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Mining of Egypt's Red Sea invertebrates for potential bioactive producers

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Abstract

Objective The objective of this work was to isolate bacteria from Red Sea invertebrates, determine their antimicrobial activity, and screen for the biosynthetic gene clusters [polyketides (PKs) and nonribosomal peptides (NRPs)] which could be involved in the production of bioactive secondary metabolites.

Result Eleven different samples of marine invertebrates' were collected from Egypt's Red Sea (El-Tor-Sharm El-Sheikh and Hurghada) by scuba diving, and a total 80 isolates of the associated microorganisms were obtained from the cultivation on six different cultural medium. Seven isolates of them showed an antimicrobial activity against five pathogenic reference strains, while the most active antimicrobial agent was isolate number HFF-8 which was 99% identical to *Bacillus amyloliquefaciens*. HFF-8's extract showed positive results against Gram negative bacteria, Gram positive bacteria and yeast. Moreover, the isolates gave positive bands when screened for the presence of PK synthase (PKS) I and II and NRP synthetase (NRPS) I and II biosynthetic genes, those biosynthetic fragments when cloned and sequenced were primitively predicted as biosynthetic fragments for kirromycin and leinamycin production by NaPDoS program with 56 and 55%, respectively.

Conclusion The Red Sea can provide a sustainable solution to combat bacterial resistance. The contribution of this work is that *B. amyloliquefaciens* was isolated from *Heteroxenia fuscescens*, Red Sea, Egypt. Moreover, the bacterial extract showed a broad spectrum with a potent antimicrobial activity.

Keywords *Bacillus amyloliquefaciens* · Non ribosomal peptides · Phylogenetic analysis · Polyketide synthase · Red Sea · Soft corals · Sponges

Accession numbers: for 16S sequences: MG757672.1–MG757678.1; for PKS and NRPS sequences: MG975081–MG975083.

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Introduction

The health and well-being of people (sustainable development goal 3) is nowadays being threatened by the rise of antibiotic-resistant bacteria (World Health Organization 2014). Multidrug resistant patterns in Gram-positive and negative bacteria have resulted in untreatable infections using conventional antimicrobials. As a result of the emergence of these resistant infections, mortality and morbidity is on a rise; hence an urgent need of find solutions to combat bacterial resistance is required (Khameneh et al. 2016).

Many laboratories globally are devoted to the discovery of novel antimicrobials derived from both plant and animal sources. The marine ecosystem has been also considered recently as an important source for antimicrobial compounds due to the presence of a diverse number of microorganisms (Ponnappan et al. 2015). The Red Sea in specific serves as an epicenter for marine bio-diversity, with a high endemic biota. In addition, more than 180 soft coral species identified world-wide, approximately 40% are native to the Red Sea (McFadden et al. 2010).

The diverse environmental conditions, unique coral reef systems and seasonal fluctuation of air of the Red Sea (Temraz et al. 2006) facilitates the arising of diverse marine organisms which are a rich source of as-yet untapped bioactive molecules (Ponnappan et al. 2015). Marine microorganisms associated with invertebrates have recently shown to produce a large range of unique pharmaceutically and biotechnologically metabolites with antimicrobial, anticancer, anti-inflammatory, antioxidant effect and a potential for treating drug-resistant infections (Abdelmohsen et al. 2017).

Secondary metabolites like nonribosomal peptides (NRPs) and polyketides (PKs) in many cases act as antibiotics such as vancomycin, rifamycin and bleomycin (Amos et al. 2015). Genes for each NRP and/or PK synthesis are generally organized into a gene fragment. They are found to be synthesized by multimodular enzymes named NRP synthetases (NRPS) and types-I and II PK synthases (PKS I and II), respectively (Kleigrewe et al. 2016).

In this study, bacterial isolates were isolated from different Red Sea marine invertebrates, then were taxonomically identified, tested for their antimicrobial activity and were screened for biosynthetic gene clusters (PKs and NRPs) which have an important role in the production of bioactive secondary metabolites.

Materials and methods

Invertebrates' collection and processing

Specimens of eleven different marine invertebrates were collected randomly by hand using scuba diving from two different location in the Red Sea: A, El-Tor (Sharm El-Sheikh), Sinai, Egypt at GPS location (28°13′42.8″ N 33°37′19.4″ E) and B, Safaga, Egypt at GPS location (26°45′55.1″ N, 33°56′50.0″ E) in a water depth of 2–3 m. Then, transferred to plastic bags containing sea water and were transported to the laboratory. Specimens were rinsed with sterile seawater, cut into 1 cm^3 pieces with sterile scalpels, and then thoroughly homogenized in a sterile mortar with sterile sea water. The supernatant was diluted in 10-fold series (10^{-1}, 10^{-2}, and 10^{-3}) using autoclaved environmental water. Hundred microliters of each dilution were plated on different types of agar plates (Aboul-Ela et al. 2012).

Isolation of bacterial community associated with the selected invertebrates

Six types of growth media were prepared to isolate a wide range of the associated bacteria; marine agar (Difco™, USA), Actinomycetes media (Difco™, USA), ISP2 agar (4 g l^{-1} yeast extract, 10 g l^{-1} malt extract, 4 g l^{-1} dextrose, 15 g l^{-1} agar), starch casein gar (10 g l^{-1} soluble starch, 1 g l^{-1} casein sodium salt, 0.5 g l^{-1} K_2HPO_4, 15 g l^{-1} agar), R2A agar plates containing 2% (w/v) NaCl (Difco™) and M1 media (Mincer et al. 2002), served as specific and general rich media. All media were prepared by using (NSW) except marine and R2A agar, and all except marine agar were supplemented with 0.2 μm pore size filtered cycloheximide (100 μg/ml), nystatin (25 μg/ml) to inhibit fungal growth and nalidixic acid (25 μg/ml) to inhibit fast-growing Gram-negative bacteria (Webster et al. 2001). The inoculated plates were incubated at 30 °C for 5–20 days. Distinct colony morphotypes were picked and restreaked until visually free of contaminants.

Identification of morphotype isolates using MALDI-TOF

Preparation of samples was performed by using a sterile wooden tip to pick an isolated bacterial colony freshly grown on defined agar medium and then smearing a thin film in duplicate onto a ground steel MALDI biotarget 96 plate (direct transfer procedure). The microbial films were overlaid directly with 1.0 µl α-cyano-4-hydroxycinnamic acid (MALDI-TOF HCCA) matrix solution. For each plate, a bacterial test standard (Bruker Daltonics) was included to calibrate the instrument and validate the run. Spectra were analyzed using MALDI Biotyper automation control and the Bruker Biotyper 2.0 software and library (version 2.0, 3740 entries; Bruker Daltonics). Identification score criteria were performed as recommended by Bruker Daltonics and shown in Supplementary Table 1 (Blosser et al. 2016).

Screening the culture extracts for antimicrobial activity: crude extract preparation

Marine isolates were cultured in 500 ml flasks containing 300 ml marine broth medium. Flasks were incubated at 25–32 °C on an incubator shaker at 220 rpm. After 5–7 days of incubation, the fermented broths were extracted twice with ethyl acetate (300 ml * 2). The solvent extracts were evaporated. The extracts obtained were weighed and stored at − 20 °C (Chen et al. 2012).

Antibacterial activity against indicator strains by well-cut diffusion technique

The bacterial extracts were dissolved in dimethyl sulfoxide (DMSO, each one milligram "1 mg" from the extract was dissolved in 1 ml DMSO) and tested for antibacterial activity using the well-cut diffusion technique: Mueller–Hinton medium inoculated with indicators bacteria (Gram-positive ***Bacillus subtilis*** ATCC 6633 and ***Staphylococcus aureus*** ATCC 9144, Gram-negative ***Escherichia coli*** ATCC 10536 and ***Pseudomonas aeruginosa*** ATCC 25619, and ***Candida albicans*** ATCC 90028) were poured into the plates. After solidifying, wells were punched out using 0.7 cm cork borer. Then, 100 µl of tested extracts dissolved in DMSO were pipetted into each well. All plates were incubated at (35–37 °c) for 18–24 h (Abou-Elela et al. 2009). After incubation period, the radius of the inhibition zone was measured.

Sequencing for marine isolates 16S ribosomal RNA (16S rRNA) gene

Cells were pelleted from liquid medium by centrifugation and genomic DNA was extracted from cell pellets using the EZ-10 Spin column genomic DNA minipreps kit (Bio Basic, Inc., Markham, Ontario, Canada) according to the manufacturer's instructions. The 16S rRNA gene fragment was PCR amplified from the purified genomic DNA of each isolate using the universal primer set 27F 5′-GAGTTTGATCCT GGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTAC GACTT-3′ (Reysenbach et al. 1992). Thermal cycling using the Qiagen Taq DNA Polymerase kit (Qiagen, Crawley, West Sussex, UK) was initiated with denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 92 °C for 30 s, annealing at 48 °C for 2 min, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 5 min. Thermal cycling was performed with an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany). The amplified 1500 bp bands were confirmed and visualized on a 1% agarose gel amended with ethidium bromide. Positive bands were excised and purified using the QIAQuick gel extraction kit (Qiagen). Then, purified PCR product was sequenced in the forward and/or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817, Perkin-Elmer/Applied Biosystems, Foster City, CA).

Phylogenetic analysis for 16S ribosomal RNA (16S rRNA) gene

16S rRNA gene sequences from isolates were analyzed using BLASTn tool at the National Center for Biotechnology Information (NCBI) GenBank. Closely related 16S rRNA gene sequences were retrieved from GenBank database and included in the analysis. 16S rRNA gene sequences were aligned using the muscle multiple sequence alignment program and subsequently a phylogenetic tree was constructed by neighbor-joining with 10,000 bootstrap replicates using MEGA 6.06.

Screening for biosynthetic PKS-I, II and NRPS I, II genes

Screening for the genes associated with secondary metabolites is helpful in evaluating the biosynthetic potential of the microorganisms. Five sets of degenerate primers were used for amplification of PKS I, PKS II, NRPS I and NRPS II specific domains (Jiang et al. 2007; Kennedy et al. 2009). The primers used are listed in Supplementary Tables 2 and 3. PCR amplifications were performed in a final volume of 25 µl containing 12.5 µl Emerald Amp GT PCR master mix (2 × premix), 4.5 µl PCR grade water, 1 µl of each primer (20 pmol) and 6 µl genomic DNA as template. Amplification was done using the protocol in Supplementary Table 3. Purification of the PCR products using QIAquick PCR product extraction kit (Qiagen, Inc., Valencia, CA) and the amplified bands were confirmed and visualized on a 1% agarose gel amended with ethidium bromide using ladder 3000 bp (promega) as reference.

Cloning and sequencing of PKS and NRPS genes of *Bacillus amyloliquefaciens*

The cloning of the PCR products was performed with a TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen, US). Thereafter, the PCR product insertion was tested by selecting each colony and mixing it with 12.5 µl master mix, then the inserts were amplified from the plasmid templates using standard M13 multiple-cloning site primers and PCR conditions. The *E. coli* DH5α strains possessing plasmids with unique fragments of the predicted sizes were grown for 18 h in LB broth at 37 °C, and the plasmids extracted using a plasmid miniprep kit I (Omega Biotek, USA). The sequencing was performed in the forward and/or reverse directions using the M13F and M13R reverse primers, on an Applied Biosystems 3130 automated DNA sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA) with Cat. No. 4336817.

Sequence analysis for PKS and NRPS

Vector contamination in the nucleotide sequences For NRPS and PKS, was detected using VecScreen tool at the NCBI and removed, then the nucleotide sequences were translated into peptide sequences using BioEdit version 5.0.7. The percentage similarity and identity to other translated sequences were then determined using BLASTx in conjunction with the NCBI. To construct a phylogenetic tree, the nucleotide sequences of interest were aligned with other sequences obtained from GenBank using ClustalW, and unrooted neighbor joining phylogenies constructed using MEGA version 6.06 with a bootstrap consisting of 10,000 replications.

Results

Marine invertebrates' collection

In October 2015, 11 different samples of marine invertebrates were collected from Egypt's Red Sea (Sharm El-Sheikh-El-Tor and Hurghada). Six samples were soft corals (*Heteroxenia fuscescens*, *Litophyton arboretum*, *Sacrophyton trocheliophorum*, *Sinularia* sp., *Sacrophyton acutum and Lobophytum pauciflorum*), four samples were sponges (*Spongia officinalis*, *Callyspongia viridis*, *Suberea* sp. and *Biemna ehrenbergi*) and one sample only was from tunicates (*Didemnum moseleyi*) as shown in Fig. 1.

Isolates are classified according to the growth on different types of media

A total 80 isolates were obtained after the cultivation of the invertebrates' associated microorganisms on six different types of media. Twenty six isolates on Difco marine agar, 6 isolates on specific actinomycetes agar media, 21 isolates were obtained from R2A agar medium and 27 isolates were recovered by M1 medium. Unfortunately, no growth was observed on both of ISP medium 2 Agar and starch casein agar as presented in Supplementary Table 4.

Identification of characteristic isolates using MALDI-TOF MS

After preliminary identification (macroscopically and microscopically), nine morphologically characteristic isolates were analyzed using MALDI-TOF for further identification. The results were described in Supplementary Table 5 showed high biodiversity of the Red Sea marine invertebrates associated bacteria as they were found to belong to several bacterial groups

Fig. 1 Three different types of invertebrates (sponges, soft corals and tunicate) were collected from Red Sea as follow: 1 *Spongia officinalis*, 2 *Litophyton arboretum*, 3 *Callyspongia viridis*, 4 *Lobophytum pauciflorum*, 5 *Heteroxenia fuscescens*, 6 *Suberea* sp., 7 *Didemnum moseleyi*, 8 *Sinularia* sp., 9 *Biemna ehrenbergi*, 10 *Sacrophyton acutum*, 11 *Sacrophyton trocheliophorum*

represented in three major phyla: Gammaproteobacteria, Actinobacteria and Firmicutes. Gammaproteobacteria was comprised of one main genera which was *Vibrio*, where Actinobacteria consist of two genera which were *Kytococcus* and *Kocuria*. Finally, *Staphylococcus* and *Bacillus* from Firmicutes phylum were found to be isolated from the marine invertebrates.

Antimicrobial activity of the bacterial extracts against standard pathogenic strains

Isolates were tested for their antibacterial and anti-candidal activity and seven of them (HHF-1, HHF-8, HLA-17, HLA-19, HCV-33, HDM-39 and HS-64) were active and showed different diameters of the inhibition zones as shown in Table 1. Strain number **HHF-8** was the most active strain and showed broad spectrum activity against (Gram-positive *B. subtilis* ATCC 6633 and *S. aureus* ATCC 9144, Gram-negative *E. coli* ATCC 10536 and *P. aeruginosa* ATCC 25619, and against *C. albicans* ATCC 90028).

The rest of the isolates showed variable levels of the antimicrobial activity, isolate number HS-64 showed also a broad spectrum of activity against (Gram-positive *B. subtilis*, Gram-negative *E. coli* and *C. albicans*), but unfortunately did not show activity against *P. aeruginosa* and *S. aureus*. In case of isolate numbers HFF-1 and HCV-33, they showed activity against Gram positive strains only (*S. aureus* and *B. subtilis*). Moreover, isolates number HLA-17, HLA-19 and HDM-39 showed activity against one strain

Table 1 Antimicrobial activity of marine isolates against standard strains and analysis of 16S rRNA using BLASTn: isolates were tested for their antibacterial and anticandidal activity. Seven isolates were active, isolate number HHF-8 which related to *Bacillus amyloliquefaciens strain M4* was the most active strain and showed activity against Gram positive. The rest of the stains showed variable levels of the antimicrobial activity, the activity (zone of inhibitions) were measured in cm

Isolate nos.	HFF-1	HHF-8	HLA-17	HLA-19	HCV-33	HDM-39	HS-64
Origin	*Heteroxenia fuscescens*	*Heteroxenia fuscescens*	*Litophyton arboretum*	*Litophyton arboretum*	*Callyspongia viridis*	*Didemnum moseleyi*	*Suberea* sp.
Medium used	Difco marine agar	M1 agar	R2A agar	M1 agar	R2A agar	Difco marine agar	M1 agar
Accession numbers	MG757677.1	MG757675.1	MG757672.1	MG757678.1	MG757676.1	MG757673.1	MG757674.1
Length of 16S RNA gene	1439	1400	1399	845	993	1420	1400
Name of closely related strains	*Bacillus methylotrophicus* strain HB26	*Bacillus amyloliquefaciens* strain SB1	*Bacillus altitudinis* strain GVK 38	*Staphylococcus xylosus* strain B36	*Staphylococcus* sp. YNUTCM YJ244	*Staphylococcus saprophyticus* subsp. *saprophyticus* strain IIF2SW-B4	*Bacillus safensis* strain S-I10
Coverage (%)	99	100	100	95	98	99	100
Identity (%)	99	99	99	93	97	99	99
Staphylococcus aureus (cm)	1.2	1.3	1.8	0	1.3	0	0
Candida albicans (cm)	0	1.4	0	0	0	0	2.4
E. coli (cm)	0	1.5	0	0	0	0	1.5
Pseudomonas aeruginosa (cm)	0	1.4	0	2	0	2.3	0

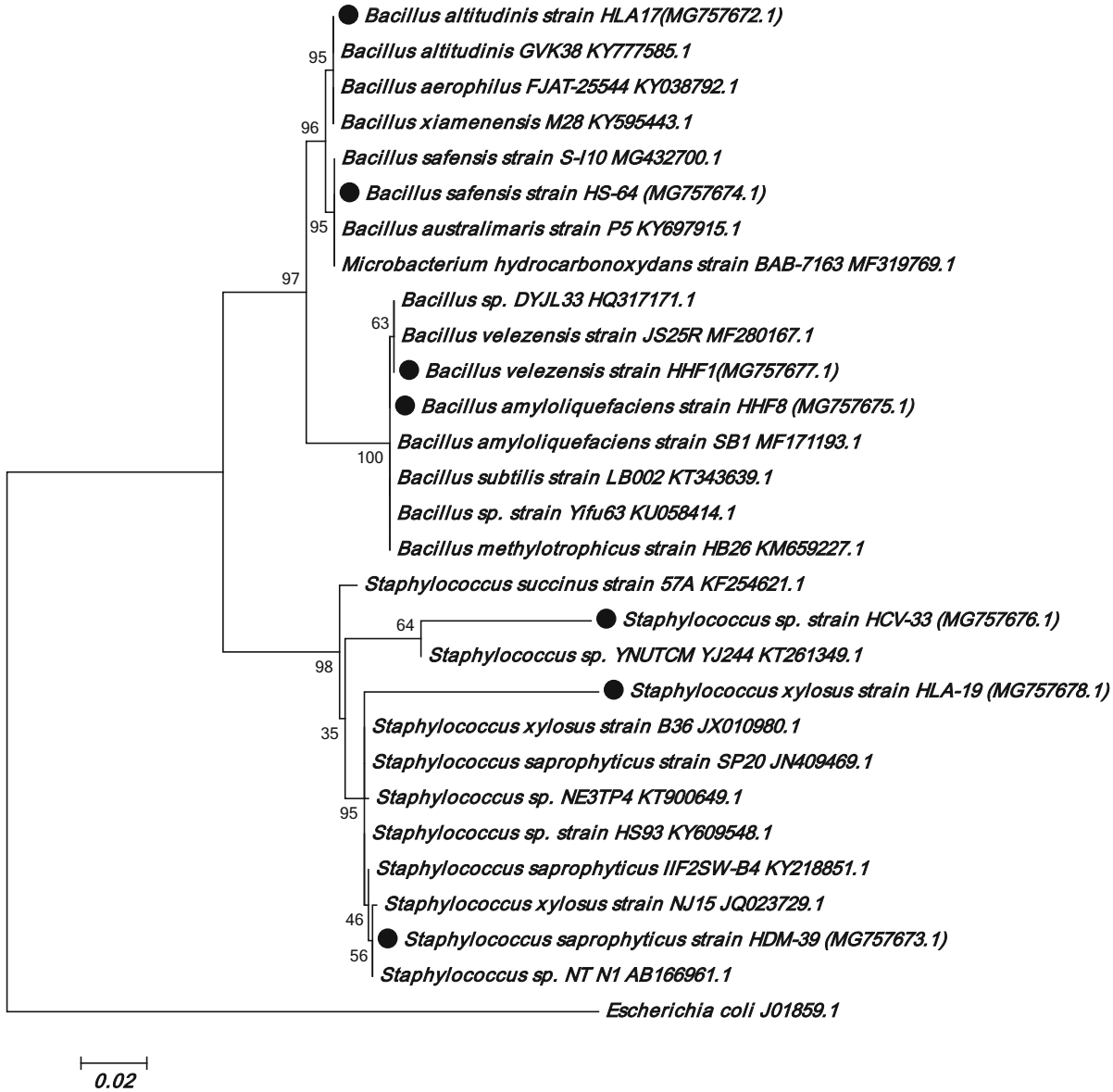

Fig. 2 The evolutionary history for 16S rRNA gene was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. All positions with less than 95% site coverage were eliminated. The support values are based on 10,000-fold bootstrapping. Evolutionary analyses were conducted in MEGA6. (●): the marine isolates with antimicrobial activity

only which was *S. aureus* in case of HLA-17 and *P. aeruginosa* in case of HLA-19 and HDM-39.

Molecular identification by 16S rRNA gene sequence and phylogenetic analysis of invertebrates' associated bacteria with antimicrobial activity

Invertebrates' associated bacteria with the antimicrobial activity were further identified by partial 16S rRNA gene sequence analysis. The bacteria with the

Table 2 Screening of the isolates for biosynthetic gene fragments PKS I and II and NRPS I and II showed that seven isolates gave positive results

Name of the host	The cryptic isolate nos.	Most related organism as revealed by 16S BLAST searches	NRPS I (Primer 1) (bp)	NRPS II (Primer 2) (bp)	PKS I (Primer 3) (bp)	PKS I (Primer 4) (bp)	PKS II (Primer 5) (bp)
Heteroxenia fuscescens	HHF-1	*Bacillus methylotrophicus strain HB26*	−	+	+	+	+
Heteroxenia fuscescens	HHF-8	*Bacillus amyloliquefaciens strain M4*	+	+	+	+	+
Litophyton arboretum	HLA-17	*Bacillus pumilus strain BPR1*	−	−	−	+	+
Litophyton arboretum	HLA-19	*Staphylococcus xylosus strain B36*	+	−	+	+	+
Callyspongia viridis	HCV-33	*Staphylococcus* sp. *YNUTCM YJ239 16S*	+	−	−	+	+
Didemnum moseleyi	HDM-39	*Staphylococcus* sp. *NCCP-1297*	−	−	−	+	−
Suberea sp.	HS-64	*Bacillus* sp. *CMU2*	+	+	+	+	+

Isolates number HFF-8 and HS-64 were positive to all five primers used. Isolates number HFF-1 and HLA-19 were positive to four primers only. Finally, isolates HCV-33, HLA-17 and HDM-39 gave positive results to three, two and one primer, respectively

antimicrobial activity were all from phylum Firmicutes and were comprised of the two genera, *Bacillus* ($n = 4$; 57.2%) and *Staphylococcus* ($n = 3$; 42.8%) with identity ranged from 93 to 99% as shown in Table 1. Phylogenetic analysis was carried out using the neighbor-joining method presented in Fig. 2.

Searching for PKs and NRPs genes

It is well known that many bioactive metabolites in microorganisms associated with the invertebrates are produced by PKS (KS domain specifically) and NRPS gene fragments. So, isolates when were screened for the presence of PKS I and II and NRPS I and II genes and the seven isolates gave positive results with at least one primer, while isolates number HHF-8 and HS-64 gave positive results with all five primers used as shown in Table 2, Supplementary Table 6 and Supplementary Figs. 1, 2 and 3.

Cloning, sequencing and phylogenetic analysis of PKS and NRPS gene fragments

The most active isolate as antimicrobial agent was HHF-8 which was related to *B. amyloliquefaciens* and showed positive results using all five primers to screen for PKS I and II and NRPS I and II genes. The PCR products were cloned and sequenced. In the case of NRPS, The fragment was screened using **Primer 2** and gave positive band which was similar to a NRPS in *Bacillus velezensis* specifically AFD class I and AMP (adenosine monophosphate) binding super family with 100 identity (%).

Regarding to the PKS fragments Primers 3 and 4 were used for the screening. **Primer 3** resulted in a single band which sequence was similar to PKS (condensing enzymes super family) found in *Bacillus* sp. *WPySW2* with 99% identity. While **Primer 4** gave positive result which is similar to the sequences of the PKS in *Bacillus* sp. *WPySW2* with 99% identity as shown in Table 3 and Figs. 3 and 4.

Primitive prediction of the chemical structure using NaPDoS domain seeker

The structures of the secondary metabolite gene fragments in the genomes of isolate HFF-8 were predicted by NaPDoS (Machado et al. 2015). Kirromycin (Weber et al. 2008) and leinamycin (Kara et al. 1990) were the two important secondary metabolites that were related to the biosynthetic

Table 3 BLASTx results for biosynthetic genes of isolate HHF-8 which was related to *Bacillus amyloliquefaciens*

Primer nos.	Name of closely related strain	Fragment length (bp)	The closet PKS/NRPS homology				Accession numbers
			Maximum scores	Query cover (%)	E values	Identity (%)	
NRPS II Primer 2 900	Non-ribosomal peptide synthetase [*Bacillus velezensis*] AFD class I and AMP (adenosine monophosphate) binding super family	704	404	89	4e−127	100	WP_069007535.1
PKS I Primer 3 800	Polyketide synthase [*Bacillus* sp. WPySW2] condensing enzymes super family	847	454	80	9e−161	99	ACG70841.1
PKS I Primer 4 1500	Polyketide synthase PksJ [*Bacillus velezensis*] condensing enzymes super family	732	366	73	1e−120	98	ASB65997.1

fragment with 56 and 55% identity, respectively as shown in Table 4.

Discussion

MALDI-TOF results represented in Supplementary Table 5 and were interpreted in Supplementary Table 1 revealed biodiversity of the Red Sea marine invertebrates associated bacteria which have been being categorized in Gammaproteobacteria, Actinobacteria and Firmicutes bacterial groups, this biodiversity is in agreement to what have been reported by Ngugi et al. (2012). Firmicutes phylum (*Bacillus* and *Staphylococcus* genus) in specific showed a broad spectrum antimicrobial activity when compared to the other two bacterial groups presenting a good potential antimicrobial metabolites produced by bacteria from the marine environment which was previously confirmed by Mani et al. (2016).

Antimicrobial activity test and phylogenetic analysis showed that seven isolates revealed antimicrobial activity against at least one pathogenic reference strain. Isolate number HFF-8 which was similar to *B. amyloliquefaciens* in specific was the most active isolate against Gram positive, Gram negative and *C. albicans* (Table 1; Fig. 2). This organism was isolated from the marine environment, it has been however previously isolated from plant (Yan et al. 2016), soil (Boottanun et al. 2017) and healthy chicken intestines (Chen et al. 2016). Moreover, when five primers were used for screening the giant gene clusters NRPSs and PKSs all isolates showed activity with at least one primer; while the two isolates HHF-8 and HS-64 showed positive results with all five primers used (Tables 2, 3; Figs. 3, 4). The biosynthetic gene fragments are assumed to be in coherence to the antimicrobial activity of the isolates which is in agreement with Ndlovu et al. (2017) who confirmed that there could be a direct relationship between the biosynthetic genes and the strong antifungal, antimicrobial activities and the production of the biosurfactant compounds. Even though, the two isolates HHF-8 and HS-64 showed positive results with all five primers, they exhibited different antibacterial activity. HHF-8 was active against all tested pathogenic reference strains while HS-64 did not, HS-64 showed no activity against *S. aureus* and *P. aeruginosa* in our study which contradicted with what Mukherjee et al. (2017) reported. The reason for this is that antibacterial activity is not necessarily directly linked to the gene expression of the biosynthetic genes PKS and NRPS only as confirmed by Ayuso et al. (2005).

Regarding to the phylogenetic analysis of the 16S rRNA sequences, the results revealed that the isolates were related to sequences in the NCBI database with 93–99% homology. Isolate number HLA-19 showed low similarity (93%) to (*Staphylococcus xylosus* strain

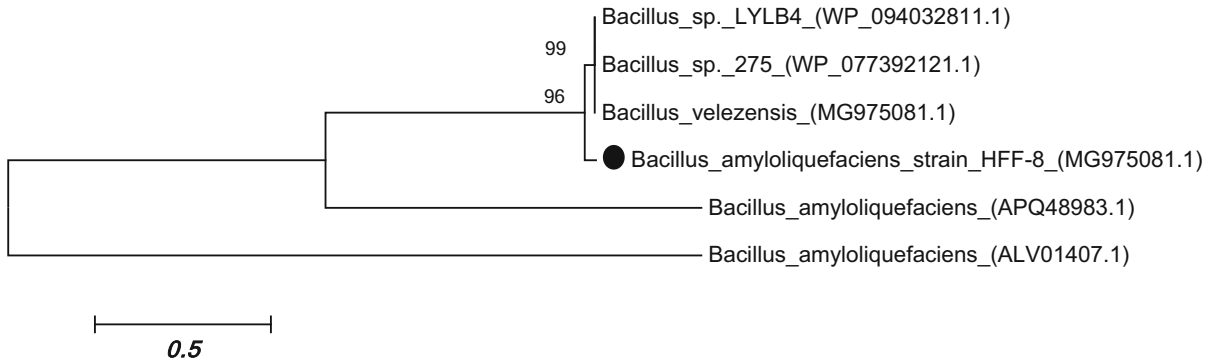

Fig. 3 Phylogenetic analysis was conducted based NRPS II (MG975081.1) from *Bacillus amyloliquefaciens* clone. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The percentage of trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA6

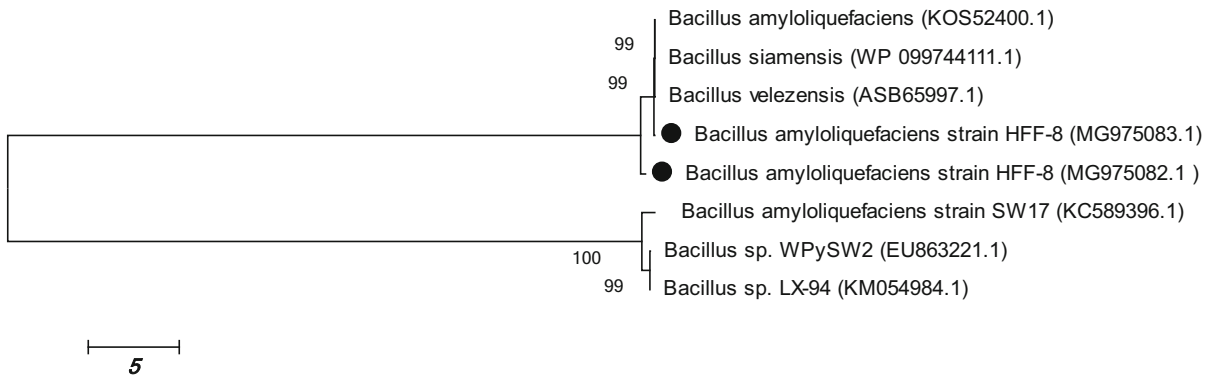

Fig. 4 Phylogenetic analysis was conducted based PKS domain [(MG975082.1) and (MG975083.1)] from *Bacillus amyloliquefaciens* clones. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman + Freq. model [1]. The supported values are based on 10,000-fold bootstrapping. Evolutionary analyses were conducted in MEGA6

B36), according to Al-Amoudi et al. (2016) 16S rRNA gene sequences deposited in GenBank with a similarity of $\leq 98\%$ is considered novel.

Conclusion

The diversity of bacteria associated with marine invertebrates and their potential to produce bioactive compounds have received much attention in recent years. This study showed a rich biodiversity of culturable organisms with a potent antimicrobial activity from Red Sea ecosystems on Egypt coastline. Also, the presence of the biosynthetic gene clusters (PKS or NRPS or both) in the strains confirmed the production of bioactive metabolites with antimicrobial activity, however some strains which showed positive results with PKS and NRPS did not show antimicrobial activity against some pathogenic reference strains. Finally, structures of the antibiotics were preliminary predicted by NaPDoS program to be kirromycin and leinamycin. However, further studies and experimental analysis using (NMR spectroscopic, HPLC, FTIR

Table 4 Prediction of the structure of the bioactive compound as revealed from the biosynthetic genes using the NaPDoS program: kirromycin and leinamycin were the two important secondary metabolites

Genes	Acc. nos.	Database match i.d.	Identity (%)	Align length	E-values	Pathway product	Structure
PKS I Primer 4	MG975082	KirAIV_CAN89634_1T *Streptomyces collinus*	56	170	4e−55	Kirromycin 18291322	Domain class: Trans
PKS I Primer 3	MG975083	LnmJ_AF484556_4T *Streptomyces atroolivaceus*	55	234	2e−74	Leinamycin ref. ID 15112993	Domain class: Trans

and mass spectrometric data) are needed to predict the final structure of the antibiotic.

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Supporting information Supplementary Table 1—Identification score criteria by Bruker Daltonics.

Supplementary Table 2—The primers used in screening or biosynthetic genes PKS I, II and NRPS I, II genes.

Supplementary Table 3—The amplification protocol of the primers used in screening for biosynthetic PKS I, II and NRPS I, II genes.

Supplementary Table 4—Classification of the isolates according to the type of the invertebrate and the type of the medium.

Supplementary Table 5—Identification of characteristic isolates using MALDI-TOF MS.

Supplementary Table 6—Screening of the isolates for biosynthetic gene fragments PKS I&II and NRPS I&II.

Supplementary Figure 1—Screening of the isolates for biosynthetic gene fragments NRPS I (primer 1) and NRPS II (primer 2).

Supplementary Figure 2—Screening of the isolates for biosynthetic gene fragments PKS I (primer 3) and PKS I (primer 4).

Supplementary Figure 3—Screening of the isolates for biosynthetic gene fragments PKS II (primer 5).

Compliance with ethical standards

Conflict of interest None.

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