intermediate resistance to most of the antibiotics tested; the P1165 isolate presented the profile with the lowest level of antibiotic resistance among the clinical isolates (Figure 7). In contrast, the marine isolates showed susceptibility or intermediate resistance to most of the antibiotics tested; the LP35 isolate presented the profile with the highest level of antibiotic resistance among the marine isolates (Figure 7). These results indicate that the marine isolates studied have low antibiotic resistance compared to clinical isolates; interestingly, three marine isolates presented resistance to piperacillin, meropenem, or imipenem.

3.5. Anthropogenic Impact on Marine and Clinical P. aeruginosa Isolates Tested. The impact of human activities on bacteria can be evaluated by the presence of class 1 integrons; these genetic elements mediate the recruitment and mobilization of antibiotic resistance genes, their abundance can change rapidly in response to selection pressure from pollutants such as biocides and antibiotics, and they can be found in both pathogenic and non-pathogenic bacteria [67, 68]. To analyze the anthropogenic impact on the marine and clinical *P. aeruginosa* isolates studied, we detected the *intI1* gene by PCR, which encodes the integrase of class 1 integrons [52, 69]. The *intI1* gene was found in one out of the seven marine isolates and in six out of the seven clinical isolates (Figure 8). As could be expected, these results reveal a higher anthropogenic impact on the clinical isolates than on the marine isolates tested. Interestingly, the marine isolate positive for *intI1* (LP35) had

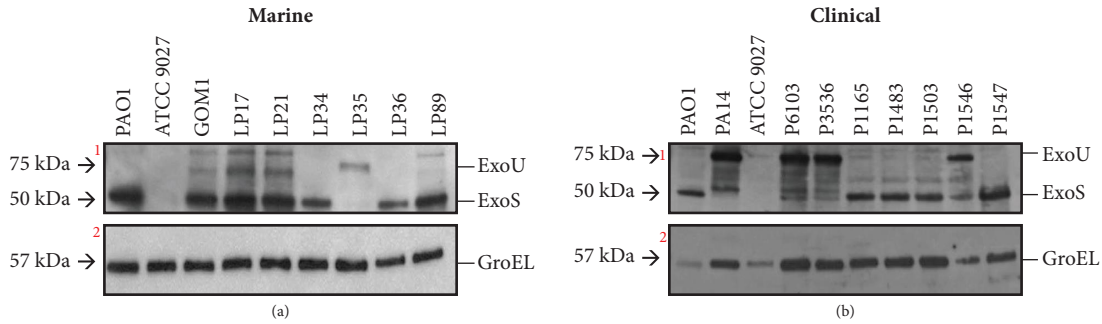

Figure 3: Detection of ExoS and ExoU toxins produced by *P. aeruginosa* isolates. The presence of ExoS or ExoU toxins in the culture supernatant of the marine (a) and clinical (b) *P. aeruginosa* strains was assessed by the Western blot using anti-ExoS and anti-ExoU polyclonal antibodies. As a load control, GroEL was detected by using anti-GroEL polyclonal antibodies. Positive controls for ExoS and ExoU were the *P. aeruginosa* PAO1 and PA14 strains, respectively, whereas the negative control was the *P. aeruginosa* ATCC9027 strain, which does not produce any of these toxins. Blots marked with numbers 1 and 2 were obtained with a mix of the anti-ExoS and anti-ExoU antibodies, and only with anti-GroEL antibodies, respectively.

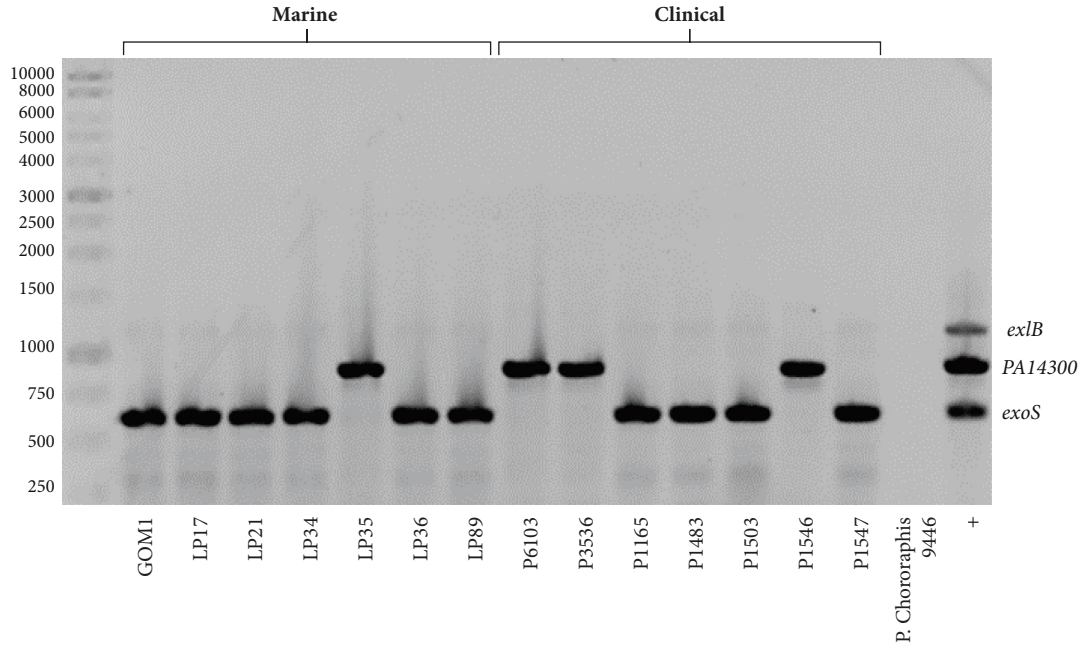

Figure 4: Identification of phylogroups in marine and clinical *P. aeruginosa* isolates. Amplification by PCR of *exoS* or *PA14300* genes in *P. aeruginosa* isolates. Strains from group 1 amplify a 621-bp fragment corresponding to the *exoS* gene, strains from group 2 amplify an 888-bp fragment corresponding to the *PA14300* gene. *P. chlororaphis* 9446 strain was used as a negative control in these assays. PCR products for *exoS* (group 1), *PA14300* (group 2), and *exlB* (group 3) genes are shown (lane +).

the highest antibiotic resistance profile among the marine isolates; conversely, the clinical isolate negative for *intI1* (P1165) had the lowest antibiotic resistance profile among the clinical isolates (Figures 7 and 8).

4. Discussion

P. aeruginosa is an opportunist pathogen ubiquitously distributed; thus, it is important to know if its presence in the environment can represent a risk for human health. In this study, we found that all tested *P. aeruginosa* isolates from the GoM exhibit virulence properties similar to those observed in clinical isolates.

We found that both the marine and clinical isolates of *P. aeruginosa* tested can kill *G. mellonella* larvae at similar infection doses and mortality rates. Previous studies have also shown that environmental strains of *P. aeruginosa* are virulent in the *G. mellonella* model [70, 71]. In addition, our results showed that the marine and clinical isolates of *P. aeruginosa* tested form biofilm at different levels. Biofilm serves as a resistance mechanism against environmental challenges, including antibiotics, and is an important virulence factor for tissue and medical device colonization [72, 73]. Biofilm is the major mode of bacteria life in the environment, including the marine habitats, enabling the formation of complex microbial

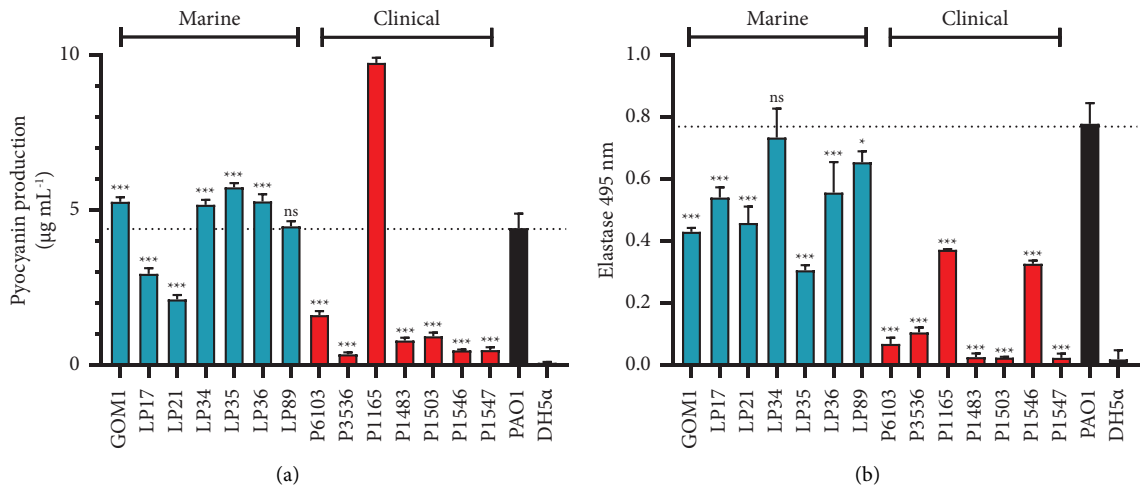

FIGURE 5: Pyocyanin and elastase production by marine and clinical *P. aeruginosa* isolates. Pyocyanin (a) and elastase (b) production was determined in the marine (blue bars) and clinical (red bars) *P. aeruginosa* isolates assessed. The *P. aeruginosa* PAO1 and *E. coli* DH5α strains (both indicated by black bars) were used as positive and negative controls, respectively. The bars represent the average of three independent experiments ± SD. *P* value was calculated using one-way ANOVA combined with Dunnett's test for comparison of the isolates with the *P. aeruginosa* PAO1 strain. ***, $P < 0.001$. ns: nonsignificant; *, $P < 0.05$.

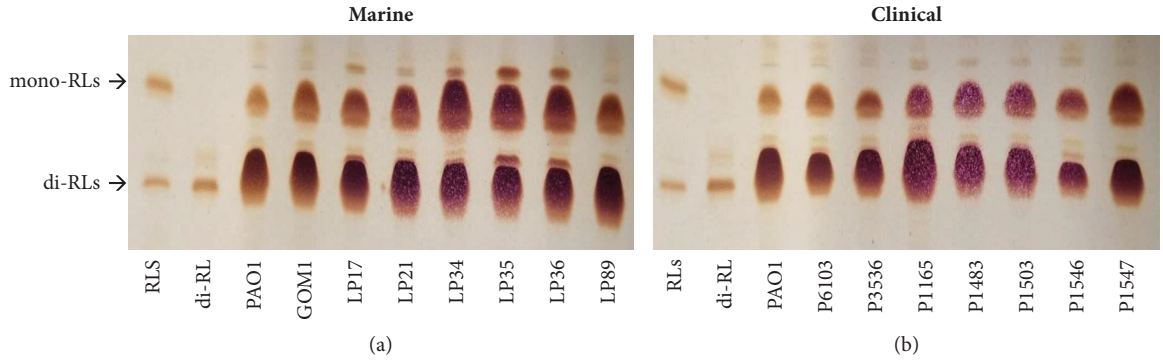

FIGURE 6: Rhamnolipid production by marine and clinical *P. aeruginosa* isolates. Production of RLs was determined by TLC in the culture supernatants of the marine (a) or clinical (b) *P. aeruginosa* isolates. As controls, a mixture of mono-RL and di-RLs, only di-RLs, and the *P. aeruginosa* PAO1 strain (produces both types of RLs) were assessed.

communities [74, 75]. Several studies have reported that marine strains of *P. aeruginosa* are capable of forming biofilm [76, 77]. Furthermore, our results indicate that the marine and clinical isolates of *P. aeruginosa* tested express factors related to the virulence of this bacterium (secretion of ExoS and ExoU toxins through the T3SS, as well as production of pyocyanin, elastase, and rhamnolipids). Even, the marine isolates tested produced higher levels of pyocyanin and elastase than the clinical isolates, supporting that these factors not only contribute to virulence but also have an ecological function, enabling the bacteria to thrive in diverse environments [78].

Of the seven marine isolates tested, six secreted ExoS toxin and only one isolate secreted ExoU, while of the seven clinical isolates tested, four secreted ExoS and three secreted ExoU. Consistent with these results, it has been reported that ExoS is more prevalent than ExoU in both environmental and clinical isolates of *P. aeruginosa* [79]. The secretion of the ExoS and ExoU effectors has a phylogenetic significance since the phylogroup 1 strains produce ExoS, while those of phylogroup 2 ExoU [17]. Our results also confirm these findings.

The secretion of toxins such as ExoS, ExoT, ExoU, and ExoY, through the T3SS, has been associated with increased virulence in *P. aeruginosa* [80]. The secretion of ExoS and ExoU toxins further indicates that both the marine and clinical *P. aeruginosa* isolates tested have a functional T3SS. The T3SSs are associated with virulence but are also found in nonpathogenic bacteria [81]. A previous genomic analysis identified T3SS genes in 109 different bacterial genera, including environmental bacteria unrelated to eukaryotic hosts, beneficial plant bacteria like rhizobia, and some *Pseudomonas* isolates [82].

As described above, the 14 marine and clinical *P. aeruginosa* isolates tested in our study have a functional T3SS. In agreement with our results, a previous whole-genome analysis showed a very low prevalence (1.5%) of

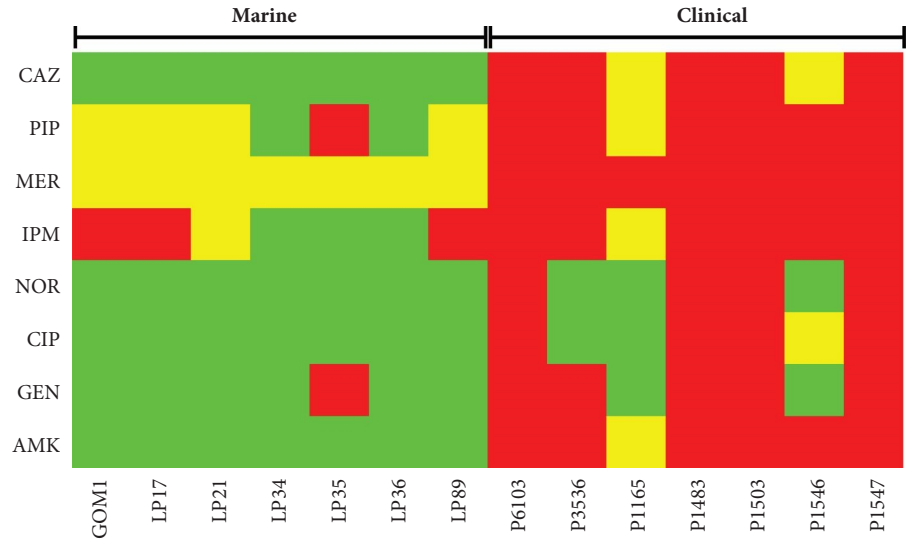

Figure 7: Antibiotic susceptibility of marine and clinical *P. aeruginosa* isolates. Heatmap of antibiotic susceptibility to selected antibiotics. Strains were classified as resistant (red), intermediate resistant (yellow), or susceptible (green) by determining the minimum inhibitory concentration (MIC) as indicated by CLSI guidelines. All tests were performed in triplicate. The tested antibiotics were ceftazidime (CAZ), piperacillin (PIP), meropenem (MER), norfloxacin (NOR), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IMI), and amikacin (AMI).

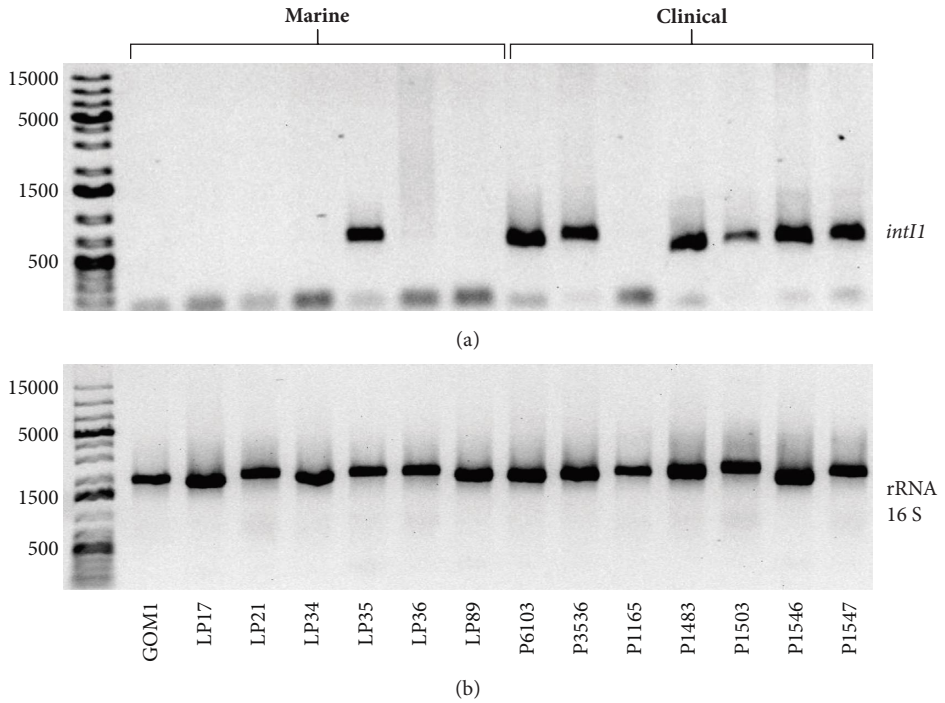

Figure 8: Detection of the *intI1* gene in marine and clinical *P. aeruginosa* isolates. Agarose gel electrophoresis of the PCR products of the *intI1* gene (a) and the rRNA 16S gene used as a positive control (b). The same results were obtained in three different assays.

P. aeruginosa strains lacking the T3SS and producing Exolysin A; interestingly, these PA7-like strains present a moderate virulence [83].

Different studies have reported the production of pyocyanin and elastase in environmental strains of *P. aeruginosa* [37, 84, 85]. Pyocyanin is a redox-active pigment that acts as an electron acceptor and has ecological functions related to signaling [86]. Elastase B is the predominant protease in the *P. aeruginosa* secretome, and its ecological function is related to nutrient acquisition through protein degradation [87]. On the other hand, the production of RLs has also been reported in marine strains of *P. aeruginosa* [37, 88–90] and contributes to various biofilm-related processes such as formation, maturation, dispersal, and bacterial propagation within the biofilm [91, 92].

In summary, our findings support previous research, indicating that all *P. aeruginosa* strains possess the potential to be pathogenic regardless of their ecological habitats, including marine environments [30].

In addition to their virulence properties, we also compared the antibiotic susceptibility of the analyzed marine and clinical isolates of *P. aeruginosa*. Evolution of antibiotic resistance is a complex process influenced by numerous factors. The primary driver of resistant bacteria is the selective pressure from human antibiotic usage, particularly in clinical environments [93]. The marine *P. aeruginosa* isolates we tested were more susceptible to antibiotics than clinical isolates, which is consistent with previous reports, indicating a lower prevalence of antibiotic resistance in environmental isolates than in clinical isolates of *P. aeruginosa* [94, 95]. However, some of the marine *P. aeruginosa* isolates we analyzed showed resistance to carbapenems (meropenem or imipenem). Previous studies indicate that the increased efflux system activity contributes to antibiotic resistance of environmental *P. aeruginosa* strains, including those from aquatic environments [96, 97]. Furthermore, previous research has identified genetic factors for antibiotic resistance in bacteria from pristine natural environments such as caves, deep water, and subsurface regions [98–101].

Antibiotic resistance arises mainly from antibiotic exposure caused by human activities. Class 1 integrons contribute to the acquisition and dissemination of antibiotic resistance genes and serve as markers to evaluate the anthropogenic impact in bacteria [67, 102]. Numerous studies have demonstrated a strong correlation between the prevalence of class 1 integrase *intI1* and the degree of anthropogenic impact and pollution in various environments [68, 103, 104]. We determined the presence of the class 1 integron in the isolates of *P. aeruginosa* in the study. As could be expected, the *intI1* gene was found in nearly all the clinical isolates (6 out of 7) and in only one of the seven marine isolates, indicating a greater anthropogenic influence in the clinical isolates than in the marine isolates tested. Notably, the marine isolate with the lowest antibiotic susceptibility, LP35, possessed the class 1 integron. The aquatic environment is recognized as a major reservoir and dissemination route for antibiotic resistance worldwide [105]. Several studies have documented the presence of class 1 integrons in antibiotic-resistant *P. aeruginosa* strains from various environments, including aquatic environments [106, 107]. Consistently, a previous study found a higher prevalence of class 1 integrons in clinical *P. aeruginosa* compared to environmental isolates [108].

5. Conclusions

Our study, together with previous reports, provides evidence supporting that *P. aeruginosa* marine isolates possess similar virulence properties but less antibiotic resistance than clinical strains. Additionally, as could be expected, our results show a lower presence of class 1 integrons, a marker of anthropogenic impact, in the *P. aeruginosa* marine isolates tested than in clinical strains.

Data Availability

All data supporting the findings of this study are available within the paper.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

L.E.R.-G. conducted the investigation, data collection, analysis, writing, review, editing, and wrote the first draft of the manuscript. L.F.M.-M, A.G.-V., and S.E.Q.-M. contributed to the methodology, investigation, data curation, review, and editing. M.C.-Y, G.S.-C, R.F.-C., and L.P.-L. contributed to the resources, project administration, writing, review, and editing. V.H.B. contributed to the conceptualization, funding acquisition, writing, review, editing, and supervision of the study. All the authors have read and approved the final version of the manuscript.

Acknowledgments

We thank Dr. Ulises Garza-Ramos for providing the *P. aeruginosa* isolates P6103 and P3536, Dr. Bertha González-Pedrajo for supplying anti-ExoS and anti-ExoU antibodies, and Dr. Adrian Ochoa for his valuable suggestions on the analysis of class 1 integrons. We also thank Dr. Deyanira Pérez-Morales for supporting during the early stages of this study and Biol. Javier Castañeda and Dr. Luis F. Muriel-Millán for technical assistance. L.E.R.-G. extends special thanks to M.C. Mariela Serrano-Gutiérrez for proofreading the manuscript. This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT)/México [PN 2017-01-5182] to V.H.B., CONACYT–Mexican Ministry of Energy-Hydrocarbon Trust [201441] to L.P.-L, and Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT)-DGAPA/UNAM to M.C.-Y. [IA200823] and G.S.-Ch. [IN201222]. L.E.R.-G. and S.E.Q.-M. are doctoral students of the Programa de Doctorado en Ciencias Bioquímicas, Universidad Nacional Autónoma de México (UNAM), and received a predoctoral fellowship from the CONACYT [818121, 1044595], respectively.

References

[1] S. Crone, M. Vives Flórez, L. Kvich et al., "The environmental occurrence of *Pseudomonas aeruginosa*," *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 128, no. 3, pp. 220–231, 2020.

[2] S. P. Diggle and M. Whiteley, "Microbe profile: *Pseudomonas aeruginosa*: opportunistic pathogen and lab rat," *Microbiology*, vol. 166, no. 1, pp. 30–33, 2020.

[3] J. Botelho, F. Grosso, and L. Peixe, "Antibiotic resistance in *Pseudomonas aeruginosa*– mechanisms, epidemiology and evolution," *Drug Resistance Updates*, vol. 44, Article ID 100640, 2019.

[4] G. Mancuso, A. Midiri, E. Gerace, and C. Biondo, "Bacterial Antibiotic Resistance: the most critical pathogens," *Pathogens*, vol. 10, p. 1310, 2021.

[5] G. Jander, L. G. Rahme, and F. M. Ausubel, "Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects," *Journal of Bacteriology*, vol. 182, no. 13, pp. 3843–3845, 2000.

[6] T. S. Walker, H. P. Bais, E. Déziel et al., "*Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, Biofilm formation, and root exudation," *Plant Physiology*, vol. 134, no. 1, pp. 320–331, 2004.

[7] D. J. Saikia, P. Chattopadhyay, G. Banerjee, B. Talukdar, and D. Sarma, "Identification and Pathogenicity of *Pseudomonas aeruginosa* DJ1990 on tail and fin rot disease in spotted snakehead," *Journal of the World Aquaculture Society*, vol. 49, no. 4, pp. 703–714, 2018.

[8] S. L. Gellatly and R. E. W. Hancock, "*Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses," *Pathogens and Disease*, vol. 67, no. 3, pp. 159–173, 2013.

[9] N. Sathe, P. Beech, L. Croft, C. Suphioglu, A. Kapat, and E. Athan, "*Pseudomonas aeruginosa*: infections and novel approaches to treatment "Knowing the enemy" the threat of *Pseudomonas aeruginosa* and exploring novel approaches to treatment," *Infectious Medicine*, vol. 2, no. 3, pp. 178–194, 2023.

[10] A. C. Pelegrin, M. Palmieri, C. Mirande et al., "*Pseudomonas aeruginosa*: a clinical and genomics update," *FEMS Microbiology Reviews*, vol. 45, no. 6, 2021.

[11] S. Qin, W. Xiao, C. Zhou et al., "*Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics," *Signal Transduction and Targeted Therapy*, vol. 7, pp. 199–227, 2022.

[12] R. Q. Notti and C. E. Stebbins, "The structure and function of type III secretion systems," *Microbiology Spectrum*, vol. 4, no. 1, 2016.

[13] A. R. Hauser, "The type III secretion system of *Pseudomonas aeruginosa*: infection by injection," *Nature Reviews Microbiology*, vol. 7, no. 9, pp. 654–665, 2009.

[14] A. Kaminski, K. H. Gupta, J. W. Goldufsky, H. W. Lee, V. Gupta, and S. H. Shafikhani, "*Pseudomonas aeruginosa* ExoS induces intrinsic apoptosis in target host cells in a manner that is dependent on its GAP domain activity," *Scientific Reports*, vol. 8, no. 1, Article ID 14047, 2018.

[15] K. S. Hardy, M. H. Tessmer, D. W. Frank, and J. P. Audia, "Perspectives on the *Pseudomonas aeruginosa* Type III secretion system Effector ExoU and its subversion of the host Innate immune response to infection," *Toxins*, vol. 13, no. 12, p. 880, 2021.

[16] G. Horna and J. Ruiz, "Type 3 secretion system of *Pseudomonas aeruginosa*," *Microbiological Research*, vol. 246, Article ID 126719, 2021.

[17] E. A. Ozer, E. Nnah, X. Didelot, R. J. Whitaker, and A. R. Hauser, "The Population Structure of *Pseudomonas aeruginosa* is characterized by genetic isolation of exoU+ and exoS+ Lineages," *Genome Biology and Evolution*, vol. 11, no. 7, pp. 1780–1796, 2019.

[18] A. Crousilles, E. Maunders, S. Bartlett et al., "Which microbial factors really are important in *Pseudomonas aeruginosa* infections?" *Future Microbiology*, vol. 10, no. 11, pp. 1825–1836, 2015.

[19] C. D. Morin, E. Déziel, J. Gauthier, R. C. Levesque, and G. W. Lau, "An organ system-based synopsis of *Pseudomonas aeruginosa* virulence," *Virulence*, vol. 12, no. 1, pp. 1469–1507, 2021.

[20] M. T. T. Thi, D. Wibowo, and B. H. A. Rehm, "*Pseudomonas aeruginosa* biofilms," *International Journal of Molecular Sciences*, vol. 21, no. 22, pp. 8671–8725, 2020.

[21] K. Lee and S. S. Yoon, "*Pseudomonas aeruginosa* Biofilm, a programmed bacterial life for fitness," *Journal of Microbiology and Biotechnology*, vol. 27, no. 6, pp. 1053–1064, 2017.

[22] C. Liao, X. Huang, Q. Wang, D. Yao, and W. Lu, "Virulence Factors of *Pseudomonas aeruginosa* and antivirulence strategies to combat its drug resistance," *Frontiers in Cellular and Infection Microbiology*, vol. 12, pp. 926758–926817, 2022.

[23] S. W. Miranda, K. L. Asfahl, A. A. Dandekar, and E. P. Greenberg, "*Pseudomonas aeruginosa* quorum sensing," *Advances in Experimental Medicine and Biology*, vol. 1386, pp. 95–115, 2022.

[24] T. Bjarnsholt and M. Givskov, "The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*," *Analytical and Bioanalytical Chemistry*, vol. 387, no. 2, pp. 409–414, 2007.

[25] S. D. Ahator and L. Zhang, "Small is mighty—chemical communication systems in *Pseudomonas aeruginosa*," *Annual Review of Microbiology*, vol. 73, no. 1, pp. 559–578, 2019.

[26] Z. Pang, R. Raudonis, B. R. Glick, T. J. Lin, and Z. Cheng, "Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies," *Biotechnology Advances*, vol. 37, no. 1, pp. 177–192, 2019.

[27] J. M. Munita and C. A. Arias, "Mechanisms of antibiotic resistance," *Microbiology Spectrum*, vol. 4, no. 2, pp. 1–37, 2016.

[28] T. de Sousa, M. Hébraud, M. L. N. E. Dapkevicius et al., "Genomic and metabolic characteristics of the pathogenicity in *Pseudomonas aeruginosa*," *International Journal of Molecular Sciences*, vol. 22, no. 23, Article ID 12892, 2021.

[29] M. C. Wolfgang, B. R. Kulasekara, X. Liang et al., "Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*," *Proceedings of the National Academy of Sciences*, vol. 100, no. 14, pp. 8484–8489, 2003.

[30] M. V. Grosso-Becerra, C. Santos-Medellín, A. González-Valdez et al., "*Pseudomonas aeruginosa* clinical and environmental isolates constitute a single population with high phenotypic diversity," *BMC Genomics*, vol. 15, no. 1, 2014.

[31] L. Freschi, J. Jeukens, I. Kukavica-Ibrulj et al., "Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium," *Frontiers in Microbiology*, vol. 6, 2015.

[32] L. Freschi, A. T. Vincent, J. Jeukens et al., "The *Pseudomonas aeruginosa* Pan-Genome provides new insights on its population structure, horizontal gene transfer, and pathogenicity," *Genome Biology and Evolution*, vol. 11, no. 1, pp. 109–120, 2019.

[33] A. A. Cunningham, P. Daszak, and J. L. N. Wood, "One Health, emerging infectious diseases and wildlife: two decades of progress?" *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 372, no. 1725, Article ID 20160167, 2017.

[34] A. V. Manwar, S. R. Khandelwal, B. L. Chaudhari, J. M. Meyer, and S. B. Chincholkar, "Siderophore production by a marine *Pseudomonas aeruginosa* and its antagonistic action against phytopathogenic fungi," *Applied Biochemistry and Biotechnology*, vol. 118, no. 1-3, pp. 243–252, 2004.

[35] N. Kimata, T. Nishino, S. Suzuki, and K. Kogure, "*Pseudomonas aeruginosa* isolated from marine environments in Tokyo Bay," *Microbial Ecology*, vol. 47, no. 1, pp. 41–47, 2004.

[36] N. H. Khan, M. Ahsan, W. D. Taylor, and K. Kogure, "Culturability and survival of marine, freshwater and clinical *Pseudomonas aeruginosa*," *Microbes and Environments*, vol. 25, no. 4, pp. 266–274, 2010.

[37] M. Cocotl-Yañez, M. P. Soto-Aceves, A. González-Valdez, L. Servín-González, and G. Soberón-Chávez, "Virulence factors regulation by the quorum-sensing and Rsm systems in the marine strain *Pseudomonas aeruginosa* ID4365, a natural mutant in lasR," *FEMS Microbiology Letters*, vol. 367, no. 12, 2020.

[38] L. F. Muriel-Millán, J. L. Rodríguez-Mejía, E. E. Godoy-Lozano et al., "Functional and genomic characterization of a *pseudomonas aeruginosa* strain isolated from the south-western gulf of Mexico reveals an enhanced adaptation for long-chain alkane degradation," *Frontiers in Marine Science*, vol. 6, pp. 1–15, 2019.

[39] L. E. Romero-González, J. Rojas-Vargas, L. F. Muriel-Millán, J. Bustos-Martínez, V. H. Bustamante, and L. Pardo-López, "Genomic and phenotypic characterization of *Pseudomonas* sp. GOM7, a novel marine bacterial species with antimicrobial activity against multidrug-resistant *Staphylococcus aureus*," *PLoS One*, vol. 18, no. 7, Article ID e0288504, 2023.

[40] U. Garza-Ramos, H. Barrios, F. Reyna-Flores et al., "Widespread of ESBL- and carbapenemase GES-type genes on carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates: a multicenter study in Mexican hospitals," *Diagnostic Microbiology and Infectious Disease*, vol. 81, no. 2, pp. 135–137, 2015.

[41] U. Garza-Ramos, P. Tinoco, T. Rojas et al., "Molecular characterization of integron class 1 (In196) encoding the VIM-2 metallo-β-lactamase of *Pseudomonas aeruginosa* isolated from a hospital environment," *Journal of Chemotherapy*, vol. 21, no. 5, pp. 590-591, 2009.

[42] L. G. Rahme, E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel, "Common virulence factors for bacterial pathogenicity in plants and animals," *Science*, vol. 268, no. 5219, pp. 1899–1902, 1995.

[43] S. H. Yoon, S.-M. Ha, S. Kwon et al., "Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies," *International Journal of Systematic and Evolutionary Microbiology*, vol. 67, no. 5, pp. 1613–1617, 2017.

[44] S. Stepanović, D. Vuković, V. Hola et al., "Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*," *Apmis*, vol. 115, no. 8, pp. 891–899, 2007.

[45] D. W. Essar, L. Eberly, A. Hadero, and I. P. Crawford, "Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthase and evolutionary implications," *Journal of Bacteriology*, vol. 172, no. 2, pp. 884–900, 1990.

[46] Clsi, *Performance Standards for Antimicrobial Susceptibility Testing*, CLSI, Wayne, PA, USA, 30th edition, 2020.

[47] A.-P. Magiorakos, A. Srinivasan, R. B. Carey et al., "Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance," *Clinical Microbiology and Infection*, vol. 18, no. 3, pp. 268–281, 2012.

[48] J. M. S. Loh, N. Adenwalla, S. Wiles, and T. Proft, "*Galleria mellonella* larvae as an infection model for group A *streptococcus*," *Virulence*, vol. 4, no. 5, pp. 419–428, 2013.

[49] Y. Zhang and R. M. Miller, "Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant)," *Applied and Environmental Microbiology*, vol. 58, no. 10, pp. 3276–3282, 1992.

[50] T. Matsuyama, M. Sogawa, and I. Yano, "Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents," *Applied and Environmental Microbiology*, vol. 53, no. 5, pp. 1186–1188, 1987.

[51] M. P. Soto-Aceves, M. Cocotl-Yañez, E. Merino et al., "Inactivation of the quorum-sensing transcriptional regulators LasR or RhlR does not suppress the expression of virulence factors and the virulence of *Pseudomonas aeruginosa* PAO1," *Microbiology*, vol. 165, no. 4, pp. 425–432, 2019.

[52] H. W. Stokes, C. L. Nesbø, M. Holley, M. I. Bahl, M. R. Gillings, and Y. Boucher, "Class 1 integrons potentially predating the association with tn402-like transposition genes are present in a sediment microbial community," *Journal of Bacteriology*, vol. 188, no. 16, pp. 5722–5730, 2006.

[53] S. E. Quiroz-Morales, S. García-Reyes, G. Y. Ponce-Soto, L. Servín-González, and G. Soberón-Chávez, "Tracking the origins of *Pseudomonas aeruginosa* phylogroups by diversity and evolutionary analysis of important pathogenic marker genes," *Diversity*, vol. 14, no. 5, p. 345, 2022.

[54] M. Andrejko, A. Zdybicka-Barabas, and M. Cytryńska, "Diverse effects of *Galleria mellonella* infection with entomopathogenic and clinical strains of *Pseudomonas aeruginosa*," *Journal of Invertebrate Pathology*, vol. 115, pp. 14–25, 2014.

[55] M. L. Beeton, D. R. Alves, M. C. Enright, and A. T. A. Jenkins, "Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model," *International Journal of Antimicrobial Agents*, vol. 46, no. 2, pp. 196–200, 2015.

[56] G. W. Lau, H. Ran, F. Kong, D. J. Hassett, and D. Mavrodi, "*Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice," *Infection and Immunity*, vol. 72, no. 7, pp. 4275–4278, 2004.

[57] K. Brindhadevi, F. LewisOscar, E. Mylonakis, S. Shanmugam, T. N. Verma, and A. Pugazhendhi, "Biofilm and quorum sensing mediated pathogenicity in *Pseudomonas aeruginosa*," *Process Biochemistry*, vol. 96, pp. 49–57, 2020.

[58] T. Strateva and I. Mitov, "Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections," *Annals of Microbiology*, vol. 61, no. 4, pp. 717–732, 2011.

[59] L. V. Silva, A. C. M. Galdino, A. P. F. Nunes et al., "Virulence attributes in Brazilian clinical isolates of *Pseudomonas aeruginosa*," *International Journal of Medical Microbiology*, vol. 304, no. 8, pp. 990–1000, 2014.

[60] C. Juan, C. Peña, and A. Oliver, "Host and pathogen biomarkers for severe *Pseudomonas aeruginosa* infections," *The Journal of Infectious Diseases*, vol. 215, no. 1, pp. S44–S51, 2017.

[61] I. Jurado-Martín, M. Sainz-Mejías, and S. McClean, "*Pseudomonas aeruginosa*: an audacious pathogen with an adaptable arsenal of virulence factors," *International Journal of Molecular Sciences*, vol. 22, no. 6, pp. 3128–3137, 2021.

[62] S. Hall, C. McDermott, S. Anoopkumar-Dukie et al., "Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*," *Toxins*, vol. 8, p. 236, 2016.

[63] F. Casilag, A. Lorenz, J. Krueger, F. Klawonn, S. Weiss, and S. Häussler, "The LasB Elastase of *Pseudomonas aeruginosa* Acts in concert with alkaline protease apra to prevent flagellin-mediated immune recognition," *Infection and Immunity*, vol. 84, no. 1, pp. 162–171, 2016.

[64] A. Muggeo, C. Coraux, and T. Guillard, "Current concepts on *Pseudomonas aeruginosa* interaction with human airway epithelium," *PLoS Pathogens*, vol. 19, no. 3, 2023.

[65] G. Soberón-Chávez, F. Lépine, and E. Déziel, "Production of rhamnolipids by *Pseudomonas aeruginosa*," *Applied Microbiology and Biotechnology*, vol. 68, no. 6, pp. 718–725, 2005.

[66] V. M. D'Costa, C. E. King, L. Kalan et al., "Antibiotic resistance is ancient," *Nature*, vol. 477, no. 7365, pp. 457–461, 2011.

[67] M. R. Gillings, W. H. Gaze, A. Pruden, K. Smalla, J. M. Tiedje, and Y. G. Zhu, "Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution," *The ISME Journal*, vol. 9, no. 6, pp. 1269–1279, 2015.

[68] L. Ma, A.-D. Li, X.-L. Yin, and T. Zhang, "The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments," *Environmental Science & Technology*, vol. 51, no. 10, pp. 5721–5728, 2017.

[69] M. R. Gillings, S. Krishnan, P. J. Worden, and S. A. Hardwick, "Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples," *FEMS Microbiology Letters*, vol. 287, pp. 56–62, 2008.

[70] M. Andrejko, A. Zdybicka-Barabas, M. Wawrzoszek, and M. Cytryńska, "Diverse susceptibility of *Galleria mellonella* humoral immune response factors to the exoproteinase activity of entomopathogenic and clinical strains of *Pseudomonas aeruginosa*," *Zoological Science*, vol. 30, no. 5, pp. 345–351, 2013.

[71] E. Kaszab, J. Radó, B. Kriszt et al., "Groundwater, soil and compost, as possible sources of virulent and antibiotic-resistant *Pseudomonas aeruginosa*," *International Journal of Environmental Health Research*, vol. 31, no. 7, pp. 848–860, 2021.

[72] O. Ciofu, C. Moser, P. Ø Jensen, and N. Høiby, "Tolerance and resistance of microbial biofilms," *Nature Reviews Microbiology*, vol. 20, no. 10, pp. 621–635, 2022.

[73] A. Ghafoor, I. D. Hay, and B. H. A. Rehm, "Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture," *Applied and Environmental Microbiology*, vol. 77, no. 15, pp. 5238–5246, 2011.

[74] L. Hall-Stoodley, J. W. Costerton, and P. Stoodley, "Bacterial biofilms: from the Natural environment to infectious diseases," *Nature Reviews Microbiology*, vol. 2, pp. 95–108, 2004.

[75] P.-Y. Qian, A. Cheng, R. Wang, and R. Zhang, "Marine biofilms: diversity, interactions and biofouling," *Nature Reviews Microbiology*, vol. 20, no. 11, pp. 671–684, 2022.

[76] S. Gholami, M. Tabatabaei, and N. Sohrabi, "Comparison of biofilm formation and antibiotic resistance pattern of *Pseudomonas aeruginosa* in human and environmental isolates," *Microbial Pathogenesis*, vol. 109, pp. 94–98, 2017.

[77] K. U. Mahto and S. Das, "Microscopic techniques to evaluate the biofilm formation ability of a marine bacterium *Pseudomonas aeruginosa* PFL P1 on different substrata," *Microscopy Research and Technique*, vol. 84, no. 10, pp. 2451–2461, 2021.

[78] P. Laborda, F. Sanz-García, S. Hernando-Amado, and J. L. Martínez, "*Pseudomonas aeruginosa*: an antibiotic resilient pathogen with environmental origin," *Current Opinion in Microbiology*, vol. 64, pp. 125–132, 2021.

[79] H. Feltman, G. Schulert, S. Khan, M. Jain, L. Peterson, and A. R. Hauser, "Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*," *Microbiology*, vol. 147, no. 10, pp. 2659–2669, 2001.

[80] M.-H. Park, S. Y. Kim, E. Y. Roh, and H. S. Lee, "Difference of Type 3 secretion system (T3SS) effector gene genotypes (*exoU* and *exoS*) and its implication to antibiotics resistances in isolates of *Pseudomonas aeruginosa* from chronic otitis media," *Auris Nasus Larynx*, vol. 44, pp. 258–265, 2017.

[81] A. Zboralski, A. Biessy, and M. Filion, "Bridging the gap: type III secretion systems in plant-beneficial bacteria," *Microorganisms*, vol. 10, no. 1, p. 187, 2022.

[82] Y. Hu, H. Huang, X. Cheng et al., "A global survey of bacterial type III secretion systems and their effectors," *Environmental Microbiology*, vol. 19, no. 10, pp. 3879–3895, 2017.

[83] M. Medina-Rojas, W. Stribling, E. Snesrud et al., "Comparison of *Pseudomonas aeruginosa* strains reveals that Exolysin A toxin plays an additive role in virulence," *Pathogens and Disease*, vol. 78, no. 1, 2020.

[84] A. Bel Hadj Ahmed, M. Salah Abbassi, B. Rojo-Bezares et al., "Characterization of *Pseudomonas aeruginosa* isolated from various environmental niches: new STs and occurrence of antibiotic susceptible high-risk clones," *International Journal of Environmental Health Research*, vol. 30, no. 6, pp. 643–652, 2020.

[85] L. Ruiz-Roldán, B. Rojo-Bezares, M. de Toro et al., "Antimicrobial resistance and virulence of *Pseudomonas* spp. among healthy animals: concern about exolysin ExlA detection," *Scientific Reports*, vol. 10, no. 1, Article ID 11667, 2020.

[86] D. V. Mavrodi, J. A. Parejko, O. V. Mavrodi et al., "Recent insights into the diversity, frequency and ecological roles of phenazines in fluorescent *Pseudomonas* spp," *Environmental Microbiology*, vol. 15, no. 3, pp. 675–686, 2013.

[87] M. J. Everett and D. T. Davies, "*Pseudomonas aeruginosa* elastase (LasB) as a therapeutic target," *Drug Discovery Today*, vol. 26, no. 9, pp. 2108–2123, 2021.

[88] T. Cheng, J. Liang, J. He, X. Hu, Z. Ge, and J. Liu, "A novel rhamnolipid-producing *Pseudomonas aeruginosa* ZS1 isolate derived from petroleum sludge suitable for bioremediation," *AMB Express*, vol. 7, no. 1, p. 120, 2017.

[89] J. Chakraborty and S. Das, "Characterization of the metabolic pathway and catabolic gene expression in biphenyl degrading marine bacterium *Pseudomonas aeruginosa* JP-11," *Chemosphere*, vol. 144, pp. 1706–1714, 2016.

[90] J. Du, A. Zhang, X. Zhang, X. Si, and J. Cao, "Comparative analysis of rhamnolipid congener synthesis in neotype *Pseudomonas aeruginosa* ATCC 10145 and two marine isolates," *Bioresource Technology*, vol. 286, Article ID 121380, 2019.

[91] M. Laabei, W. D. Jamieson, S. E. Lewis, S. P. Diggle, and A. T. A. Jenkins, "A new assay for rhamnolipid detection—important virulence factors of *Pseudomonas aeruginosa*," *Applied Microbiology and Biotechnology*, vol. 98, no. 16, pp. 7199–7209, 2014.

[92] A. M. Abdel-Mawgoud, F. Lépine, and E. Déziel, "Rhamnolipids: diversity of structures, microbial origins and roles," *Applied Microbiology and Biotechnology*, vol. 86, no. 5, pp. 1323–1336, 2010.

[93] D. I. Andersson and D. Hughes, "Selection and transmission of antibiotic-resistant bacteria," *Microbiology Spectrum*, vol. 5, no. 4, 2017.

[94] K. A. Ramsay, S. J. T. Wardell, W. M. Patrick et al., "Genomic and phenotypic comparison of environmental and patient-derived isolates of *Pseudomonas aeruginosa* suggest that antimicrobial resistance is rare within the environment," *Journal of Medical Microbiology*, vol. 68, no. 11, pp. 1591–1595, 2019.

[95] S. Kim, S. Masai, K. Murakami et al., "Characteristics of antibiotic resistance and tolerance of environmentally endemic *Pseudomonas aeruginosa*," *Antibiotics*, vol. 11, no. 8, p. 1120, 2022.

[96] A. Alonso, F. Rojo, and J. L. Martinez, "Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin," *Environmental Microbiology*, vol. 1, no. 5, pp. 421–430, 1999.

[97] P. Olga, V. Apostolos, G. Alexis, V. George, and M. Athena, "Antibiotic resistance profiles of *Pseudomonas aeruginosa* isolated from various Greek aquatic environments," *FEMS Microbiology Ecology*, vol. 92, no. 5, p. fiw042, 2016.

[98] K. Bhullar, N. Waglechner, A. Pawlowski et al., "Antibiotic resistance is prevalent in an isolated cave microbiome," *PLoS One*, vol. 7, no. 4, 2012.

[99] M. Toth, C. Smith, H. Frase, S. Mobashery, and S. Vakulenko, "An antibiotic-resistance enzyme from a deep-sea bacterium," *Journal of the American Chemical Society*, vol. 132, no. 2, pp. 816–823, 2010.

[100] M. G. Brown and D. L. Balkwill, "Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface," *Microbial Ecology*, vol. 57, no. 3, pp. 484–493, 2009.

[101] P. Suárez, A. V. Gutiérrez, V. Salazar et al., "Virulence properties and antimicrobial resistance of *Pseudomonas aeruginosa* isolated from cave waters at Roraima Tepui, Guayana Highlands," *Letters in Applied Microbiology*, vol. 70, no. 5, pp. 372–379, 2020.

[102] S. R. Partridge, G. Tsafnat, E. Coiera, and J. R. Iredell, "Gene cassettes and cassette arrays in mobile resistance integrons," *FEMS Microbiology Reviews*, vol. 33, no. 4, pp. 757–784, 2009.

[103] W. H. Gaze, L. Zhang, N. A. Abdouslam et al., "Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment," *The ISME Journal*, vol. 5, no. 8, pp. 1253–1261, 2011.

[104] R. Lucassen, L. Rehberg, M. Heyden, and D. Bockmühl, "Strong correlation of total phenotypic resistance of samples from household environments and the prevalence of class 1 integrons suggests for the use of the relative prevalence of intI1 as a screening tool for multi-resistance," *PLoS One*, vol. 14, no. 6, 2019.

[105] S. Suzuki, A. Pruden, M. Virta, and T. Zhang, "Editorial: antibiotic resistance in aquatic systems," *Frontiers in Microbiology*, vol. 8, 2017.

[106] M. Nardelli, P. M. Scalzo, M. S. Ramírez, M. P. Quiroga, M. H. Cassini, and D. Centrón, "Class 1 integrons in environments with different degrees of urbanization," *PLoS One*, vol. 7, no. 6, 2012.

[107] G. Corno, T. Ghaly, R. Sabatino et al., "Class 1 integron and related antimicrobial resistance gene dynamics along a complex freshwater system affected by different anthropogenic pressures," *Environmental Pollution*, vol. 316, Article ID 120601, 2023.

[108] L. Ruiz-Martínez, L. López-Jiménez, E. Fusté, T. Vinuesa, J. P. Martínez, and M. Viñas, "Class 1 integrons in environmental and clinical isolates of *Pseudomonas aeruginosa*," *International Journal of Antimicrobial Agents*, vol. 38, no. 5, pp. 398–402, 2011.