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Abundance and Antagonistic Interactions among Bacterioplankton in Suez Gulf

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Abstract: Abundance and antagonestic interactions existing among bacterioplankton in Suez Gulf (Egypt) were investigated. Counts of different bacterial groups (aerobic heterotrophes, spore-formers, Cytophaga-Flavobacteria and Staphylococci) and some chemical parameters in water samples were estimted in different sites selected along the Gulf .Restriction fragment length polymorphism (RFLP) technique was employed to group selected isolates based on genotypic fingerprint. The antagonestic interactions of the isolates were assayed by the well-cut diffusion technique. Antagonestic isolates were assigned to two phylogenetically different phyla. Firmicutes harboured five strains (*Bacillus subtilis AM2373342* (SH1), *Bacillus pumilus AM237349*, *Staphylococcus aureus BX571856, Staphylococcus equorum AM237374* and *Staphylococcus succinius AJ421446*) and Actinobacteria harboured one strain (*Kocuria* sp. *DCO358872*). All identified isolates were Gram-positive bacteria; this may be related to specific features of Suez Gulf which resulted from human interferences.

Key words: Antagonism · Bacterioplankton · Firmicutes · Actinobacteria · Sea water

INTRODUCTION

The biodiversity of microbial communities and the functional roles they play in the marine environment are hugely significant [1]. The competition for substrates is a major evolutionary driving force in the microbial world. A much more precise knowledge of these processes occurring on the highly diverse particles in the ocean will be important for understanding the complexity of global carbon-flux pathway [2]. Antagonistic interactions among bacteria represent an interesting evolutionary strategy, conferring a selective advantage in competition for food and space in the environment and acting as an effective control of microbial populations inhabiting the same ecological niche [3]. Marine bacteria have been intensely screened for their inhibitory effect against terrestrial micro-organisms [4]. Conversely, few reports have regarded the inter-specific interactions among bacteria of the same or related marine environments, but they certainly demonstrate that antagonestic effects, expressed by phylogenetically different bacterial groups, are a widespread trait in marine habitats [5-7].

The aim of this work was to investigate the antagonistic interactions among bacterioplankton isolated from Suez Gulf, Egypt.

MATERIALS AND METHODS

Chemical Analysis of Water: The oxidizable organic matter was expressed as mgl^{-1} according to Ellis *et al.* [8]. Total dissolved nitrogen and phosphate were expressed as μg at. N_2-Nl^{-1} and μg at.PO_4-Pl^{-1} respectively according to Grasshoff [9].

Estimation of Culturable Bacteria from Sampling Sites: Water samples were collected from the different sites (Suez North, Suez middle, Adabia Harbour, Ras Sidr, Ain Sokhna, Ras Gharib, El-Tour and Ras Shokheir) along the Gulf (Fig.1). Samples were collected in 500 ml sterile screw-caped bottles as described by Austin [10]. Serial dilutions (10^{-2}-10^{-6}) were made using sterilized sea water. A portion (0.1 ml) from each appropriately diluted sample was used to inoculate plates prepared with sea water agar for counting aerobic heterotrophs. For counting spore-forming bacteria, a portion from each sample was boiled in water bath for 10 min to kill vegetative cells. The plating procedure was carried out in the same manner. Plates of mannitol salt agar and *Cytophaga-Flavobacterium* (CF) isolation medium were inoculated with 1ml of appropriately dilution sample for counting *Staphylococci* and

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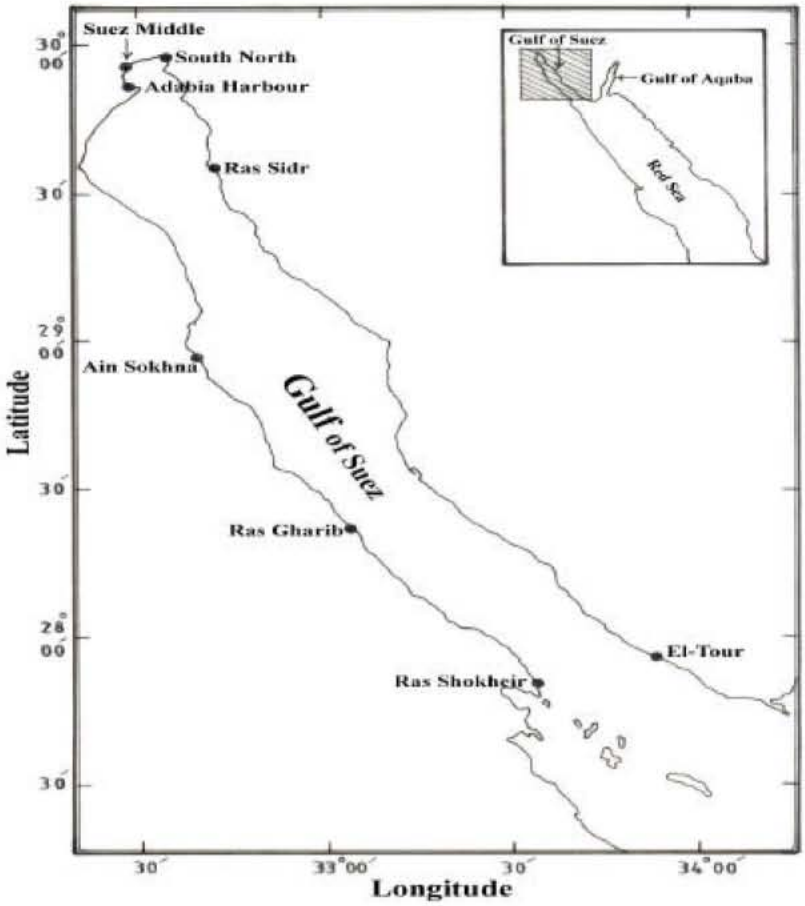

Fig. 1: Distribution of sampling sites along the Suez Gulf

Table 1: Primers used in PCR amplification and sequencing

Primers	Sequence (5` to 3`)
16S 357 F	ACT CCT ACG GGA GGC AGC AG
16S 907R	CCG TCA ATT CAT TTG AGT TT

Cytophaga- Flavobacterium group respectively. Plates were incubated at 30°C for 24 h. The morphologically different bacterial strains were selected for antagonistic studies and identified by 16S rRNA gene sequence analysis.

Screening for Antagonistic Interactions: Thirty two isolates were selected to screen for antagonism. Tooth picking technique [11] was used to test the ability of isolated bacteria to inhibit the growth of each others. The well-cut diffusion technique was used to test the ability of the bacterial isolates to inhibit the growth of indicator microbes.After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm, where dividing Y^2 over X^2 determines an absolute unit (AU) for the clear zone. The absolute unit of each antagonistic isolate, which indicates a positive result in the antagonistic action, was calculated according to the following equation: $AU= Y^2/X^2$[12].

Molecular Characterization of Bacterial Isolates: The genomic DNA of selected strains was isolated using the GFX genomic DNA purification kit (Amersham Bioscience) according to the manufacturer instruction. The DNA was analyzed using 0.7% agarose gel electrophoresis. The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using the following primers (Table 1). RFLP (Restriction Fragment Length Polymorphism) was carried out by digestion of the purified PCR fragments (550 bp) using *Bsp* 1431 (*Sau*3A) /*Msp*I (double digestion). The genetic fingerprint patterns were visualized by electrophoretic separation on 1% agarose gels stained with ethidium bromide. These RFLP patterns were analyzed by the image analysis software Total Lab to obtain dendrogram that reflects genotypic relationships between examined isolates.

The PCR primers (F357 and R907), were designed using Primer 3 software to amplify approximately a 550-base pair fragment of the 16S rDNA region according to the *Escherichia coli* genomic DNA sequence. The PCR reaction mixture contained 200 μM of each dNTP, .5 μM primers, 10mM Tris-HCl pH 8.3, 1.5mM magnesium chloride, 50 mM potassium chloride, 2.5 units Tag polymerase, m and 1 μl of template DNA. Amplicons were obtained with a PCR cycling program of 94°C for 1 min followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 2 min. At the end of thermocycling, PCR reaction was incubated at 72°C for 7 more min. As described by Ausubel *et al.* [13] amplicons were visualized by electrophoretic separation on 1% agarose gels stained with ethidium bromide. PCR fragments were purified from amplification reactions with QlAquick PCR purification reagents (QlAGEN) according to the kit manual. DNA sequence was determined using ABI Prism™ DNA automated sequencer and dye terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied BioSystems). Primers, described previously, were separately used for sequencing of the amplified 16S rDNA fragments.

RESULTS

Viable Count of Heterotrophic Bacteria: Data compiled in Table 2 reveal no strong correlation between chemical parameters and bacterial count in water samples. The highest organic matter content (6.72 mg l^{-1}) was observed in Ras Gharib, which exhibited the highest count (2400 X 10^4 CFU ml^{-1}) for spore formers only. On the contrary, samples from Ras Shokeir harboured the highest count of aerobic heterotrophes (700X 10^4 CFU ml^{-1}) and contained only 4 mgl^{-1} organic matter and 0.6 μg $at.^{-1}$ total nitrogen. CF and Staphylococci showed highest counts (880 and 4000 CFU ml^{-1}, respectively) in Suez Middle samples characterized by highest total nitrogen content (50.96 μg $at.^{-1}$).

Selection and RFLP Fingerprinting of the Bacterial Isolates: The RFLP results were analyzed by the image analysis software Total Lab. to obtain a simplified dendrogram that reflects genotypic relationships between examined isolates (Fig. 2). As shown in the dendrogram, mixed *Bsp* 1431 (*Sau*3A) and *Msp* I digestion generated two main clusters at a similarity level of approximately 30% with SH29 as a reasonable outgroup. The first cluster included only 8 isolates while the second comprised the majority (23 isolates).

Table 2: Aerobic plate count (CFU ml^{-1}) of different bacterial groups and some chemical parameters in sea water samples collected from sites under investigation

Sampling site	Aerobic heterotrophs X 10^4	Aerobic spore formers X 10^2	CF X 10^2	Staphylococci X 10^2	Organic mater mg l^{-1}	Total nitrogen μg $at.^{-1}$	Dissolved phosphate μg at. l^{-1}
El-tour	250	1700	38	160	2.56	0.65	0.05
Suez North	600	200	410	80	2.72	24.99	<0.03
Ras Gharib	300	2400	110	50	6.72	48.41	4.8
Suez Middle	300	19	880	4000	4.0	50.96	0.2
Adabia Harbour	320	40	490	3000	2.08	8.5	<0.03
Ras Shokeir	700	34	200	40	4.0	0.60	<0.03
Ain Sokhna	6	30	95	ND	0.46	5.59	<0.03
Ras Sidr	460	20	8	>30	4.8	0.90	0.03

Table 3: Screening for antagonism among experimental bacterial isolates

Antagonistic isolate	Number of positive records	Antagonized isolate	Antagonism %*
SH1	8	SH4, SH7, SH12, SH15, SH23, SH27, SH28 and SH32	25.8
SH10	2	SH23 and SH28	6.5
SH11	5	SH5,SH7, SH10, SH23 and SH28	16.1
SH14	2	SH5 and SH27	6.5
SH23	1	SH15	3.2
SH24	1	SH25	3.2

Table 4: 16S rRNA gene sequence affiliation, with their closest phylogenetic neighbours

Representative isolate	Site of isolation	Isolating medium	Accession no.	Next relative by Gene Bank alignment	Sequence homology	Phylum
SH1	Ain Sokhna	Sea water agar	EU107759	*Bacillus subtilis AM2373342*	100%	Firmicutes
SH10	Adabia Harbour	Sea water agar	EU107760	*Bacillus pumilus AM237349*	100%	Firmicutes
SH11	Suez North	Sea water agar	EU107764	*Kocuria DCO358872*	99%	Actinobacteria
SH14	Ain Sokhna	Sea water agar	EU107762	*Staphylococcus aureus BX571856*	100%	Firmicutes
SH23	Ras Gharib	Cytophaga medium	EU107761	*Staphylococcus equorum AM237374*	100%	Firmicutes
SH24	Adabia Harbour	Mannitol salt agar	EU107763	*Staphylococcus succinius AJ421446*	100%	Firmicutes

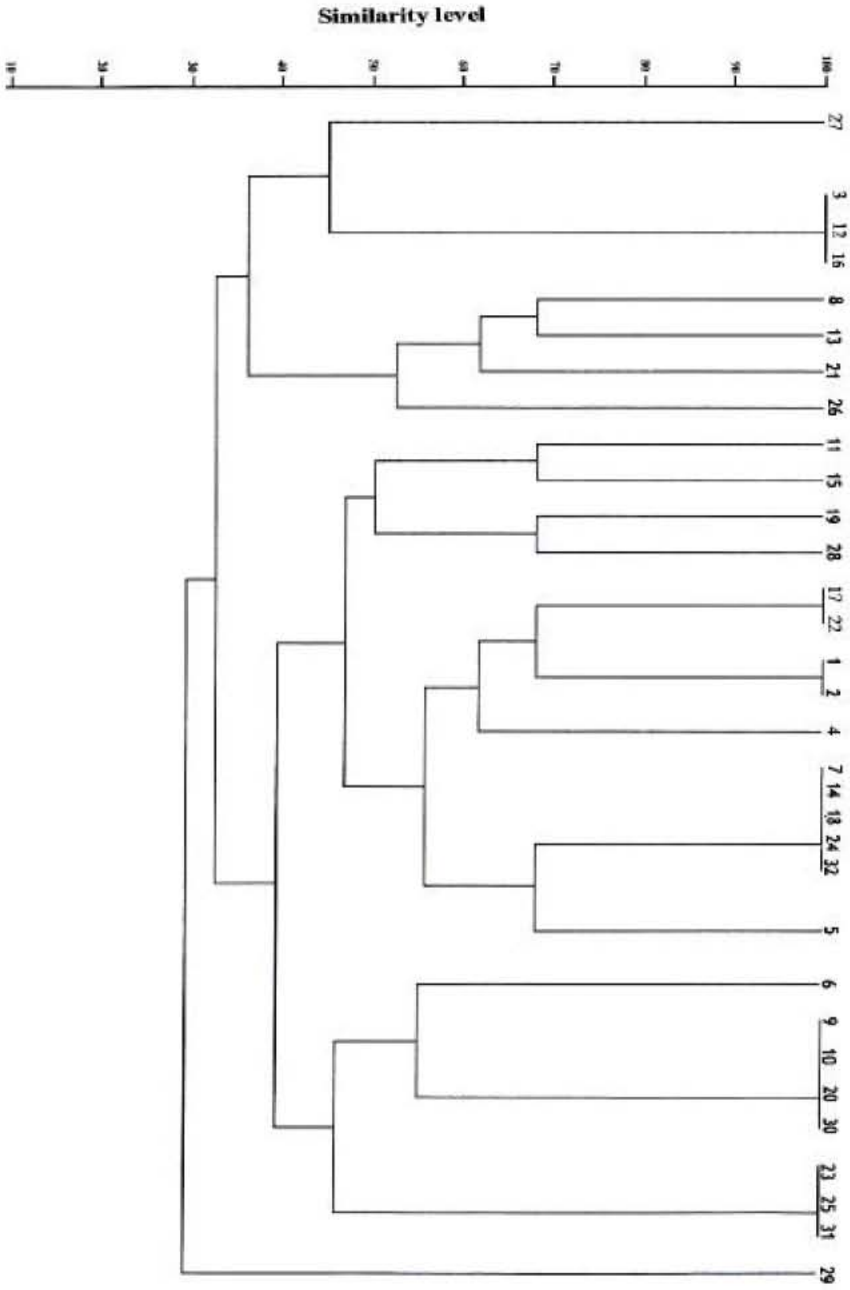

Fig. 2: RFLP dendrogram developed on the basis of *Bsp* 1431/ *Msp*I, (double digestion) showing the similarity level between different bacterial isolates. Full isolate code includes SH prior to each number (1-32)

Antagonistic Interactions: The selected isolates were examined for production of compounds with antimicrobial activities against each other. The results in Table 3 confirm the occurrence of antagonistic interactions. Production of the inhibitory compounds was found in 23% of the isolates. Strain SH1 isolated from Suez North sea water displayed the highest antagonistic activity causing growth inhibition to (~ 25% of tested strains). Strain SH11 showed antagonism against (~ 16%). The test was extended to evaluate the antagonistic effect of the selected bacterial isolates against some pathogens. The data in Fig. 3 depict that only 6 marine bacterial isolates showed antimicrobial activity against one or more of the test organisms.

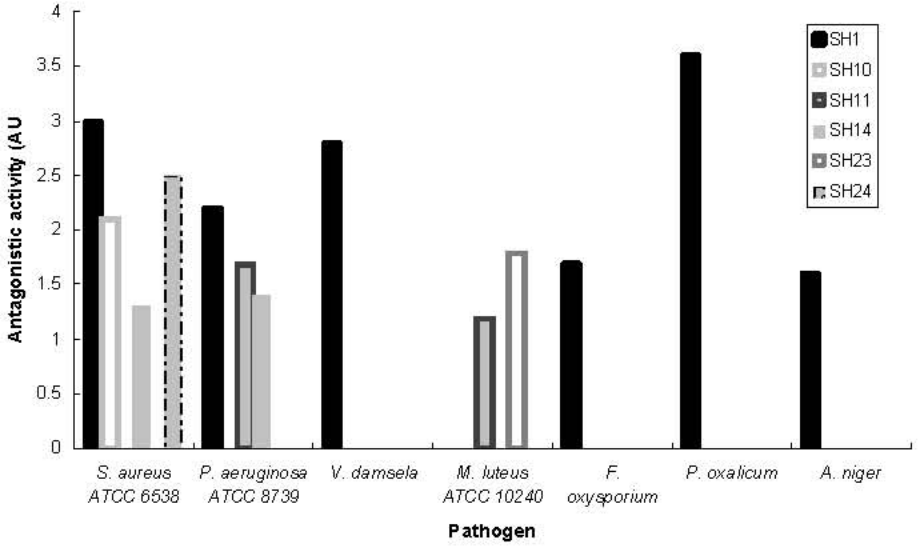

Fig. 3: Antagonistic activity of selected isolates against some reference bacterial pathogens expressed as absolute unit. Absolute unit {AU} = Y^2/X^2.

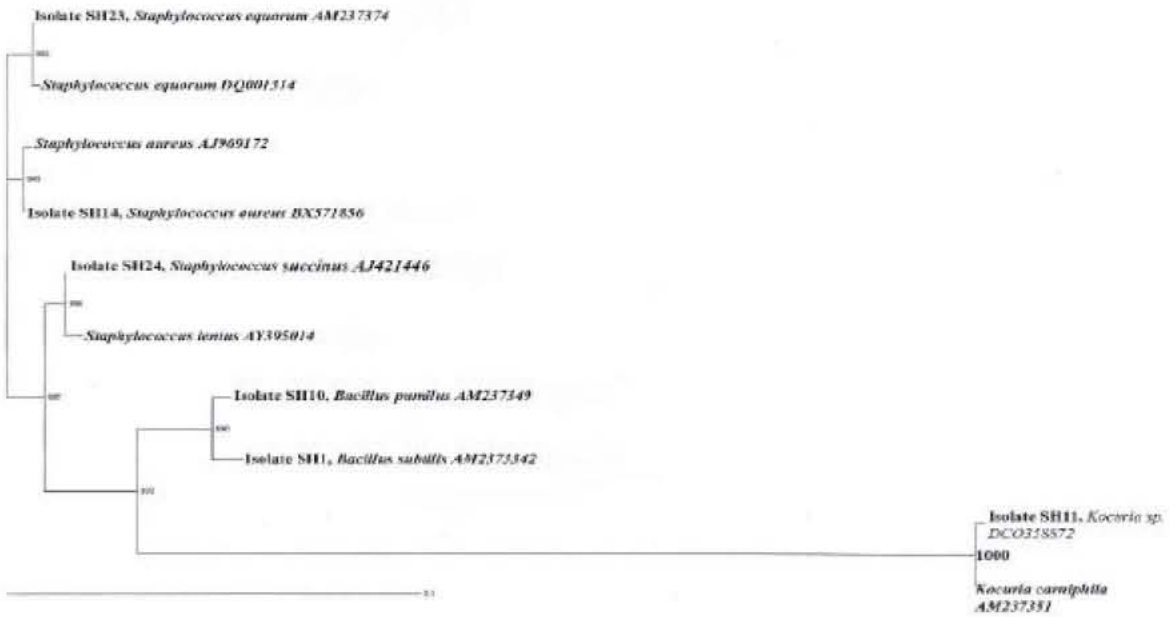

Fig. 4: Phylogenetic analysis of 6 novel isolates based on partial sequencing of 16Sr DNA. The scale indicates substitutions per site.

Molecular Phylogeny of the Selected Isolates: Based on the results of antagonistic interactions, the six most promising bacterial isolates were selected for identification and molecular phylogenetic analysis. The phylogenetic relationships of these isolates and their closely related relatives were analyzed using the PHYLIP (maximum-likelihood) program in Biology WorkBench software (http:biology.ncsa.edu/) and summarized in (Fig. 4 and Table 4).

DISCUSSION

An important step towards understanding the roles of various bacteria in the marine environment is determining the numbers and relative abundances of different bacterial groups. In addition, quantification of bacterial cells and their physiological state is essential for understanding the ecological scope of their global magnitude [14]. The sites selected for the present

investigation are located along Suez Gulf, which is considered as one of the most interesting areas in Egypt. The observed chemical characteristics of sea water in the sites under investigation are similar to those obtained in previous studies [15-17]. The aerobic heterotrophic bacterial counts obtained in this study for aerobic heterotrophs are similar to those obtained by Cavello *et al.* [18] in Lonian Sea in Italy. The variation of count observed in different sites represents the responses of heterotrophic bacteria to environmental changes. Bacterial abundance is now known to vary at the millimeter scale [19].

The highest counts observed in Suez North ($6x10^6$ CFUml^{-1}) and Ras Shokier ($7x10^6$ CFUml^{-1}) appear to be a result of combination of continuous effluent input and hydrographic dynamics which affect *in situ* microbial community. These two sites experience 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 [20]. They grow rapidly by transforming organic matter available in excess. In addition, organic particles are sites of intense microbial activity [21] and bacterial abundance increases with proximity to nutrient-rich particles, reaching concentrations up to three orders of magnitude greater than those in ambient waters [22]. On the contrary, the lowest count observed in seawater at Ain Sokhna ($5.5 x10^4$ CFUml^{-1}) simply reflects the clean nature of this area.

A representative sort of genetic fingerprint was then required to discriminate between the different bacterial isolates. Genetic diversity refers to the variation at the level of individual genes [23]. Restriction fragment length polymorphism (RFLP) has been applied to type a wide range of organisms including bacteria [24,25]. 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 [21]. It is hypothesized that bacteria use chemically mediated defenses to compete for space and nutrients in these micro environments [11]. Grossart and Colleagues have further suggested that between species antagonistic interactions are a micro-scale factor that can influence particle colonization rates [21].

The production of inhibitory substances is a common phenomenon among bacteria isolated from bacterial bio-film, giving them a competitive advantage over other bacteria [26]. A large fraction (25%) of the examined bacterial isolates exhibited antagonistic properties against other pelagic bacteria. Much lower (5 to 8%) detected by Nair and Simidu [27] and much higher (35-53.5%) percentage estimated by Long and Azam [11] were reported in previous studies. Based on 16Sr DNA sequences, two isolates were affiliated as members of the genus *Bacillus*. Strains SH1 and SH10 showed 100% similarity to *B. subtilis* and *B.pumilus*, respectively. *Bacillus* species are widely distributed in nature and have remarkable ability to survive strong environmental stresses. Moreover; members of the genus *Bacillus* have been isolated from aquatic habitats and marine ecosystems [28]. Borsodi *et al.* [28] isolated 40 *Bacillus* and related strains from aquatic habitats. They produce a wide variety of antibiotics, enzymes, surfactants ....etc. [29]. A wide variety of antibacterial and antifungal agents are known to be produced by *B.subtilis* genotypes [30-32]. Similarly, *B. pumilus* produces antimicrobial agents to bacteria and fungi [33].

Three isolates were found to be members of the genus *Staphylococcus*; SH14 showed 100% similarity to *S. aureus*, whereas strains SH23 and SH24 were 100% similar to *S. equorum* and *S. succinus*, respectively. They showed antagonistic actions against *Candida albicans* ATCC 14053 and *S. aureus* ATCC 6538, respectively. Members of this genus have been reported to be isolated from sea water [34]. Several antimicrobial substances, especially bacteriocins, were isolated and purified from *Staphylococcus* species. dos Santos Nascimento *et al.* [35] produced a bacteriocin-like inhibitory substance from *S. aureus* 188 known as staphylococcin 188. It has a broad-activity spectrum against several bacterial pathogens. On the basis of phenotypic and phylogenetic properties, strain SH11 was identified as an actinobacterium; a member of the genus *Kocuria*. The genus *Kocuria* was divided from the genus *Micrococcus* on the basis of the phylogenetic and chemotaxonomic dissection [36]. All of recognized species of *Kocuria* are coccoid, Gram-positive, non-endospore-forming, aerobic non-halophilic microorganisms [37]. At the time of writing and up to the knowledge obtained by searching, there are nine *Kocuria* species with validly published names. Of these species only *K. marina* isolated from a high-salinity environment [38].

Surprisingly, 16S rRNA gene sequencing revealed that all isolates were Gram-positive bacteria (phylum Actinobacteria and Firmicutes). Even though this finding might be considered unusual

for marine water column, similar results have been reported by Grossart *et al.* [39] and Lo Giudice *et al.* [6]. This might be due to the environmental characteristics of the sites under investigation which have been suffered from pollution of human interference (domestic/industrial/fishing).

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