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ARTICLE

Characterization of Biofilm Forming Marine *Pseudoalteromonas spp*

Samia S. Abouelkheir[1*] Eman A. Abdelghany[2] Hanan A. Ghozlan[2] Soraya A. Sabry[2]

1. National Institute of Oceanography and Fisheries (NIOF), Marine Microbiology Lab., Kayet Bay, El-Anfushy, Alexandria, Egypt
2. Faculty of Science, Alexandria University, Egypt

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ABSTRACT

Biofilm forming bacteria are omnipresent in the marine environment. *Pseudoalteromonas* is one of the largest within the γ-proteobacteria class, and a member of marine bacteria. Species of *Pseudoalteromonas* are generally found in association with marine eukaryotes. In this work, we present the isolation and characterization of two strains forming biofilm on rock surface and associated with marine sponge. They were identified using 16SrDNA as *Pseudoalteromonas prydzensis* alex, and *Pseudoalteromonas* sp. alex. They showed the highest titer in biofilm formation quantified using the test tube method using crystal violet.

1. Introduction

Bacterial biofilms form a highly structured community of cells attached to each other and/or a surface enclosed in a complex matrix of extracellular polymeric substances. These biofilms have several terms, including periphyton and mycrophytobenthos [1]. This phenomenon enables bacteria to colonize and prevalent in natural, industrial, and medical environments [2]. In marine environments, bacteria play important roles including driving biogeochemical cycles [3] and supplying materials and energy to higher trophic levels [4]. The phenotypic plasticity of bacteria is responsible for their success and ubiquity [5]. Biotic and abiotic surfaces in various marine environments are rapidly colonized by microorganisms, and surface colonization by marine microbes often involves biofilm formation [6]. Marine bacterial cells produce an extracellular polymeric substance (EPS) matrix after adhesions that establish the formation of a biofilm [1]. These biologically active compounds adapt bacteria to resist the extreme environmental conditions, such as high pressure, hydrothermal vent, and depletion of micronutrients [7].

The genus Pseudoalteromonas has attracted the attention of scientists because they are widely distributed in the marine environment and they are associated with a variety of marine organisms such as corals, sponges and others [8]. *Pseudoalteromonas* is a genus of Gammaproteobacteria that is widespread in the world's ocean and have been shown to produce bioactive compounds that inhibit settling of several fouling invertebrates and algae during biofilm formation [9].

It was thus aimed in this study to isolate, explore, and

**Corresponding Author:*
Samia S. Abouelkheir,
National Institute of Oceanography and Fisheries (NIOF), Marine Microbiology Lab., Kayet Bay, El-Anfushy, Alexandria, Egypt;
Email: samiaabouelkhir@yahoo.com

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identify some marine biofilm forming bacteria attached to natural surfaces in sea water.

2. Materials & Methods

2.1 Bacterial Strains

The biofilm forming *Pseudoalteromonas prydzensis* alex, and *Pseudoalteromonas* sp. alex, were isolated from the surface rocks and sponge at Mediterranean seawater, Alexandria, Egypt. They were identified using 16SrDNA.

2.2 Media

Seawater (SW) medium was used for enrichment and isolation of biofilm forming bacteria. It contained (g/L): yeast extract, 1; peptone, 1; casamino acids, 1. Luria Bertani medium (LB) [10] composed of (g/L): tryptone, 10; yeast extract, 5; and NaCl, 10. Modified **Väätä**nen Nine Salts Solution (VNSS) [11] contained (g/l): NaCl, 17.6; $NaHCO_3$, 0.08; KBr, 0.04; $CaCl_2$. $2H_2O$, 0.41; $SrCl_2$. $6H_2O$, 0.008; Na_2SO_4, 1.47; KCl, 0.25; $MgCl_2$. $6H_2O$, 1.87; H_3BO_3, 0.008; $FeSO_4.7H_2O$, 0.01; Na_2HPO_4, 0.01; Peptone, 1.0; Starch, 0.5; Glucose, 0.5; Yeast extract, 0.5 that modified by replacing inorganic salts by seawater. Synthetic medium (SM) [12] had the following composition (g/L) NaCl, 24.53; $CaCl_2$. $2H_2O$, 1.54; KBr, 0.10; NaF, 0.003; KCl, 0.70; H_3BO_3, 0.03; Na_2SO_4, 4.09; $NaHCO_3$, 0.20; $SrCl_2$. $6H_2O$, 0.017; $MgCl_2$. H_2O, 11.1; KH_2PO_4, 1; K_2HPO_4, 1; $NaNO_3$, 1; NH_4Cl, 1; glycine, 1; glucose, 5; biotin, 5×10^{-8}, thiamine-HCl, 1×10^{-4}.

2.3 Bacterial Isolation

Biofilm forming bacteria were isolated by scrapping the surface of rock submerged in seawater and streaking on seawater agar plates. Sponge was cut into pieces in a flask containing seawater and shacked for 2h before streaking on SW agar plates and incubated at 30°C for 24h.

2.4 Biofilm Development in Glass Tubes

Biofilm formation technique was adopted by Hassan et al., (2011) [13]. Pure mucoid bacterial colonies showing different morphotypes were cultured onto SW agar plates for 48h then transferred into 5ml SW broth and left for another 48h under static condition at 30°C. The cells (OD_{600} of 0.15) were then harvested by centrifugation (6000x g, 5min) and re suspended in 5ml SW broth in test tubes to give a final volume of 5ml .Tubes were then incubated statically at 30°C for 24h to allow bacteria to form biofilms and used for biofilm assay . A negative control contained 5ml SW medium.

2.5 Biofilm Assessment

Quantification of cell adhesion and biofilm formation was performed by the method described by Haney et al., (2018) [14] based on staining biofilm with crystal violet (CV). The content of each tube was poured off after incubation and the attached bacterial cells were rinsed three times with 3ml phosphate buffer saline (PBS) prepared by dissolving NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.44 g; and KH_2PO_4, 0.24 g in 800 ml distilled H_2O and pH adjusted to 7.4 with HCl then complete to a final volume of 1L and dried. Dried tubes with attached bacteria were fixed with 3ml of an aqueous crystal violet (CV) solution (1%) for 20min. Excess CV was removed and biofilms were rinsed with PBS. For quantification of biofilms, the attached stained cells were removed and re-suspended in 3ml ethanol (95%), and the absorbance was then measured spectrophotometrically at 600nm.

2.6 Genotypic Identification of ER9 and ER11

Genomic DNA was extracted from pure biofilm forming isolates according to Sambrook et al., (1989) [15]. The purity of the isolated DNA was confirmed by gel electrophoresis and subjected to polymerase chain reaction (PCR). The 16s rDNA was amplified using primers designed to amplify 1500 bp fragment of the 16s rDNA region. The forward primer was 5'AGAGTTTGATCMTGGCTCAG3' and the reverse primer was 5'TACGGYTACCTTGTTACGACTT3'. The PCR product was sequenced using the same PCR primers and confirmed by Gel electrophoresis according to Sambrook et al., (1989) [15]. The molecular phylogeny was performed using BioEdit software [16]. Sequences of rRNA genes, for comparison, were obtained from the NCBI database. The phylogenetic tree was displayed using the TREEVIEW program.

2.7 Phenotypic Characterization

Phenotypic characteristics were determined for the selected isolates according to the standard methods described by Ventosa et al., (1982) [17].

3. Results & Discussion

3.1 Selection of Biofilm Forming Marine Bacteria

Thirty mucoid colonies developed on SW agar plates were screened for biofilm formation and quantified using the CV quantification method. This method was reported to be the most convenient for analyzing biofilm formation [18]. The method depends on that CV binds to negatively charged molecules, including nucleic acids and acidic polysaccharides, and therefore serves as an overall measure of the whole biofilm. The values of biofilm in test tubes ranged from OD_{600} 0.5 to OD_{600} 3. These values are in good agreement with those reported by other investigators in natural environments [14]. The highest values (OD_{600}= 2.7and 2.6) were recorded for

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isolates ER11 & ER9, respectively (Figure1).

Bacteria were classified according to the scheme of Hassan et al., (2011) [13] as follows: non biofilm producer (0), less than or equal to ODc; weak biofilm producer (+), OD greater than ODc and less than or equal to 2×ODc; moderate biofilm producer (++), OD greater than 2×ODc and less than or equal to 4×ODc; strong biofilm producers (+++), OD greater than 4×ODc. This classification is based upon the cutoff OD (ODc) value which was defined as three standard deviations above the mean OD of the negative control. Based on obtained data, 40% of the isolates were non-biofilm producers, whereas a 30% were classified as weak, 16.6% as moderate and 13.3 % as strong biofilm forming bacteria.

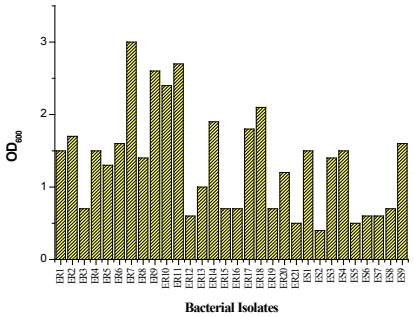


Figure 1. Quantification of biofilms formed by marine bacterial isolates grown in SW medium and incubated for 24h at 30°C

The distribution of biofilm forming bacteria on biotic and abiotic surfaces is depicted in Figure2. It is worth mentioning that the majority of biofilm forming bacteria (70%) were isolated from rock surface, whereas only 9 isolates (30%) were recovered from sponge. Biofilms cover most subtidal and intertidal solid surfaces such as rocks, ships, loops, marine animals and algae [19]. Surface roughness has been reported to be an important factor in bacterial attachment to inert surfaces. Other groups observed greater cell attachment on surfaces with high roughness [20]. In good agreement with our values, those reported by Bruhn et al., (2019) [21] for *Roseobacter* sp.

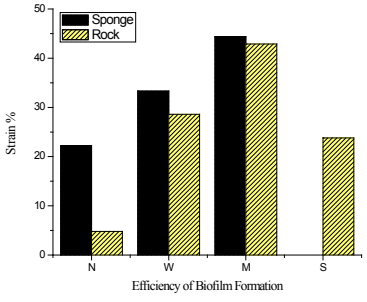
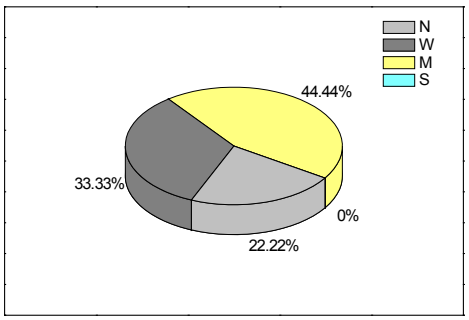


Figure 2. Percentage of biofilm forming bacteria isolated from sponge and marine rocks

Note: N= non biofilm formers; W= weak biofilm former; M= moderate biofilm former and S= strong biofilm former.

Data in Figure3a depict that according to scheme proposed by Hassan et al. (2011) [13], none of the isolates recovered from sponge were classified as strong producers, whereas, almost 44% were classified as moderate producers, 22% as normal producers and 33% as weak producers. The chart presented in Figure3b explains that the majority of isolates (42.9%) obtained from rock surface were moderate producers whereas, weak and strong producers represented 28.6 and 23.8% respectively.



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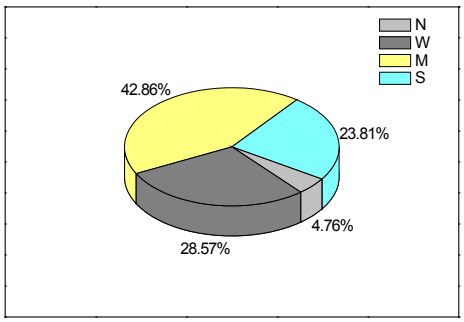

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Figure 3. A chart showing the % of biofilm forming bacteria isolated from sponge (a) and a marine rock (b)

Note: N= non biofilm formers; W= weak biofilm former; M= moderate biofilm former and S= strong biofilm former.

3.2 The Role of Nutrient Status on Biofilm Formation

Indeed, attachment of bacteria to surfaces depends on the specific micro-niche created by marine particles [22]. Therefore, four media of different formulae (SW, LB, VNSS and SM) were used to select the best nutrient composition for biofilm formation. Data in Figure4 reveal that *In vitro* biofilm formation was highly dependent on medium composition. Synthetic medium (SM) did not support biofilm formation (data not shown) whereas, media containing natural compounds enhanced biofilm formation. In general SW medium containing low concentrations of natural substances (1 g of each of yeast extract, peptone and casamino acids) supported the *In vitro* biofilm formation as

indicated by the highest values of OD_{600} ranged from 1 to 3.0 compared to other tested media. Similarly, LB showed more or less compatible biofilm values to SW depending on bacterial strain. The suitability of seawater medium might be attributed to the low nutrient contents which support the growth and biofilm formation of oligotrophic marine bacteria. Oligotrophic environments are defined by low absolute concentrations of nutrients [23].

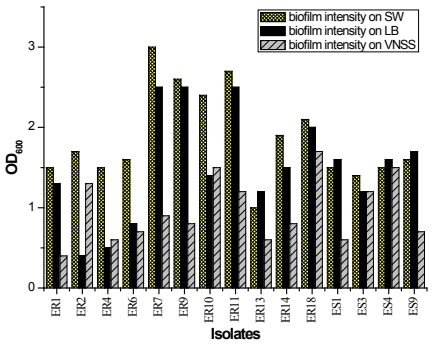

Figure 4. Values of OD_{600} as a measure of biofilm formation by marine isolates grown on different media

3.3 Microtitre Plate Versus Test Tube Method for Biofilm Formation

The microtitre plate was reported to be an easy, reliable and excellent method for screening a large number of isolates [24]. Therefore, this experiment was designed to compare the *In vitro* biofilm formation using the microtitre plate and test tube methods. From data shown in Figure5, it is concluded that under our experimental conditions the values obtained by the tube method were higher than those measured in microtitre plates. In some cases these values showed two or three fold increase compared to those of microtitre plates. Therefore, the tube method was adopted for further studies.

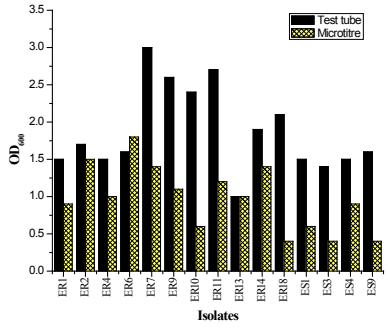

Figure 5. Quantification of biofilms formed by marine bacteria using Tube method versus Microtitre Plate

3.4 Phylogenetic Analysis of ER9 and ER11

The partial sequences of amplified 16SrDNA genes of ER9 and ER11 were aligned with closest relatives of sequences on database. Partial sequence of ER9 showed the highest homology (99%) to *Pseudoalteromonas* sp. OC-A5-12 and 98% to *Pseudoalteromonas prydzensis* strain CAIM381, whereas ER11 sequence analysis showed 97% similarity to several *Pseudoalteromonas* strains. Members of this genus have been most frequently isolated from marine biofilm, marine eukaryotes, seawater, sea ice, etc. [25, 8]. The two strains are members of Family *Pseudoalteromonadacea*, *Gammaproteobacteria*, and Phylum *Proteobacteria*. The sequences were deposited in GenBank with accession numbers JF965506 and JN592714 respectively. Members of *Gammaproteobacteria* have been reported to be one of the most abundant groups of readily cultivable heterotrophs from marine environment [26]. They possess a versatile pathway which explains their coexistence and survival in diverse environments [26]. Figures 6, 7 show the phylogenetic trees of the two isolates and their relation to other related species. Therefore, isolate ER9 was designated as *Pseudoalteromonas prydzensis* alex and ER11 was designated as *Pseudoalteromonas* sp. alex.

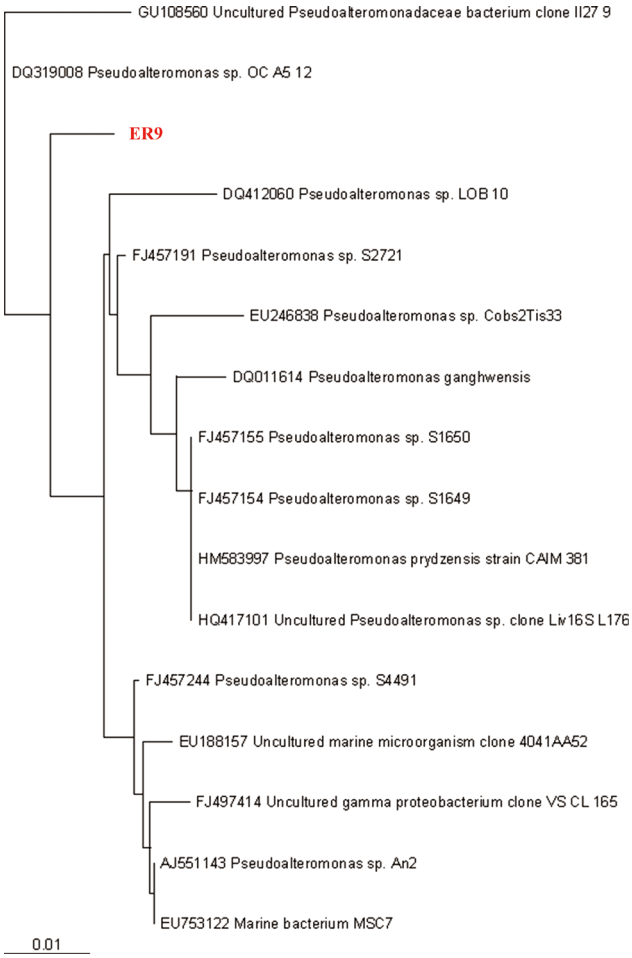

Figure 6. Phylogenetic tree of isolate ER9

Note: The dendogram based on partial 16S rDNA gene sequencing

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shows the phylogenetic position of the isolate among representatives of related bacterial species

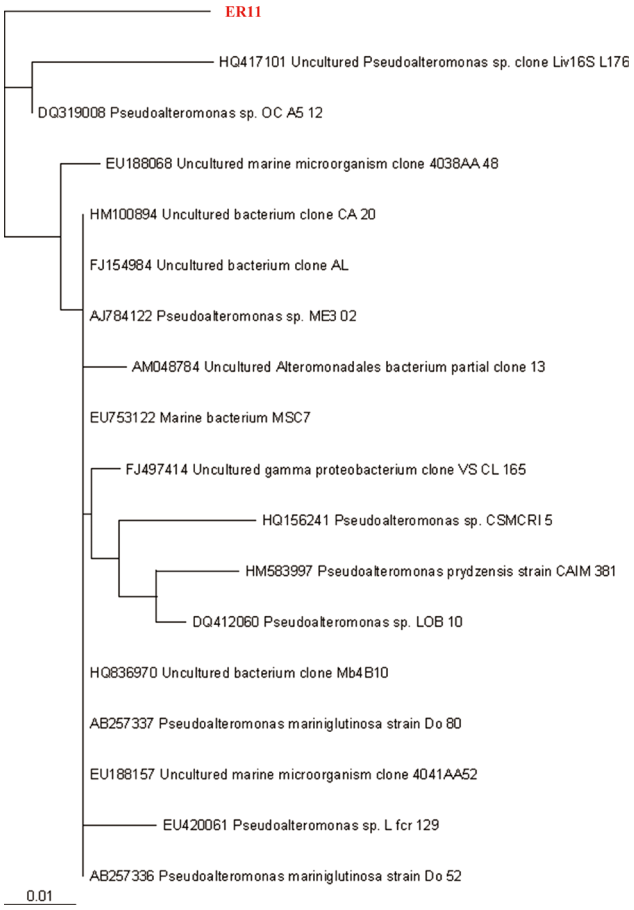

Figure 7. Phylogenetic tree of isolate ER11

Note: The dendogram based on partial 16S rDNA gene sequencing shows the phylogenetic position of the isolate among representatives of related bacterial species

3.5 Phenotypic Characterization

Phenotypic characterization of bacteria is complementary to phylogenetic data. Therefore, the two strains were subjected to some morphological, physiological and biochemical tests. Data are summarized in Table 1. It is worth mentioning that despite the difference in generic identification between the two *Pseudoalteromonas* strains, they both shared the same characteristic performed in this study.

Table 1. Differential characteristics of biofilm forming bacteria

Character	*P. prydzensis* alex	*P.* sp. alex
Colony morphology	Mucoid off-white	Mucoid off-white
Cell morphology	Short rods solitary	Short rods solitary
Gram stain	-	-
Metabolism		
Growth without sea water salts	-	-
Halotolerance (% NaCl)	10	10
Growth at:		
10°C	-	-
15°C	+	+
20°C	+	+
30°C	+	+
37°C	+	+
Biochemical		
Catalase	+	+
Oxidase	+	+
H_2S production	-	-
Nitrate reduction	-	-
Hydrolysis of		
Dextrin	+	+
Starch	+	+
Cellulose	-	-
Casein	+	+
Gelatine	-	-
Chitin	+	+
Tween 20	+	+
Tween 80	+	+
Oil	+	+
Utilization		
D-Glucose	-	-
D-Fructose	-	-
Maltose	+	+
Sucrose	-	-
Lactose	-	-
Galactose	-	-
Reaction to antibiotic		
Ofloxacin	R	R
Cefuroxime	S	S
Levofloxacin	R	R
Imipenem (Tinam)	S	S
Ampicillin/Sulbactam	S	S
Vancomycin	S	S
Augmentin	S	S
Ciprofloxacin	R	R
Amikacin	S	S
Ampicillin	S	S
Netilmicin	R	R
Meropenem	S	S
Cefotaxime	S	S
Chloramphnicol	R	R
Nitrofurantin	S	S
Norfloxacin	R	R

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4. Conclusion

Marine microbes live attached to surfaces forming biofilms which are of significant importance from the biotechnological point of view. Two *Pseudoalteromonas* isolated from rock surfaces were the most potent in biofilm formation as detected by the tube method using crystal violet. This genus is very important in producing valuable molecules, thus further investigations are planned in the future.

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