JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 379-382

Acinetobacter baylyi Biofilm Formation Dependent Genes

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(Received: 08 December 2013; accepted: 29 January 2014)

Acinetobacter baylyi **is a gram-negative aerobic bacterium. *A. baylyi* ADP1 has gained popularity and has been used repeatedly in research studies because of its high capability for natural transformation. The whole genome sequencing of *A. baylyi* (ADP1) was published in 2004. Because of the good potential of this microorganism as a model for genetic studies in *Acinetobacter* spp., we decided to study putative genes that may play a role in biofilm formation. In this study, we investigated *Acinetobacter baylyi* ADP1 wild type and several mutant genes (pili assembly chaperone, Fimbrial-biogenesis Protein, putative fimbrial usher protein, putative surface protein) for their ability to form abiotic biofilms. Biofilm formation of *A. baylyi ADP1* was characterized by using an in vitro biofilm formation assay on polystyrene surfaces. Biofilms were formed optimally when bacteria were grown in a minimal medium under aerobic conditions. Our investigation has shown that at least two mutant genes fimbrial-biogenesis Protein (3317) and putative surface protein (PEVL 389) may be involved in biofilm formation. Biofilm formation of Fimbrial-biogenesis Protein and putative surface protein were significantly reduced in mutants compared to wild type by t-test, suggesting that these genes may play a partial role in biofilm formation.**

Key words: Acinetobacter baylyi ADP1, Deletion mutants, Biofilm, Genes, Drug resistant.

The genus *Acinetobacter* is a Gram-negative coccobacilli, *Acinetobacter* species are strictly aerobic oxidase-negative, non-motile, nitrate-negative and non-fermentative. *Acinetobacter baylyi* ADP1 is naturally soil bacterium. Because of its metabolic versatility and high competency for natural genetic transformation, it is a model organism of choice for genetic and metabolic investigations. ADP1 also shares about a third of its genes with *E. coli*, thus providing a convenient and complementary model for studying metabolism. *A. baylyi* ADP1 genome has been sequenced and annotated in 2004 and full isogenic mutants were created by Genoscope in 2008[1]. The study of microbial development has shown that many microorganisms are capable of forming surface-attached microbial communities, known as biofilms. Biofilms comprise a single microbial species or multiple microbial species and can be formed on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants[2]. To identify and characterize the bacterial elements and genetic determinants that are necessary for biofilm development, Construction of mutants has been a method of choice for many researchers for studying genetic phenotypes. A complete set of single-gene deletions of all non-essential genes have also been created in *Acinetobacter baylyi* ADP1 by replacing coding regions with a kanamycin resistance cassette. Furthermore, a simple colorimetric assay screen has been used by a number of groups to

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facilitate such study[3-7]. This screen uses the well of a Microtiter plate as the chamber in which a biofilm is formed. Biofilms are visualized with a variety of dyes (such as crystal violet). Mutants that are unable to form or weakly form a biofilm under these conditions are termed surface attachment defective as opposed to wild type. A phylogenetically closely related genus to *Acinetobacter baylyi* ADP1 is *Acinetobacter baumannii*[8] which is a significant worldwide nosocomial pathogen with a particular ability to develop antimicrobial resistance and cause nosocomial outbreaks of infection in hospitals[9]. This organism frequently causes infections associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts or Foley catheters. Biofilm formation is a well-known pathogenic mechanism in such infections. In addition, the environmental survival of some microorganisms may be facilitated by biofilm formation on abiotic surfaces[9]. In this study we decided to use *A. baylyi* as a model to study biofilm formation. A wild-type and several mutant strains of *A. baylyi* ADP1 were used to investigate the biofilm formation under the same conditions. We report on this work two genes that may play a partial role in biofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The bacterial strains *A. Baylyi* ADP1 strains (wild type and mutants were gifted by Genoscope, Evry, France) and were grown aerobically at 37°C on Luria–Bertani (LB) broth and agar (Sambrook *et al*., 1989). LB media supplemented with 30 mg/ml kanamycin was used with all mutant strains.

Optimization of Solvent Solubilization Of Stained Biofilm

Bacterial cells are inoculated directly into sterile 96 microtiter plates filled with 100 µl of the LB medium (LB-kanamycin for mutants or LB for wild type) per well. Plates were incubated overnight at 37°C. Bacterial growth on microtiter plates were removed by pipetting and the wells were washed with dd H_2O. About 125 µl of 0.1% crystal violet solution to each well was added. Followed by staining for 10 min at room temperature. Plates were shaked and the crystal violet solution was removed. Microtiter plates were inverted and vigorously tapped on paper towels to remove any excess liquid and were allowed to air-dry. 200 µl of each solvent 95% ethanol, 100% dimethyl sulfoxide known as DMSO, 80% ethanol/ 20% acetone, 33% glacial acetic acid was added to each stained well to solubilize the satin by covering plates and incubating 10 to 15 min at room temperature. The contents of solution of each well was briefly mixed and then transferred to a separate well in an optically clear flat-bottom 96 well plate. The optical density (OD) of each well was measured at a wavelength of 595 nm.

Biofilm assays

Briefly, the assay was performed according to O'toole method[2]. One milliliter of fresh medium, either LB or LB-Kanamycin in polystyrene or polypropylene sterile tubes was inoculated with 0.01 ml of an overnight culture. Duplicate cultures for each sample were incubated for 12 h either shaking (at 200 r.p.m. in an orbital shaker) or stagnant at 37 °C. Bacterial cells were centrifuged briefly at 5000 r.p.m. for 1 min and the supernatant of the other tube was aspirated and rinsed thoroughly with distilled water. The cells attached to the tube walls were visualized and quantified by

Table 1. Depiction of raw biofilm formation assay average reads at different OD595 nm

Cell growth at OD 595	Wild type	3335	3317	1639	PEVL389
0.05	0.0900	0.0653	0.0190	0.0266	0.0283
0.1	0.1366	0.0723	0.0073	0.0316	0.0313
0.2	0.1263	0.0606	0.0070	0.0343	0.0200
0.4	0.0970	0.0500	0.0073	0.0303	0.0170
0.6	0.0840	0.0426	0.0066	0.0333	0.0136
0.8	0.0523	0.0433	0.0123	0.0373	0.0150
Average	0.0977	0.0557	0.0099	0.0322	0.0208

staining with crystal violet (CV) and solubilization with 33% glacial acetic acid[10]. The OD595 was used to assess the level of biofilm formed. All assays were done in triplicates on separate days using fresh samples each time.

RESULTS

In this study, the biofilm formation of gram-negative bacterium, *A. baylyi* ADP1, was examined using an assay based on the method described in a previous study[2]. In this assay, staining with 1% CV for 15 min enables the visualization of attached cells after 12 h of bacterial biofilm formation. Unattached, planktonic cells are removed by rinsing with ddH_2O. Cells are stained purple with CV. These biofilm assays were carried out under various conditions and with different solubilization solvent to determine the optimum experimental conditions. Biofilms were readily apparent on polystyrene surfaces, and both growth and biofilm formation were greater under aerobic conditions (Fig. 1). Biofilm visualization and formation were considerably enhanced when 33% glacial acetic acid was used as solubilization solvent with all strains. Biofilm formation was reduced among all mutants. Notably, it was statistically significantly reduced by students' t-test ($P < 0.05$) among mutant (3317) Fimbrial-biogenesis Protein & (PEVL 389) putative surface protein (Fig. 1). This observation may suggest a partial role in biofilm formation by these two genes in *A.baylyi*.

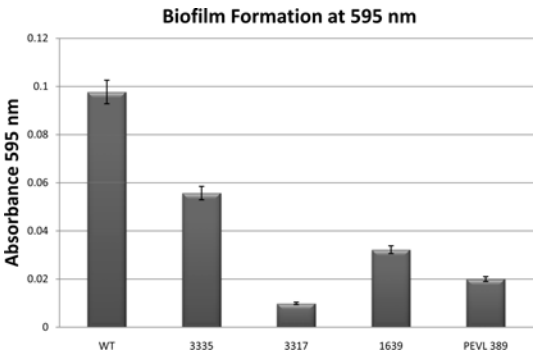

Fig. 1. Quantification of biofilm formation by viable bacteria attached to polystyrene surface at OD 595 nm. Biofilms were readily apparent on polystyrene surfaces, biofilm formation was statistically significantly reduced by students' t-test ($P < 0.05$) among mutant (3317) Fimbrial-biogenesis Protein & (PEVL 389) putative surface protein. This observation may suggest a partial role in biofilm formation by these two genes in *A.baylyi*. Error bars, 1 SD

DISCUSSION

Increasing involvement of *Acinetobacter* infections in hospitals and their multi drug resistance nature has been an important observation[15,14]. *Acinetobacter* strains are associated with pathogenicity, bacterial adhesion and biofilm formation[12]. Biofilm formation is an important feature of most clinical isolates of *Acinetobacter* spp. Biofilms are formed by the assembling of surface microbial cells that are enclosed in an extracellular polymeric matrix[11]. *Acinetobacter* biofilms play a role in infectious diseases such as cystic fibrosis, periodontitis in bloodstream and UTI because of their ability to adhere to medical devices made of plastic or glass[16,13,17].

The environmental effect such as temperature and pH on biofilm formation, and factors like cell surface hydrophobicity (CSH) and production of lectins are important in biofilm formation. The genetic and molecular analysis of these factors could explain the resistance and survival of this pathogen under adverse conditions such as those found in patients and nosocomial environments. This could prove the significance of biofilm formation and antibiotic resistance.

In the present study, biofilms were readily apparent on polystyrene surfaces, and both growth and biofilm formation were greater under aerobic conditions. Visualization and formation of the biofilms were considerably enhanced when 33% glacial acetic acid was used as solubilization solvent with all strains. Biofilm formation was greatly reduced in all mutants. Notably statistical analysis using students' t-test ($P < 0.05$) also revealed a significant reduction in biofilm formation among mutant (3317) Fimbrial-biogenesis Protein and (PEVL 389) putative surface protein. This observation may suggest a partial role in biofilm formation by these two genes.

CONCLUSIONS

Acinetobacter baylyi is a gram negative bacteria that shares common genetic characteristics with *Acinetobacter baumannii* which is known for their multi-drug resistance phenotypes and a major nosocomial infection in health care setting. The availability of whole genome sequencing and the ability of their natural competency could make *A. baylyi* as an easy genetic model for studying *A. baumannii* mutagenesis to enhance our understanding of the nature of multi-drug resistance phenotypes. In this work, we aimed to understand which of *A. baylyi* genes may be involved in biofilm formation. We found that two possible genes may be important in biofilm formation in *A. baylyi*. We suggest that these two genes may play the same role in *A. baumannii* biofilm formation. However, these two genes will need to be verified empirically in *A.baumannii*.

ACKNOWLEDGMENTS

We would like to acknowledge Genoscope for sending us *A. baylyi* ADP1 mutants and for providing us with all information that we need for this project. This work was supported by the national center for biotechnology at KACST, Riyadh, Saudi Arabia.

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