Arch Microbiol
DOI 10.1007/s00203-015-1086-3

ORIGINAL PAPER

The Kolumbo submarine volcano of Santorini island is a large pool of bacterial strains with antimicrobial activity

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Received: 28 November 2014 / Revised: 13 January 2015 / Accepted: 16 January 2015

Abstract Microbes in hydrothermal vents with their unique secondary metabolism may represent an untapped potential source of new natural products. In this study, samples were collected from the hydrothermal field of Kolumbo submarine volcano in the Aegean Sea, in order to isolate bacteria with antimicrobial activity. Eight hundred and thirty-two aerobic heterotrophic bacteria were isolated and then differentiated through BOX-PCR analysis at the strain level into 230 genomic fingerprints, which were screened against 13 different type strains (pathogenic and nonpathogenic) of Gram-positive, Gram-negative bacteria and fungi. Forty-two out of 176 bioactive-producing genotypes (76 %) exhibited antimicrobial activity against at least four different type strains and were selected for 16S rDNA sequencing and screening for nonribosomal peptide (NRPS) and polyketide (PKS) synthases genes. The isolates were assigned to genus *Bacillus* and Proteobacteria, and 20 strains harbored either NRPS, PKS type I or both genes. This is the first report on the diversity of culturable mesophilic bacteria associated with antimicrobial activity from Kolumbo area; the extremely high proportion of antimicrobial-producing strains suggested that this unique environment may represent a potential reservoir of novel bioactive compounds.

Communicated by Erko Stackebrandt.

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Keywords Kolumbo hydrothermal vents · Mesophiles · Bioactivity

Introduction

The increasing number of antibiotic multi-resistant bacterial pathogens makes urgent need for the discovery of new antibacterial agents. Since microbes have been revealed as an excellent source of new drugs and the majority of antibiotics used to date derive from molecular scaffolds of natural products or their semisynthetic derivatives (Newman and Cragg 2007), efforts for novel scaffold discovery are focused on the mining of largely unexplored microbial niches (Fischbach and Walsh 2009). It seems that the oceans are a prolific and (unlike the terrestrial environment) relatively unexploited source of novel natural products. Since the 1960s, many novel pharmaceutical compounds produced by a diverse range of marine bacteria have been isolated with antitumor, antiviral, antimicrobial or generally cytotoxic properties (Gerwick and Fenner 2013; Mayer et al. 2013). Therefore, marine microorganisms may represent an efficient and successful source for natural products research.

Deep-sea hydrothermal ecosystems, lying below 200-m depth (Tarasov et al. 2005), are colonized by dense communities living in complete darkness and surrounded by an almost desert sea bottom (Thornburg et al. 2010). They are considered to be a promising resource of novel chemotypes with medicinally relevant biological activities as they represent one of the most extreme and dynamic environments on Earth. The hot and reduced conditions at the vent outlets are replaced by cooler and more oxidized conditions at the outer chimney walls and sediment–seawater interface, with significant variations recorded in physical (pH, temperature) and chemical parameters (O_2, nutrient and

Published online: 28 January 2015

metal concentrations), which provide multiple ecological niches (Pettit 2011). The most abundant bacteria in vents are members of mesophilic communities adapted to life at temperatures near the ambient seawater (Prieur 2005) and capable of utilizing both vent-derived reductants and seawater-derived oxidants as nutrients (Olins et al. 2013). The adaptation strategies of vent microbial colonizers for survival against such fluctuating conditions induce genomic innovation (Sogin et al. 2006) and metabolic pathways with promising unique bioactivities (Skropeta 2008; Wilson and Brimble 2009).

So far, diverse deep-sea hydrothermal fields such as Okinawa Trough, East Pacific Rise and mid-Atlantic Ridge have been proved a new source of unique exopolysaccharides and unusual secondary metabolites with medical applications of bacterial origin (Blunt et al. 2014). The glycosaminoglycan-like polysaccharide produced by the bacterium *Vibrio diabolicus* exhibits effective results on the enhancement of bone regeneration in vivo while the novel secondary metabolites Ammonificins C 105 and D 106 isolated from a *Thermovibrio ammonificans* strain induce apoptosis in a cell-based screen (Senni et al. 2013). In addition, genes encoding for bioactive compounds of nonribosomal peptide synthetases and polyketide synthases type I (PKS-I) were detected from *Actinobacteria* isolates retrieved from Mariana Trench sediments (Pathom-aree et al. 2006).

The aim of the present study was to isolate mesophilic bacteria with potential antimicrobial activity from microbial mats of chimney and sediment samples retrieved from the active hydrothermal field of Kolumbo submarine volcano (500-m depth) about 7 km off the northeastern coast of Santorini, in the Aegean Sea. According to previous studies (Carey et al. 2011; Kilias et al. 2013), Kolumbo active chimneys were characterized by a permeable framework of sulfides and sulfates with vent plumes emitting mainly CO_2 at 210 °C and seawater pH as low as 5.0, while the Fe-rich crater floor was mantled by white and reddish-orange microbial mats with temperature recorded between 16.2 and 17 °C. To our knowledge, the present study is the first to survey the diversity of deep-sea hydrothermal vent bacteria that produce antimicrobial agents. The study was based on the isolation of bacteria according to their activity against pathogenic and nonpathogenic bacteria and fungi. In addition, attempts were made to identify specific genes, such as NRPS and PKS type I, known to be responsible for those bacterial responses.

Materials and methods

Samples collection

During *E/V Nautilus* 2010 expedition in the Aegean Sea (Carey et al. 2011), sediment and chimney samples were collected on *Hercules* ROV (Remote Operating Vehicle), Dive 1705 from the hydrothermal field in the north part of the crater of Kolumbo submarine volcano (500-m depth). The in situ temperature (17 °C) was determined using the external *Hercules* temperature probe. The sediment sample was collected by a push core (Core No. 12, Fig. 1a) (36:31.5698N and 25:29.1432E) and the chimney sample (36:31.5413N and 25:28.5181E) by the ROV manipulator arm (Fig. 1b). Once on board, the sediment sample was extracted with an autoclaved glass cylinder, while the surface of the chimney spires was scraped with a sterile scalpel and then loaded into sterile tubes (Falcon Type) of 50 mL volume. In both sediment and chimney samples, sterilized filtered seawater was added containing 0.05 % (w/v) Na_2S before storage at +4 °C until use.

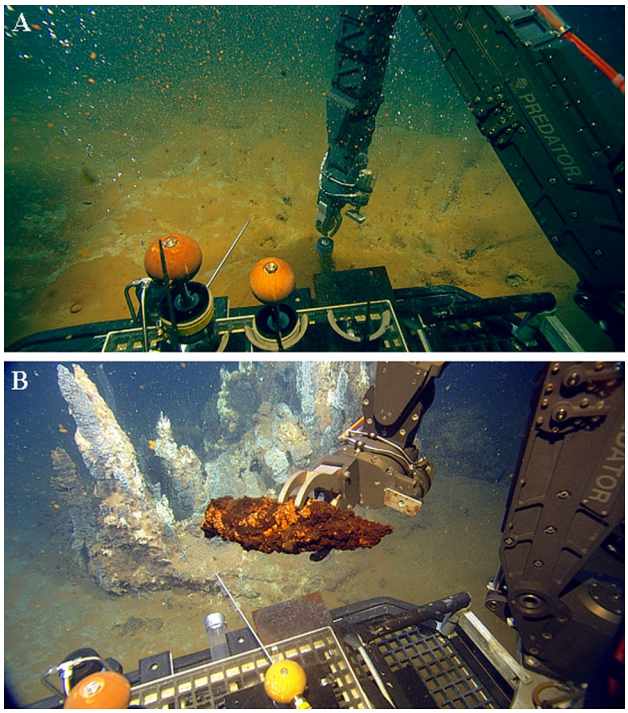

Fig. 1 **a** Sediment sampling by the ROV manipulator arm using a push core (36:31.5698N and 25:29.1432E). **b** Sampling from the chimney by the ROV manipulator arm (36:31.5413N and 25:28.5181E)

Isolation of the bacterial strains

For the isolation of microorganisms, the samples were serially and decimally diluted from 10^{-1} to 10^{-7} with filtered sterile seawater as diluent. Aliquots (100 μL) were subsequently spread onto agar plates with diverse media, pH adjusted to 7.2 before autoclaving and incubated aerobically up to 6 weeks at room temperature for the retrieval of heterotrophic mesophiles (Inagaki et al. 2003; Gontang

et al. 2007). Three different media were used for isolation: (1) Marine Agar medium (2216 Difco, BD), (Joint et al. 2010), (2) tryptone–yeast extract–glucose agar (TYGA) (Kontro et al. 2005) modified by replacing yeast extract with 0.1 % K_2HPO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.001 % $Fe_2(SO_4)_3{\cdot}6H_2O$, 0.0001 % $CuSO_4{\cdot}5H_2O$, 0.0001 % $ZnSO_4{\cdot}7H_2O$ and 0.0001 % $MnSO_4{\cdot}H_2O$ and (3) glycerol–arginine agar medium (Kontro et al. 2005) modified as follows: 0.5 % tryptone, 1.25 % glycerol, 0.1 % NaCl, 0.1 % K_2HPO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.001 % $Fe_2(SO_4)_3{\cdot}6H_2O$, 0.0001 % $CuSO_4{\cdot}5H_2O$, 0.0001 % $ZnSO_4{\cdot}7H_2O$ and 0.0001 % $MnSO_4{\cdot}H_2O$. Three agar plates were inoculated from each dilution and culture media. After incubation, plates were examined and colonies were selected and transferred to new agar plates several times for isolation and purification. The cultures were stored in 30 % (w/w) glycerol solutions at −80 °C.

DNA extraction and whole-genome bacterial fingerprinting (BOX-PCR)

Genomic DNA extraction was performed following the protocol of Moore et al. (2004). Electrophoresis of the DNA obtained was performed using 1 % (w/v) agarose gels (Agarose Basic, Applichem GmbH). Genomic DNA was eluted from the spin column with 100 μL of elution buffer for immediate use or storage at −20 °C.

The BOX element (BOX1A) was amplified as described by Rademaker et al. (2004), using the BOXA1R primer 5′-CTACGGCAAGGCGACGCTGACG-3′. Amplification procedures were performed as follows: The samples were denatured at 95 °C for 10 min, amplified in 30 cycles of 94 °C for 30 s, 52 °C for 1 min, 65 °C for 6.5 min followed by a final extension step of 65 °C for 16 min. The BOX products were electrophorized in a 1.5 % (w/v) agarose gel for 240 m at 110 V in 1xTAE. The PCR for each isolate was repeated three times for reproducibility.

In vitro antimicrobial activity of the isolates corresponding to the different BOX genomic fingerprints

The antimicrobial activity was tested by diffusion method against indicator strains of Gram-positive bacteria [*Bacillus subtilis* (DSM 10), *Micrococcus luteus* (DSM 1790), *Kocuria rhizophila* (DSM 348), the pathogens *Staphylococcus aureus* (DSM 1104), *Enterococcus faecalis* (DSM 2570), *Streptococcus pneumoniae* (DSM 24048)], Gram-negative bacteria [*Acinetobacter radioresistens* (DSM 6976), *Pseudomonas fluorescens* (DSM 50090), the pathogens *Neisseria gonorrhoeae* (DSM 9189), *Pseudomonas aeruginosa* (DSM 1117), *E. coli* (DSM 1103)], the filamentous fungus *Aspergillus niger* (DSM 1957) and the pathogenic yeast *Candida albicans* (DSM 1386).

Aliquots (10 μL) of overnight liquid cultures of isolates were plated onto seawater agar plates (Gontang et al. 2007) incubated for 96 h at 25 °C and then overlaid by soft medium containing 0.7 % sterilized soft agar (kept at 50 °C) inoculated with overnight liquid culture of the indicator strain (exception: *Asp. niger*, 3 days culture). The following media were used for overlay: 0.8 % NB (Nutrient Broth, HiMedia M002) for *B. subtilis*, *M. luteus*, *K. rhizophila* and *P. fluorescens*, 0.24 % PDB (Potato Dextrose Broth, DIFCO 254920) for *A. niger*, 0.21 % YM (Yeast Mold, DIFCO 271120) for *C. albicans*, 0.3 % TSB (Tryptone Soya Broth, Oxoid CM0131) for *A. radioresistens* and 0.21 % ISTB (Iso SensiTest Broth, Oxoid CM047) for *N. gonorrhoeae, S. aureus, Enterococcus faecalis, P. aeruginosa, S. pneumonia* and *E. coli*. To allow the growth of the indicator strains, the overlaid plates were incubated for 24 h at 30 °C or 37 °C (for bacterial pathogens). After incubation, the plates were checked for growth inhibition zones. Experiments were carried out in triplicate, and similar results were obtained. The activity was considered to be weak if the inhibition zone was ≤1 cm wide.

Partial 16S rRNA gene sequencing of the isolates shown to have strong inhibitory activity

Amplification of the 16S rRNA gene was performed in 50 μL reaction mix containing 50 ng of extracted genomic DNA, 5 μL 1× reaction buffer, 2.5 μL 50 mM $MgCl_2$, 1 μL dNTP Mix 10 mM each, 20 pmol of each primer, 4 % (v/v) DMSO, 5 unit Taq DNA polymerase (Biotools, England). Nuclease-free water was used to bring the reaction volume to 50 μL. The 16S rRNA gene primers used were pA 5′-AGAGTTTGATCCTGGCTCAG-3′ and R1492 5′-TACGGYTACCTTGTTACGACT-3′ (Devereux and Wilkinson 2004). The PCR conditions were 95 °C for 3 min, then 35 cycles each of 30 s at 94 °C, 1 min at 56 °C and 1.5 min at 72 °C. A final extension step of 72 °C for 5 min was then performed. The amplified 16S rRNA gene fragments were analyzed on 1.2 % (w/v) agarose gel, purified with Nucleospin Extract PCR kit (Macherey–Nagel, Germany) and used as sequencing templates. Sequencing was carried out by VBC-BIOTECH Service GmbH (Austria) using R1492 primer. Homology searches were performed, and close relatives were determined in the GeneBank database using the nucleotide BLAST with default parameters within National Center for Biotechnology Information (NCBI). The sequences were aligned against reference sequences by MUSCLE, and the aligned dataset was used as input for phylogenetic analysis performed with program Molecular Evolutionary Genetics Analysis (MEGA), version 6.0 (Tamura et al. 2013). Tree topology was evaluated by bootstrap analyses based on 1,000 replicates, and

the phylogenetic tree was inferred using the neighbor-joining method. The obtained 16S rRNA gene sequences were deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/ena/) under Accession Numbers HG764607–HG764648.

Screening of the most active isolates for nonribosomal peptide synthetase (NRPS) and polyketide synthase type I (PKS) genes

For the PKS and NRPS genes screening, gene fragments of the most conserved domains (ketosynthase KS domain of PKS and adenylation A domain of NRPS) were amplified as recommended by Zhang et al. 2008, 2009, using the following degenerate oligonucleotide primers: for KS domain, GBf (5′-RTRGAYCCNCAGCAICG-3′)–GBr (5′-VGTNCCNGTGCCRTG-3′), GCf (5′-GCSATGGAYCCSCARCARCGSVT-3′)–GCr (5′-GTSCCSGTSCCRTG SSCYTC SAC-3′), KSDPQQf (5′-MGNGARGCNNWN SMNATGGAYCCNCARCANMG-3′)–KSHGTGr (5′-GGRTCNCCNARNSWNGTNCCNGTNC-CRTG-3′). For A domain, MTF (5′-GCNGGYGGYGC-NTAYGTNCC-3′)–MTR (5′-CCNCGDATYTTNACY TG-3′), NS1 (5′-CAACCCCTATGCCTTTTGAA-3′) - NS2 (5′-TAAACAACCCATGCTCCACA-3′), NP1 (5′-CCTA ATTCAATACGAAAACCACGAADYTTNAYYTG-3′)–NP2 (5′-TGTATGTTATTTATACTTCTGGTTCTACTG-GTMRNCCANARG G-3′). A reaction volume of 50 μL contained 5 μL of 1× reaction buffer, 2 μL of $MgCl_2$ 50 mM, 5 μL DMSO 100 %, 1 μL dNTPs Mix 10 mM each, 2 μL of each primer 100 mM, 0.25 μL of unit Taq DNA polymerase (Biotools, Spain) and 1 or 2 μL (50–100 ng) of template DNA. Amplification cycles consisted of an initial denaturation step of 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 50 °C for PKS and 55 °C for NRPS for 1 min, 72 °C for 2 min for amplification and a final extension step of 72 °C for 10 min. The amplicons were visualized, and the ones of the correct size (1,000 and 700 bp for NRPS and PKS genes, respectively) were gel purified (Nucleospin Extract PCR kit, Macherey–Nagel, Germany) and cloned into pTZ57R/T vectors (Fermentas, USA). Sequencing of the cloned inserts was carried out by VBC-BIOTECH Service GmbH (Austria) using M13/pUC F (5′-GTTTTCCCAGTCACGAC-3′) primer. The deduced amino acid sequences of A and KS domains were used as queries to search related proteins in the nr protein database at NCBI using the BlastP algorithm with default parameters. Phylogenetic trees based on the 19 amino acid sequences of A domains and the six amino acid sequences of KS domains were constructed using the multiple sequence alignment tools in MUSCLE and mega package. Neighbor-joining method was adopted with 1,000 bootstrap reiterations. The gene sequences were deposited in GenBank under the Accession Numbers KM186546–KM186570.

Results

Diversity of mesophilic bacteria in the Kolumbo hydrothermal field

Eight hundred and thirty-two mesophilic heterotrophic bacteria were recovered using three different culture media. Six hundred and forty-four strains were isolated from the sediment sample, 34 % of which were isolated using marine agar medium and the rest (66 %) from modified TYGA medium. One hundred and eighty-eight strains were isolated from the chimney sample; 50 % of the isolates derived from the marine agar medium, 19 % from the modified TYGA medium and 31 % from the modified glycerol–arginine agar medium. Whole-genome genotyping using the BOX-PCR methodology was performed, and differentiation of all isolated bacteria based on their BOX-PCR genomic fingerprints revealed 230 distinct genotypes, 38 of which derived from the chimney sample. The representatives of the different genomic fingerprints detected were principally present up to eight times, forming a variety of small different genotypic groups, except six major groups containing from 20 to 47 isolates.

Antimicrobial activity of the isolated strains

One hundred and seventy-six out of the 230 different genotypes showed antimicrobial activity against at least one of the 13 indicator strains. Fifty-four of the active isolates were active only against Gram-positive bacteria, three isolates exhibited activity only against Gram-negative bacteria and 13 isolates were active only against fungi. Twenty-six isolates inhibited the growth of both Gram-positive and Gram-negative bacteria, 39 isolates exhibited activity against both Gram-positive bacteria and fungi, five isolates were active against both Gram-negative bacteria and fungi and 36 strains were active against Gram-positive and Gram-negative bacteria and fungi. The most frequently inhibited targets were the Gram-positive pathogens *S. pneumonia, S. aureus* and the fungus *A. niger*. 21.6 % of the isolates tested inhibited the growth of the yeast test strain, *C. albicans*.

Taxonomy of the most bioactive isolated strains by 16S rDNA analysis

Forty-two of the isolates, deriving from both chimney and sediment samples, exhibited strong antimicrobial activity after the repeated transfer to new media (Table 1) and were

Table 1 Antimicrobial activity of the multi-active isolates

Isolate	*A. radioresistens*	*Ps. fluorescens*	*Ps. aeruginosa*	*N. gonorrheae*	*E.coli*	*B. subtilis*	*M. luteus*	*K. rhizophila*	*St. aureus*	*Ent. faecalis*	*Str. pneumoniae*	*Asp. niger*	*C.albicans*
Inhibitory activity													
S446	++	+	−	−	−	−	−	+++	++	++	++	−	++
S250	++	−	−	++	−	−	+++	−	−	++	++	−	−
S610	−	−	+	+	−	−	−	−	−	−	−	+++	+++
C764	−	−	−	++	++	++	++	−	−	−	+	+	−
C47	−	−	−	−	−	−	+++	+++	−	+	+	+	−
S601	++	−	−	++	−	−	+++	−	+++	−	+++	−	−
C705	−	−	−	−	+	−	+	−	−	−	+	+++	+++
S38	−	−	−	−	−	−	++	+++	+	+	+	+++	+++
S595	−	−	−	−	−	−	−	−	+++	++	−	+++	++
S463	+	−	−	−	−	−	+++	+++	+++	−	+++	−	−
C126	−	−	−	−	−	−	+++	+++	+	+	+	−	+
S104	+	−	−	−	−	−	++	+	+	+	+	+	−
S36	−	−	−	−	−	−	+++	+++	++	++	++	++	+++
S264	−	−	−	−	−	−	+	+	++	++	++	++	−
S252	−	−	−	−	−	−	++	+++	+	+	+	+	−
S71	++	++	−	++	−	−	+++	−	++	++	++	−	−
S30	++	−	−	+	−	−	++	+++	−	−	++	−	−
S278	−	−	−	++	++	++	−	−	++	++	−	++	+++
S163	−	−	−	−	−	++	++	+++	−	−	+	−	−
S286	+	−	−	+	−	−	−	+	−	−	+	+	+
S301	+	−	−	+	−	++	+++	−	−	−	++	++	+
S572	−	−	−	−	−	+++	+++	+++	−	−	+++	+++	+++
S336	+	+	−	+	−	+	+	−	+	+	+	+	+
S122	−	−	−	−	−	+	+	+	−	−	+	+	+
C799	−	−	−	+	−	−	+	+	−	−	+	+	−
C701	+	−	−	−	+	+++	+++	−	−	−	−	−	+++
S141	+	−	−	−	−	−	−	+	−	−	+	++	−
S99	+	−	−	−	−	++	+++	−	+	−	−	+	−
S396	−	+	−	+	−	−	−	−	−	−	−	+++	+++
S93	++	++	−	−	−	−	+++	+++	−	+++	+++	+++	−
C816	+	−	−	+	+	+++	+++	+++	−	++	++	+++	+++
C737	−	−	−	++	++	−	−	−	++	−	−	+++	+++
S638	−	−	−	−	−	−	+++	++	+++	++	++	−	++
S652	+++	+++	−	−	−	−	−	−	−	−	+++	−	+++

Table 1 continued

Isolate	*A. radioresistens*	*Ps. fluorescens*	*Ps. aeruginosa*	*N. gonorrheae*	*E.coli*	*B. subtilis*	*M. luteus*	*K. rhizophila*	*St. aureus*	*Ent. faecalis*	*Str. pneumoniae*	*Asp. niger*	*C.albicans*
S604	−	−	−	−	−	−	+	+	+	−	+	−	++
C54	++	++	++	−	−	−	−	−	−	−	++	−	−
S453	−	−	−	−	−	−	+++	++	−	++	+++	−	−
S567	+	+	−	+	+	+	+	+	−	+	+	−	−
S287	++	−	−	−	−	++	++	++	++	−	++	−	−
S222	+	−	−	−	−	−	−	−	+	−	−	+	++
S175	+	−	−	+	+	+	−	−	−	−	+	++	−
S461	++	+	−	−	−	−	+	++	++	++	++	−	++

−, no or weak inhibition; +, inhibition zone ≤1 cm; ++, inhibition zone ≤2 cm; +++, inhibition zone ≤3 cm

chosen for sequencing and phylogenetic analysis (Table 2). They were submitted to partial 16S rDNA analysis, followed by BLAST search of GenBank database and phylogenetic tree calculation by using the neighbor-joining algorithm. Isolate 16S rRNA gene sequences were grouped into two distinct clusters (Fig. 2). Eleven different 16S rRNA gene OTUs were found, seven of which were identified at the genus level as *Bacillus* (98–100 % identity) and comprised 35 isolates derived from sediment and chimney samples. Three OTUs were assigned to the γ-*Proteobacteria*, genera *Pseudomonas* and *Halomonas* (97–99.9 % identity) corresponding to six strains, isolated from the sediment sample and one OTU was affiliated with a strain isolated from the chimney sample, belonging to α-*Proteobacteria*, genus *Loktanella* (99.3 % identity).

Abundance of NRPS and PKS genes of the most bioactive isolates

In the present study, sequences of PKS type I and NRPS genes were obtained from 6 and 19 isolates, respectively; the rest of the multi-active strains tested, either may not contain these genes, or the genes were not amplified by the primers used. For phylogenetic analysis of PKS genes, reference strains were selected from NRPS-PKS, *trans*-acyl transferase (*trans*-AT) PKS, *cis*-acyl transferase (*cis*-AT) PKS and FAS (fatty acid synthase) families, and representatives of type II PKS were included as out-groups. Two KS domain sequences were related to hybrid NRPS-PKS genes, and four were grouped into the *trans*-acyltransferase (AT) type I polyketide synthases (Fig. 3), and interestingly, all of them showed a close relationship with reported *Actinomycetales* and *Bacillus* PKS genes of marine origin (Table 2).

Three *Pseudomonas* isolates showed high similarity to *Pseudomonas* NRPS-related genes and the rest (1 *Pseudomonas* and 15 *Bacillus*) of the isolates matched with NRPS genes isolated from *Bacillus* spp. (Table 2). The translated sequences showed similarities of 84–100 % to previously described NRPS adenylation domains and 87–100 % to PKS ketosynthase protein domains. All of the deduced NRPS A domain amino acid sequences exhibited high similarity to nonribosomal peptide synthetase subunits with relatives involved in the biosynthesis of tyrocidine (TycC), bacitracin (BacA) and linear gramicidin (LgrD) clustered with the saframycin A-like synthases, while the potent biosurfactant lichenysin (LchAA) clustered into the surfactin domain (SrfA-C)-like group. The latter group contained the majority of the A domains recovered as shown from phylogenetic analysis using reference strains of known NRPS genes including surfactin (SrfA-C) and saframycin (Sfm-A)-like A domain families (Fig. 4).

Table 2 Similarity of 16S rDNA sequences, NRPS adenylation and PKS β-ketosynthase domain amino acid sequences to their relatives in GenBank

Strain	16S rDNA accession no.	Closest relative (accession no.)	Identity (%)	NRPS gene accession no.	Closest relative (accession no.)	Identity (%)	PKS gene accession no.	Closest relative (accession no.)	Identity (%)
C54	HG764612	*L. hongkongensis* NR_029121.1	99.3						
S453	HG764633	*H. sulphidaeris* NR_027185.1	99.9						
S222	HG764622	*Ps.phychrotolerans* NR_042191.1	99.8						
S287	HG764628	*Ps. moraviensis* NR_043314.1	97	KM186546	LchAA *B.licheniformis* U95370.1	99			
S567	HG764636	*Ps. moraviensis* NR_043314.1	99.7	KM186559	*Ps.moraviensis* WP024012355.1	96			
S175	HG764621	*Ps. moraviensis* NR_074599.1	99.5	KM186564	LgrD *Ps. protegens* WP01563425.1	99			
S461	HG764634	*Ps. moraviensis* NR_074599.1	99.6	KM186563	LgrD *Ps. protegens* WP01563425.1	100			
S572	HG764637	*B. licheniformis* NR_074923.1	99.9	KM186555	BacA *B. licheniformis* WP020452079.1	98			
S336	HG764630	*B. licheniformis* NR_074923.1	99.8	KM186556	BacA *B. subtilis* ABB80123.1	98			
S122	HG764617	*B. licheniformis* NR_074923.1	99.8	KM186547	TycIII *B. sonorensis* WP006640279.1	99			
C799	HG764648	*B. megaterium* NR_074290.1	99.5						
C701	HG764644	*B. safensis* NR_041794.1	100	KM186551	*B. pumilus* WP025092822.1	95	KM186569	*B. subtilis* ABR19764.1	99
S141	HG764619	*B. safensis* NR_041794.1	99.9	KM186548	*B. safensis* WP024424295.1	98			
S99	HG764615	*B. safensis* NR_041794.1	99.9	KM186557	*B. pumilus* WP025092819.1	97			
S396	HG764631	*B. safensis* NR_041794.1	99.9	KM186554	*B. pumilus* KDE51553.1	96			
S93	HG764614	*Br. halotolerans* NR_042638.1	99.8	KM186552	*B. subtilis* WP015252053.1	84	KM186567	*Bacillus sp. WPhG3* ACG70843.1	96
C816	HG764643	*B. subtilis* NR_075005.1	100	KM186562	*B. amyloliquefaciens* EYB38008.1	90	KM186570	*B.amyloliquefaciens* AGL92433.1	87
C737	HG764646	*B. subtilis* NR_075005.1	99.9	KM186560	*B. subtilis* WP029946301.1	99			
S638	HG764642	*B. subtilis* NR_075005.1	100	KM186553	*B. pumilus* KDE51551.1	97	KM186566	*Actinomycetales* ABR19779.1	97
S652	HG764607	*B. subtilis* NR_075005.1	99.7	KM186561	*B. subtilis* EXF52074.1	100			
S604	HG764640	*B. halmapalus* NR_026144.1	99.5						
S446	HG764632	*B. pumilus* NR_043242.1	99.8	KM186549	*B. pumilus* KDE51553.1	98			
S250	HG764623	*B. pumilus* NR_043242.1	99.8						
S610	HG764641	*B. pumilus* NR_043242.1	100						
C764	HG764647	*B. pumilus* NR_043242.1	99.8	KM186558	*B. pumilus* KDE51553.1	97	KM186565	*B. subtilis* ABR19764.1	99
C47	HG764611	*B. pumilus* NR_043242.1	100						

Table 2 continued

Strain	16S rDNA accession no.	Closest relative (accession no.)	Identity (%)	NRPS gene accession no.	Closest relative (accession no.)	Identity (%)	PKS gene accession no.	Closest relative (accession no.)	Identity (%)
S601	HG764639	*B. pumilus* NR_043242.1	100	KM186550	*B. pumilus* KDE51553.1	98			
S163	HG764620	*B. pumilus* NR_043242.1	99						
S286	HG764627	*B. pumilus* NR_043242.1	98						
S301	HG764629	*B. pumilus* NR_043242.1	98						
C705	HG764645	*B. pumilus* NR_043242.1	100				KM186568	*Actinomycetales* ABR19779.1	97
S38	HG764610	*B. pumilus* NR_043242.1	100						
S595	HG764638	*B. pumilus* NR_043242.1	99.9						
S463	HG764635	*B. pumilus* NR_043242.1	100						
C126	HG764618	*B. pumilus* NR_043242.1	99.9						
S104	HG764616	*B. pumilus* NR_043242.1	99.9						
S36	HG764609	*B. pumilus* NR_043242.1	99.9						
S264	HG764625	*B. pumilus* NR_043242.1	99.9						
S252	HG764624	*B. pumilus* NR_043242.1	99.8						
S71	HG764613	*B. pumilus* NR_043242.1	99.8						
S30	HG764608	*B. pumilus* NR_043242.1	99.8						
S278	HG764626	*B. pumilus* NR_043242.1	99.8						

Discussion

According to previous studies, Kolumbo hydrothermal vent field was characterized by an atypical geodynamic setting with unique features among seafloor hydrothermal deposits. From small pockmark-like craters in the Fe-rich sediment and chimney spire outlets, CO_2 gas bubbles were discharged (Kilias et al. 2013), suggesting that carbon fixation under such CO_2-rich conditions is operated by vent mesophiles inhabiting the niches between reducing hydrothermal vents and toxic peripheral zones (Van Dover et al. 2002). The culture-based analysis of Kolumbo-associated mesophilic bacterial community revealed a rich mesophilic community, in contrast with previous culture-dependent studies of other deep-sea ecosystems (Kaye and Baross 2000; Gärtner et al. 2011; Castro da Silva et al. 2013). Whole-genome genotyping revealed that there were few major groups of similar fingerprints, implying high diversification at the strain level. The representatives of the most dominant genotypes showed no or weak antimicrobial activity. Phylogenetic analysis of the multi-active isolates indicated that the majority was affiliated with the phylum *Firmicutes* while previously, culture-independent studies revealed *Proteobacteria* as the dominant members of the bacterial mesophilic community (Kilias et al. 2013).

The results of the antimicrobial screening assays demonstrated an intense and broad metabolic activity by most of the isolates. Comparison with the proportion of bioactivity-producing bacteria isolated from other habitats like free-living planktonic communities (Zheng et al. 2005), sponges (Penesyan et al. 2009) and marine sediments (Nithya and Pandian 2010) highlighted an extremely high percentage of active isolates (76 %) from sediment and chimney microbial mats against bacterial and eukaryotic (filamentous fungi and yeasts) indicator strains. This finding may be connected to the formation of microbial aggregations in surface-attached biofilms that offer protection to microorganisms, but also represent a highly competitive environment in terms of space and nutrients. The production of antimicrobial metabolites may serve to maintain a stable

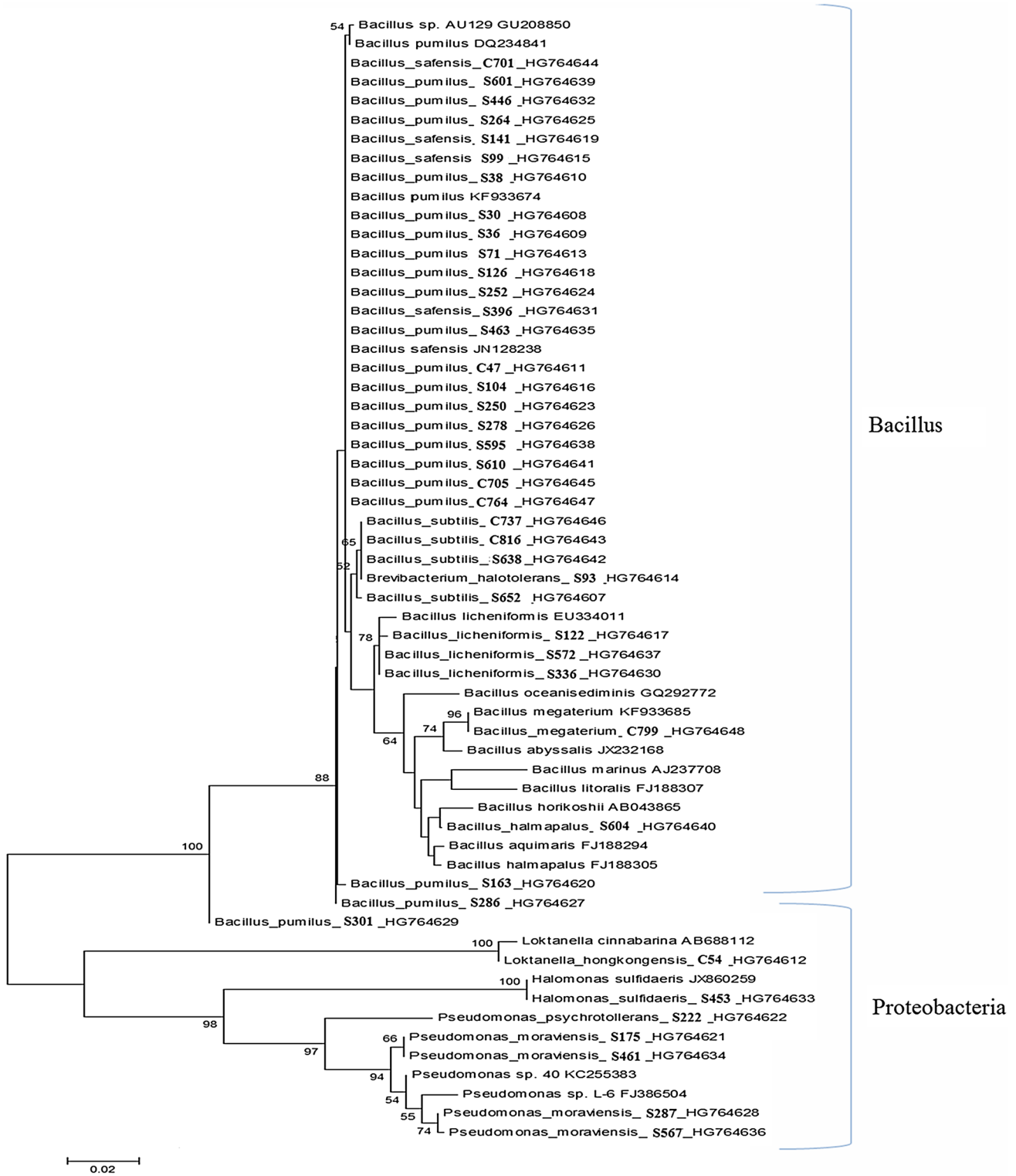

Fig. 2 Neighbor-joining phylogenetic tree based on the aligned partial 16S rRNA gene sequences (c. 700 nucleotide positions) of the antimicrobial strains and close relatives isolated from marine sediments. Sequences from the present study are highlighted in *bold* type (S for sediment and C for chimney isolates). Percentage bootstrap values (1,000 resamplings) are given for major nodes. The *scale bar* indicates the number of substitutions per nucleotide position

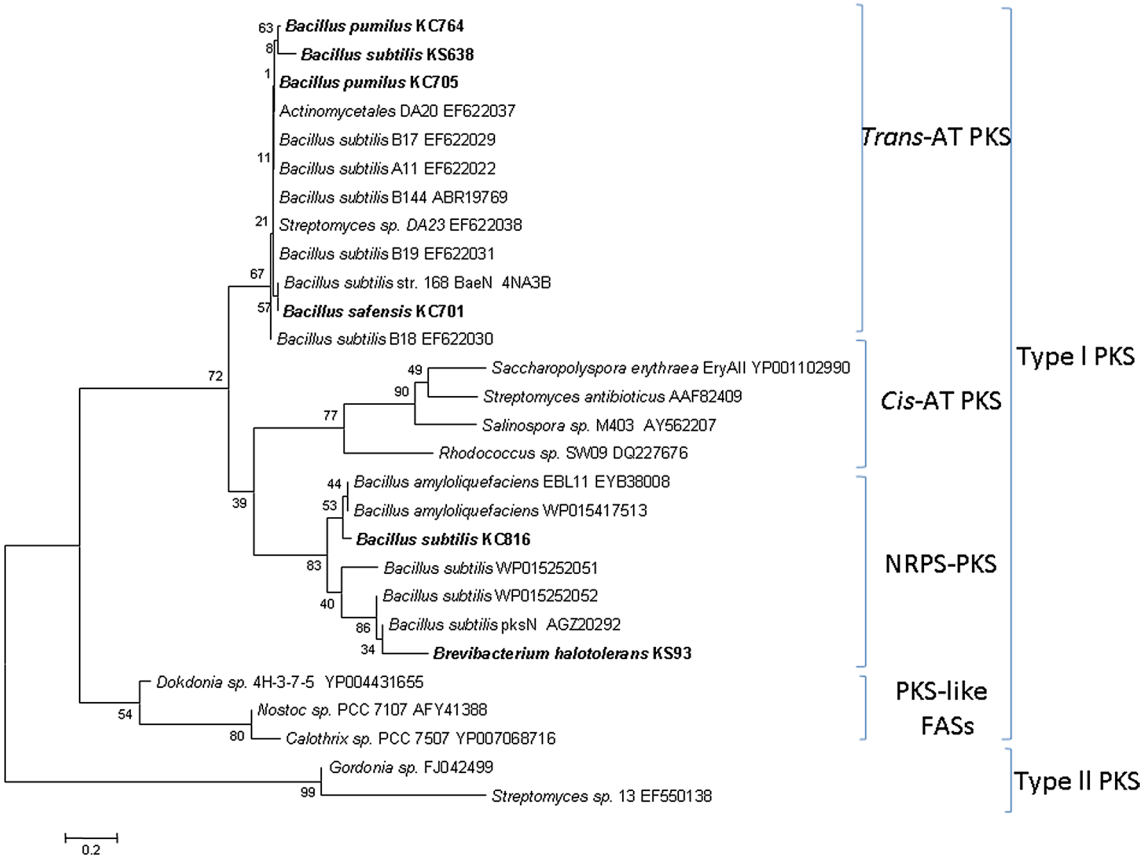

Fig. 3 Neighbor-joining phylogenetic tree of KS domain aa sequences of Kolumbo most bioactive isolates (highlighted in *bold* type with KS for sediment and KC for chimney isolates) and reference sequences including NRPS-PKS, *trans*-acyl transferase (*trans*-AT) PKS, *cis*-acyl transferase (*cis*-AT) PKS, FAS (fatty acid synthase) families. Representatives of type II PKS were added as out-groups. Percentage bootstrap values of neighbor-joining analysis from 1,000 resamplings are indicated at the nodes. The *scale bar* represents 0.2 amino acid substitutions per site

composition of biofilm communities (Penesyan et al. 2010) and provide chemical defense against eukaryotic colonizers or predators (Matz et al. 2008). Moreover, the detection of high bioactivity against bacterial and fungal pathogens is significant given the increasing occurrence of multi-drug resistant human pathogens and the necessity for novel antibiotics.

Forty-two isolates with strong antimicrobial activity were chosen for phylogenetic analysis. Although it is known that in the absence of bacterial interactions, the loss of certain metabolic capabilities due to a loss of plasmids or the lack of trigger substances or stress factors is frequently observed (Laatsch 2006), the selected isolates from Kolumbo under laboratory conditions exhibited strong activity even after repeated transfer to fresh media. That result suggested that those isolates may be amenable to large-scale production of antimicrobial agents.

Thirty-five out of the forty-two multi-active strains belonged to the genus *Bacillus*; marine sediments are the primary oceanic habitats from which *Bacillus* strains have been recovered (Austin 1988). In particular, *B. pumilus* and *B. subtilis* spp. are found to be the most abundant surface associated bacilli isolated from marine environment (Liu et al. 2013). Strains of *Bacillus* sp. isolated from the shallow hydrothermal field of D. João de Castro Seamount (DJCS) Azores (Mohandass et al. 2012) and Guaymas Basin, Gulf of California at 1,500-m depth (Dick et al. 2006), were studied for their adaptation strategies and their extended survival in toxic conditions. Their sporulation mechanism is correlated with antibiotic synthesis (Fickers 2012), which seems to be involved in quorum-sensing, biofilm and swarming development besides the antimicrobial action (Stein 2005). Seven isolates were included in the classes of α- and γ-*Proteobacteria*. Strain C54 is affiliated with the genus *Loktanella* of the *Roseobacter* clade: the only clade containing culturable members of the α-*Proteobacteria*, which comprises one of the largest fractions of heterotrophic bacteria in the marine environment (Cottrell and Kirchman 2000). Previous studies on the ecological role of roseobacters have

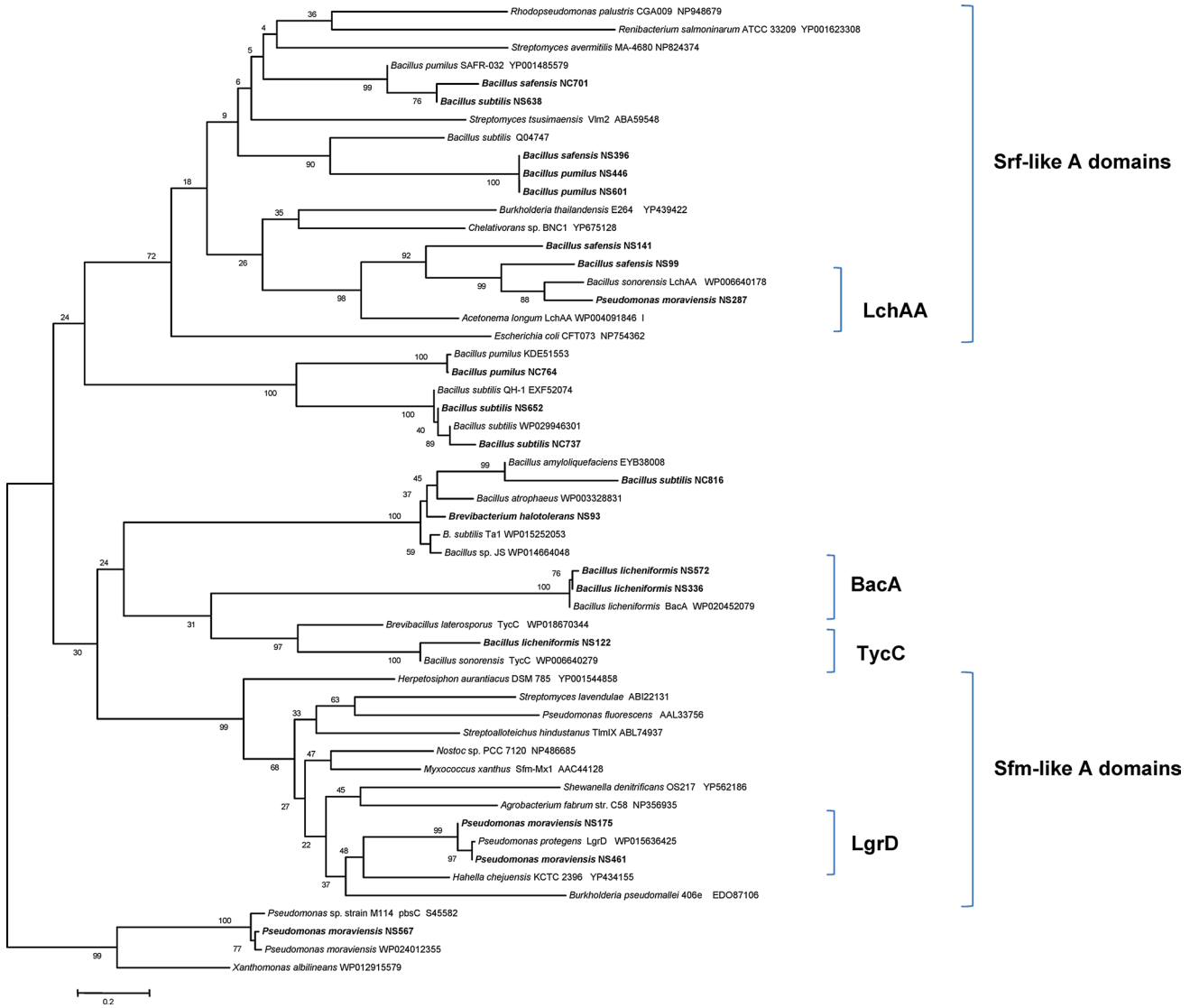

Fig. 4 Neighbor-joining phylogenetic tree from analysis of A domain amino acid sequences of Kolumbo most bioactive isolates (highlighted in *bold* type with NS for sediment and NC for chimney isolates) and reference sequences of known NRPS sequences including saframycin A and surfactin A families. Percentage bootstrap values of neighbor-joining analysis from 1,000 resamplings are indicated at nodes. The *scale bar* represents 0.5 AA substitutions per site

demonstrated that they prevent surface colonization due to the production of secondary metabolites with antimicrobial activities against a variety of marine bacteria (Wagner-Dobler et al. 2004), pointing to a competitive advantage for these organisms. Particle-associated strains of the *Roseobacter* clade were demonstrated to be 10 times more likely to produce antimicrobial compounds than were free-living ones (Long and Azam 2001). Strain S453 is phylogenetically related to the genus *Halomonas* of the γ-*Proteobacteria*. *Halomonas* strains have been isolated from deep-sea volcanic weathered basalt and sulfide rocks in low-temperature hydrothermal vent fields and can oxidize Fe(II), Mn(II) as well as an extensive variety of organic compounds (Kaye and Baross 2000). *Halomonas* strain LOB-5 collected from Loihi Seamount (east of Hawaii, 1,174 m depth) produces the most hydrophilic of the reported amphiphilic peptidic siderophores, the loihichelins A–F (Homann et al. 2009). The rest of the most bioactive bacteria are affiliated with the genus *Pseudomonas*, which is considered among the important groups able to produce antibiotics. Strains of this genus have been also isolated from deep-sea environments since the 1960s (Quigley and Colwell 1968) at a variety of depths (Li et al. 1999) including the West Pacific Ocean (>5,000-m depth) (Xu et al. 2007) and Mariana Trench, the deepest part of the ocean floor (Kato et al. 1997).

Among various environmental niches, microbial communities at the limits of life include giant genes encoding multi-modular synthases, for the production of polyketides (PKSs) and nonribosomal peptides (NRPSs), two of the larger classes of marine microbial natural products exhibiting antimicrobial, antifungal and antiparasitic activities (Gulder and Moore 2009). The materialization of the information contained in such giant genes, commonly considered strain- or clone-specific properties (Fischbach and Walsh 2006), is associated with huge costs in terms of energy and time for the microbial producer, and this might explain their synthesis preferentially by nonpathogenic environmental bacteria with typically slow reproduction cycles to constitute weapons against predators and competitors for the same niche (Reva and Tummler 2008). According to our results and phylogenetic analyses, from the six isolates phylogenetically related to *Bacillus* group containing PKS genes, two were included in the NRPS/PKS group while four contained *trans*-AT PKS of type I genes in close relationship with previously isolated genes from South China Sea sponges (Zhang et al. 2008). *trans*-AT PKS assemblages derive from multiple recombinatorial events through horizontal recruitment of ketosynthase (KS) domains and display enzymatic features which do not fit to canonical architecture of *cis*-AT systems (Nguyen et al. 2008). They are mostly found in bacteria with unusual lifestyles and led to the discovery of novel polyketides with antibiotic (bacillaene, mupirocin and streptogramin) and antitumor (bryostatin) activities (Piel 2010). In addition, many pharmacologically important peptides derive from NRPS enzymology with remarkable structural diversity of natural products produced. This diversity depends on the different number of modules employed, the variety of activated amino acid substrates by A domains and the addition of modifying domains (Schwarzer et al. 2003). All the obtained adenylation domain gene sequences from Kolumbo isolates showed high similarity to NRPS A domains some of which are closely related to subunits of known peptides exhibiting antibiotic activity. Two aa sequences from *Pseudomonas* isolates matched with linear gramicidin D, characterized by a relatively rare type of chain termination strategy by a reductase (R) domain (Du and Lou 2010). NRPS gene sequences deriving from three *Bacillus* isolates were closely related to bacitracin A and tyrocidine III synthases, which are released by diverse macrocyclization strategies, catalyzed by thioesterase (TE) domains (Kopp and Marahiel 2007). The majority of the A domains recovered from *Bacillus* isolates showed close similarity to *B. subtilis* surfactin domain. A *Pseudomonas* NRPS gene sequence was very close to *Bacillus* lichenysin A synthetase. Lipopetides surfactin and lichenysin exhibit antibacterial, antiviral and hemolytic activities (Rodriguez et al. 2006) by a detergent-like mechanism, which allows for modification of membrane permeability and solubilization. Surfactin suspends biofilm formation of other bacteria by preventing the attachment of the cells to surfaces (Roongsawang et al. 2011).

The high percentage of Kolumbo isolates exhibiting a wide antimicrobial activity as revealed by bioassays and detection of PKS and NRPS genes indicated a broad chemical diversity of biologically active metabolites. It seems that Kolumbo hydrothermal field provides an environment which hosts interesting bacteria with important characteristics like the production of bioactive natural compounds, and it warrants further exploration. The potential for discovery of novel bioactive molecules with activity against fungal and bacterial pathogens is evident.

Acknowledgments We thank Prof. Steven Carey and Dr. Katherine L. Croff Bell (Chief Scientist) for inviting us to participate in the *E/V Nautilus* 2010 expedition, the crew of *E/V Nautilus* and the pilots of the remotely operated vehicle *Hercules* for their help in obtaining the samples. We also thank the members of Microbiology Group Prof. George Dialinas, Dr. Alexandros Savvidis and Dr. Sotiris Amillis for their precious help. This research has been co-financed by the European Union (European Social Fund—ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF)—Research Funding Program: Heracleitus II.

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