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Original Article

Molecular Study of Acinetobacter baumannii that Lacking Some Essentials Genes Responsible of Toxin-Antitoxin System

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Abstract

Acinetobacter genus has various species that are widespread in different environments and can exist in non-living environment samples as well. *Acinetobacter baumannii* (*A. baumannii*) is known to be one of the main causes of nosocomial infection. Few studies have examined the possibility of the presence of this opportunistic pathogen in non-living environment samples. In this study, *A. baumannii* strain cl-2 was isolated from dishwasher basket samples and it was identified by 16S ribosomal RNA sequencing analysis. The present study also investigated the presence of some important genes responsible for toxin-antitoxin (TA) systems necessary for the resistance of this bacterium in improper environmental conditions. Additionally, attempts were made to study some essential virulence factors, such as hemolysin, lipase, protease, and lecithinase production, as well as biofilm formation and surface motility. The findings revealed that the isolate belongs to the *A. baumannii* strain cl-2. The isolate was deposed in the National Center for Biotechnology Information, (NCBI) and the data can be accessed via the NCBI accession number (MW642251). The results of screening the TA system by *higBA*, *mazEF*, and *relBE* genes showed the isolate did not contain these genes. The hemolysin toxin activity (phenotypic test) was performed by using the streaking and spot methods on blood agar. It was found that the *A. baumannii* strain cl-2 had the ability to hemolyze red blood cells and produce lecithinase and protease enzymes. Finally, it was revealed that the *A. baumannii* strain cl-2 had surface motility based on the concentric diffusion ring of growth observed on Luria Broth agar (0.3%). In conclusion, the isolates under study showed association patterns between their ability to produce hemolysin, lipase, lecithinase, as well as protease, and other virulence factors, including surface motility and biofilm formation.

Keywords: *Acinetobacter baumannii*, Dishwasher, Hemolysin, Surface motility Toxin-antitoxin system

1. Introduction

There are 23 species of *Acinetobacter* genus, including environmental and clinical isolates, and all of them can cause diseases for humans. One of the most common and clinically concerning species around the world is *Acinetobacter baumannii (A. baumannii*), which has been diagnosed as the causative agent of many outbreaks and a troublesome pathogen (1).

The *A. baumannii* is a gram-negative bacterium with a coccobacilli shape. It is considered one of the most prominently widespread bacteria in hospital infections (2). In addition, it has been known as an important factor in pneumonia, food contamination, meningitis, urinary tract, and wound infections. It is also associated with a high mortality rate. Infection with *A. baumannii* can occur as a result of contact with contaminated hospital staff or exposure to contaminated hospital equipment. There is a lot of evidence that this germ can be found on many removable medical devices, such as catheters, arterial pressure monitoring devices, and respiratory equipment. It may also be present on dry environmental items, including mattresses, pillows, and electrical devices' remote controls (3, 4). In South Korea, researchers recorded the presence of *A.*

baumannii on non-living surfaces that are in direct contact with humans, such as garden tables and game consoles. In the UK, it was also recovered from manure agricultural soil. These environmental studies all included a molecular study depending on the 16S ribosomal RNA (16S rRNA) sequencing technique; therefore, the presence of *A. baumannii* outside the living organisms requires more studies (5).

The pathogenicity of *A. baumannii* and its ability to resist improper environmental conditions are related to its possession of many virulence factors, such as the ability to form biofilm and quorum sensing. However, the molecular basis for the increasingly prevalent strains of this bacterium and its resistance to antibiotics is not fully understood, and thus, are sometimes attributed to its possession of toxin-antitoxin (TA) systems (4, 6). The TA system is the genetic loci found in low-copy plasmids and chromosomes, which is widespread in bacteria and archaea. This system is composed of toxin and antitoxin forming a complex in which the activity of toxin is inhibited by the antitoxin. In some conditions, the antitoxin destroys, while toxin, the more stable component, remains unchanged. The latter activates when some vital functions are inhibited, such as replication, translation, and biosynthesis of the adenosine triphosphate and the cell wall. The TA system has been suggested to perform many functions, including giving the plasmid stability to regulate growth and death under stress conditions (6). Therefore, this study was designed to identify the *A. baumannii* strains isolated from an extreme environment (i.e., the dishwasher) and to detect some unique characteristics related to some essential genes for the TA systems and their ability to produce some virulence toxins. Furthermore, the present study aimed to explain adherence and biofilm formation as factors contributing to existence in such extreme environments.

2. Materials and Methods

2.1. Isolation of *Acinetobacter baumannii*

Isolates of *A. baumannii* species were collected from 10 dishwasher basket samples during different periods using sterile swabs. Samples were cultured on MacConkey agar, nutrient agar, as well as methylene blue agar, and they were incubated for 24 to 48 h. After the end of the incubation period, the bacterial culture was purified until pure colonies were obtained. Additionally, a gram stain was used and then examined microscopically.

2.2. Identification by 16S Ribosomal RNA Gene Sequencing

Total DNA was extracted from *A. baumannii* strain cl-2 by using a G-spin DNA extraction kit (Intron Biotechnology, South Korea). Afterward, electrophoresis was employed to determine DNA pieces. The polymerase chain reaction (PCR) occurred in the final volume of 25 µl composed of 5µl of Taq PCR Pre Mix, 1µl of forward primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 1µl reverse primer (5'- GGTTACCTTGTTACGACTT-3'), 1.5µl of total bacterial DNA, and 16.5µl of distilled water to complete volume into 25µl. The conditions of PCR for the detection of 16S rRNA gene were as follows: one cycle of initial denaturation at 95˚C for 5 min, 35 cycles of denaturation-2 at 95˚C for 45 sec, annealing at 58˚C for 45 sec, extension-1 at 72˚C for 45 sec, and one cycle of extension-2 at 72˚C for 7 min. The search of homology was accomplished by using programs available at the NCBI and BioEdit program. Subsequently, the isolate was recorded in the NCBI and taken a specialized accession number.

2.3. Molecular Phylogenetic Bioinformatics Analysis

The present study investigated the evolutionary relationships between *A. baumannii* strain cl-2 and other closely-related strains from different countries and locations by the neighborhood-joining method. The optimal tree with the sum of branch length =0.06449594 is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of

1,467 positions in the final dataset. Evolutionary analyses were conducted in the MEGA 6 software.

2.4. Screening of Toxin-Antitoxin System

The *A. baumannii* strain cl-2 was investigated for the TA system. It underwent the amplification process by the PCR of *higBA*, *mazEF*, and *relBE* genes either on chromosome or plasmid DNA by using specific primers for each gene (Table 1), according to Ghafourian, Good (7). The DNA of bacteria was extracted by Geneaid kit (Geneaid Biotech Ltd., Taiwan). The PCR reaction was employed in conditions as follows: one cycle of initial denaturation at 95˚C for 3 min, 30 cycles of denaturation at 94˚C for 1 min, 30 cycles of annealing at 54˚C for 1 min, 30 cycles of extension at 72˚C for 1 min, and finally, one cycle of the final extension at 72˚C for 10 min.

Table 1. Primers of toxin-antitoxin systems

Gene	Sequence of gene	Length (bp)
higBA	**Forward**: AGCACATCCGTACGATCTACTGC **Reverse:** TGCACTCCTGCGATGCGGCGAA	440 bp
mazEF	**Forward:** ACCTTCGAAGGAACTACGTCAGTAG **Reverse:** ATAGGCGAACATGCAAGAAAAGGCAGC	436 bp
relBE	**Forward:** ATGAAGTGAACGGTCAACAATA **Reverse:** ACAGACCTCGGAAAGTGGTCG	578 bp

2.5. Detection of Some Toxins in *A. baumannii* Ctrain cl-2

2.5.1. Hemolysin Production

The production of hemolysin toxin was detected by two methods: the traditional method by using streaking on blood ager, as well as incubation at 37˚C for 24 h, and another method depending on the spot inoculating of the bacterial suspension. In the second method, the ability of bacteria to produce hemolysin was tested by inoculating the center of blood agar plate in a bacterial suspension set to 10 6 CFU/ML and incubating the plate at 37˚C for 6 days with daily monitoring of the culture (8).

2.5.2. Lipase, Phospholipase Toxins, and Protease Activity

The ability of this isolate to produce lipase, lecithinase toxins (enzyme), and protease activity was studied using an egg-yolk agar medium which consisted of sterile nutrient agar (85mL) and egg-yolk suspension (15 mL). In this medium, the agar was sterilized, and then, it was cooled to 55˚C. Afterward, the egg yolk was added and poured into plates (9).

2.6. Detection of Surface Motility and Biofilm Formation

2.6.1. Surface Motility

To identify surface motility, which is a significant feature of some *A. baumannii* strains, one colony was cultured on Luria Broth (LB) agar at 37˚C for 18 h. The 0.5 Mcfarland standard was used to adjust the turbidity of the bacterial suspension as a result of obtaining 108 CFU/mL. A sample of 1 mL was taken from the bacterial suspension and inoculated on LB agar (0.3%). The LB agar plates were incubated at 37˚C for 14 h. After incubation, the diameter of circular growth was measured and recorded (10).

2.6.2. Biofilm Assay Test

Its ability to form biofilms was tested by two methods, namely tube and Congo red agar methods. In the tube method, a glass tube of tryptic soy broth (1% glucose) was inoculated by the fresh culture of bacteria and incubated at 37˚C for 24 h. Tryptic soy broth (1% glucose) was prepared by dissolving 30 g of the medium in a liter of distilled water, and the acidity was then set at 7. Glucose was pasteurized in a water bath at 63˚C for half an hour, then added to the sterilized, cooled, and prepared medium at a ratio of 1%. After incubation, the suspension was shed, and the glass tube was washed by phosphate buffer saline and left to dry. The dry tube was treated with crystal violet (1%), and it was then washed with deionized water and

dried (6, 11). In Congo red agar method, the fresh culture of bacteria was inoculated into the Congo red agar, and the plate was incubated at 37˚C for 24 h (11).

2.7 Statistical Analysis.

For the comparison of categorical variables, Fisher's exact test was utilized (GraphPad Software, San Diego, CA, USA). Additionally, a *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Identification by 16S Ribosomal RNA Gene Sequencing and Accession Number

The *A. baumannii* strain cl-2 was analyzed by universal primers in 16S rRNA (Figure 1). The partial sequence of 16S rRNA was then compared to the NCBI data. The identification percent was 99%, which represents the relationship of isolate with standard isolates of *A. baumannii*. Accordingly, the isolate belonged to the *A. baumannii* strain cl-2. The isolate was deposed in the NCBI, and the data can be accessed via the NCBI accession number MW642251.

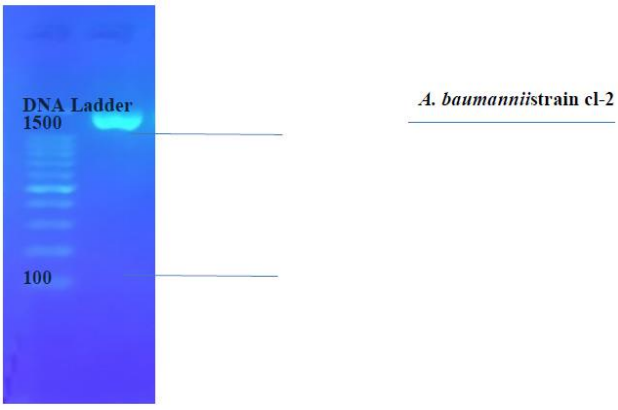

Figure 1. Agarose electrophoresis for *A. baumannii* strain cl-2 by using 16S rRNA

3.2. Molecular Phylogenetic Analysis

As illustrated in the neighbor-joining tree in figure 2, based on the similarity of 16S rRNA sequences, a relationship appeared between *A. baumannii* strain cl-2 (MW642251) and *A. baumannii* strain TERI SID (KX822160). The evolutionary history was inferred using the neighbor-joining method. The optimal tree is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [2], which are presented in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,467 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 software.

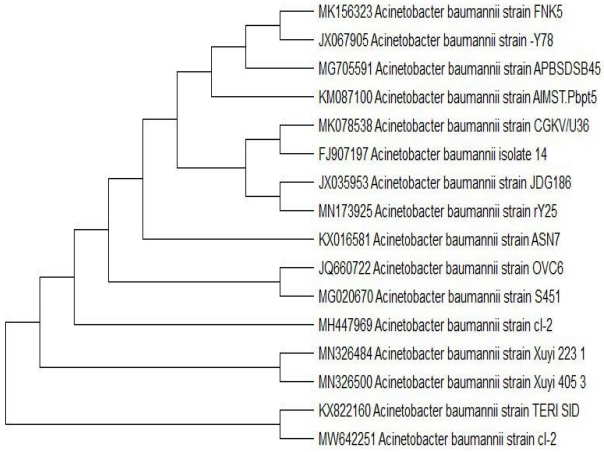

Figure 2. Phylogenetic tree for *A. baumannii* strain cl-2

3.3. Toxin-Antitoxin System

For screening the TA system by using *higBA*, *mazEF*, and *relBE* genes, the results showed that the isolate did not contain these genes. To the best of the researchers' knowledge, there are very few studies on the presence and spread of TA system genes and their effectiveness in bacteria, especially *A. baumannii*. Therefore, the present study aimed to investigate some essential genes in the TA system to be the first building block for broader investigations on this topic.

3.4. Production of Hemolysin, Lipase, Lecithinase, and Protease

The hemolysin toxin activity (phenotypic test) was conducted by the streaking and spot methods on blood agar. *A. baumannii* strain cl-2 had the ability to hemolyze red blood cells depending on the appearance of a greenish growth (α hemolysis), surrounded by a lightly clearing zone (β hemolysis) in the streaking method (Figure 3). In the spot method, α and β

hemolysis were visible, based on the appearance of a gray-greenish growth surrounded by a visible clear zone (Figure 4). When increasing the period of incubation, the result remained the same.

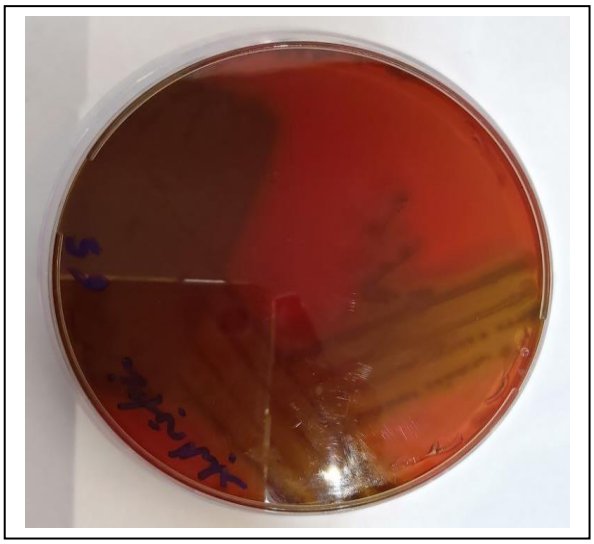

Figure 3. Hemolysis of the *A. baumannii* strain cl-2 by the streaking method

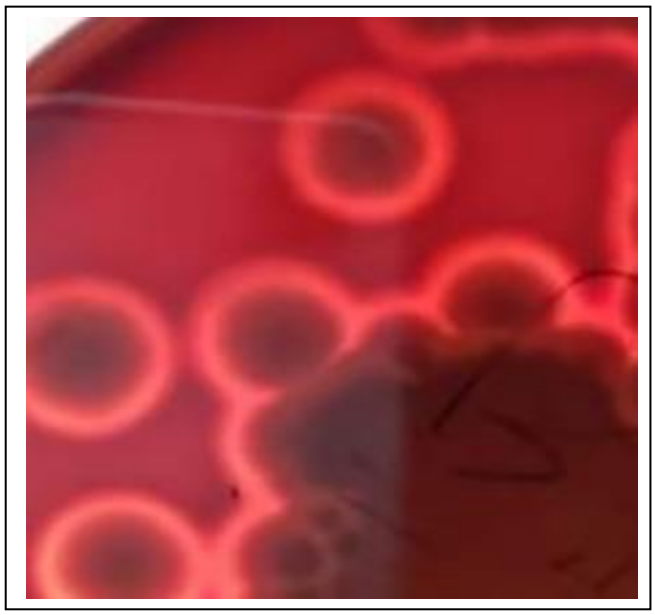

Figure 4. Hemolysis of the *A. baumannii* strain cl-2 by the spot method

The *A. baumannii* strain cl-2 showed an ability to produce lecithinase (appearing as a white precipitation growth) and protease enzyme, which was detected by a clear zone surrounding growth on the same egg-yolk agar (Figure 5). The strain also showed an ability to produce lipase based on the appearance of blue color after the addition of a saturated solution of copper sulfate to the egg-yolk agar (Figure 6).

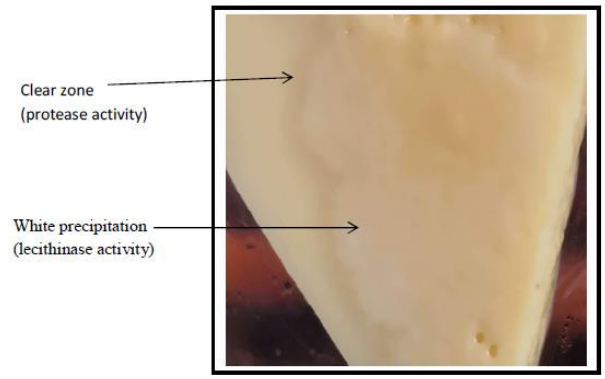

Figure 5. Lecithinase and protease activity of the *A. baumannii* strain cl-2

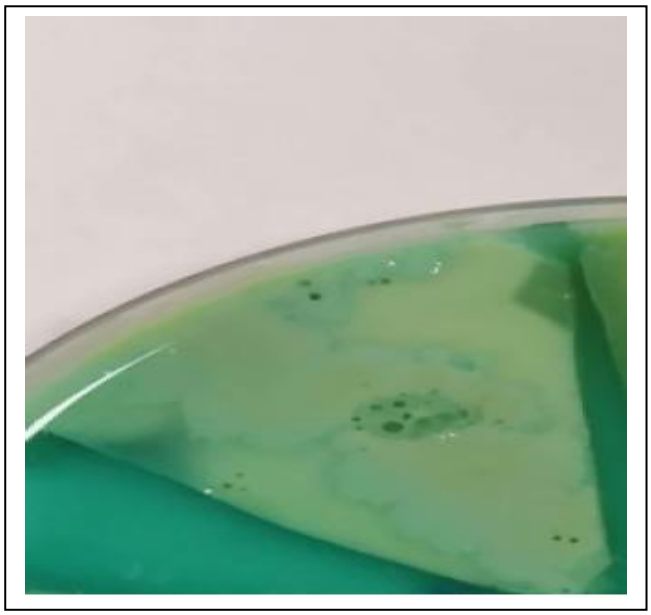

Figure 6. Lipase activity of the *A. baumannii* strain cl-2

3.5. Surface Motility and Biofilm Formation

The *A. baumannii* strain cl-2 had the evidence of surface motility based on the concentric diffusion ring of growth that was observed on LB agar as (0.3%) (Figure 7). The strain under study also showed an ability of adherence and biofilm formation depending on the tube and Congo red methods. In the tube method, crystal violet precipitation was observed in the lower part of the glass tube (Figure 8), while in the Congo red agar method, the growth had a black appearance (Figure 9).

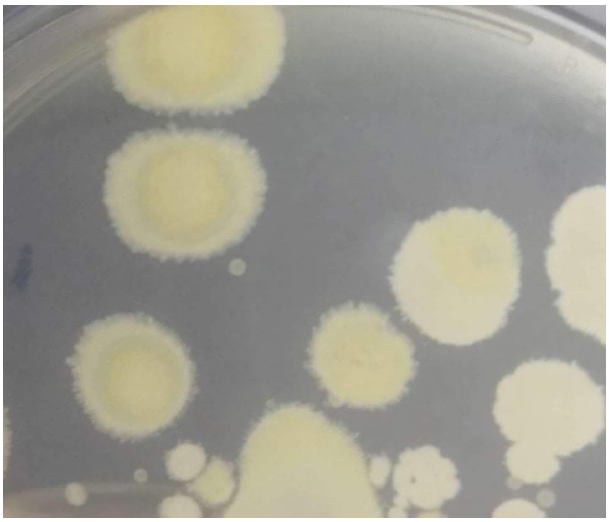

Figure 7. Surface motility of the *A. baumannii* strain cl-2

Figure 8. Biofilm formation of *A. baumannii* strain cl-2 by tube method

Figure 9. Biofilm formation of the *A. baumannii* strain cl-2 by the Congo red method

4. Discussion

Based on the results of 16S rRNA sequencing analysis and the study of some phenotypic futures and the *A. baumannii* strain cl-2, as well as *higBA*, *mazEF*, and *relBE* toxin-antitoxin genes, it can be expected that this strain is a novel strain despite having a close relationship with *A. baumannii* strain TERI SID based on phylogenetic analysis. The findings of the present study are not in agreement with those of a study by Ghafourian, Good (7) that showed most strains of the clinical *A. baumannii* must have at least one of *higBA*, *mazEF*, and *relBE* toxin-antitoxin genes, especially *mazEF* gene which is present in all clinical strains. The difference between the results of the two studies can be explained by the difference between clinical and environmental strains of *A. baumannii*. The detection of the toxin-antitoxin system in most bacteria is assumed an important assay for identifying the ability of bacteria to resist stress conditions. Since dishwashers are extreme environments in terms of high temperature, as well as high salt concentration and pressures, the presence of *A. baumannii* is surprising, especially when isolated from baskets. Therefore, this isolate must have specific strategies to be able to exist in such environments. The presence of surface motility

indicated the true motility of the *A. baumannii* strain cl-2, which may be acquired through lateral gene transfer from other bacteria. The movement model that was observed in the strain under study showed some similarities with the motility model that appeared on the same media in a study by Dahdouh, Hajjar (12). However, it is not similar to the kinetic model that was observed in a study by Clemmer, Bonomo (10). The difference may be due to the type of agar used, as well as the strain under study. Dahdouh, Hajjar (12) attributed the differences in surface motility patterns of the *A. baumannii* to the experimental factors, the commercial mark of agar, and specific characteristics related to the concerned strain; therefore, this topic needs further investigations (12).

It is well known that bacteria within a biofilm, such as the *A. baumannii*, have the ability to resist extracellular environmental stress, as biofilm formation bacteria *A. baumannii* have surface advantage structures, such as polysaccharide, which contribute to biofilm formation and maintenance (13, 14). The ability of *A. baumannii* strain cl-2 regarding biofilm formation is considered one of the most important strategies to resist stress conditions in the dishwasher.

The isolate under study showed the association patterns between its ability to produce hemolysin, lipase, lecithinase, protease, as well as other virulence factors, including surface motility and biofilm formation. This finding is in agreement with the results of a previous study by Dahdouh, Hajjar (12), revealing that the isolates with the highest motility diffusion diameter (144.5 mm on a 15-cm square petri dish) produced strong biofilm, caused hemolysis of blood in an agar plate, and had proteolytic activity (12). The results of a previous study also showed a relationship between the ability to form biofilms and motility in the *A. baumannii* (11).

Authors' Contribution

Study concept and design: A. H. A.
Acquisition of data: A. H. A.
Analysis and interpretation of data: A. H. A.
Drafting of the manuscript: A. W. A.
Critical revision of the manuscript for important intellectual content: A. H. A.
Statistical analysis: A. Y. M. A.
Administrative, technical, and material support: A. H. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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