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Halophytes-associated endophytic and rhizospheric bacteria: diversity, antagonism and metabolite production

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ABSTRACT

In Saudi Arabia, halophytes occupy tidal and intertidal forest ecosystems. They and their associated microflora have immense potential to yield novel and important useful natural products. Three halophytes (*Avicennia marina*, *Halocnemum strobilaceum*, *Zygophyllum qatarense*) were targeted for the isolation and identification of populations of endophytic and rhizospheric bacteria having antimicrobial potential. A total 554 bacterial isolates were initially screened against oomycetes fungal pathogens, *Phytophthora capsici* and *Pythium ultimum*. Of these, only 57 rhizospheric and endophytic bacteria exhibited inhibition against the targeted bioassay oomycetes. Tentative identification of the bacteria was on the basis of 16S rRNA gene sequences which revealed 92–100% sequence identity to type strains of related species and placed these organisms in six major classes: *Actinobacteria*, γ -*Proteobacteria*, *Firmicutes*, α -*Proteobacteria*, *Flavobacteriia* and β -*Proteobacteria*. When checked for lytic enzyme production, mostly the isolates of *Actinobacteria* and *Firmicutes* were potential enzyme producers. Detection of secondary metabolite biosynthetic genes – type I polyketide synthases, type II polyketide synthases and nonribosomal peptide synthetases – confirmed that 21 (35.5%) isolates were positive for at least one type of the biosynthetic gene. In order to identify metabolites, three isolates, *Alteromonas australica* (EA73), *Aidingimonas halophila* (EA105) and *Halomonas zindurans* (EA127), were selected and subjected to chemical analyses using liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry. Both analyses showed the presence of different bioactive compounds in the culture extracts of isolates some of which are already reported for their diverse biological activities such as 2, 4-Diacetylphloroglucinol. Our results demonstrated that halophytes represent an important source of potentially active bacteria producing antifungal metabolites of medical significance.

Introduction

Halophytes inhabit the intertidal areas of Saudi Arabia and they consist of populations of grasses, epiphytes, shrubs and trees. Among halophytes, mangroves exist in tropical climates requiring warm conditions and can tolerate salinity, anaerobic conditions, tides, winds and high temperatures. Their habitat enables them to grow with rich biodiversity. Mangroves under these stressful conditions are able to produce different kinds of active metabolites with diverse biological functions. Until now, more than 200 active metabolites have been isolated from mangroves (Bandaranayake, 2002).

Mangroves are a good source of steroids, saponins, flavonoids, triterpenes, alkaloids, tannins and have been used in different traditional medicines due to the antimicrobial activities of their extracts (Eldeen & Effendy, 2013). Previous studies reported a wide range of biological activities such as antiviral, antibacterial and antifungal properties of plant extracts (Chandrasekaran, Kannathasan, Venkatesalu, & Prabhakar, 2009; Premnathan et al., 2009). Due to their biological activities, they are an ideal material for investigation of associated microorganisms and their bioactive compounds. Both rhizospheric and endophytic bacteria seem to play important roles for the survival and growth of host plants. They provide several beneficial effects including plant growth promotion and resistance against pathogens by producing different substances such as volatile and antifungal compounds (Chung et al., 2008; Ryu et al., 2003). Halophytes also harbour different groups of bacteria, and these bacteria perform different functions such as growth promotion and disease resistance against different pathogens (Thatoi, Behera, Mishra, & Dutta, 2013). Bacterial communities associated with halophytes are beneficial for the host whether as rhizospheric bacteria (Roy, Hens, Biswas, Biswas, & Kumar, 2002) or in endophytic form (Garcias-Bonet, Arrieta, de Santana, Duarte, & Marbà, 2012; Hu, Li, & He, 2010). These bacteria are also an excellent source of different useful enzymes and antibiotics (Thatoi et al., 2013).

Many halophytes have associated endophytic bacteria that possess a broad spectrum of antifungal activities against different pathogenic bacteria (Hu et al., 2010; Jose & Christy, 2013). Recently, new marine compounds such as isocoumarin, furanocoumarin and kandenol have been reported from halophyte-associated bacteria (Xu, 2015); however, there are limited studies on isolation of bacteria from halophytes in coastal areas of the Red Sea and their associated identification and biological activities. In a recent study from Saudi Arabia, microbial communities from a mangrove (*Avicennia marina*) were identified using a metagenomic approach but no functional aspects of the microbes involved have been studied (Alzubaidy et al., 2016). Therefore, we designed a study to isolate both rhizospheric and endophytic bacteria from different parts of halophytes, *A. marina*, *Halocnemum strobilaceum* and *Zygophyllum qatarense*, and to perform a tentative identification of the strains using 16S rDNA molecular techniques and screened them against different phytopathogens. We also report enzyme production, secondary metabolite biosynthetic genes and the identification of metabolites from selected bacterial isolates. Three potential bacteria were selected for identification of metabolites and antimicrobial compounds such as 2,4-Diacetylphloroglucinol (DAPG), prednicarbate, benzydamine, methyl jasmonate, cephaloridine and triamcinolone acetonide were discovered.

Materials and methods

Sample collection and isolation of rhizospheric bacteria from halophytes

Three different halophytes specimens [*A. marina* (mangrove), *H. strobilaceum* and *Z. qatarense*] were collected from the coastal area of Thuwal, Jeddah, Saudi Arabia (22° 15' 54" North, 39° 6' 44" East). All of the plant specimens were placed in a sterile bag after collection and transferred to the laboratory for bacterial isolation. Soil, roots, leaves and pneumatophores of halophytes were used for the isolation of rhizospheric and endophytic bacteria. To isolate bacteria from soil adhering to roots of the plants, they were dipped in sterile distilled water and serial dilutions were made (10^{-3} , 10^{-4} and 10^{-5}) in filtered autoclaved seawater (FAS) and spread in triplicates on four different media used for culturing of bacteria. Half strength R2A ($\frac{1}{2}$ R2A) [0.25 g yeast extract; 0.25 g Proteose Peptone No. 3 (Difco Laboratories, Detroit, MI); 0.25 g casamino acid; 0.25 g dextrose; 0.25 g soluble starch; 0.15 g sodium pyruvate; 0.15 g K_2HPO_4 ; 0.03 g $MgSO_4$], half strength Tryptic soy agar ($\frac{1}{2}$ TSA) [pancreatic digest of casein 7.5 g; papain digest of soybean 2.5 g; NaCl 2.5 g; agar 15.0 g], marine agar (MA) [peptone 5.0 g; yeast extract 1.0 g; Ferric Citrate 0.1 g; NaCl 19.45 g; MgCl 8.8 g; Na_2SO_4 3.24 g; CaCl 1.8 g; KCl 0.55 g; $NaHCO_3$ 0.16 g; KBr; 0.08 g; $SrCl_2$ 34.0 mg; H_3BO_3 22.0 mg; Na_2SiO_3 4.0 mg; NaF 2.4 mg; NH_4NO_3 1.6 mg; Na_2HPO_4 8.0 mg; agar 15.0 g] and half nutrient agar ($\frac{1}{2}$ NA) [beef extract 1.5 g; peptone 2.5 g; agar 15.0 g] (Difco Laboratories) for bacterial culturing in 1000 mL filtered seawater for bacterial culturing. Roots and leaves tissues were also used for isolation of bacteria.

Isolation of endophytic bacteria from halophytes

For isolation of endophytic bacteria from plant roots, leaves and pneumatophores surface sterilisation was performed. Roots, leaves and pneumatophores segments were washed 5–6 times with tap water and then further sterilised by washing with disinfectants as described previously (Bibi, Yasir, Song, Lee, & Chung, 2012). After sterilisation of roots, leaves and pneumatophores segments, small pieces of sterilised roots, leaves and pneumatophores segments were ground in FAS using sterile mortar and pestle. Aliquots were further serially diluted (10^{-3} , 10^{-4} and 10^{-5}) and plated in triplicate on the four different media mentioned above. To suppress fungal growth, 50 μ g/mL cycloheximide was mixed to the medium before pouring. Plates were incubated at 25°C for 2 weeks for bacterial growth. Individual colonies were further streaked to check the purity of the strains, and all these bacterial strains were further subcultured and stored in 15% (v/v) glycerol stock of strains at -70°C in King Fahd Medical Research Centre and were given lab numbers from EA73-EA131, respectively.

Screening of bacteria for antifungal activity

Rhizospheric and endophytic bacteria isolated from halophytes were further screened for their antifungal potential. Five different fungal pathogens: *Phytophthora capsici* (*P. capsici*), *Pythium ultimum* (*Py. ultimum*), *Magnaporthe grisea* (obtained in this laboratory), *Alternaria mali* (KCTC 6972) and *Fusarium moniliforme* (KCTC 6149), were

obtained from the Korean type culture collection centre (KCTC). Antagonistic activity against fungal pathogens was determined by using the cross streak method (Bibi et al., 2012). All isolates were streaked on PDA media supplemented with ½ R2A in seawater. Each 6-mm mycelial disc of test fungal pathogens was placed in the centre of the plate perpendicular to the streak of isolates at 4 cm from the edges of the plate and was incubated for 4–6 days at 28°C. All strains were checked twice for antagonistic activity. The antagonistic activity was then evaluated by measuring the inhibition zone of fungal mycelia around the bacterial colony.

Extraction of bacterial DNA and 16S rRNA gene sequencing

Genomic DNA was extracted from antagonistic bacteria using a DNA extraction kit (Thermo Scientific, Waltham, MA, USA). To identify antagonistic bacteria, 16S rRNA gene sequencing was performed. Using bacterial universal primers 27F (5'-AGAGTTT-GATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT -3'), the 16S rRNA gene fragment was amplified. Amplifications were performed under following conditions: one cycle of 95°C for 5 min followed by 28 cycles of 95°C for 1 min, an annealing of 58°C for 50 s and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Using PCR purification kit (Thermo Scientific), the PCR products were purified and sequenced commercially by Macrogen (Seoul, Korea). Sequences obtained after 16S rRNA gene similarity were identified using the EzTaxon server (<http://eztaxon-e.ezbiocloud.net/>) (Kim et al., 2012) to identify the bacteria. To determine the phylogenetic position of the antagonistic bacteria and related type strains, the 16S rRNA gene sequences of type strains were obtained from the National Center for Biotechnology Information. For the phylogenetic analysis, CLUSTALX (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) multiple alignments of the bacterial sequences were performed and BioEdit software (Hall, 1999) was used to edit the gaps. The neighbour-joining method in the MEGA6 Programme with bootstrap values based on 1000 replications was used for constructing the phylogenetic tree (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

Evaluation of hydrolytic enzymatic activity

Protease activity was checked using skim milk ½ R2A agar plates. Bacteria producing protease made clear zone on skim milk agar plates. Amylase production was checked on starch media. Amylase producing bacteria showed starch hydrolysis as a clear zone on starch ½ R2A agar plates (Kumar, Karan, Kapoor, Singh, & Khare, 2012). For lipase activity, tributyrin ½ R2A agar media was used. After 48 h of incubation at 28°C, a clear zone was detected around bacteria that were hydrolysing tributyrin. To check cellulase activity, CMC agar (carboxymethylcellulose agar) media was used. Bacteria were streaked on plates of the respective enzyme media and incubated at 28°C for 2 days. After these plates were flooded with a solution of 0.1% Congo red and put on an orbital shaker for 15 min and washed with 1M NaCl (Hendricks, Doyle, & Hugley, 1995). Positive activity was seen as a halo zone around bacterial colonies on CMC agar.

Detection of secondary metabolite biosynthetic genes

The detection of a biosynthetic gene, polyketide synthetase I (PKS-I) and nonribosomal peptide synthetase (NRPS) genes were checked using primer pairs K1F/M6R (5'-TSAAGTCSAACATCGGBCA-3'; 5'-CGCAGGTTSCSGTACCAGTA-3') and A3F/A7R (5'-GCSTACSYSATSTACACSTCSGG-3'; 5'-SASGTCVCCSGTSCGGTAS-3'), respectively (Ayuso-Sacido & Genilloud, 2005). The polyketide synthetase II (PKS-II) gene was amplified using primer pair KSa/Kβ (5'-TSGCSTGCTTGGAYGCSATC-3'; 5'-TGGAANCCGCCGAABCCTCT-3') (Metsä-Ketelä et al., 1999). PCR amplification products were then checked by using 0.8% agarose gel electrophoresis, and bands of 1.2–1.4 kb, 600 bp and 700–800 bp were detected as products of PKS-I, PKS-II and NRPS genes, respectively.

Optimisation of bacterial culture condition for production of antifungal activity

To optimise culture conditions of the selected bacterial strains for the production of antifungal activities, an appropriate medium for culturing was selected. Four different media, i.e. ½ R2A broth, ½ TSA, ½ NA in seawater and Marine broth in distilled, were used for culturing. After every 24 h, optical density (OD) was checked and antifungal activity was assessed against *P. capsici* and *Py. ultimum* using disc diffusion method. The effect of temperature was checked at different ranges of temperatures (20–40°C) in ½ R2A broth. For pH optimisation, different pH value ranges (5–12) were used for the growth and antifungal compound production in ½ R2A broth.

Gas chromatography–mass spectrometry analysis for identification of metabolites

All chemicals and solvents were of MS grade. *N,O*-bis(trimethylsilyl) trifluoroacetamide + trimethylchlorosilane (TMCS) (99:1), Methoxyamine hydrochloride and Pyridine (R.G., Reag. ACS, Reag. Ph Eur) were purchased from Sigma-Aldrich, and methanol was purchased from Thermo Fisher. Bacterial cultures (1 mL) were lysed and centrifuged at 10,000g for 10 min. The supernatant (200 µL) was transferred to a 1.5-mL Eppendorf tube and 600 µL acetonitrile was added and vortex for 30 s. Samples were further centrifuge at 13,000g for 10 min and 200 µL supernatant was transferred to glass vials. The solvent was removed by a nitrogen dry machine. Further 80 µL methoxy amine pyridine hydrochloride (15 mg/mL) was added to the glass-derived bottle, vortexed for 2 min and incubated at 80°C for 30 min; *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) 80 µL containing 1% (v/v) TMCS was added to each vial and the mixture was vortexed for 2 min and then derivatised at 80°C for 30 min. Mixtures were centrifuged for 10 min (13,000g), then 100 µL supernatant was used for gas chromatography–mass spectrometry (GC-MS) metabolomics analysis. The derivatised samples were analysed on Shimadzu GCMS-QP2010 Ultra, including a capillary column (30 m × 0.25 mm × 0.25 µm) used to separate the derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min through the column. The injector temperature was maintained at 260°C and injection volume was 1 µL in spoutless mode. The initial oven temperature was 80°C and was held for 2 min, ramped to 300°C at a rate of 10°C/min, and finally held at

300°C for 5 min. The temperature of ion source (electron impact) was set to 200°C. The collision energy was 70eV. Mass data were acquired in a full-scan mode (m/z 45–800), with an acquisition rate of 100 spectrum/s.

Liquid chromatography–mass spectrometry analysis of bacterial culture

Five millilitres of bacterial culture was placed on –80°C for 5 min and then transferred to a 37°C water bath for 5 min, and this procedure was repeated for five times. Centrifugation was done at 15,000g for 10 min, 3 mL supernatant was transferred to a tube and 12 mL acetonitrile was added and vortexed for 30 s. Again, centrifugation was done at 15000g for 10 min and 300 µL supernatant was taken for liquid chromatography–mass spectrometry (LC-MS) metabolomics analysis. Injection volume was 3 µL and the samples are analysed on Agilent 6540B TOF/Q-TOF Mass Spectrometer coupled with Agilent 1290 UPLC and Dual AJS ESI ion source. An ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 µm) column and pre-column (Phenomenex Security Guard™) was used to separate sample. The column temperature was set to 45°C and the flow rate was 0.5 mL/min. Acquisition range was from 50 to 1500 m/z , and scan rate was 1.00 spectrum/s. MS parameters were set as follows: capillary voltage 3500 V, nebulizer pressure 35 psi, drying gas 10 L/min, gas temperature 325°C, vaporizer 200 V, voltage charge 1000 V; negative-ion mode capillary voltage 3500 V, corona negative 15.0 V, fragmentor 175 V, skimmer1 65.0 V, octopole RF Peak 750 V; positive ion mode capillary voltage 3500 V, corona positive 4.0 V, fragmentor 175 V, skimmer1 65.0 V and octopole RF Peak 750 V. Raw data were imported to Agilent MassHunter Qualitative Analysis B.06.00 software. Metabolites were identified by the in-house database.

Nucleotide sequence numbers

All of the nucleotide sequences of the bacterial isolates from this study have been deposited in the GenBank database under accession numbers KY436446–KY436504.

Statistical analysis

Metabolites were identified by the NIST database. Briefly, after alignment with Statistic Compare component, the CSV file was obtained with three-dimensional data-sets including sample information, peak name, retention time, m/z and peak intensity. Internal standard peak and peaks caused by noise, column bleed and BSTFA derivatisation procedure, were removed from the data-set, and the peaks from the same metabolites were combined.

Results

Isolation of rhizospheric and endophytic bacteria from halophytes

In this study, three halophytes were collected from the coastal area of Thuwal, Jeddah (Fig. S1) and were identified as *A. marina* (mangrove), *H. strobilaceum* and *Z. qatarense* (Fig. S2(a–d)). The diversity of rhizospheric and endophytic was evaluated from soil, roots, leaves and pneumatophores of halophytes. A total of 554 colony-forming rhizospheric

and endophytic bacteria were isolated from these three halophytes (Table 1). We have used five different culturing media for isolation of rhizospheric and endophytic bacteria were used. These different media that are in high and low nutrient contents favour different groups of bacteria to grow. Some of the bacterial colonies were so small and required 2–3 weeks to grow.

Screening of bacteria against pathogenic fungi

Rhizospheric and endophytic bacteria were tested for their antagonism against pathogenic oomycetes, *Py. ultimum* and *P. capsici*. Of 554 bacteria, 57 (10.2%) displayed inhibition to both oomycete plant pathogens. The proportion of antagonistic bacteria varied, being highest in *A. marina* ($n = 28$; 12.3%) and *Z. qatarense* ($n = 16$; 11.8%), followed by *H. strobilaceum* ($n = 13$; 6.7%) (Table 1). These antagonistic bacteria were then tested for other three fungal pathogens. Different groups of antagonistic rhizospheric and endophytic bacteria were identified from halophytes, where γ -Proteobacteria was the dominant phylum (Table 1). Antagonistic activity of all bacterial isolates is summarised in Table 2. Some strains showed strong inhibition with 7–9 mm against oomycetes pathogens. Among 57 antagonistic bacteria, only 4 (6.7%), *Alteromonas* sp. (EA73) *Streptomyces* sp. (EA91), *Bacillus* sp. (EA115) and *Halomonas* sp. (EA127), exhibited inhibitory activity against all pathogenic fungi tested, whereas *Bacillus* sp. (EA78), *Bacillus* sp. (EA81), *Mycobacterium* sp. (EA84), *Bacillus* sp. (EA98) and *Halomonas* sp. (EA127) had strong inhibition only against oomycetes (Table 2). Some antagonistic bacteria were active against only oomycetes and were inactive for other pathogenic fungi used for screening in this study (Table 2). *Halomonas* was the dominant genus in this study among 30 different genera of antagonistic bacteria isolated from halophytes.

Phylogenetic analysis of antagonistic bacteria based on 16S rRNA gene sequence

Antagonistic bacteria ($n = 57$) were identified by using 16S rRNA gene sequence analysis. Twenty different genera of bacteria were identified and belonged to five major classes: *Actinobacteria* ($n = 12$; 21%), γ -Proteobacteria ($n = 24$; 42%), *Firmicutes* ($n = 10$; 18%), α -Proteobacteria ($n = 8$; 14%), and *Flavobacteriia* ($n = 3$; 5%) (Figure 1). The proportion of different genera in each part of plant sample and soil is shown in Figure 1(b–e). Sequence identity of antagonistic bacteria was from 92.8% to 100% (Table 2). The phylogenetic tree inferred using 16S rRNA gene data showed that branching patterns remained constant. High bootstrap values were recorded in the phylogenetic tree using 16S rRNA

Table 1. Distribution of rhizospheric and endophytic antagonistic bacteria isolated from halophytes.

Plant species ^a	Isolates ^b	Antagonists ^c	Antagonists ^d (%)	Dominant phylum ^e
<i>Avicennia marina</i>	226	28	12.3	<i>Actinobacteria</i>
<i>Halocnemum strobilaceum</i>	193	13	6.7	γ -Proteobacteria
<i>Zygophyllum qatarense</i>	135	16	11.8	γ -Proteobacteria
	554	57	10.3	γ -Proteobacteria

^aScientific names of halophytes collected from coastal area for isolation of bacteria.

^bTotal number of bacteria isolated from halophytes.

^cTotal number of antagonistic bacteria against *P. capsici* and *Py. ultimum* by confrontation bioassay.

^dPercentage of antagonistic bacteria from total bacteria.

^eDominant phylum in all antagonistic bacteria to oomycetes.

Table 2. Taxonomic identification, antifungal activity and enzymes production of rhizospheric and endophytic bacteria from halophytes.

Lab no	Closely related type strain ^d	Antifungal activity against ^a							Enzymatic activities ^b				Detection of ^c		
		Accession number	% identity ^e	<i>Py. ultimum</i>	<i>P. capsici</i>	<i>M. grisea</i>	<i>A. mali</i>	<i>F. moniforme</i>	Protease	Amylase	Lipase	Cellulase	NRPS	PKS-I	PKS-II
Avicennia marina															
Rhizospheric bacteria (Soil)															
EA73	<i>Alteromonas australica</i> H 17 ^T	KY436446	98.6	+++	++	+	+++	—	—	—	++++	—	—	—	—
EA74	<i>Alteromonas macleodii</i> ATCC 27126 ^T	KY436447	98.7	+	+	—	—	—	—	—	++++	—	—	—	—
EA75	<i>Alteromonas australica</i> H 17 ^T	KY436448	97.3	++	+	—	—	—	—	—	++++	—	—	—	—
EA76	<i>Pseudoalteromonas flavipulchra</i> NCIMB 2033 ^T	KY436449	97.7	++	+	—	—	—	—	—	—	—	—	—	—
EA77	<i>Halomonas smymensis</i> AAD6 ^T	KY436450	99.7	+	+	—	—	—	—	—	—	—	—	—	—
EA78	<i>Bacillus licheniformis</i> ATCC 14580 ^T	KY436451	99	+++	+++	++	—	—	++++	++++	—	—	—	—	—
EA79	<i>Halomonas denitrificans</i> M29 ^T	KY436452	99.4	+	+++	—	—	—	—	—	—	—	—	—	—
EA80	<i>Alteromonas gracilis</i> 9a2 ^T	KY436453	92.8	++	+	—	—	—	—	—	—	—	—	—	—
EA81	<i>Bacillus persicus</i> B48 ^T	KY436454	97.2	+++	+++	—	—	—	—	—	—	—	—	—	—
Endophytic bacteria (Pneumatophores)															
EA82	<i>Devosia subaequoris</i> HST3-14 ^T	KY436455	98.3	++	W	—	—	—	—	—	++++	—	—	—	—
EA83	<i>Nocardioides aromaticivorans</i> H-1 ^T	KY436456	99.4	+	+	—	—	—	++++	—	++++	—	+	—	—
EA84	<i>Mycobacterium wolinskyi</i> ATCC 700010 ^T	KY436457	99.9	+++	+++	—	—	—	—	—	++	++++	+	—	—
EA85	<i>Streptomyces spectabilis</i> NBRC 13424 ^T	KY436458	95.9	+	++	—	—	—	—	—	—	—	+	+	+
EA86	<i>Erythrobacter citreus</i> RE35F/1 ^T	KY436459	98.2	W	+	—	—	—	—	—	—	—	—	—	—
EA87	<i>Nocardioides albus</i> KCTC 9186 ^T	KY436460	99.1	++	+	—	—	—	++++	—	++++	—	—	+	—
EA88	<i>Halomonas denitrificans</i> M29 ^T	KY436461	99.2	+	++	+	—	—	+	—	—	—	—	—	—
EA89	<i>Nocardioides luteus</i> KCTC 9575 ^T	KY436462	100	+	+	—	—	—	++++	—	+++	—	—	—	—
EA90	<i>Marinobacter mobilis</i> CN46 ^T	KY436463	98.3	+	++	+	—	—	+	—	—	—	—	—	—
EA91	<i>Streptomyces enissocaesilis</i> NBRC 100763 ^T	KY436464	100	++	++	++	+++	++	+++	—	+	—	+	+	+
Endophytic bacteria (Roots)															
EA94	<i>Nocardioides luteus</i> KCTC 9575 ^T	KY436467	100	++	W	—	—	—	—	—	—	—	—	—	—
EA95	<i>Nocardioides albus</i> KCTC 9186 ^T	KY436468	96.8	W	+	+	—	—	—	—	—	—	+	—	—
EA96	<i>Nitratireductor shengliensis</i> 110399 ^T	KY436469	97.4	++	+	+	++	—	—	—	—	—	—	—	—
EA97	<i>Inquilinus limosus</i> DSM 16000 ^T	KY436470	96.4	+++	+	+	—	—	—	—	—	—	—	—	—
EA98	<i>Bacillus berinqensis</i> BR035 ^T	KY436471	97.5	+++	+++	—	—	—	—	—	—	—	—	—	—

EA99	<i>Amycolatopsis thermalba</i> SF45 ^T	KY436472	98.9	+	+	++	-	-	-	-	-	-	+	-	+
Endophytic bacteria (Leaf)															
EA100	<i>Micrococcus flavus</i> LW4 ^T	KY436473	99.8	+	+	-	-	-	-	-	-	-	-	-	-
EA101	<i>Brevibacterium casei</i> NCDO 204 ^T	KY436474	99.3	W	+	-	-	-	-	-	-	-	+	-	+
EA102	<i>Staphylococcus epidermidis</i> ATCC 14990 ^T	KY436475	99.5	++	+	-	-	-	-	-	-	-	-	-	-
<i>Halocnemum strobilaceum</i>															
Rhizospheric bacteria (Soil)															
EA103	<i>Oceanicola litoreus</i> M-M22 ^T	KY436476	96.3	+	+	-	-	-	-	-	+++	-	-	+	-
EA104	<i>Roseisalinus antarcticus</i> EL-88 ^T	KY436477	94.1	+	+	-	-	-	-	-	-	-	-	-	-
EA105	<i>Aidingimonas halophila</i> YIM 90637 ^T	KY436478	97.8	+++	+	++	++	-	-	-	+++	-	-	-	-
EA106	<i>Halomonas anticariensis</i> FP35 ^T	KY436479	97.7	++	++	++	+	-	-	-	-	-	+	+	+
EA107	<i>Halomonas lutea</i> DSM 23508 ^T	KY436480	99	++	+++	+	-	-	-	-	+++	-	-	-	-
EA108	<i>Halomonas smymensis</i> AAD6 ^T	KY436481	99.8	+	++	-	-	-	-	-	-	-	-	-	-
Endophytic bacteria (Roots)															
EA109	<i>Marinobacter lacisalsi</i> FP2.5 ^T	KY436482	98.2	++	+	-	-	-	-	-	-	-	+	-	-
EA110	<i>Bacillus timonensis</i> MM10403188 ^T	KY436483	96.9	+	++	+	+	-	-	-	-	-	+	-	-
EA111	<i>Halomonas lutea</i> DSM 23508 ^T	KY436484	96.9	+	++	W	-	-	-	-	-	-	-	-	-
EA112	<i>Salipiger mucosus</i> DSM 16094 ^T	KY436485	99.6	++	+++	+	+	-	-	-	-	-	-	-	-
EA113	<i>Rubrimonas cliftonensis</i> OCh317 ^T	KY436486	92.9	+	+	+	-	-	-	-	-	-	+	-	-
Leaf															
EA114	<i>Staphylococcus hominis</i> subsp. novobiosepticus GTC 1228 ^T	KY436487	99.7	++	++	-	-	-	-	-	-	-	-	-	-
EA115	<i>Bacillus subtilis</i> subsp. inaquosorum KCTC 13429 ^T	KY436488	99.9	+++	++	+	+	+	-	-	-	-	-	-	+
<i>Zygophyllum qatarense</i>															
Rhizospheric bacteria (Soil)															
EA116	<i>Muricauda lutimaris</i> SMK-108 ^T	KY436489	97.6	+	++	+	-	-	-	++	++++	-	-	-	-
EA117	<i>Tamlana crocina</i> HST1-43 ^T	KY436490	99.4	+	+	-	-	-	++	-	-	-	-	-	-
EA118	<i>Marinimicrobium haloxylanilyticum</i> SX15 ^T	KY436491	96.9	+	++	W	-	-	-	-	-	-	-	-	-
EA119	<i>Marinobacter salsuginis</i> SD-14B ^T	KY436492	97.3	++	+++	+	-	-	++	-	-	-	+	+	-
EA120	<i>Halobacillus trueperi</i> DSM 10404 ^T	KY436493	99.4	+++	+++	-	-	-	-	-	-	-	-	-	-
EA121	<i>Zunongwangia mangrovi</i> P2E16 ^T	KY436494	98.9	+	+	-	-	-	-	-	-	-	-	-	+
Endophytic bacteria (Roots)															
EA122	<i>Marinobacter mobilis</i> CN46 ^T	KY436495	97.9	+	++	++	-	-	-	++	++++	-	+	-	+
EA123	<i>Halomonas smymensis</i> AAD6 ^T	KY436496	100	+	+	+	-	-	-	-	-	-	+	-	-
EA124	<i>Marinobacter lacisalsi</i> FP2.5 ^T	KY436497	96.5	+	++	-	-	-	-	+	-	-	+	+	-

(Continued)

Table 2. Continued.

Lab no	Closely related type strain ^d	Antifungal activity against ^a							Enzymatic activities ^b				Detection of ^c		
		Accession number	% identity ^e	<i>Py. ultimum</i>	<i>P. capsici</i>	<i>M. grisea</i>	<i>A. mali</i>	<i>F. moniforme</i>	Protease	Amylase	Lipase	Cellulase	NRPS	PKS-I	PKS-II
											++++				
EA125	<i>Microbulbifer celer</i> ISL-39 ^T	KY436498	99.3	++	W	—	—	—	—	—	++	—	+	—	+
EA126	<i>Bacillus licheniformis</i> ATCC 14580 ^T	KY436499	99.3	++	+++	++	+	—	++++	+	+++	—	—	—	—
EA127	<i>Halomonas zindurans</i> B6 ^T	KY436500	96.7	+++	+++	+	+	+++	—	—	—	—	—	—	—
	Endophytic bacteria (Leaf)														
EA128	<i>Arthrobacter crystallopoietes</i> DSM 20117 ^T	KY436501	97.3	+	+	—	—	—	—	—	—	—	—	—	—
EA129	<i>Halomonas smymensis</i> AAD6 ^T	KY436502	99.7	+	+	+	—	—	—	—	—	—	+	—	—
EA130	<i>Staphylococcus warneri</i> ATCC 27836 ^T	KY436503	99.8	++	++	—	—	—	—	—	+++	—	—	—	—
EA131	<i>Marinobacter algicola</i> DG893 ^T	KY436504	97.7	++	++	++	—	—	—	—	+++	—	+	—	—

^aAntagonistic activity of all bacteria isolated in this study. The activity was measured after 3–5 days incubation at 28°C by measuring the clear zone of mycelial growth inhibition: —, Negative; +, 3 mm; ++, between 4 and 6 mm; +++, between 7 and 9 mm

^bProduction of protease, amylase, lipase and cellulase was determined by plate assay. Enzymatic activity was estimated as zone of halo formed around bacterial colonies: —, Negative; +, 3 mm; ++, between 4 and 6 mm; +++, between 7 and 9 mm; +++, between 10 and 12 mm; +++++, between 13 and 15.

^cDetection of NRPS and PKS-I and PKS-II genes in antagonistic bacteria.

^dIdentification based on partial 16S rRNA gene sequence analyses of all antagonistic bacteria.

^e% similarity with closely related type strain

Figure 1. (a) Percentage composition of different phyla of antagonistic rhizospheric and endophytic bacteria isolated from four halophytes on the basis of 16S rRNA gene sequence similarity. Percentage composition of different phyla in (b) soil, (c) roots, (d) leaves and (e) pneumatophores.

gene sequences data (Figure 2). Antagonistic strains of class *Firmicutes* were placed in a separate cluster recovered with higher bootstrap values. Antagonistic bacteria in *Firmicutes* mainly belonged to the genera *Bacillus*, *Staphylococcus* and *Halobacillus*. Three different clusters have been generated for isolates of class *Actinobacteria*. Antagonistic bacteria in these three clusters were identified with high bootstraps values (54–100%). Representative isolates in this class belong to four different genera, i.e. *Nocardioides*, *Arthrobacter*, *Streptomyces* and *Mycobacterium*. Three strains of *Flavobacteriia* were identified with a separate cluster also showing high bootstrap values (64–100%). The strains of *α-Proteobacteria* comprising eight different genera were placed in one cluster in the phylogenetic tree with high bootstrap values. The representative strains of *γ-Proteobacteria* belong to five different genera. All strains of the genus *Halomonas* make a distinct cluster with the closely related type strain *Halomonas denitrificans* M29^T with a bootstrap value of 99–100%. In this study, some novel and new antagonistic rhizospheric and endophytic bacterial strains were also identified in addition to common and already known bacteria (Table 2). Some antagonistic rhizospheric and endophytic bacteria were recovered showing low 16S rRNA gene sequence similarity (<95%). Two isolates of *α-Proteobacteria*, i.e. *Roseisalinus* sp. (EA104) and *Rubrimonas* sp. (EA113), and one isolate belonging to *γ-Proteobacteria*, i.e. *Alteromonas* sp. (EA80), were identified as novel species with similarities of <95% to the closest type strains in GenBank (Table 2). In addition to these novel isolates, many other novel antagonistic rhizospheric and endophytic bacteria

Figure 2. Phylogenetic distribution of antagonistic bacteria isolated from halophytes on the basis of 16S rRNA gene sequences obtained from bacteria and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene by using the neighbour-joining method from distances computed with the Jukes–Cantor algorithm. Bootstrap values (1000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar represents 0.01 accumulated changes per nucleotide. Isolates selected for bioactive metabolites identification are highlighted.

belonging to different genera were identified in this study. More potential antagonistic candidates have been identified from the class *γ-Proteobacteria*. Some strains of *γ-Proteobacteria* with high antagonistic activity against pathogenic fungi were common and had a sequence similarity of 100% with the relative type strain. *Alteromonas* sp. (EA73), *Aidinimonas* sp. (EA105) and *Halomonas* sp. (EA127) and had moderate to strong inhibitory activity and were identified as novel strains with sequence similarity of 98.6%, 97.8% and 96.7%, respectively, with relative type strains. We have selected these three antagonistic strains depending on their 16S rRNA sequence similarity and antagonistic activity for secondary metabolite identification.

Enzymatic activities of antagonistic bacteria

Antagonistic rhizospheric and endophytic bacteria were evaluated for their ability to produce cell wall lytic enzymes. We have tested protease, amylase, lipase and cellulase activities (Table 2). The number of antagonistic rhizospheric and endophytic bacteria exhibiting lipase activity ($n = 19$; 32%) was high as compared to other enzymatic activities.

Most of lipase-producing bacteria were endophytes ($n = 17$) and were isolated from leaves and root tissues of halophytes. Three rhizospheric bacteria, strains EA73–EA75 belonging to *Alteromonas*, displayed high lipase activity (Table 2). Protease production was observed lower ($n = 8$; 13.5%) than lipase activity. Most of the endophytes ($n = 5$) were potential protease enzyme producers. Mostly *Actinobacteria* and *Firmicutes* strains were recorded for high protease production. Strains of *Bacillus* sp. (EA78), *Nocardioides* sp. (EA87), *Nocardioides* sp. (EA89) and *Bacillus* sp. (EA126) had strong protease production. Amylase activity was recorded for 6 (10%) antagonistic bacteria. Most of the bacterial strains with strong amylase activity are species of the class *Firmicutes*. Endophytic strain of *Bacillus* sp. (EA78) exhibited high production of amylase. Cellulase production was observed in only 1 (1.6%) bacterial strains. A strain of *Mycobacterium* sp. (EA84) exhibited cellulase production and it belongs to *Actinobacteria*. Some antagonistic strains were negative ($n = 24$; 40.6%) for production of all tested enzyme activities.

Detection of NRPS and PKS-I systems

All antagonistic bacterial isolates were subjected to genetic screening for the presence of NRPS and PKS genes. Of 57 isolates tested, PCR amplification revealed that 21 strains (36.8%) were positive for at least one of the biosynthetic gene tested (Table 2). And for the NRPS gene, 18 (31%) strains were positive. Most of the strains were positive for NRPS genes and belonged to the class *Actinobacteria*. For the gene PKS-I, only 7 (12%) antagonistic bacterial strains were positive. Similarly, PKS-II gene cluster was detected in only 9 (15.2%) strains. Both these genes PKS-I and PKS-II were mostly detected in the strains of *Actinobacteria*. Three bacterial isolates, *Erythrobacter* sp. (EA85), *Streptomyces* sp. (EA91) and *Halomonas* sp. (EA106), in this study possess all of the biosynthetic genes (NRPS, PKS-I and PKS-II). Two isolates belonging to *γ-Proteobacteria*, *Marinobacter* sp. (EA119) and *Marinobacter* sp. (EA124), showed the presence of both NRPS and PKS-I genes. Similarly, two isolates of *Actinobacteria*, *Amycolatopsis* sp. (EA99) and *Brevibacterium* sp. (EA101), were positive for the presence of both NRPS and PKS-II system.

Culture condition optimisation and identification of metabolites by GC-MS and LC-MS

In all tested media, ½ R2A broth was found to be the best culturing media. Selected strains showed the best growth at pH 7.5 and 28°C in the shaking incubator (140 rpm). After optimisation of conditions, selected bacterial strains were grown in 5 mL ½ R2A broth in seawater for 36–48 h until OD₆₀₀ reached 0.9. From these 57 antagonistic bacteria, only 3 antagonistic isolates were selected belonging to the class *γ-Proteobacteria*, i.e. *Alteromonas* sp. (EA73), *Aidingimonas* sp. (EA105) and *Halomonas* sp. (EA127) due to their low sequence similarity and high antifungal activity. GC-MS and LC-MS analyses of culture of these three strains identified different chemical constituents (Figures 3(a–c) and 4(a–e)). For GC-MS, different chemical compounds were identified by comparing their mass spectra with the NIST library. Isolate *Alteromonas* sp. (EA73) exhibited the presence of bioactive compounds like aminoguanidine, styramate, azetidine and cyacetacide (Figure 3(a))

Figure 3. Bioactive compounds detected by GC-MS analysis. (a) GC-MS spectra of *Alteromonas* sp. (EA73), (b) *Aidingimonas* sp. (EA105) and (c) *Halomonas* sp. (EA127).

which have been already known for their use in the pharmaceutical industry (Harrington, 2011). The culture extract of strain *Aidingimonas* sp. (EA105) showed the presence of chemical compounds of biological significance such as benzopyrazine, benzofurazan, isoborneol, betamethasone borneol and thalidomide valerate (Figure 3(b)). Chemical analyses of *Halomonas* sp. (EA127) resulted in the identification of different active metabolites namely silanamine, cephaloridine and oxiraneundecanoic (Figure 3(c)).

LC-MS analysis was performed to identify metabolites from culture extract. LC-MS analysis of *Alteromonas* sp. (EA73) identified peaks for only nine secondary metabolites in both the positive- and negative-ion mode (Figure 4(a,b)). These compounds include DAPG, methyl jasmonate, prednicarbate, methoxamedrine, benzydamine, 5-formylsalicylic acid, geranylgeraniol, stearamide and erucamide. For *Aidingimonas* sp. (EA105), 26 different peaks were identified where 4 active compounds including allopurinol, trigonelline, triamcinolone acetone and dodecanedioic acid were detected in the negative-ion mode (Figure 4(c)). *Halomonas* sp. (EA127) showed the presence of four bioactive compounds in their culture extract, i.e. DAPG, benzydamine and dodecanedioic acid (Figure 4(d,e)). In this study, the antibiotic DAPG is produced by two strains *Alteromonas* sp. (EA73) and *Halomonas* sp. (EA127) that is already known for its antiphytopathogenic and biocontrol properties.

Figure 4. Spectra of LC-MS analysis showing detection of various bioactive metabolites. *Alteromonas* sp. (EA73) (a) Positive mode LC-MS analysis and (b) negative mode LC-MS analysis; (c) Negative mode *Aidingimonas* sp. (EA105) LC-MS analysis; (d) *Halomonas* sp. (EA127) Positive mode and (e) negative mode LC-MS analysis.

Discussion

Halophytes exist under extreme environmental conditions which lead to the production of certain metabolites enabling them to survive under these harsh conditions (De Carvalho & Fernandes, 2010). In this study, we have isolated 57 different antagonistic bacteria active against different common fungal pathogens. For the isolation of bacteria, we used four

Figure 4. Continued

different types of media in low nutrient concentration which results in the isolation of diverse groups of antagonistic bacteria. We have also used this methodology in our previous studies and this has resulted in the isolation of many novel isolates with unique bioactivities (Bibi et al., 2012, 2017). To our knowledge, this is the first study on the isolation, screening, characterisation and metabolite identification of rhizospheric and endophytic bacteria from halophytes in Saudi Arabia.

In this study, we have used a culture-dependent approach to explore the diversity of antagonistic rhizospheric and endophytic bacteria. Screening of 554 rhizospheric and endophytic bacteria against oomycetes and identification on the basis of 16S rRNA gene sequence resulted in the identification of 57 different bacteria comprising five major classes. Bioactive compounds production by rhizospheric and endophytic bacteria is one of the techniques used by bacteria to defend the host against different pathogens (Coombs, Michelsen, & Franco, 2004). Several previous studies have reported the production of antimicrobial compounds by bacteria isolated from mangrove plants (Eldeen & Effendy, 2013; Hu et al., 2010). We have isolated a high number of species of antagonistic rhizospheric and endophytic bacteria from these plants. High percentage of antagonistic bacteria may be resulted as different culturing media, isolation source (soil, roots and leaves) and only oomycetes pathogens as target organisms were used for screening. Some previous studies reported the final limited outcome of bacterial diversity when only soil was used as a microbial isolation source (Mitra, Santra, & Mukherjee, 2008; Rameshkumar & Nair, 2009). Most of the antagonistic bacteria were recovered from the root ($n = 27$; 46%) tissue samples. Similarly, the

number of antagonistic bacteria was higher in soil ($n = 23$; 39%) as compared to leaf tissues ($n = 9$; 15%) (Figure 1(b–d)). The importance of endophytes from mangrove plants has been previously reported (Eldeen & Effendy, 2013; Menpara & Chanda, 2013), and in our study, endophytes exhibited strong antagonistic activities against different fungal pathogens. These bacterial endophytes belong to genus *Bacillus* and can be considered as potential candidates for use as biocontrol agents.

Identification of these antagonistic bacteria based on the 16S rRNA gene resulted in five major classes of bacteria. Phylogenetic analysis yielded a tree topology that grouped all antagonistic rhizospheric bacteria and endophytic into five different groups: *Actinobacteria* (*Nocardioides*, *Arthrobacter*, *Streptomyces*, *Mycobacterium*), γ -*Proteobacteria* (*Alteromonas*, *Pseudoalteromonas*, *Halomonas*, *Marinobacter*, *Aidingimonas*), *Firmicutes* (*Bacillus*, *Staphylococcus*, *Halobacillus*), α -*Proteobacteria* (*Devosia*, *Erythrobacter*, *Nitratireductor*, *Inquilinus*, *Oceanicola*, *Aidingimonas*, *Salipiger*, *Rubrimonas*), and *Flavobacteriia* (*Muricauda*, *TamLana*, *Zunongwangia*) (Figure 1 and 2). Some genera were represented by several species in these halophytes while some other genera were represented by only single species. Our results demonstrated that class γ -*Proteobacteria* was dominant (41%) among all the antagonistic bacterial communities isolated from four different halophytes (Tables 1 and 2). In this study, five different genera of γ -*Proteobacteria* (*Pseudomonas*, *Alteromonas*, *Pseudoalteromonas*, *Halomonas*, *Marinobacter*, *Aidingimonas*) were isolated. Among Gram-negative marine bacteria, γ -*Proteobacteria* produced the highest number of secondary metabolites with functional diversity (Long & Azam, 2001). In this study, we also choose isolates *Alteromonas* sp. (EA73), *Aidingimonas* sp. (EA105) and *Halomonas* sp. (EA127) belong to γ -*Proteobacteria* for secondary metabolite identifications.

The second dominant class of bacteria was *Actinobacteria* (20%) and *Firmicutes* (20%). Today, the marine actinomycetes are considered as a major source for discovery of novel natural products. Marine *Actinobacteria* are a source of novel antimicrobial compounds, many of them such as salinosporamides are in clinical trials for use as anticancer agents (Fenical & Jensen, 2006). Previously, different strains of *Actinobacteria* isolated from mangrove sediments showed antimicrobial activity against different human pathogenic bacteria (Lee et al., 2014). In this study, most of the antagonistic rhizospheric and endophytic bacteria belonging to the class *Actinobacteria* in this study such as *Nocardioides*, *Arthrobacter*, *Pseudonocardia*, *Streptomyces*, *Agromyces* and *Mycobacterium* are already known for their antagonism and production of antimicrobial metabolites (Fenical & Jensen, 2006). Bacteria belonging to class *Firmicutes* are commonly found both as rhizospheric and endophytic bacteria as they are easily cultivated. In marine environments, strains of *Bacillus* are the dominant producers of antibacterial, antifungal antibiotics, enzymes and surfactants (Mondol, Shin, & Islam, 2013). The representative of *Firmicutes* in our study belonged to *Bacillus*, *Staphylococcus* and *Halobacillus* species are already known to produce antifungal substances (Wagner-Döbler, Beil, Lang, Meiners, & Laatsch, 2002). Previously two endophytic bacterial strains of *Bacillus* were isolated from mangrove plants and showed inhibition against bacterial and fungal plant pathogens (Menpara & Chanda, 2013). In this study, 14% antagonistic rhizospheric and endophytic bacteria were related to class α -*Proteobacteria* including eight different genera. Several strains of α -*Proteobacteria* isolated from marine sources live in symbiosis and nitrogen fixers, and produce antibiotics (Castro et al., 2014; Wagner-Döbler et al., 2002). In this study, three strains of *Flavobacteriia* were isolated from soil samples

adhering roots of host halophyte, *Z. qatarense*. Two antagonistic strains of *Flavobacteriia*, *Muricauda* sp. (EA116) and *Marinimicrobium* sp. (EA118) seems novel based on their 16S rRNA similarity to related type strains.

Marine bacteria are already known for the production of different hydrolytic enzymes exhibiting diverse enzymatic activities and catalysing different biochemical processes using these enzymes (Thatoi et al., 2013). Most of the bacteria in our study produced lipase, protease and amylase enzymes. Only two strains were found positive for production of cellulase. Mostly *Actinobacteria* and *Firmicutes* strains were able to produce different enzymes. *Bacillus* sp. (EA126) showed protease, amylase and lipase activity. This strain is endophytic and isolated from roots of halophyte *Z. qatarense*. These hydrolytic enzymes are necessary for antagonism as well as for intracellular colonisation of bacteria to host plant. Castro et al. (2014) have evaluated mangrove plants for isolation and screening of bacteria for enzymatic activities. They found different groups of endophytic enzymatic bacteria where *Firmicutes* was the dominant class consistent with our study.

Marine bacteria possess PKS and NRPS genes which are hallmarks for the production of secondary metabolites. Of 57 isolates tested for PKS-I, PKS-II and NRPS gene, only 12% were positive for PKS-I, 15% for PKS-II and 31% for NRPS genes. Mostly strains of *Actinobacteria* were positive for the presence of PKS-I and PKS-II genes. Two endophytic isolates, *Streptomyces* sp. (EA85) and *Streptomyces* sp. (EA91), and one rhizospheric bacteria, *Halomonas* sp. (EA106), possess all of the biosynthetic genes (NRPS, PKS-I and PKS-II) tested. The PCR-based detection of PKS-I, PKS-II and NRPS genes is important in screening and identification of bacterial isolates capable of producing active secondary metabolites. In our study, some isolates including three strains chosen for chemical analysis were negative for amplification of these genes and may be due to the use of degenerate primers which were not suitable for these genes (Qin et al., 2009; Qin, Li, Dastager, & Hozzein, 2016; Salomon, Magarvey, & Sherman, 2004). Detection of PKS-I and PKS-II genes was high in strains of *Actinobacteria*, providing a confirmation for the high possibility of *Actinobacteria* producing active secondary metabolites.

The antifungal potential of selected isolates was influenced by culturing in R2A as culture media using optimum culture conditions. The maximum antifungal activity was observed after 48 h of growth at 28°C with pH 7.5. Three selected Gram-negative bacteria belong to class γ -*Proteobacteria* were analysed using LC-MS and GC-MS for secondary metabolites present in their culture extract. We used LC-MS in addition to GC-MS as LC technique mainly focuses on polar metabolites (especially phosphate-containing compounds), most of which cannot be analysed using GC-MS. Both analyses confirm the presence of various bioactive compounds, although not novel but already known for their bioactivity. The antibiotic DAPG was detected in two isolates *Alteromonas* sp. (EA73) and *Halomonas* sp. (EA127) culture extracts when analysed by LC-MS. DAPG is an antibiotic with broad-spectrum antibacterial and antifungal activities produced by *Pseudomonas fluorescens* strain that has been used as a biocontrol agent against several plant pathogens including *Py. ultimum* (Delany et al., 2000) which is also a test pathogen in our study. Both these strains, *Alteromonas* sp. (EA73) and *Halomonas* sp. (EA127), showed strong inhibition against *Py. ultimum* in an *in vitro* assay when we tested. Detection of DAPG in both these strains suggests its role in these bacteria as an antifungal agent against tested pathogens. Members of genus *Alteromonas* usually associated with marine sponges produce compounds such as a macrolactam and amide ester with cytotoxic and

antimicrobial activity (Thomas, Kavlekar, & LokaBharathi, 2010). Similarly, strains of *Halomonas* from a marine source are able to produce siderophore, Loihichelins (Giddings & Newman, 2015). But no previous study has reported the production of DAPG by marine strains of these two genera. *Alteromonas* sp. (EA73) also showed the presence of some anti-inflammatory compounds such as prednicarbate and benzydamine are used as drugs where benzydamine has antibacterial activity (Fanaki & El-Nakeeb, 1992). In addition to these compounds, methyl jasmonate, volatile compounds produced by plants as a defence mechanism (Gimenez-Ibanez, Chini, & Solano, 2016), were also detected in *Alteromonas* sp. (EA73) culture extract. Aminoguanidine and styramate have medical uses in diabetic and anticonvulsant drugs, respectively, were also detected. No such compound has been reported previously in strains of *Alteromonas*.

Cephaloridine is a semisynthetic cephalosporin antibiotic, and was detected in *Aidingimonas* sp. (EA105). This derivative of cephalosporin C is used in respiratory tract infections. This is first report of cephaloridine detection in bacteria. *Aidingimonas* sp. (EA105) was also able to produce benzydamine in addition to triamcinolone acetonide and dodecanedioic acid. Triamcinolone acetonide is synthetic corticosteroid and used in various skin infections while dodecanedioic acid has various industrial uses. Like *Aidingimonas* sp. (EA105), triamcinolone acetonide and dodecanedioic acid also were detected in *Halomonas* sp. (EA127). Furthermore, *Halomonas* sp. (EA127) also exhibited production of allopurinol and thalidomide valerate which have their use in pharmaceutical industry. According to LC-MS and GC-MS analyses, all compounds detected in these three selected strains have their medicinal use and are reported as anti-inflammatory, antibacterial and antifungal compounds. These compounds seem to be responsible for antifungal activities of these three strains.

Conclusions

In this study, 57 antagonistic rhizospheric and endophytic bacteria inhabiting halophytes have been isolated and partially characterised. Among all, 21 bacterial strains have at least one type of biosynthetic gene cluster, indicating their importance and potential for discovery of new bioactive compounds. Moreover, these antagonistic bacteria exhibited enzymatic activities indicating their industrial and biotechnological potential. Analyses of selected potential strains exhibited the presence of various bioactive metabolites including known antibiotics and bioactive compounds of synthetic nature not reported from bacteria before. These results suggest that halophytes are a potential source of unexplored metabolites. Antagonistic bacteria from halophytes growing in the coastal area of the Red Sea in Saudi Arabia have the potential to produce a diverse range of antimicrobial compounds that can be used in medicine and as a biocontrol agent in agriculture.

Disclosure statement

No potential conflict of interest was reported by the authors.

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