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FULL LENGTH ARTICLE

Antagonistic interactions and phylogenetic diversity of antimicrobial agents producing marine bacteria in Suez Bay

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Abstract Estimation of the total viable bacterial counts and some physicochemical parameters in different sites selected along the Suez Bay was carried out. The highest bacterial density is positively correlated with pollution strength and is localized at the end of the Suez Bay on the one hand of Suez Gulf. It is also function of pollution strength at different examined sites. Antagonistic interactions among the most dominating twenty-two bacterial isolates were assayed. The marine isolate AB12 isolated from sea water of NIOF station displayed the highest antagonistic activity (42.8%). Antagonistic isolates were assigned to phylogenetically 4 different phena which were identified as *Staphylococcus*, *Micrococcus*, *Enterococcus* and *Enterobacter* species in addition to 5 single clusters which were identified as *Acinetobacter* sp. and *Pseudomonas* sp. The promising strain was identified at the molecular level as *Pseudoalteromonas piscicida*.

Introduction

Bacteria and microorganisms are ubiquitous in the marine environment. They are taxonomically diverse, biologically active and colonize all marine habitats from the deep ocean to the shallowest estuarine (Kelman et al., 2006).The ocean is characterized by physical and chemical gradients that may affect species diversity (Yawata et al., 2014).

Competition among microbes for space and nutrient in marine environment is a powerful selection pressure to marine microorganisms to produce marine natural products processing medical and industrial values. Different substances exhibiting antimicrobial and antifouling activity have been found among these kinds of bacteria due to the specialized role they play in their respective hosts (El-Amraoui et al., 2014).

Changes in the bacterial species composition, such as those potentially caused by microscale antagonism, could alter the hydrolytic activity exerted by bacteria on organic particles. Furthermore, the species richness and diversity on particles could be influenced by bacterium-bacterium antagonism, and

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in turn, this could affect the nature and rates of biogeochemical transformation of the particles. Thus, bacterium-bacterium antagonisms could be important variables in the ecology of pelagic bacteria and in bacterium-mediated carbon cycling in the ocean (Long and Azam, 2001).

- A small number of marine plants, animals and microbes have already yielded more than 12,000 novel chemicals with hundreds of new compounds are still being discovered every year (Anand et al., 2006). Few reports have regarded the inter-specific interactions among bacteria of the same or related marine environments, but they certainly demonstrate that antagonistic effects, expressed by phylogenetically different bacterial groups, are a widespread trait in marine habitats (Long and Azam, 2001; Grossart et al., 2004; Bhattarai et al., 2006).
- The aim of the present study is to study the distribution of heterotrophic bacteria along different sites along the Suez Bay and to investigate the antagonistic interactions among marine bacteria isolated from these sites in addition to identification of the most promising isolates using different techniques.

Materials and methods

Sampling sites

Water samples were collected from the surface of 10 different sampling locations along the sea shore of the Suez Bay (Egypt), as shown in (Fig. 1).

Sampling and physicochemical analysis

Water samples were collected for four successive seasons (winter 2012–autumn 2012) along the Suez Bay seashore. Different physicochemical parameters (Temperature, pH, salinity, dissolved oxygen, ammonia, nitrites, nitrates, phosphates and organic matter content) were estimated according to the standard procedures (Strickland and Parsons, 1972; Grasshoff, 1976; Clesceri et al., 1999).

Indicator microbes

Different pathogenic strains such as *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 8739), *Escherichia coli* (ATCC 8739), *Aeromonas hydrophila*, *Vibrio anguillarum* and *Candida albicans* were used as indicator strains. These strains were all kindly provided by Prof. Dr. Yossry Gohar Prof. of Microbiology at Faculty of Science Alexandria University.

Estimation of heterotrophic bacteria

Total count of heterotrophic bacteria was estimated employing the standard plate count technique (Pawsey, 1974; Clesceri et al., 1999).

Statistical analyses

The bacterial level was further studied as a function of various factors of water quality using stepwise multiple regression analysis. The factors under study were: temperature, pH, salinity, DO, NH_4, NO_2, NO_3, PO_4 and organic matter.

Antagonistic interactions

Cross antagonism between the bacterial isolates

Tooth picking technique was used to test the ability of isolated bacteria to inhibit the growth of each other. Two nutrient agar plates were used in this test. One plate was freshly inoculated

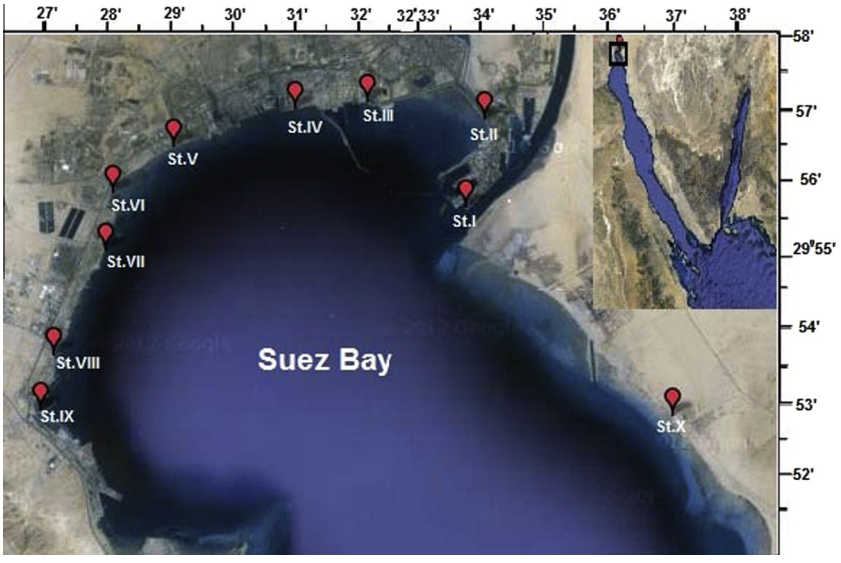

Figure 1 The study area and sampling sites of the Suez Bay (I. The Entrance Channel, II. El-Kornesh, III. Marine High School, IV. El-Zitia, V. Kabanon, VI. Electric power station, VII. National Institute of Oceanography and fisheries, VIII. Attaka, IX. Adabyia, X. Ayoun Moussa).

with 0.5% (v/v) of 24 h old culture (10^8 cell m^{-1}) of the indicator strain. The second plate was used as a template without being seeded with the indicator strain. The nutrient agar plates were stabbed consequently using a sterile clean tooth pick each time with a single colony of each of the tested bacteria. The uninoculated nutrient agar plate was firstly stabbed, and then the seeded nutrient agar plate with the indicator strain was secondly stabbed with the same tooth pick and incubated at 30 °C for 24–48 h. The clear zone around the tooth picked isolates indicates a positive result in the antagonistic action (El-Masry et al., 2002).

Antagonistic action against indicator microorganisms

The antagonistic activity was detected using well cut-diffusion technique in which cut (5 mm) was punched upon the surface of nutrient agar plates inoculated with indicator strains mentioned before. The radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm to calculate the activity unit (AU), which was calculated according the following equation:

$$AU = Y^2/X^2$$

where, Y is the radius of the clear zone around each well and X is the radius of the well itself. This equation was applied according to (El-Masry et al., 2002) to evaluate the activity unit. This test was done in duplicates and repeated twice.

Phenotypic characterization of bacterial isolates

The active isolates were identified based on morphological and physiological characteristics and some biochemical standard methods (Staley et al., 1989; Williams et al., 1989).

Numerical analysis

Taxonomic characters were coded in a binary form of the presence/absence type. Similarities among the tested strains were estimated with simple matching (Sokal and Michenu, 1958), (UPGMA), single linkage and complete linkage (Sneath and Sokal, 1973).

Molecular identification of the most promising bacterial isolate

DNA was isolated, purified and the region of 16S rDNA was amplified using either universal primers or species specific primers. Genotypic characterization was performed using 16S sequence analysis. Multiple alignments with sequences of most close members and calculations of levels of sequence similarity were carried out using Bioedit (Hall, 1999). Sequences of rRNA genes, for comparison, were obtained from the NCBI database.

Results

The annual mean ranges of temperature, pH, salinity, dissolved oxygen, NH_4,NO_2,NO_3, PO_4 and organic matter content (data not shown) in the studied sites are 22.05–28.15 °C, 8.2–8.4, 41–42.62‰, 3.22–4.7 mg/l, 0.09–0.37 μg/l, 0.03–0.22 μg/l, 10.32–37.97 μg/l, 10.43–28.98 μg/l and 14.08–19.42 mgO_2/l respectively. In general, results indicated that Kabanon and Adabyia are the most polluted sites.

Estimation of heterotrophic bacteria

Water samples were analyzed for total viable bacterial counts as shown in Fig. 2. Sea water samples of Adabyia characterized by the highest annual mean of bacterial count (14×10^5 CFU/ml) followed by El-Zitia (7×10^5 CFU/ml) while the lowest annual mean was recorded in Ayoun Moussa (31×10^3 CFU/ml).

Clear seasonal variations were observed in the bacterial densities of the selected sites. In that respect, total viable count of bacteria during Spring 2012 ranged from 1.20×10^4 CFU/ml at Electric power station to 1.45×10^6 CFU/ml at Attaka. These counts increased during summer 2012 ranging from 1.05×10^4 CFU/ml at Kabanon to 2×10^6 CFU/ml at Adabyia which represented an intermediate to high bacterial count population. This was followed by a general decrease in the bacterial TVC at most of the sites during autumn 2012 although counts still represent high population except for Marine High School that showed the lowest counts (75×10^2 CFU/ml), while the highest count was recorded at Kabanon (2×10^6 CFU/ml). Winter 2012 recorded the maximum densities of bacterial populations at most sites. In that season bacterial TVC ranged from 5×10^3 CFU/ml at Ayoun Moussa to 2.25×10^6 CFU/ml at Attaka.

It is clear from these results that the highest bacterial counts in the water samples are localized at the end of the Suez Bay on the one hand of Suez Gulf (Adabyia and Attaka) and are function of pollution strength at different examined sites compared to the east and westwards the coast.

Statistical analysis

Multiple regression analysis was applied to reflect the relationship between all the environmental factors and the counts of bacteria in seawater during different seasons. The regression equation is: bacteria = −51685745.34 − 219361.98 pH + 1667383.37 temperature − 1253302.85 dissolved oxygen − 62812.86 phosphate + 2336574.97 nitrite − 103180.78 nitrate + 243740.18 organic matter − 6182241.37 ammonia + 498862.89 salinity. These results show that, the counts of bacteria are positively correlated with temperature, dissolved nitrite, organic matter and salinity, and negatively correlated with pH, dissolved oxygen, dissolved phosphates, dissolved nitrate and dissolved ammonia.

Antagonistic interactions

In this experiment, a 22×22 array of tests (484) was performed using the tooth pick technique to study antagonism between isolates. The results in (Table 1) confirmed the occurrence of antagonistic interactions among the isolated bacteria. Production of the inhibitory compounds was found in 69.2% of the isolates. The marine isolate AB12 isolated from sea water of NIOF station displayed the highest antagonistic activity causing growth inhibition of 9 other strains (≃42.8% of the tested strains) followed by isolate AB8 causing growth inhibition to 7 strains (≃33.3% of the tested strains). Strain AB22 showed antagonistic activity against 6 bacterial isolates (≃28.6% of the tested strains). On the other hand strains AB10, AB17 and AB6 showed antagonism against 4, 3 and 2 of the bacterial isolates representing ≃19.1%, 14.2% and

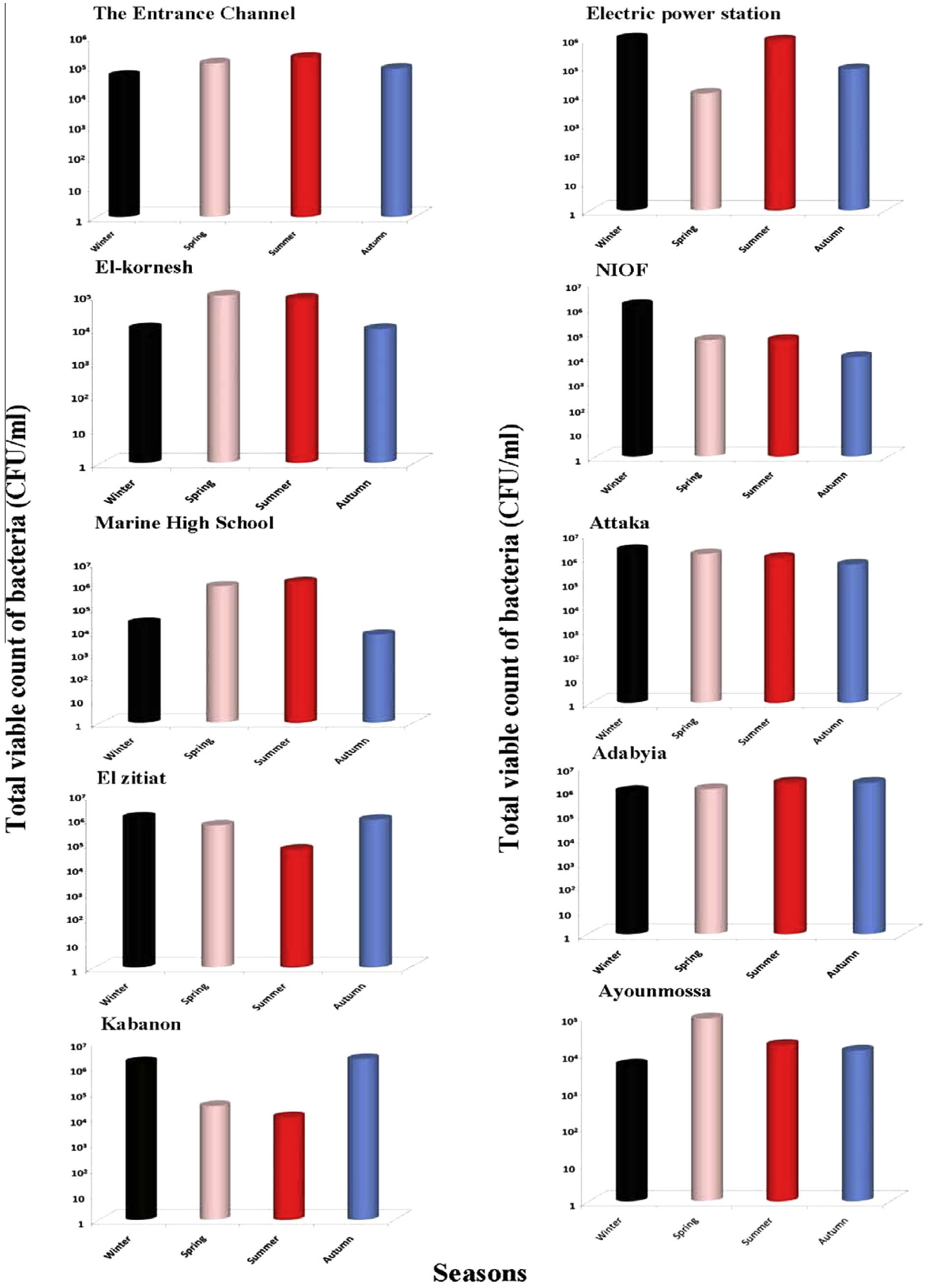

Figure 2 Total viable count (CFU/ml) of bacterial isolates from sea water of the study area during the period of study.

Table 1 Screening for antagonism among experimental bacterial isolates.

Antagonistic isolate	Number of positive records	Antagonized isolate	Antagonism%
	1	AB7	4.7
AB6	2	AB5 & AB20	9.5
AB8	7	AB3, AB4, AB6, AB7, AB9, AB19 & AB22	33.3
AB10	4	AB7, AB12, AB19 & AB22	19.1
AB11	1	AB7	4.7
AB12	9	AB3, AB4, AB5, AB6, AB7, AB9, AB10, AB19 & AB22	42.8
AB17	3	AB10, AB16 & AB20	14.2
AB21	1	AB5	4.7
AB22	6	AB3, AB5, AB8, AB10,AB14 & AB16	28.6

9.5% of the tested strains respectively, while strains AB2, AB11 and AB21 showed antibacterial activities against one isolate only (≃4.7%).

The test was extended to evaluate the antagonistic effect of the selected bacterial isolates against some pathogenic microorganisms. The data in (Fig. 3) revealed that only 9 marine bacterial isolates (AB2, AB6, AB8, AB10, AB11, AB12, AB17, AB21 and AB22) showed varied antimicrobial activity against one or more of the test pathogens. The highest antimicrobial activity (17 mm) was for AB12 against *E. coli* ATCC 8739, followed by 15 mm against *A. hydrophila*, *V. anguillarum*, *P. aeruginosa* ATCC 8739 and *C. albicans* and thus was chosen to complete the study.

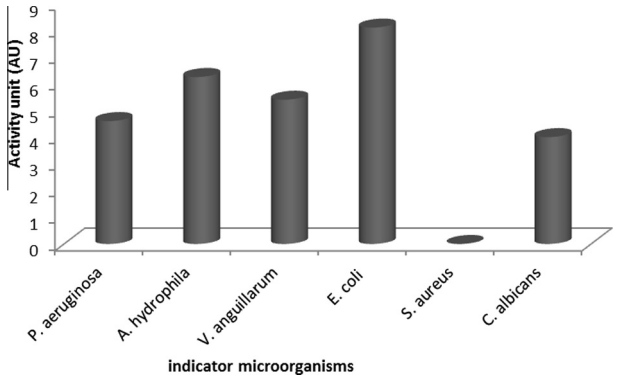

Figure 3 Antimicrobial activities of bacterial isolate AB12 against some indicator microorganisms.

Identification of bacteria producing antimicrobial agent(s)

All bacterial isolates (Gram + ve) and (Gram − ve) with promising antimicrobial activity were subjected to morphological, physiological and biochemical characterization as shown in (Table 2). They were numerically clustered (Fig. 4a and b). As shown in the dendogram (Fig. 4a), Gram positive bacterial isolates (9 strains) are grouped into 3 phena (A, B and C) at 59% similarity level. The phenotypic characteristics of the three phena are listed in (Table 2).

Phenon A: This group included only 3 strains which were clustered at 66.67% similarity level. One strain AB15 was isolated from NIOF station and the others from El-Kornesh station.

Phenon B: This phenon was the minor group that harbored 2 strains which clustered at 70% similarity level. One strain AB13 was isolated from NIOF station and the second AB10 from Attaka station.

Phenon C: This phenon included 3 strains which clustered at 64% similarity level. One strain AB14 was isolated from NIOF station, the strain AB11 from Ayoun Moussa station, while the Entrance channel included only one strain AB3.

One strain of each phenon was selected and identified by the aid of Bergey's Manual of Systematic Bacteriology. Member of phenon A was identified as *Staphylococcus* sp. and member of phenon B was identified as *Micrococcus* sp. while member of phenon C was identified as *Enterococcus* sp. In addition to the three mentioned phena, the dendogram contained one single cluster at 53% similarity level, which was un identified.

On the other hand, at 47% similarity level, the Gram negative bacterial isolates (13 strains) were clustered into one **phenon (a)** as shown in the dendogram (Fig. 4b), which harbored 8 strains identified as *Enterobacter* sp. and there were 5 single clusters. AB18 and AB20 were separated at 47% similarity level and were identified as *Acinetobacter* sp. AB12 strain was separated at 56% similarity level and was identified as *Pseudomonas* sp. while the AB7 and AB1 were clustered at (53 and 55)% similarity level and was not identified.

Molecular identification of the most promising bacterial isolate

Partial sequencing of *Pseudoalteromonas piscicida* DNA (1500 bp) amplification product (1396 base pair) is shown in (Fig. 5). This sequence was compared with those which gave the highest homology using Blast search computer based program. (Fig. 6) and revealed that the highest homology was with JX861209.1 *P. piscicida* with 99% similarity. The sequence was deposited in the GenBank database with accession number KF776137. Its phylogenetic relationships (Fig. 7) confirmed the identification of the most active marine bacterium, The resulting data indicated that the isolate AB12 under study was identified as *P. piscicida*.

Table 2 Comparison of the frequencies of positive and negative characters of five phena obtained by numerical taxonomy analysis.

Characters	Gram positive isolates			Gram negative isolates
	Phenon A	Phenon B	Phenon C	Phenon a
	(3 strains)	(2 strains)	(3 strains)	(8 strains)
Morphological characters				
Cell shape				
Rods	0	0	33	100
Cocci	100	100	66	0
Presence of capsules	0	0	0	0
Presence of spores	0	0	0	0
Diffusible pigments	66	0	0	0
Motility	0	100	100	0
Biochemical characters				
Citrate utilization	0	0	33	37.5
Production of				
Catalase	66	100	0	100
Urease	0	100	33	25
Lipase	33	50	33	25
Protease	33	0	66	0
Gelatinase	0	50	66	50
Chitinase	66	0	0	50
Amylase	100	0	66	62.5
H_2S	0	0	0	0
B.Esc.	66	0	0	37.5
Indole test	0	0	0	0
VP test	0	50	66	12.5
MR test	0	0	33	0
Nitrate reduction	0	100	100	75
Antibiosis against				
E. coli	33	100	0	12.5
S. aureus	66	50	33	25
A. hydrophila	0	0	100	25
P. aeruginosa	66	100	0	37.5
V. anguillarum	33	0	0	12.5
C. albicans	0	0	100	37.5
Number of identified strains	1	1	1	1
Expected identification	*Staphylococcus* sp.	*Micrococcus* sp.	*Enterococcus* sp.	*Enterobacter* sp.

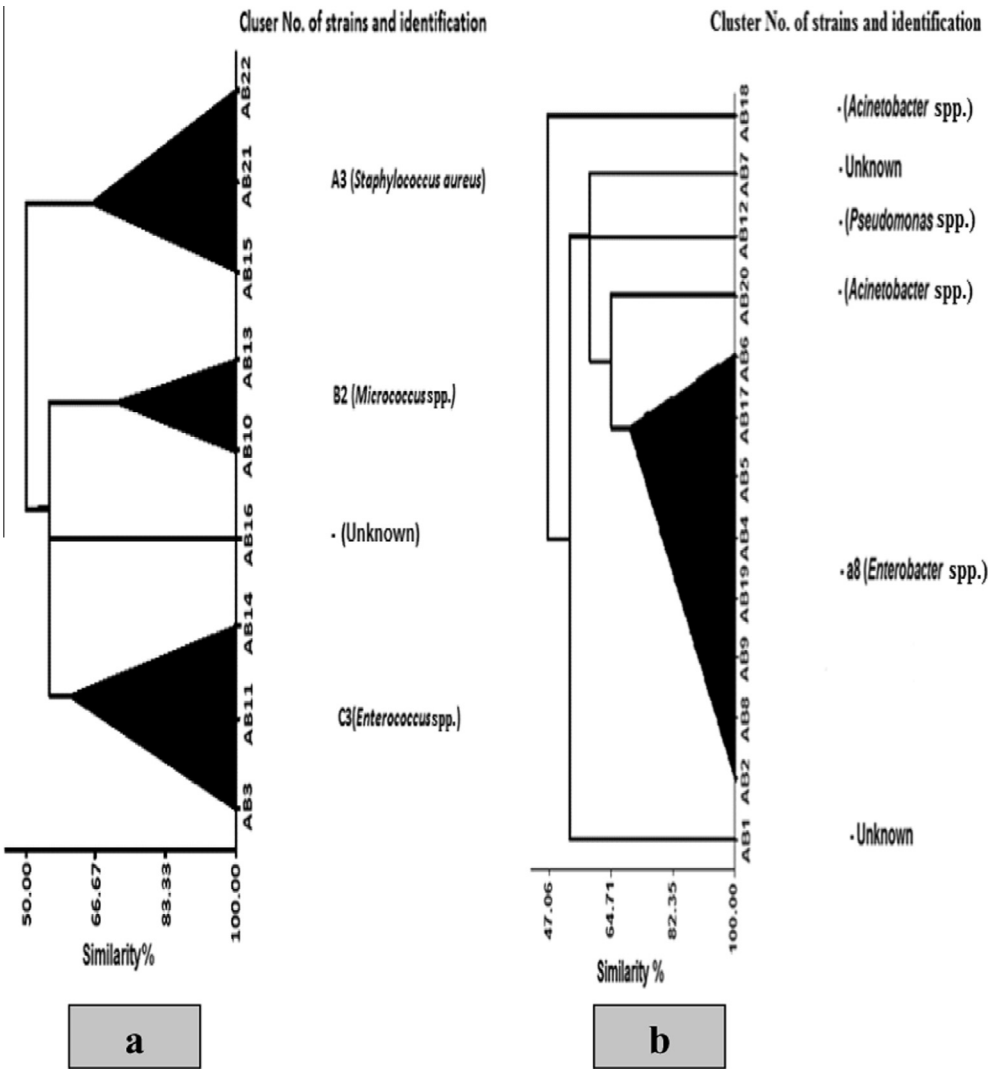

Figure 4 Simplified dendogram showing the relationships among clusters of Gram positive (a) and Gram negative (b) strains based on the $S_s\mu$-UPGMA analysis.

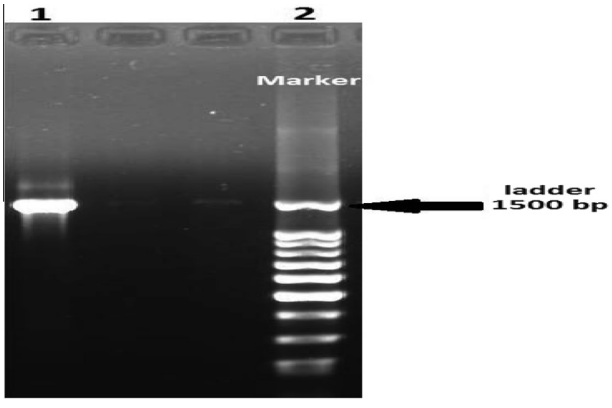

Figure 5 16S agarose gel electrophoresis of the amplified 16S rRNA gene of the isolate under study. Lane 1 is the purified PCR product and lane 2 is 100 bp DNA ladder.

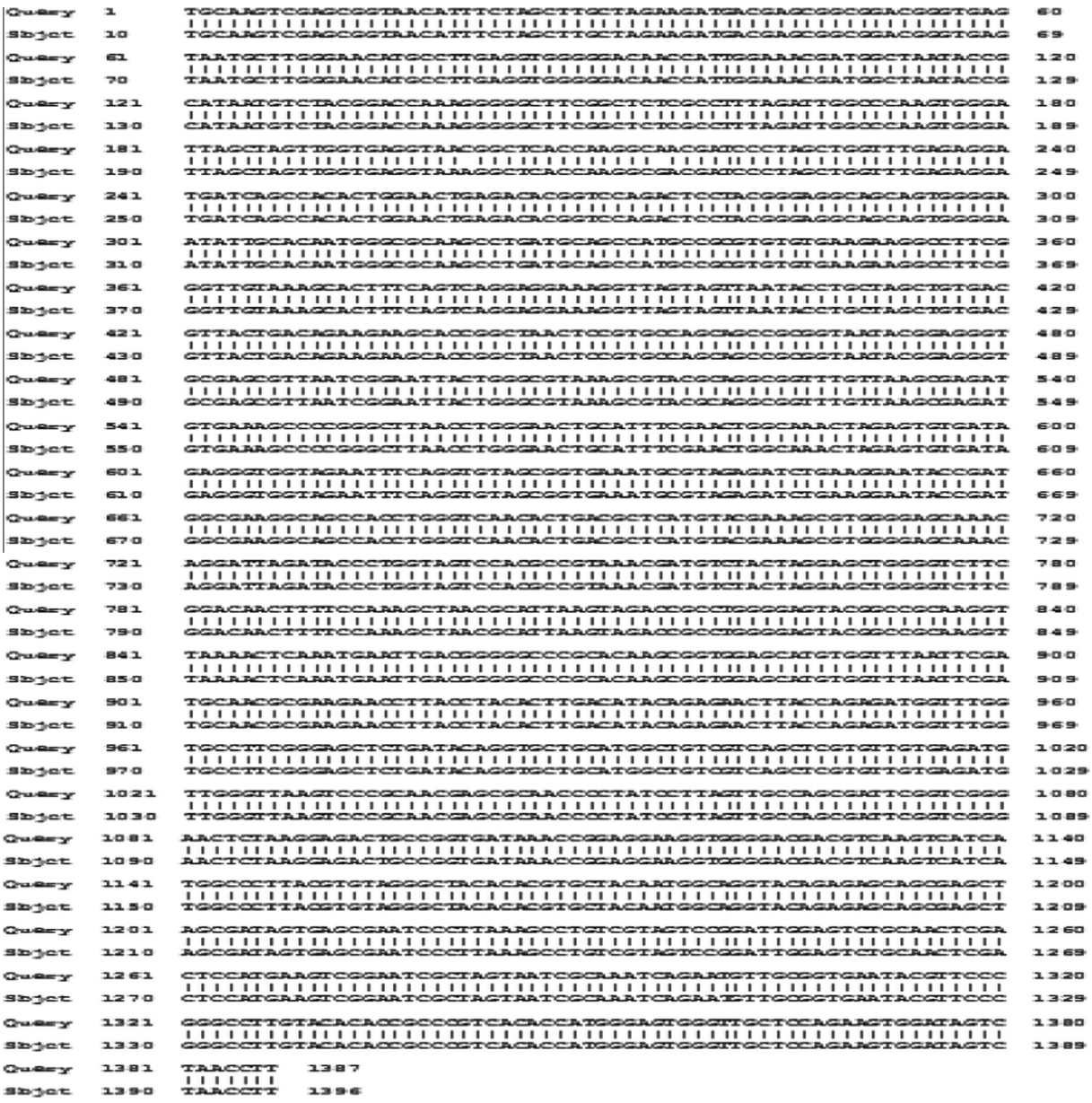

Figure 6 16S rRNA nucleotide sequence obtained from the representative experimental strain.

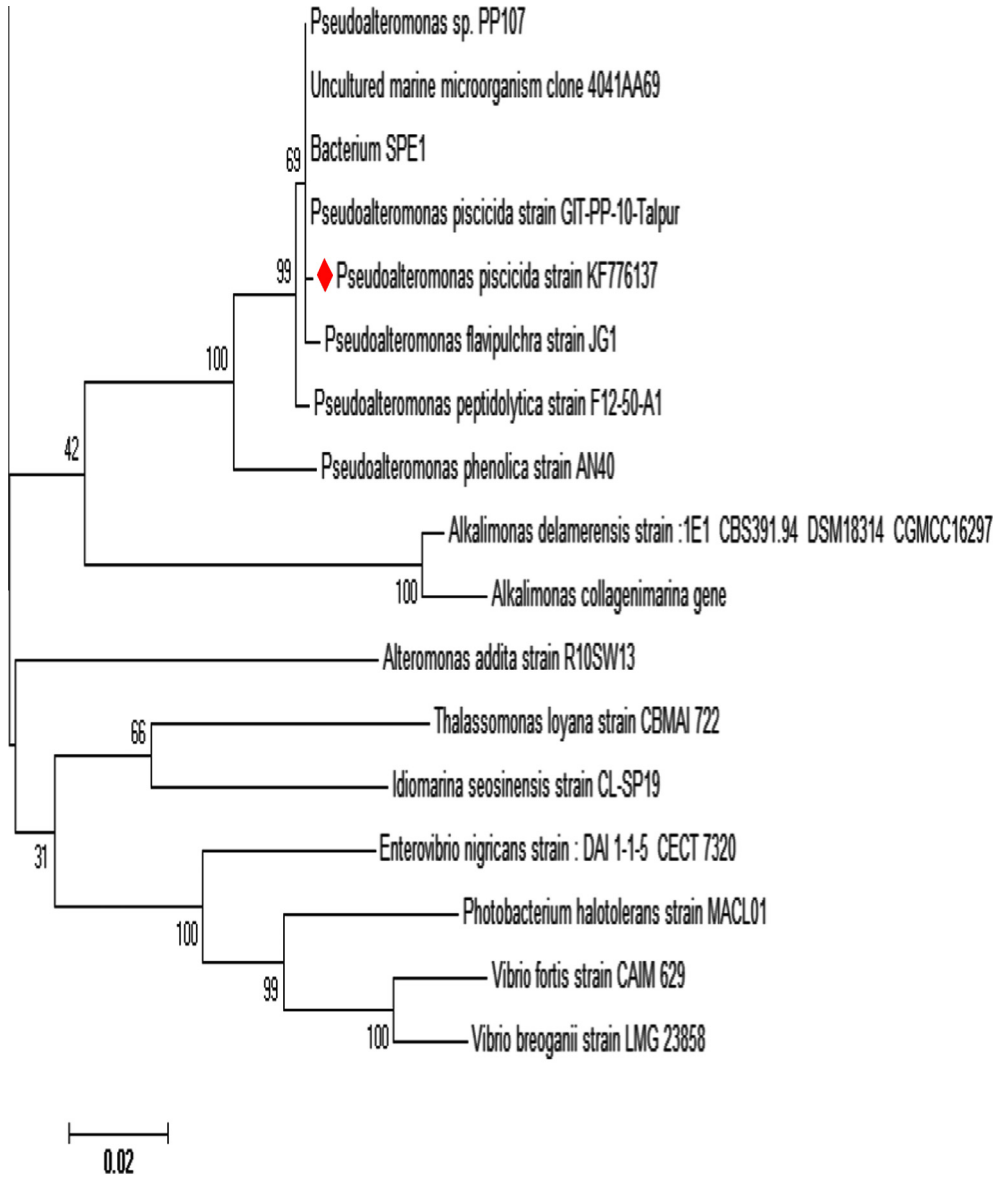

Figure 7 Phylogenetic relationships among the representative experimental strain and the most closely related *pseudomonas* species. The dendogram was generated using tree view program.

Discussion

Marine microbiology is developing strongly in several countries with a distinct focus on bioactive compounds (Kornprobst, 2010). Numerous studies have indicated that diverse marine microbes appear to have the capacity to produce an impressive array of marine natural products exhibiting a wide variety of biological activities such as antimicrobial, anti-tumor, anti-inflammatory and anti-cardiovascular agents (Zhi-Qiang et al., 2013).

Marine microbial natural products (MMNPs) have attracted increasing attention from microbiologists, taxonomists, ecologists, agronomists, chemists and evolutionary biologists during the last few decades (Blunt et al., 2013).

In the present study, different physicochemical parameters were investigated. The physicochemical characteristics of the studied area are consistent with those reported in previous studies (Mohamed et al., 2007; El-Sawy, 2009).

An important step toward understanding the roles of various bacteria in the marine environment is determining the numbers and relative abundances of different bacterial groups (Giovannoni and Rappe, 2000). In addition, quantification of bacterial cells and their physiological state is essential for understanding the ecological scope of their global magnitude (Quéric et al., 2004).

The counts obtained in this study for aerobic heterotrophs are similar to those obtained by Hassan (2008) in the Suez Gulf. The variation of count observed in different sites represents the responses of heterotrophic bacteria to environmental changes. Several studies have concluded that, the marine microbial communities are influenced by environmental variables such as temperature, nutrient availability, water salinity,

length of day, algal bloom and hydrostatic pressure (Uphoff et al., 2001).

The highest annual mean of bacterial counts observed in Adabyia (14×10^5 CFU/ml) and El-Zitia (7×10^5 CFU/ml) appears to be a result of combination of continuous effluent input and hydrographic dynamics which affect in situ microbial community. These two sites show varieties of human interference (domestic/industrial/fishing). Dispersion and dilution of industrial and/or domestic wastes create a favorable situation for bacteria and other microbial heterotrophs (Ramaiah et al., 2002). They grow rapidly by transforming organic matter available in excess. On the contrary, the lowest annual mean of bacterial count observed in seawater was at Ayoun Moussa (31×10^3 CFU/ml) which simply reflects the clean nature of this area. Regarding seasonal distribution of the sea water TVC, high counts of TVC were detected in winter season, while low counts were recorded during autumn as explained by Hassan (2008).

Taking into account the pervasive nature of antibiosis, we tested the hypothesis of antagonistic interactions among experimental isolates. Previous studies of antagonistic interactions between marine bacteria have focused on isolates from pelagic particles, including marine snow (Grossart et al., 2004). It is hypothesized that bacteria use chemically mediated defenses to compete for space and nutrients in these micro environments (Long and Azam, 2001). The production of inhibitory substances is a common phenomenon among bacteria isolated from bacterial biofilm, giving them a competitive advantage over other bacteria (Avendano-Herrera and Riquelme, 2007; Rypien et al., 2010; Matthias et al., 2013).

The occurrence of antagonistic interactions among experimental bacterial isolates obtained in this study was proven. Nine isolates showed a prominent antimicrobial effect against 59.1% of the total tested bacteria.

A large fraction (40.9%) of the examined bacterial isolates exhibited antagonistic properties against other pelagic bacteria. Lower percentages of bacterial antagonism (35–53.5%) were reported in other previous studies (Long and Azam, 2001).

Although marine microorganisms have been increasingly of interest as a source of new bioactive molecules (Asha et al., 2011), a great percentage of them have not been described. To discover novel by-products from marine environments, maintenance of not simply abundant but diverse microorganisms is necessary. Early schemes for systematic were mainly determinative and made no attempts to mirror presumed "natural" relationships of these microorganisms. There was a heavy reliance on morphological criteria. The morphological diversity of marine microorganisms was used for the purpose of defining taxa at the genus level (El-Sersy et al., 2010).

Nine bacterial isolates were selected for antagonistic studies against six identified pathogens, they showed varied antimicrobial activity against one or more of the test pathogens while only one strain (AB12 from NIOF station) had broad spectrum effect against out of six tested organisms. These organisms were subjected to morphological, physiological and biochemical characterization for numerical identification.

The use of numerical identification and the development of matrices for routine have been successfully applied to various groups of bacteria (Oscar et al., 2009).

It was found that the majority of these microorganisms were Gram negative (59.1%), while (40.9%) represented the Gram positive group. This finding was reported in other studies (Gauthier, 1969). Nonetheless, later studies confirmed that Gram-positive producers also occur (Hassan, 2008). Different studies reported that pigments have been associated with antibacterial activity (Azamjon et al., 2011).

All the identified species were documented as producers for antagonistic agents which can be used in different applications. These include *Micrococcus* sp. (Awais et al., 2007); *Enterococcus* sp. (Campos et al., 2006; Miles, 2007) and *Pseudomonas* sp. (Cardozo et al., 2013; Sader et al., 2014).

AB12 strain was separated at 56% similarity level and was identified as *Pseudomonas* sp. The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria, this genus found in natural habitats like soil, fresh water, marine environments etc., (Franzetti and Scarpellini, 2007).

It is well recognized that *Pseudomonas* sp. produce bioactive compounds such as phenazine compounds, quinolones, hydrogen cyanide, 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin (Mashburn-Warren et al., 2009).

In the present study, this cluster represented the promising strain for production of the antimicrobial agents against the selected pathogens and this strain was chosen for further characterization including molecular characterization.

Speciation of *Pseudomonas* in environment studies can be particularly problematic; therefore protocols for unambiguous, DNA-based analysis could receive wide use in applications ranging from water quality to microbial source tracking. PCR-based method for confirming the identity of *P. piscicida* in water samples was employed as a rapid and inexpensive method. The genus was proposed by Cress et al. (2013), who divided *Alteromonas* into two genera, *Alteromonas* and *Pseudoalteromonas*, based on 16S rDNA sequences. Several species of *Pseudoalteromonas* were isolated from sea water and are known to be producers of bioactive substances (Bowman, 2007).

The phylogenetic relationships among the new experimental isolate (AB12) and the closely related *Pseudomonas* species have been described in the present work and revealed that, strain AB12 was taxonomically positioned within the *Pseudoalteromonas* group representing 96% identity. The data obtained by 16S rRNA coincide with those found by traditional, morphological, physiological and biochemical methods. This strain was identified as *P. piscicida* AB12.

Conclusion

Suez Bay is a rich source of antimicrobial agents producing bacteria and provides ecofriendly, renewable and economic alternatives to the traditional antibiotics.

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