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# Biofilm Formation by Marine *Cobetia marina* alex and *Pseudoalteromonas* spp: Development and Detection of Quorum Sensing N-Acyl Homoserine Lactones (AHLs) Molecules

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ABSTRACT

Surfaces submerged in seawater are colonized by various microorganisms, resulting in the formation of heterogenic marine biofilms. This work aims to evaluate the biofilm formation by *Cobetia marina* alex and doing a comparative study between this promising strain with the two bacterial strains isolated previously from the Mediterranean seawater, Alexandria, Egypt. Three strains; *Cobetia marina* alex, *Pseudoalteromonas* sp. alex, and *Pseudoalteromonas prydzensis* alex were screened for biofilm formation using the crystal violet (CV) quantification method in a single culture. The values of biofilm formed were OD<sub>600</sub> = 3.0, 2.7, and 2.6, respectively leading to their selection for further evaluation. However, factors affecting biofilm formation by *C. marina* alex were investigated. Biofilm formation was evaluated in single and multispecies consortia. Synergistic and antagonistic interactions proved in this work lead to the belief that these bacteria have the capability to produce some interesting signal molecules N-acyl Homoserine Lactones (AHLs).

## 1. Introduction

In marine environments, bacteria play roles that include driving biogeochemical cycles<sup>[1]</sup> and supplying materials and energy to higher trophic levels<sup>[2]</sup>. Although genotypic evolution may contribute a significant selective advantage to environmental stimuli, phenotypic plasticity allows bacteria to grow and thrive under fluctuating, challenging conditions<sup>[3]</sup>. In addition, bacterial biofilm forms a highly structured community of cells that are attached to each other and/or on a surface through the

production of an extracellular polymeric substance (EPS) matrix that enable bacteria to colonize different habitats<sup>[4,5]</sup>. Biofilms have several terms, including periphytons and Microphytobenthos<sup>[6]</sup>.

The cell-to-cell communication process between bacteria, generally known as quorum sensing (QS), controls several vital features of biofilm development between other phenomena<sup>[7]</sup>. The bacterial cell envelope plays a crucial role in the intercellular signalling as well as communication between neighbouring cells in small microcolonies that help in decision-making processes. Part

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of this communication occurs through quorum sensing (QS), a phenomenon that involves cell density-dependent control of gene expression. For QS to be possible, a minimum number of bacteria must be aggregated within a specific volume sensed by increasing the concentration of autoinducers [8].

*Cobetia marina* is a Gram-negative, aerobic, slightly halophilic, rod-shaped bacterium extensively used as a model in marine biofouling research and as multi-species biofilm formation in natural seawater [9]. It is distinct from the genus *Halomonas* and is one of eleven genera of the family Halomonadaceae [10]. Historically, *Cobetia marina* was first described as *Arthrobacter marinus* by Cobet et al. (1970) [11], but was later identified as *Pseudomonas marina* [12]. Almost 10 years later, it was reclassified once again within the genus *Deleya* [13] and then transferred to the genus *Halomonas* [14]. Delineation of the species in this genus was performed based on 16S and 23S rRNA sequence similarities and additional phenotypic evidence that supported the inclusion of *Halomonas marina* in the novel genus *Cobetia* as *Cobetia marina* [15,16]. On the other hand, *Pseudoalteromonas* is a genus of Gammaproteobacteria present in various marine habitats that is widespread in the world's ocean [17]. Many strains of this genus are surface colonizers on marine eukaryotes and produce a wide range of pigments and bioactive compounds that inhibit settling of several fouling invertebrates and algae during biofilm formation [18].

Therefore, it was thus aimed in this study to explore and identify biofilm-forming bacteria attached to rock surfaces in seawater. It was also aimed to evaluate whether synergistic or competitive interactions occur during multispecies biofilm formation. Moreover, some factors affecting biofilm formation by *C. marina* alex were studied.

## 2. Materials & Methods

### 2.1 Media

Seawater (SW), Modified Vääänen Nine Salts Solution (VNSS), Luria Bertani medium (LB) and Synthetic medium (SM) used to evaluate the biofilm formation and to explore the role of nutritional status were prepared as previously described by Abouelkheir et al. (2020) [19]. YMG Medium [20] used for screening for EPS production contained in g/L: glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5. All components were dissolved in 500 ml distilled water and 500 ml aged seawater (ASW), pH adjusted to 7.0.

### 2.2 Identification of Bacterial Isolate

Isolate ER7 was selected for phylogenetic analysis

using 16SrRNA in addition to phenotypic characterization.

### 2.3 Bacterial Strains

*Cobetia marina* alex used in this study was isolated from rock surface submerged in Mediterranean seawater, Alexandria, Egypt and identified by 16SrRNA. *Pseudoalteromonas prydzensis* alex with accession number JF965506 and *Pseudoalteromonas* sp. JN592714 were isolated and identified in a previous work [19]. *Agrobacterium tumefaciens* was secured from the faculty of Agriculture, Alexandria University. *Escherichia coli*, *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp., *Vibrio* sp. N1, *Vibrio* sp. N2, *Streptococcus* sp., and *Candida albicans* were obtained from Department of Botany and Microbiology, Faculty of Science, Alexandria University and National Institute of Oceanography and Fisheries, Alexandria branch.

### 2.4 Biofilm Development and Assessment in Glass Tubes

Biofilm formation technique was adopted after Hassan et al. (2011) [21] and quantification of biofilm was performed using the method described by Haney et al., (2018) [22] based on staining the biofilm with crystal violet (Figure 1).

### 2.5 Factors Affecting Biofilm Formation by *C. marina* alex

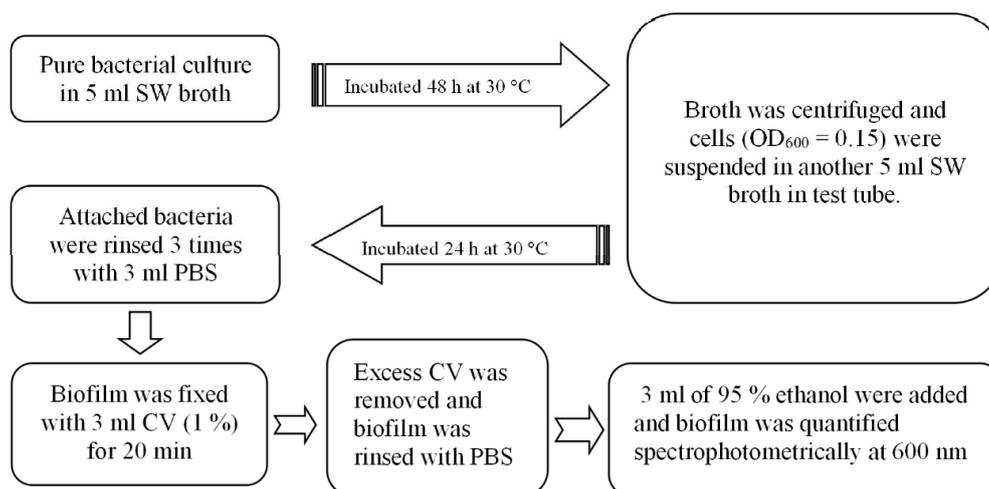
Effect of pH on biofilm formation was examined by adjusting portions of SW medium to different pHs from 5 to 7. Simultaneously, biofilm formation was evaluated at different temperatures (20, 30, and 37 °C). The role of NaCl concentration on biofilm formation was examined by supplementing the SW medium with different concentrations (5, 10, 15, 20 and 25%). Carbon sources (glucose, lactose, galactose, maltose or sucrose) were separately added to SW medium at two different concentrations (0.25 and 0.5 g/L) to elucidate their effect on biofilm formation.

### 2.6 Progression of Biofilm Formation

The biofilm formed in test tubes was quantified at different time intervals from 1 to 4 days. Biofilm formation was also monitored microscopically by examining cell attachment to glass coverslips as described by Walker & Horswill (2012) [23].

### 2.7 EPS Production

The ability of bacterial strains to produce EPS was



**Figure 1.** A schematic diagram shows the formation and quantification of biofilm using the test tube method

investigated by the method adopted by Casillo et al., (2018)<sup>[24]</sup>. Each bacterial strain was grown in 50 ml YMG broth and incubated at 25 °C for 24 h. 200 µl were used to inoculate 50 ml of the same medium and incubated at 25°C for 5 days at 120 rpm. Cells were removed by centrifugation at 10000x g for 20 min. Cold ethyl alcohol (3 ml) was added to cell free supernatant and left overnight at 10 °C. The precipitated EPS was air dried and weighed.

## 2.8 Bioassay for Acyl Homoserine Lactone (AHL) Production by Agar-plate

*Agrobacterium tumefaciens* was used as a reporter strain to screen for AHL- like signalling molecules<sup>[25]</sup>. Agar plate-based bioassay was carried out by streaking 50 µl of the reporter strain on the surface of LB medium supplemented with 50 µl X-Gal (5-Bromo-4-Chloro-3-Indolyl-D-Galactopyranoside) solution (Appli Chem. GmbH, Darmstadt, Germany) prepared by dissolving 20 mg of X-Gal in 1ml of 100 % dimethylformamide (DMF) and stored at -20 °C in the dark. 50 µl of each tested strain were spotted on top of the plates. Plates were checked after incubation at 28 °C for 48 h. The development of blue colour (catabolism of X-Gal) in the plates indicated the presence of AHL like substances.

## 2.9 Biofilm Formation by Multispecies Consortia

Species interaction was studied in single species using one bacterium, in dual combination of two strains or triple combination of the three strains (*Cobetia marina* alex, *Pseudoalteromonas prydzensis* alex, and *Pseudoalteromonas* sp. alex). This was performed by mixing equal inoculum of each organism and allowing the biofilm to be formed and quantified. Biofilm formation

was also assessed in a consortia of *Cobetia marina* alex and one of several marine bacteria. These included 3 Gram +ve (*Staphylococcus* sp., *Streptococcus* sp., and *Bacillus* sp.) and 4 Gram-ve (*E. coli*, *Pseudomonas* sp., *Vibrio* sp. N1, and *Vibrio* sp. N2), as well as *Candida* sp. In mixed species consortia, equal cell densities were used. Biofilm was measured as previously described.

## 2.10 Data Analysis

All investigations were performed in triplicates. The results were statistically analyzed and accomplished by using Past3 software and Origin Pro 8.1. The data were expressed by means ± SE and ± SD. The significant values were determined at P-value < 0.05.

## 3. Results and Discussion

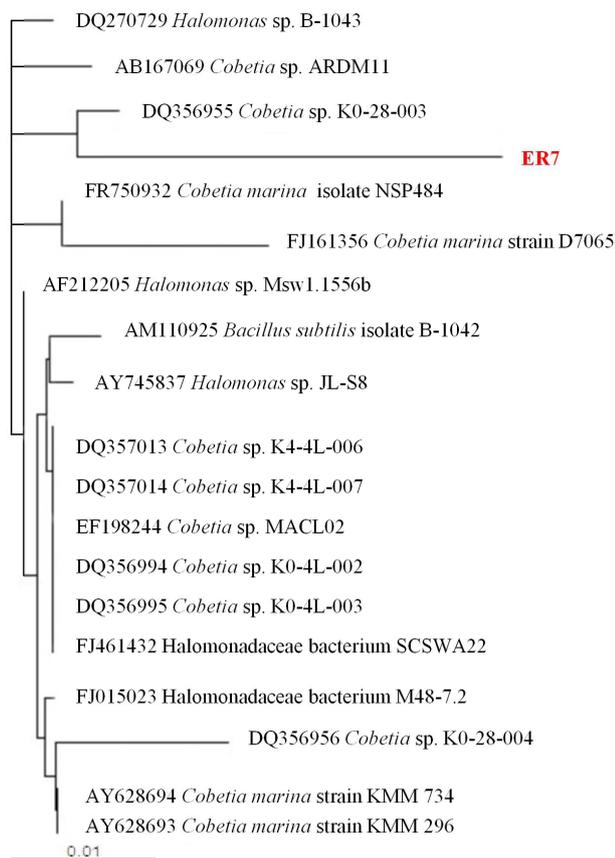
### 3.1 Biofilm Formation

*Pseudoalteromonas* sp. alex, *P. prydzensis* alex, and *Cobetia marina* alex (ER7) were screened for biofilm formation in single culture<sup>[26]</sup>. The values of biofilm formed in test tubes ranged from OD<sub>600</sub> 0.5 to 3 which are in good agreement with those reported by other investigators in natural environments<sup>[27]</sup>. The highest value (OD<sub>600</sub>= 3.0) was recorded for isolate ER7 leading to its selection for further evaluation.

### 3.2 Phylogenetic Analysis of ER7

The partial sequence of amplified 16SrRNA gene of isolate ER7 was aligned with closest relatives of sequences on NCBI database and showed 99% similarity to several sequences of *Cobetia marina* strains (Figure 2). *Cobetia marina* is a member of the family Halomonadaceae, Gammaproteobacteria together with eight other genera<sup>[28]</sup>.

Its ecophysiological diversity was studied by Tang et al. (2018)<sup>[29]</sup>. The sequence was deposited in GenBank with accession number JF965505. It was thus designated as *Cobetia marina* alex. Members of Gammaproteobacteria are the most abundant group of readily cultivable heterotrophs in the marine environment<sup>[30]</sup>.



**Figure 2.** Phylogenetic tree of ER7, the dendrogram based on partial 16S rRNA gene sequencing shows the phylogenetic position of ER7 among representatives of related bacterial species

### 3.3 Phenotypic Characterization

The three bacterial strains formed off-white mucoid colonies on seawater agar plates. Cells were Gram negative, non sporulating short rods (arranged in pairs for *Cobetia marina*) and unable to grow in absence of seawater referring to their halophilic nature. All strains tolerated up to 10% NaCl in the medium. Only *C. marina* alex grew at 10 °C indicating its psychrotolerant nature. All were catalase and oxidase positive except for *C. marina* alex (oxidase negative). Although strains of *Pseudoalteromonas* produced hydrolytic enzymes and degraded several substrates such as cellulose, carboxymethyl-cellulose (CMC), xylan, pectin,

and soluble starch, *C. marina* alex did not show any degradative capability to the same substrates.

All strains showed variability to utilize different carbon sources and sensitivity to the tested antibiotics (Figure 3). In this context, the phenotypic features of *C. marina* alex isolated from the Mediterranean Sea, and Alexandria were compared to other *C. marina* strains isolated from other habitats. Strains used for comparison were *C. marina* LMG2217T (type strain) isolated from seawater, Atlantic Ocean, *C. marina* KMM296 isolated from Mussel (*Grenomytilus grayanus*), the Sea of Japan, Pacific Ocean and five strains isolated from brown alga (*Fucus evanescens*), the Sea of Okhotsk, Pacific Ocean. Data used for comparison were all taken from Ivanova et al. (2005)<sup>[31]</sup>.

Data given in Figure 4 depict a number of phenotypic characteristics that distinguish the Mediterranean Sea strain from other *Cobetia* strains. Sodium ion was required for growth in all except for *C. sp.* KMM296. They all shared the property of reducing NO<sub>3</sub> and inability to produce lipase. Only *C. marina* alex could not grow in 20% NaCl, whereas LMG2217 and KMM734 grew at this level. Our strain shared the ability to grow at 10 °C together with KMM296. Maltose was used as a carbon source by *C. marina* alex only.

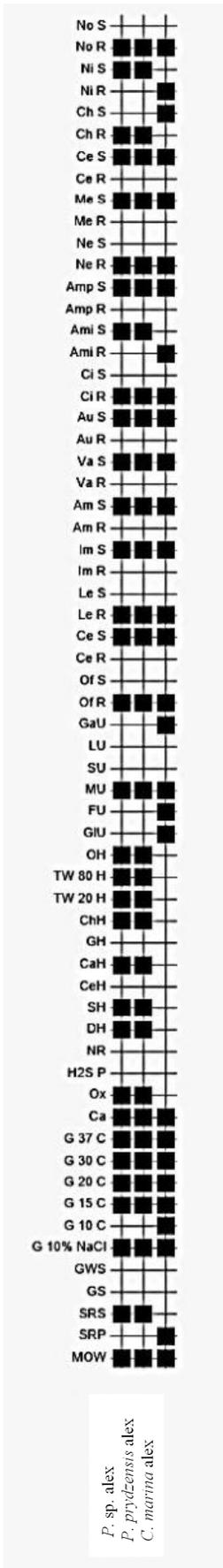
### 3.4 Factors Affecting Biofilm Formation by *C. marina* alex

#### 3.4.1 Effect of pH and Temperature

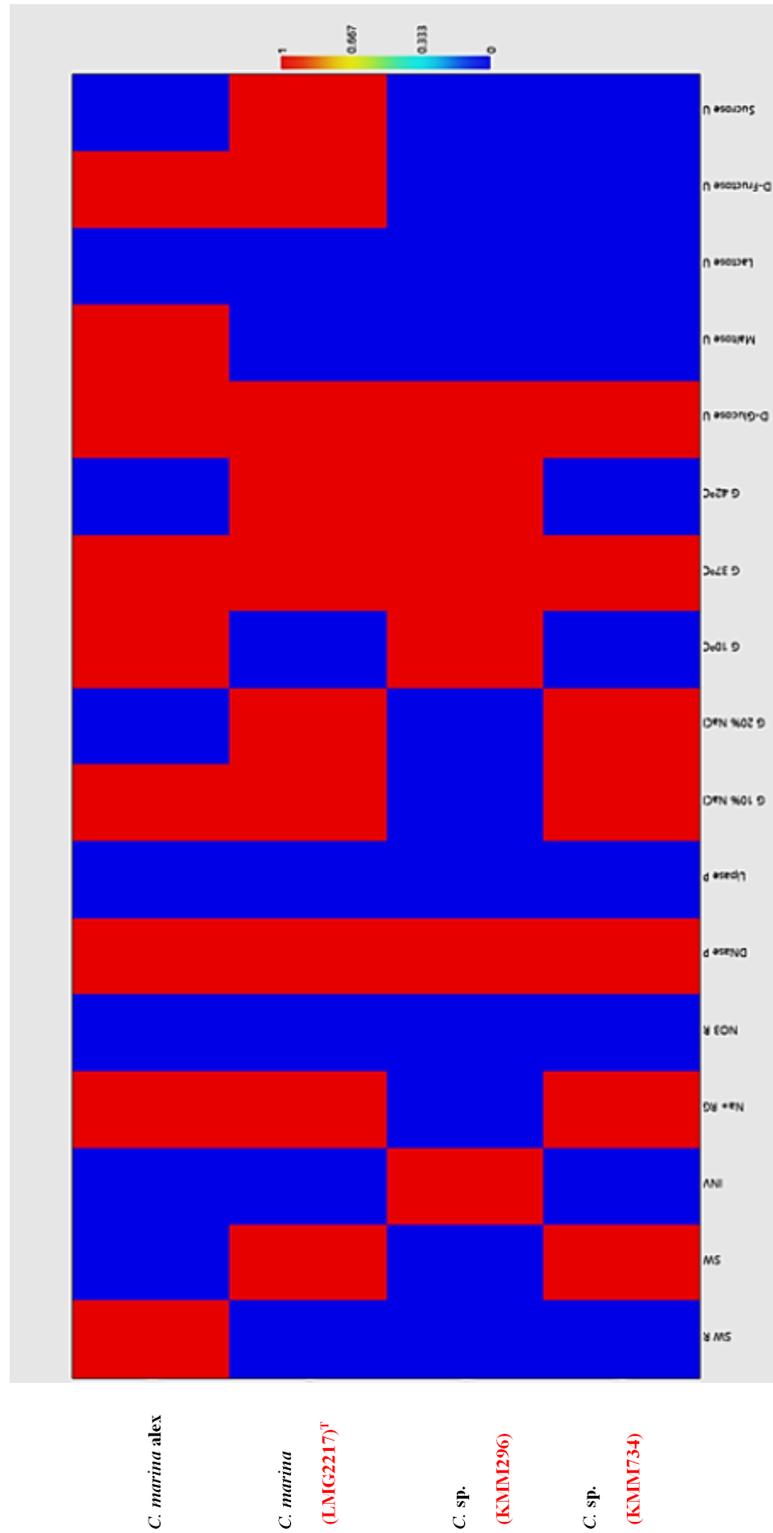
The psychrotolerant, moderate halophilic bacterium was allowed to grow stagnant on portions of SW medium adjusted to different pHs from 5 to 10 and incubated under static conditions at 20, 30 and 37°C; biofilm was measured after 24h.

Figure 5a reveals that the biofilm values formed at neutral or slightly alkaline pH were comparable (OD ~<sub>600</sub> 4.5) but a higher value (OD ~<sub>600</sub> 7.6) was recorded at alkaline pH. Similar data were obtained with *Stenotrophomonas maltophilia*<sup>[32]</sup>. On the contrary, optimal biofilm formation by *Cobetia crustatorum* sp. nov was recorded at pH 5.0-6.0. However, it was observed that biofilm formation was sensitive to pH changes with *Streptococcus gordonii* and *S. meliloti*<sup>[33]</sup>. Taken together, these results suggest that pH effect in terms of establishing a biofilm, may differ from one bacterial species to another, thereby, enabling each bacterial species to efficiently colonize its preferred environment.

Moreover, Anderson (2016)<sup>[34]</sup> examined the effect of increasing temperatures on biofilms in coastal waters and reported that the increase in water temperature enhanced biofilms at the warmer station compared to the colder one.



**Figure 3.** Differential characteristics of the three biofilm-forming bacterial strains used in this study, presence (■) and absence (□) MOW: Off-white mucoid, SRP: Short rods arranged in pairs, GS: Cells were Gram-negative, GWS: Unable to grow in absence of seawater



**Figure 4.** Plot matrix chart showing comparative phenotypic features (presence (red) and absence (blue)) of *C. marina alex* isolated in this study and other strains isolated from different ecological habitats [31]

In general, effective temperature is dependent on bacterial strain<sup>[35]</sup>.

### 3.4.2 Effect of Osmolarity

The present investigation was undertaken to study the effect of adding different concentrations of NaCl to the culture medium on biofilm formation. Data provided in Figure 5b show that biofilm formation by *C. marina alex* was dramatically affected by osmolarity. The value was doubled upon the addition of 5 % NaCl in the medium compared to medium with seawater only. Moreover, supplementing the medium with 10% NaCl caused an almost 1.5 fold increase in biofilm value. Further increase in NaCl concentration was obviously deleterious to biofilm formation. Osmolarity was reported to clearly influence *P. fluorescens* biofilm formation by Kimkes & Heinemann (2020)<sup>[36]</sup>. Similarly, Amaya-Gómez et al. (2015)<sup>[35]</sup> found that growth at high osmolarity inhibited biofilm formation. Biofilm formation by *Cobetia crustatorum* sp. nov was best in presence of 6.5 % (w/v) NaCl<sup>[10]</sup>.

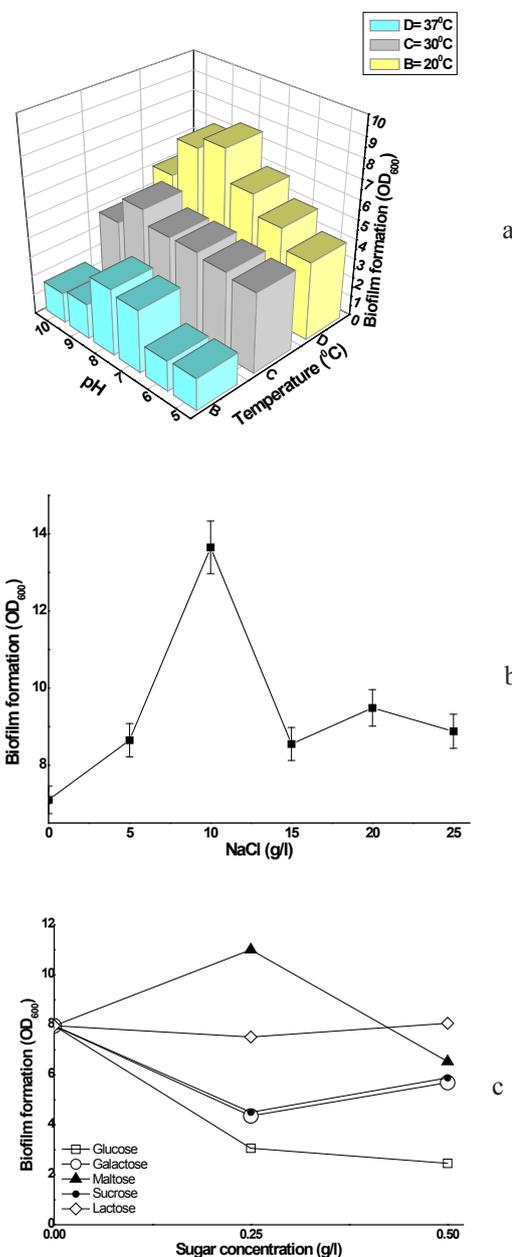
### 3.4.3 Effect of Carbon Source

Data given in Figure 5c show that among tested carbon sources, only maltose (0.25 g/L) enhanced biofilm formation with an increase of about 30 % compared to SW medium free from the carbon source. However, glucose addition did not significantly improve biofilm formation.

### 3.5 Single Species Biofilm Progression

Biofilm development was monitored in test tubes of a monoculture of *Cobetia marina alex*, *P. prydzensis alex*, and *P. sp. alex*. A similar pattern for biofilm development was observed for all strains. Cells began to attach to substratum after inoculation and well developed as indicated by OD<sub>600</sub> values (7.9, 7.2 and 6.9, respectively) after 12 h of incubation and remained stable till 18h. Detachment of cells from biofilm was clearly observed by the drop in OD<sub>600</sub> values after 16h (Figure 6).

The attachment and colonization experiments conducted to monitor biofilm formation microscopically on glass coverslips depicted that cells of *C. marina alex* formed a well-defined spherical microcolony after 6 h and was well developed after 12 h (Figure 7). The same pattern was characterized for the marine bacterium *Vibrio cholerae* which also formed microcolony<sup>[37]</sup>. On the other hand, it was observed that *P. prydzensis alex* and *P. sp. alex* cells were not randomly scattered but rather distributed in batches after 6 h of attachment. Similar observations were reported for *P. tunicola*<sup>[38,39]</sup>. The biofilm formation by *P. spongiae* was performed in static

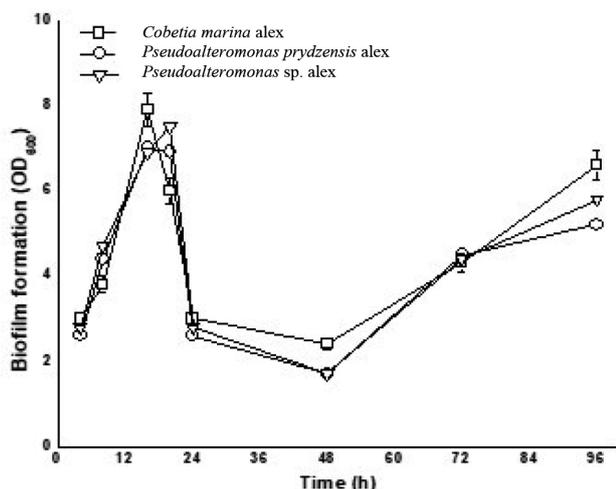


**Figure 5.** Effect of temperature and pH on biofilm formation by *C. marina alex*. (a), Effect of NaCl concentration on biofilm formation by *C. marina alex* on seawater medium at 30 °C for 24 h. The error bars represent the standard error of the means (SEMs) and the null hypothesis of the analysis of result; the samples came from the same population and biofilm formation at the 0.05 level is not significantly different. Statistics were achieved by a Kruskal-Wallis ANOVA test (b), Values of OD<sub>600</sub> as a measure of biofilm formation by *C. marina alex* grown on seawater medium supplemented with different carbon sources at 30 °C for 24 h (c); The error bars represent the standard error of the means (SEMs)

condition for a short period of time (10 h)<sup>[40]</sup>.

### 3.6 Extracellular Polysaccharides (EPS) Production

EPS is produced as a secondary metabolite that plays a role in biofilm formation<sup>[41]</sup>. The amount of EPS produced

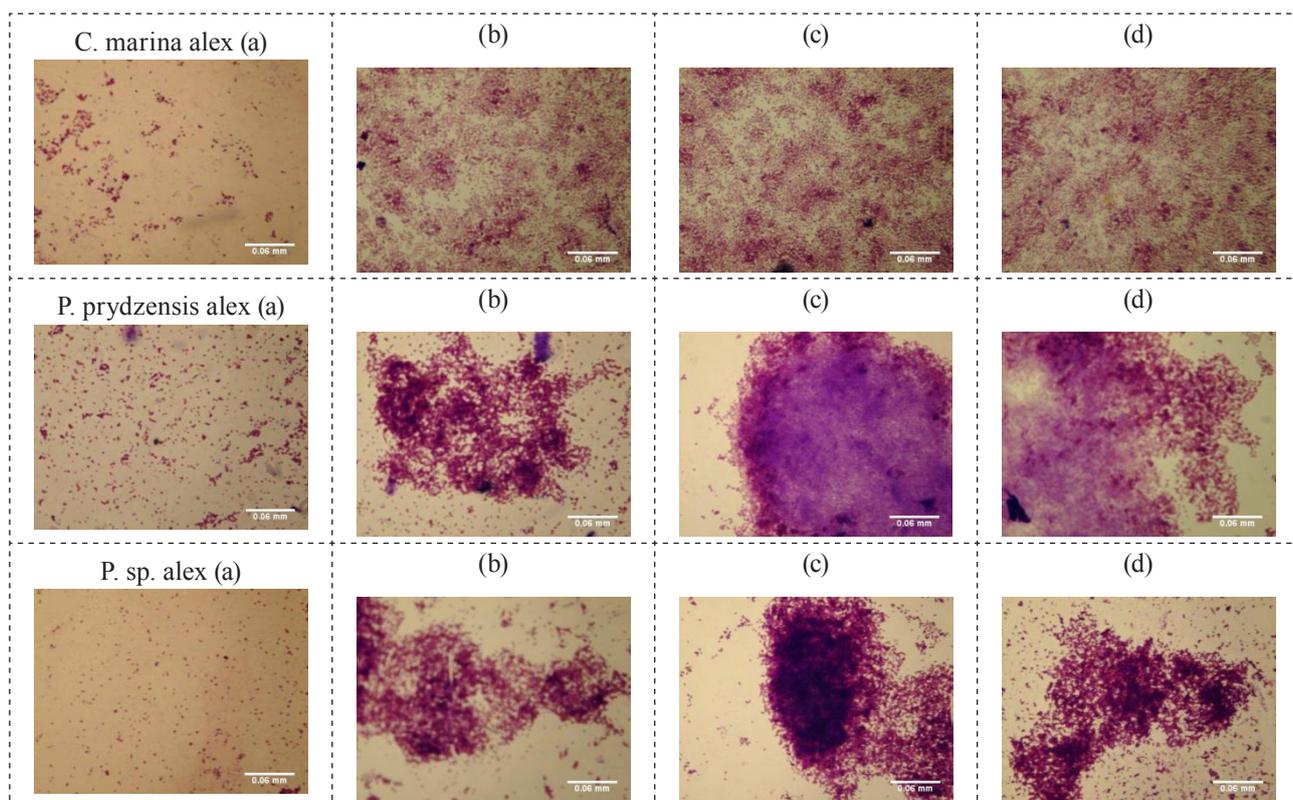


**Figure 6.** Single species biofilm progression on seawater medium at 30 °C at different time intervals; The error bars represent the standard error of the means (SEMs)

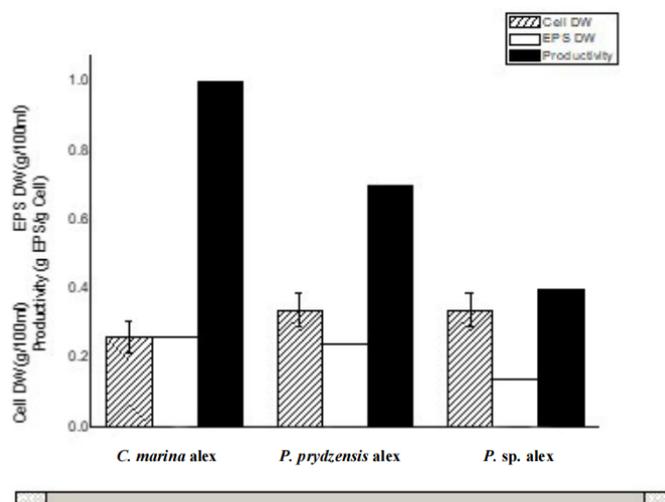
is determined by the biomass yield beside other factors, therefore, the amount of EPS produced per unit biomass (productivity) varied among the tested strains (Figure 8). Under our experimental condition, *C. marina alex* produced equal amounts of dry weight and EPS (0.26 g/100 ml) and therefore exhibited the highest productivity (1.0 g/cells) compared to other tested species. The two *Pseudoalteromonas* species gave almost the same amount of dry weight but *P. prydzensis alex* produced a higher amount of EPS (0.34 g/100 ml) compared to *P. sp. alex* (0.24 g/100 ml) and consequently, the EPS productivity of *P. prydzensis alex* was 50% higher than that of *P. sp. alex*. Same observation was reported by Al-Nahas et al. (2011)<sup>[42]</sup> for *Pseudoalteromonas* species.

### 3.7 Quorum Sensing Molecules

A variety of different molecules can be used as signals. Common classes of signalling molecules are oligopeptides in Gram-positive bacteria, N-Acyl Homoserine Lactones (AHLs) in Gram-negative bacteria and a family of autoinducers known as AI-2 in both Gram-negative and Gram-positive bacteria. AHLs signals are involved in the regulation of a range of important biological functions, including luminescence, antibiotic production, plasmid



**Figure 7.** Biofilm development of *C. marina alex*, *P. prydzensis alex*, and *P. sp. alex* grew on cover slide as observed by Phase-contrast light microscope (x1000) with scale bar of 0.06. (a) Planktonic cells and biofilm development after (b) 6 h (c) 12 h and (d) 24 h incubation



**Figure 8.** Cell mass, EPS mass, and EPS productivity after incubation on YMG broth at 25 °C for 5 days at 120 rpm; the error bars represent the standard deviation of the means (SDMs)

transfer, motility, virulence and biofilm formation<sup>[43]</sup>. In the present study as a sequential step to EPS production, detection of quorum sensing molecules was essential. The reporter strain (*Agrobacterium tumefaciens*) was used in order to test the production of AHL signalling molecules. The strain produces a blue colour in the presence of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) in response to a wide range of AHLs<sup>[44]</sup>. Positive results were obtained with the three tested bacterial strains as indicated by the appearance of blue colour surrounding the colonies (Figure 9). This only occurs as a response to the production of AHLs as quorum sensing molecules. Our data are in agreement with other data which indicate that most AHL-producing bacteria belong to *Vibrio* and *Pseudoalteromonas* species and Rhodobacteraceae family<sup>[25]</sup> frequently found to be dominant in early marine biofilm communities<sup>[45]</sup>. Moreover, Dobretsov et al. (2009)<sup>[46]</sup> screened about forty-three bacterial isolates from marine snow and associated planktonic diatoms, for production of AHLs in the laboratory using *Agrobacterium tumefaciens*

as bacterial reporter strain. Ibacache-Quiroga et al. (2013)<sup>[47]</sup> used *Agrobacterium tumefaciens* NTL4 as a biosensor in which a blue colour indicated X-gal hydrolysis was recorded as positive for presence of HSL in cell-free supernatant of *A. salmonicida*. Our results indicate that quorum sensing molecules may play a role in biofilm formation by the tested strains.

### 3.8 Interspecies Interaction

The interspecies interactions in dual, triple and mixed species biofilms were investigated to find out whether synergistic interactions occur during multispecies biofilm formation and whether multispecies biofilms offer enhanced fitness compared to single-species biofilm. Therefore, this approach was an attempt to get closer to the reality of naturally occurring biofilms. Each of the three strains was grown as single-species biofilm and in a possible combination of two or three species biofilm consortia in replicates of four. The results obtained reveal that when the three species (*C. marina alex*, *P. prydzensis*

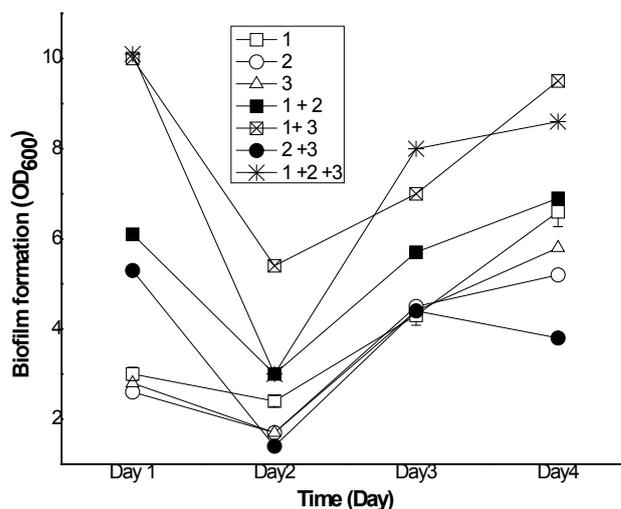


**Figure 9.** Screening for AHL molecule in *C. marina alex* (a), *P. prydzensis alex* (b) and *P. sp. alex* (c) grown on LB agar plates-based bioassay. Blue color refers to the presence of quorum sensing molecule (AHL)

alex, and *P. sp. alex*) coexisted in a biofilm, the value of biofilm after 24h increased by more than three-fold compared to the value of each single species (Figure 10). A surprising result was that a mixed culture of *C. marina* alex and *P. sp. alex* yielded a biofilm of a value similar to that of the triple biofilm. It also observed that dual-species biofilms were of more or less similar values and higher than those of single species (Figure 10).

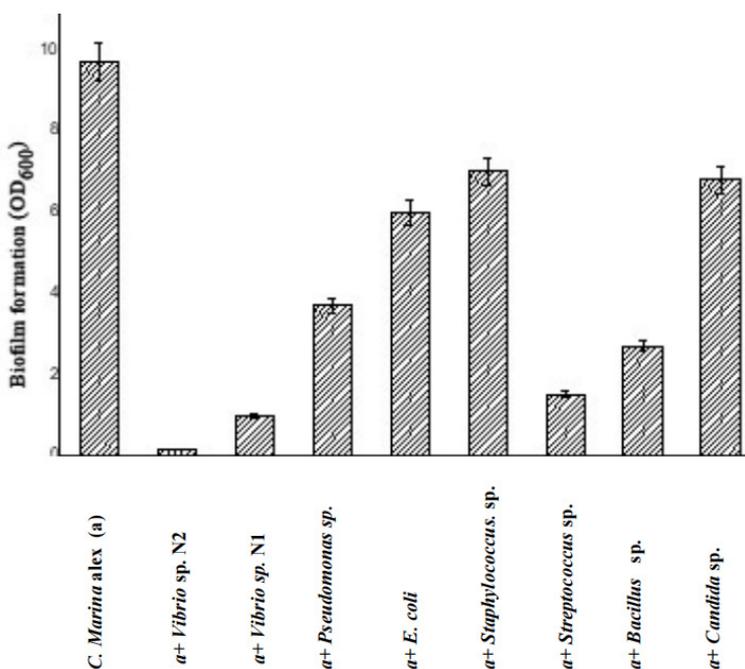
The previously obtained data refer to the presence of synergism between the three marine bacterial species and between *C. marina* alex and *P. sp. alex*. No antagonistic interaction was observed among the tested strains. These data are in agreement with that obtained by Rao et al., (2010) [48] who suggested that *Pseudolateromonas tunicata* required the presence of diverse bacteria for effective colonization. The phenomenon of cooperative biofilm formation in mixed consortia has been described [49]. Synergy between species present in dual-or multispecies biofilms has been reported several times, mostly in description of biofilm-forming bacterial isolates from the oral cavity [50]. Guillonnet al. (2018) [51] interestingly found that the two strains *Persicivirga mediterranea* TC4 and *Shewanella* sp. TC10 produced a newly secreted compound when grown in dual species versus mono-species biofilms. Moreover, the roles played by multispecies biofilms in resistance to disinfectants due to more EPS secretion compared with mono species biofilms was reviewed by Reuben et al. (2019) [52].

In an extension to the species interaction investigation, an experiment was conducted to evaluate the possible



**Figure 10.** Interaction between the three marine bacteria in single, dual and triple biofilms; (1) *C. marina* alex, (2) *P. prydzensis* alex, and (3) *P. sp. alex*. The error bars represent the standard error of the means (SEMs)

interaction that might exist between *C. marina* alex and some marine bacteria in addition to a clinical isolate of *Candida* sp. *C. marina* alex was allowed to form single and dual biofilms with each of the tested organisms. Data in Figure 11 indicate that a negative effect (antagonism) was observed with all strains. The most notably antagonism was recorded in the dual combination of *C. marina* alex and *Vibrio* spp. where almost 90-100 % inhibition in biofilm formation was found compared to the single biofilm of *C. marina* alex.



**Figure 11.** Antagonism in biofilms formed by *C. marina* alex as a control and in dual combinations with some marine bacteria. The error bars represent the standard error of the means (SEMs)

Percentages decreases of 60 and 40% were recorded with *Pseudomonas* sp. and *E. coli*, respectively. Same observation was found with the Gram- positive bacteria. Although 35% biofilm inhibition was recorded with *Staphylococcus* sp., much more existed with *Streptococcus* sp. and *Bacillus* sp. (almost 90 and 75%, respectively). Biofilm formed in a dual combination of *C. marina* alex and *Candida* sp. was 35% less than that formed by *C. marina* alex. The antagonistic interaction found in this work is surprising because all tested microorganisms were able to form biofilms in single culture. Therefore, more detailed studies are still needed to provide more explanation to such interaction.

#### 4. Conclusions

There is widespread occurrence of microbial biofilms in marine ecosystems, but the factors that affect the configuration of these microbial communities are still a matter of current consideration. This study highlights the biofilm-forming bacteria in the Mediterranean seawater, Alexandria, Egypt as well as cell-to-cell communication and interactions among species which will provide a clearer picture that could improve current understanding of these microbial structures inhabited the Mediterranean seawater in Alexandria, Egypt. Biofilm forming bacteria have gained a great attention due to the recent biotechnological applications of biofilms in wastewater treatment, bioremediation technology, and enzyme function.

#### Author Contributions

S.S.A. conceived the idea conducted the experiments, analyzed and interpreted the data, wrote the manuscript, performed the statistical analysis, designed and constructed figures and charts; E.A.A. proposed the research concept, conceived & conducted the experiments; H.A.G. and S.A.S. conceived the research idea, analyzed the data, and edited the manuscript.

#### Competing Interests

The authors declare no competing interests.

#### Data Availability Statement

Correspondence and requests for materials should be addressed to S.S.A. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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