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The sponges *Hymeniacidon perlevis* and *Halichondria panicea* are reservoirs of antibiotic-producing bacteria against multi-drug resistant *Staphylococcus aureus*

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Abstract

Aims

Evaluation of the antibacterial activity of cultivable bacteria associated with the marine sponges *Hymeniacidon perlevis* and *Halichondria panicea* against multi-drug resistant *Staphylococcus aureus*.

Methods and Results

One hundred and fourteen bacterial isolates were recovered from *H. perlevis* and *H. panicea*. Antibacterial action was demonstrated by 70% of the isolates against reference strain *Staphylococcus aureus* ATCC 29213 and by 31.6% against *Pseudomonas aeruginosa* ATCC 27853 in agar overlay assays. Antibacterial potential was further analysed against 36 multi-drug resistant hospital *Staphylococcus aureus* strains with diverse resistance profiles. Among the 80 isolates positive against *S. aureus* ATCC 29213, 76.3% were active against at least one clinical *S. aureus* pathogen and 73.6% inhibited one or more methicillin-resistant (MRSA) and vancomycin non-susceptible *S. aureus* strains. In addition, 41.3% inhibited all vancomycin non-susceptible MRSA strains.

Conclusions

Culturable bacteria associated to *H. perlevis* and *H. panicea* are promising sources of antibacterial compounds of great pharmaceutical interest.

Significance and Impact of Study

This study was the first to explore the antibacterial potential of culturable bacteria associated with the marine sponges *H. perlevis* and *H. panicea* against MDR bacteria. This is the first report of antibacterial activity by *Aquimarina*, *Denitrobaculum, Maribacter* and *Vagococcus* isolates against MDR *S. aureus* strains, including vancomycin non-susceptible and methicillin-resistant ones, against which new antibiotics are urgently needed.

Keywords: Staphylococci, Antimicrobials, Antibiotics, Resistance, Environmental

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Introduction

Staphylococcus aureus is a gram-positive commensal bacterium that can transition to pathogenicity, evading the immune system, and cause various infections (Pollitt *et al.*, 2018). Some *S. aureus* strains have been able to adapt to environmental stress and acquire resistance to most antibiotics used in human or veterinary medicine (Stefani *et al.*, 2015). Resistance to methicillin, acquired by horizontal gene transfer of the mobile genetic element SCC*mec* (Staphylococcal Cassette Chromosome *mec*), emerged in the 1960s and quickly spread (Turner *et al.*, 2019). Subsequently, the use of glycopeptide antibiotics to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections started (Purrello *et al.*, 2016). However, *S. aureus* strains less susceptible or resistant to glycopeptides (such as vancomycin and daptomycin) emerged since the late 1990's (Purrello *et al.*, 2016).

Althoughresistance to most antibacterial compounds increased with their use, big pharmaceutical companies ceased research and development of new antibiotics in the 1980s. Consequently, the number of new drugs available on the market decreased as the demand for new antibiotics increased (Duval *et al.*, 2019). Although some new classes of antibiotics have recently been discovered (Ling *et al.*, 2015; Hover *et al.*, 2018; Stokes *et al.*, 2020), there is currently an important need for new antibacterial compounds. The World Health Organization (WHO) issued in 2017 a priority list for the research and development of new antibiotics, in which it classifies methicillin-resistant, vancomycin-intermediate and -resistant *S. aureus* in the high priority group (Tacconelli and Magrini, 2017).

Marine sponges have been attracting interest as they are considered a reservoir of great microbial diversity and a rich source of bioactive substances (Laport *et al.*, 2009). Sponges (phylum Porifera) are the most primitive metazoans and simplest form of multi-cellular animals, with limited tissue differentiation. Their limited physical defences are counterbalanced by highly developed chemical defences (Hentschel *et al.*, 2012). Many of the bioactive compounds of pharmacological interest found in sponges, such as antimicrobial, anti-inflammatory and anti-cancer, are produced by and have been isolated from sponge-associated microorganisms (Thomas *et al.*, 2010; Indraningrat *et al.*, 2016). In fact, these microorganisms are considered as part of the sponge holobiont, i.e. the complex ecosystem made up of the host and its microbiota, which interacts and responds to environmental change, providing together essential functions such as nutrition, defence and immunity (Pita *et al.*, 2018). In order to lower the rate of rediscovery of known compounds, one promising strategy is the study of unexplored bacterial strains in microbial communities to access their pool of biosynthetic gene clusters and secondary metabolite chemical diversity (Clardy and Walsh, 2004). However, cultivation of host-associated bacteria is challenging (Laport,

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2017). Previous studies have reported that percentages of culturable bacteria in sponges ranged from under 1% to around 10% of the total bacterial community, in terms of abundance or diversity (Webster and Hill, 2001; Esteves *et al.*, 2016; Gutleben *et al.*, 2020). These percentages can however be increased using specialized media (Webster *et al.*, 2001; Lavy *et al.*, 2014; Wohlleben *et al.*, 2016). For example, the use of diverse oligotrophic isolation media, mimicking the sponge tissue and the marine environment, raised this percentage to 14% and allowed to recover a greater diversity of culturable strains (Sipkema *et al.*, 2011; Laport, 2018).

In this study, we isolated 114 bacterial strains associated with the marine sponges *Hymeniacidon perlevis* and *Halichondria panicea* using a variety of growth media. These two sponge species were chosen because they belong to the Halichondriidae family, one of the richest sources of bioactive compounds of pharmacological interest (Thomas *et al.*, 2010). Yet, their microbiota has barely been studied for antibiotic production. Although some bioactive compounds produced by bacteria associated to *Halichondria panicea* have been previously reported (Perovic *et al.*, 1998; Wicke *et al.*, 2000; Imhoff and Stöhr, 2003), only Actinobacterial isolates have been explored (Schneemann *et al.*, 2010). Antibacterial activity of *Hymeniacidon perlevis*-associated bacteria has only been reported twice: Xi *et al.* (2012) studied actinomycetes isolates and Zheng *et al.* (2005) identified norharman, a beta-carboline alkaloid antimicrobial compound from a *Pseudoalteromonas piscida* strain isolated from that sponge species. We first assessed the antibacterial activity of the sponge-associated bacterial strains against *S. aureus* ATCC 29213, a sensitive reference strain, and then extended the study to a gram-negative reference strain (*P. aeruginosa* ATCC 27853) and to a collection of multi-drug resistant *S. aureus* pathogens.

Materials and methods

Sponge sampling

Two *Hymeniacidon perlevis* specimens and one *Halichondria panicea* specimen were collected at low tide on the rocks of the beach of Wimereux (50° 46’ 14’’ N – 1°36’000 E), located in Nord-Pas-de-Calais, France, on October 20th 2013. A specimen of *H. perlevis* was collected at low tide on the rocks of the beach of Audresselles (50° 49’ 24’’ N – 1°36’000 E) located in Nord-Pas-de-Calais, France, on April 15th 2018. Specimens were placed in sterile bags, stored on ice and taken to the laboratory within 24 h. The specimens were identified by spicule measurement at the Department of Taxonomy and Phylogeny at the Royal Belgian Institute of Natural Sciences.

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Isolation of sponge-associated bacteria

Two separate sponge-associated bacterial strains collections were recovered from the sponge specimens sampled in 2013 and 2018. The first collection was isolated using commercial nutritional media and the second using specially formulated dilute culture media.

Sponges collected in 2013 were rinsed with distilled water and macro-organisms were removed under sterile conditions. The specimens were kept at 6°C in three commercial nutritional media: Brain Heart Infusion Broth (BHI, Bacto BD), Reasoner's 2A Broth (R2, Melford) and Marine Broth (M, Difco BD) during two and eight days in order to isolate fast and slow growing bacteria, respectively. Each individual specimen was cut into small pieces, re-immersed in the same nutritional medium and vortexed (for 20 s). From the resulting macerate, serial dilutions (10^{-3} to 10^{-5}) were made in saline solution (NaCl 0.9%) and 100-µL aliquots were plated in Petri dishes on the corresponding media. The plates were incubated for 24 h at 25°C. Colony-forming units (cfu) were selected based on colony morphology (colour, shape, size, texture, and shine). Aliquots of each isolate were stored at -80°C in 15% glycerol and 2% DMSO (dimethyl sulfoxide).

Isolation of sponge-associated bacteria from the specimen sampled in 2018 was carried out according to the method described by Esteves *et al.* (2016) with small modifications. The sponge was rinsed with distilled water and macro-organisms were removed. Sponge tissue was washed and blended in Artificial Sea Water (ASW) (33.3 g/L Instant Ocean Sea Salt in Milli-Q water) for 5 min. The homogenate was successively filtered down to 2-µm to remove eukaryotic cells and organelles and centrifuged to precipitate microbial cells. Sponge-associated bacteria were isolated on five culture media (Table 1), adapted from Esteves *et al.* (2016) to mimic physiological conditions of the sponge environment. Sponge extract was prepared by sponge homogenization and successive filtration down to 0.2 µm and was used as component of the culture media "a" and "b". The microbial cell suspension was serially diluted (10^{-3} to 10^{-5} -fold) in ASW, 100 µL of each dilution were inoculated on the culture media (Table 1) and incubated at 18°C. Visually distinct cfu were purified. Aliquots of each isolate were stored at -80°C in 15% glycerol and 2% DMSO.

Sequencing of the 16S rRNA gene of bacterial isolates

Bacterial DNA was recovered by resuspending cellular material from each colony in 25 µl sterile PCR grade water and boiling the suspension at 100°C for 15 min. PCR amplification was performed by adding 3 µL of DNA solution to 47 µL containing 1x Green GoTaq MasterMix (Promega) and 20 pmol of each universal

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primer, 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). Cycle conditions consisted of an initial denaturation step at 94°C for 6 min 30 s, followed by 30 cycles at 94°C for 30 s, 55°C for 1 min 30 s and 72°C for 2 min 30 s, and a final elongation step at 72°C for 5 min. PCR products were analysed by electrophoresis on a 0.8% agarose gel, purified using the QIAquick PCR purification kit (Qiagen) and sequenced using the universal primers 27F and 1492R at Macrogen Europe (Amsterdam, The Netherlands). Assembled 16S rRNA gene sequences were aligned and classified using the online portal of the SILVA SINA alignment service of the ARB-Silva database (http://www.arb-silva.de/aligner/). The closest 16S rRNA sequence matches were determined using the Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology (NCBI) with the Reference RNA sequences (refseq_rna) database. The sequences were deposited in GenBank under the accession numbers MT484090-MT484100, MT484136-MT484235 and MT484262-MT484264 (detailed in Supplementary Table S1).

To construct the phylogenetic tree, all 16S rRNA gene sequences were aligned in MEGA X using the ClustalW algorithm. The maximum likelihood phylogenetic tree was calculated in MEGA X, using the Tamura-Nei nucleotide substitution model. Tree validation was performed by 100 bootstrap replications. The phylogenetic tree was imported to R version 3.6.3 and plotted using the packages ggtree version 2.0.4 (Yu, 2020) and ape version 5.3 (Paradis and Schliep, 2019).

Hospital-isolated *Staphylococcus aureus* strains and antibiotic susceptibility testing

Thirty-six *S. aureus* strains isolated from prosthesis infections in hospitals (mostly from Hôpital André Mignot – Centre Hospitalier de Versailles, France) were provided by Prof. Sigrid Flahaut, Laboratory of Applied Microbiology, Université Libre de Bruxelles.

Antimicrobial susceptibility testing of the strains was performed according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) Disk Diffusion Method for Antimicrobial Susceptibility Testing v. 6.0 (2017) on Mueller-Hinton Agar (Merck). The *S. aureus* ATCC 29213 strain was used as quality control following EUCAST (Matuschek *et al.*, 2014). The following antimicrobial discs (Bio-rad) were used: amikacin (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), fusidic acid (10 µg), gentamicin (10 µg), levofloxacin (5 µg), linezolid (10 µg), minocycline (30 µg), moxifloxacin (5 µg), netilmicin (10 µg), norfloxacin (10 µg), ofloxacin (5 µg), penicillin (1 µg), quinupristin-dalfopristin (15 µg), rifampicin (5 µg), tetracycline (30 µg), tigecycline (15 µg), tobramycin (10 µg), trimethoprim (5 µg) and trimethoprim-sulfamethoxazole (1.25 µg - 23.75 µg). Susceptibility to teicoplanin and vancomycin (Sigma-Aldrich) was tested following the microdilution method for minimal inhibitory

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concentration (MIC) determination in Mueller-Hinton Broth (Merck) with a concentration range from 64 to 0.25 µg mL^{-1}. The results were interpreted using WHONET v20.12.8 (Stelling and O'Brien, 2016) with the EUCAST Clinical Breakpoint Tables v.11.0 (2020) as reference. Multidrug resistance (MDR) was defined as acquired non-susceptibility to at least one drug out of three or more antimicrobial categories (Magiorakos *et al.*, 2012). The vast majority of *S. aureus* strains (i.e. all but strains 483, LDA2 and SDD) were multi-drug resistant (MDR). The strains had diverse susceptibility profiles, including 15 methicillin-resistant strains (MRSA) (inferred from cefoxitin resistance), 15 vancomycin non-susceptible strains and six strains resistant to both compounds (383a, 399, 400, 403, 498a and MR25). A heatmap with dendrograms (Supporting Figure S1) was plotted on R version 3.6.3 using the ComplexHeatmap package version 2.2.0 (Gu *et al.*, 2016).

Assay for antibacterial activity

The antibacterial activity of sponge isolates was assessed with agar overlay assays as previously described by Marinho *et al.* (2009) with some modifications. Five µL of stationary-phase cultures (about 10^6 cfu) of each bacterial isolate were spotted onto agar plates and incubated at 25°C until the colony diameter reached 5-8 mm (24 to 48 h). The bacteria were then killed by exposure to chloroform (VEL, Leuven) vapor for 30 min. In parallel, the indicator strains were grown in BHI broth at 37°C for 18 h and diluted to 1% v/v in 3 mL of BHI soft agar, which was poured over the plates containing the killed colonies. After incubation at 37°C for 24 h, the presence of an inhibition zone above (and beyond) the spotted strain colonies was recorded. All tests were carried out in triplicate. A test was considered positive when at least one replicate was positive. The assay was performed for initial screening using two antibacterial-susceptible reference strains of the American Type Culture Collection (ATCC) as indicators: *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853. Sponge isolates inhibiting *S. aureus* ATCC 29213 were subsequently tested against the set of 36 hospital-isolated *S. aureus* strains.

Results

Phylogenetic affiliation of the bacterial strains and phylogenetic tree

A total of 114 colony-forming units were isolated, 69 on commercial culture media (BHI, R2 or M) and 45 on formulated dilute media (Table 1). Thirty of the isolates had 16S rRNA sequence matches below 97% to previously reported bacterial species on NCBI (Table 2).

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Strains isolated on commercial media belonged to two phyla: Proteobacteria (class Gammaproteobacteria, 46 isolates, 66.7%) and Firmicutes (23 isolates, 33.3%). The most abundant genus was *Pseudomonas* (20 isolates, 29.0%), followed by *Citrobacter* (13 isolates, 24.6%) and *Lactococcus* (11 isolates, 16.0%). In total, strains of 12 different genera were isolated on commercial media (Figure 1). The maximum likelihood phylogenetic tree of the strains did not show two distinct clusters of isolates obtained from the two sponge species *H. panicea* and *H. perlevis* at the genus level (Figure 1). In fact, all genera isolated on commercial media from *H. panicea* were also isolated from *H. perlevis.* Nevertheless, five genera were unique to *H. perlevis.*

Isolates obtained from *H. perlevis* using formulated dilute media belonged to 4 different phyla: Proteobacteria (29 isolates, 64.4%), of which 12 Alphaproteobacteria and 17 Gammaproteobacteria, Bacteroidetes (eight isolates, 17.8%), Actinobacteria (five isolates, 11.1%), and Firmicutes (three isolates, 6.6%). The most represented genus was *Vibrio* (16 isolates, 35.5%), followed by *Paracoccus* (six isolates, 13.3%). A total of 13 different genera were recovered using dilute media (Figure 1).

In total, 91 isolates were recovered from *H. perlevis* specimens using eight nutritional media (Figure 2). There was little overlap of isolated genera between the different media. Of the 27 recovered bacterial genera, the majority (17 genera) were isolated uniquely on one of the nutritional isolation media. Only two genera were isolated on three media and seven genera on two media. Commercial media provided 46 isolates, with a majority of Firmicutes isolated on BHI and Gammaproteobacteria on R2. Dilute media provided 45 isolates. Isolates affiliated to the genera *Dokdonia*, *Ruegeria* and *Rhodococcus* were exclusively recovered on media containing sponge extract (a and b).

Antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A great proportion (71.0%) of the isolated bacteria showed antibacterial activity against *S. aureus* ATCC 29213 and/or *P. aeruginosa* ATCC 27853 (Figure 3). They were affiliated to all four phyla.

A higher number of strains exhibited antibacterial activity against *S. aureus* ATCC 29213 (80 isolates, 70.2%,) than against *P. aeruginosa* ATCC 27853 (37 isolates, 32.5%). Interestingly, 31.6% of the isolates (36) exhibited inhibitory activity against both the gram-positive and gram-negative test strains. These included most *Vibrio* isolates and half of the *Pseudomonas* isolates, as well as some *Aquimarina, Denitrobaculum, Enterococcus*, *Lactococcus*, *Paracoccus*, *Ruegeria* and *Vagococcus* isolates.

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Antibacterial activity against multi-drug resistant *Staphylococcus aureus* strains

The 80 sponge isolates which inhibited the growth of *S. aureus* strain ATCC 29213 were further tested against a collection of 36 hospital-isolated multi-drug resistant *S. aureus* strains with varied antimicrobial susceptibility profiles, including resistance to methicillin and vancomycin (Supporting Figure S1).

Sixty-one of these strains (76.3%) inhibited at least one hospital-isolated *S. aureus* strain (Figure 4). Isolates of the genera *Vibrio, Pseudomonas* and *Vagococcus* exhibited the largest proportion of activity and range, with respectively 10, seven and four isolates inhibiting the 36 *S. aureus* strains tested. Three other isolates (of the genera *Citrobacter*, *Denitrobaculum and Lactococcus*) also inhibited all hospital-isolated *S. aureus* strains.

Two thirds of the isolates (52 out of 80) were active against at least one vancomycin non-susceptible methicillin-resistant *S. aureus* strain. In fact, 33 isolates (41.3%) (15 *Vibrio*, seven *Pseudomonas*, five *Vagococcus*, three *Denitrobaculum*, two *Lactococcus* and one *Citrobacter*) demonstrated inhibitory activity against all six vancomycin non-susceptible MRSA strains.

Discussion

Bacteria associated with two sponge species (*H. perlevis* and *H. panicea*) collected in 2013 and 2018 at Wimereux and Audresselles (FR) were isolated and studied for their antibacterial activity against *S. aureus* (one sensitive and 36 MDR strains) and *P. aeruginosa* (one sensitive strain). A total of 114 viable and culturable isolates were recovered from both sponge species using eight different nutritional media (Figure 1). The isolates were affiliated to phyla (Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes) frequently obtained from marine sponges (Thomas *et al.*, 2016) and widely present in the bacterial microbiomes of *H. perlevis* and *H. panicea* (Supporting Figure S2). Our results (Figure 2) support previous observations that different media compositions allow the recovery of distinct taxa (Sipkema *et al.*, 2011; Esteves *et al.*, 2016). Although cultivation of all host-associated bacterial taxa detected by molecular techniques remains challenging, custom media have been successfully employed to culture previously uncultured bacteria (Webster *et al.*, 2001; Sipkema *et al.*, 2011; Versluis *et al.*, 2017). The BLAST search for closest relatives of our isolates suggests that several new bacterial species were recovered in this study (Table 2) (Gevers *et al.*, 2005).

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Agar overlay assays of the 114 culturable isolates against reference strains *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 revealed the antibacterial activity of most isolates (71%) (Figure 3). The presence of a large proportion of bioactive bacteria associated to the sponges *H. panicea* and *H. perlevis* might be related to their dominant and invasive natures. Indeed, *H. panicea* is one of the most common intertidal sponges in North-Western Europe. Its establishment and maintenance is likely aided by the release of a large array of defensive secondary metabolites (Pita *et al.*, 2018). This idea is supported by previous reports of its production of antifouling (Toth and Lindeborg, 2008), neuroactive and cytotoxic compounds (Perovic *et al.*, 1998; Ferreira *et al.*, 2011) and antitumoral glycoglycerolipids (Wicke *et al.*, 2000). Although very limited information on the bioactivity of *H. perlevis* and its microbiome is available, it is known to be an invasive species (Turner, 2020). In order to successfully spread, the sponge needs rich chemical defences. For example, it has been shown that sponges found in environments with a high predator abundance possess vast chemical defences (Loh and Pawlik, 2014). Since sponge disease can be caused by bacteria (Webster, 2007), antibacterial compounds are expected to play an important defence role for these species.

A greater part of isolates was active against *S. aureus* (70.2%) than against *P. aeruginosa* (32.5%). This result was expected as gram-negative bacteria tend to be more resistant to antibiotics owing to the lower permeability of their outer membrane and expression of efflux pumps (Pang *et al.*, 2019). However, several isolates (31.6%) were active against both *S. aureus* and *P. aeruginosa* strains, exhibiting a broader spectrum of antibacterial action (Figure 3). All isolates affiliated to the genus *Denitrobaculum* and most of those affiliated to *Vibrio* exhibited such antibacterial activity. Some isolates of other genera, such as *Pseudomonas*, *Enterococcus*, *Lactococcus* or *Paracoccus*, were active against both strains. Such a percentage of isolates capable of inhibiting the growth of gram-positive and gram-negative reference strains is rare. The bulk (87.0%) of studies on sponge bacteria report a percentage under 15.0%, half of which are in fact under 5.0% (Hentschel *et al.*, 2001; Zheng *et al.*, 2005; Anand *et al.*, 2006; Chelossi *et al.*, 2007; Marinho *et al.*, 2009; Zhang *et al.*, 2009; Santos *et al.*, 2010, 2019; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Papaleo *et al.*, 2012; Esteves *et al.*, 2013; Haber and Ilan, 2014; Graça *et al.*, 2015; Laport *et al.*, 2017; Matobole *et al.*, 2017; Bibi *et al.*, 2018, 2020; Trianto *et al.*, 2019; Freitas-Silva *et al.*, 2020; Rajasabapathy *et al.*, 2020). Similar results have only been reported in three studies (Kennedy *et al.*, 2009; O' Halloran *et al.*, 2011; Zeng *et al.*, 2013). The antibacterial activity displayed by these bacterial isolates against both gram-positive and gram-negative strains may be due to their production of broad-spectrum antibacterial molecules or a cocktail of bioactive molecules with diverse modes of action.

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The percentage (70.2%) of sponge isolates inhibiting the growth of antimicrobial-susceptible *S. aureus* ATCC 29213 is also remarkable. A review of the literature on the antibacterial activity of sponge bacteria against *S. aureus* reference strains highlighted that this percentage is much higher than the one obtained in comparable studies, which ranges between 1.5% and 32.0% (Hentschel *et al.*, 2001; Zheng *et al.*, 2005; Chelossi *et al.*, 2007; Kennedy *et al.*, 2009; Marinho *et al.*, 2009; Zhang *et al.*, 2009; Santos *et al.*, 2010, 2019; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Papaleo *et al.*, 2012; Xi *et al.*, 2012; Esteves *et al.*, 2013; Zeng *et al.*, 2013; Haber and Ilan, 2014; Laport *et al.*, 2017; Bibi *et al.*, 2018; Trianto *et al.*, 2019; Freitas-Silva *et al.*, 2020; Rajasabapathy *et al.*, 2020). Only one study with a similar percentage of active strains against *S. aureus* was found (74%), but it exclusively focused on *Pseudovibrio* isolates (O' Halloran *et al.*, 2011).

Activity of the isolates was therefore further assessed against a collection of 36 *S. aureus* strains isolated from hospital infections. To the best of our knowledge, this study uses the largest panel of antibiotic-resistant *S. aureus* clinical strains to determine sponge-bacteria inhibitory activities. As observed in Figure 4, antibacterial activity against the *S. aureus* reference strain did not imply activity against clinical pathogens in 25% of the cases. Reference strain *S. aureus* ATCC 29213 is highly susceptible to all antibiotics except penicillin, making it a useful preliminary screening tool but precluding it from revealing relevant activity on its own. This result underlines the importance of conducting bioactivity assays against a large panel of clinical pathogens, with diverse antibiotic resistance mechanisms, to demonstrate their biotechnological potential.

Over 75% of isolates (61 out of 80) inhibited the growth of at least one hospital-isolated *S. aureus* strain (Figure 4). Thus, half of all isolates recovered (61 out of 114) were active against antibiotic-resistant *S. aureus* strains. These numbers are striking, as previous publications analyzing activity of sponge-associated bacteria against antibiotic-resistant clinical *S. aureus* strains reported significantly lower active fractions, ranging from 0 to 14.0% (Hentschel *et al.*, 2001; Marinho *et al.*, 2009; Santos *et al.*, 2010, 2019; Papaleo *et al.*, 2012; Laport *et al.*, 2017; Matobole *et al.*, 2017; Bibi *et al.*, 2018; Trianto *et al.*, 2019; Freitas-Silva *et al.*, 2020). Only one study by Kennedy *et al.* (2009) found results closer to ours, reporting 29.0% of 52 *Haliclona simulans* bacterial isolates active against 11 MDR *S. aureus* strains. O' Halloran *et al.* (2011) reported over 60.0% of isolates active against three MRSA/VISA strains, yet only *Pseudovibrio* strains were screened.

Active isolates inhibited the larger part (an average of 26) of the 36 *S. aureus* strains, including MRSA and vancomycin non-susceptible strains, named as high priority antibiotic-resistant bacteria for which

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research of new antibiotics is needed by WHO (Tacconelli and Magrini, 2017). The largest contributors to activity against the high priority strains were genera *Denitrobaculum, Lactococcus, Pseudomonas, Vagococcus* and *Vibrio.* Activity of *Lactococcus, Pseudomonas* and *Vibrio* strains against multi-resistant *S. aureus* has previously been described (Marinho *et al.*, 2009; Santos *et al.*, 2010; Lee *et al.*, 2013; Okuda *et al.*, 2013; Bibi *et al.*, 2018; Freitas-Silva *et al.*, 2020). But as far as we know, this is the first report of inhibition of MDR *S. aureus* strains by *Aquimarina, Denitrobaculum, Maribacter* and *Vagococcus* isolates, which have only been reported for activity against reference susceptible strains (Flemer *et al.*, 2012; Heindl and Thiel, 2012; Esteves *et al.*, 2013; Laport *et al.*, 2019; Rajasabapathy *et al.*, 2020). On top of that, all *Denitrobaculum* and *Vagococcus* isolates were active against the six vancomycin non-susceptible MRSA strains tested, indicating they could be promising sources of bioactive compounds. Furthermore, this study demonstrates for the first time the antibacterial activity of bacterial strains affiliated to the genus *Maribacter*. Algicidal activity of *Maribacter* isolates has been reported (Wang *et al.*, 2016), but bioactivity of this genus of the Flavobacteriaceae family is virtually unexplored.

In conclusion, our results show great antibacterial potential of culturable bacteria associated with *H. panicea* and *H. perlevis*. Throughout the use of increasingly problematic *S. aureus* targets, from a reference strain to vancomycin non-susceptible MRSA clinical isolates, the abundance of inhibitory sponge isolates identified in this study is exceptional. Consequently, further analysis of the bioactivity is being carried out and deeper investigation into the microbiome of both sponge species should be pursued.

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Conflict of interest

No conflict of interest declared.

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References

Anand, T.P., Bhat, A.W., Shouche, Y.S., Roy, U., Siddharth, J. and Sarma, S.P. (2006) Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. *Microbiol Res*. **161**, 252–262.

Bibi, F., Bibi, F., Alvi, S.A., Alvi, S.A., Al-Sofyani, A., Al-Sofyani, A., Yasir, M., Yasir, M., Kensarah, E.A., Kensarah, E.A., Azhar, E.I. and Azhar, E.I. (2018) Research Article Two marine sponges-associated cultivable bacteria: Diversity and biological activities. *Genet Mol Res*. **17**.

Bibi, F., Yasir, M., Al-Sofyani, A., Naseer, M.I. and Azhar, E.I. (2020) Antimicrobial activity of bacteria from marine sponge Suberea mollis and bioactive metabolites of Vibrio sp. EA348. *Saudi J Biol Sci*. **27**, 1139–1147.

Chelossi, E., Pantile, R., Pronzato, R., Milanese, M. and Hentschel, U. (2007) Bacteria with antimicrobial properties isolated from the Mediterranean sponges Chondrilla nucula and Petrosia ficiformis. *Aquat Microb Ecol*. **49**, 157–163.

Clardy, J. and Walsh, C. (2004) Lessons from natural molecules. *Nature*. **432**, 829–837.

Duval, R.E., Grare, M. and Demoré, B. (2019) Fight Against Antimicrobial Resistance: We Always Need New Antibacterials but for Right Bacteria. *Molecules*. **24**, 3152.

Esteves, A.I.S., Amer, N., Nguyen, M. and Thomas, T. (2016) Sample Processing Impacts the Viability and Cultivability of the Sponge Microbiome. *Front Microbiol*. **7**.

Esteves, A.I.S., Hardoim, C.C.P., Xavier, J.R., Gonçalves, J.M.S. and Costa, R. (2013) Molecular richness and biotechnological potential of bacteria cultured from Irciniidae sponges in the north-east Atlantic. *FEMS Microbiol Ecol*. **85**, 519–536.

Ferreira, M., Cabado, A.G., Chapela, M.-J., Fajardo, P., Atanassova, M., Garrido, A., Vieites, J.M. and Lago, J. (2011) Cytotoxic activity of extracts of marine sponges from NW Spain on a neuroblastoma cell line. *Environ Toxicol Pharmacol*. **32**, 430–437.

Flemer, B., Kennedy, J., Margassery, L.M., Morrissey, J.P., O'Gara, F. and Dobson, A.D.W. (2012) Diversity and antimicrobial activities of microbes from two Irish marine sponges, Suberites carnosus and Leucosolenia sp.: Antimicrobial activities of sponge microbes. *J Appl Microbiol*. **112**, 289–301.

Accepted Article

This article is protected by copyright. All rights reserved

Freitas-Silva, J., Silva-Oliveira, T., Muricy, G. and Laport, M.S. (2020) Bacillus Strains Associated to Homoscleromorpha Sponges are Highly Active Against Multidrug Resistant Bacteria. *Curr Microbiol*. **77**, 807–815.

Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., de Peer, Y.V., Vandamme, P., Thompson, F.L. and Swings, J. (2005) Re-evaluating prokaryotic species. *Nat Rev Microbiol*. **3**, 733–739.

Graça, A.P., Viana, F., Bondoso, J., Correia, M.I., Gomes, L., Humanes, M., Reis, A., Xavier, J.R., Gaspar, H. and Lage, O.M. (2015) The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge Erylus deficiens (Astrophorida, Geodiidae). *Front Microbiol*. **6**.

Gu, Z., Eils, R. and Schlesner, M. (2016) Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*. **32**, 2847–2849.

Gutleben, J., Loureiro, C., Ramírez Romero, L.A., Shetty, S., Wijffels, R.H., Smidt, H. and Sipkema, D. (2020) Cultivation of Bacteria From Aplysina aerophoba: Effects of Oxygen and Nutrient Gradients. *Front Microbiol*. **11**.

Haber, M. and Ilan, M. (2014) Diversity and antibacterial activity of bacteria cultured from Mediterranean *Axinella* spp. sponges. *J Appl Microbiol*. **116**, 519–532.

Heindl, H. and Thiel, V. (2012) Bacterial isolates from the bryozoan Membranipora membranacea: influence of culture media on isolation and antimicrobial activity. *Int Microbiol*. 17–32.

Hentschel, U., Piel, J., Degnan, S.M. and Taylor, M.W. (2012) Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol*. **10**, 641–654.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C. and Hacker, J. (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges Aplysina aerophoba and Aplysina cavernicola. *FEMS Microbiol Ecol*. **35**, 305–312.

Hover, B.M., Kim, S.-H., Katz, M., Charlop-Powers, Z., Owen, J.G., Ternei, M.A., Maniko, J., Estrela, A.B., Molina, H., Park, S., Perlin, D.S. and Brady, S.F. (2018) Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nat Microbiol*. **3**, 415–422.

Accepted Article

This article is protected by copyright. All rights reserved

Imhoff, J.F. and Stöhr, R. (2003) Sponge-Associated Bacteria: General Overview and Special Aspects of Bacteria Associated with Halichondria panicea. In *Sponges (Porifera)*, , Progress in Molecular and Subcellular Biology ed. Müller, W.E.G. pp.35–57. Berlin, Heidelberg: Springer Berlin Heidelberg.

Indraningrat, A., Smidt, H. and Sipkema, D. (2016) Bioprospecting Sponge-Associated Microbes for Antimicrobial Compounds. *Mar Drugs*. **14**, 87.

Kennedy, J., Baker, P., Piper, C., Cotter, P.D., Walsh, M., Mooij, M.J., Bourke, M.B., Rea, M.C., O'Connor, P.M., Ross, R.P., Hill, C., O'Gara, F., Marchesi, J.R. and Dobson, A.D.W. (2009) Isolation and Analysis of Bacteria with Antimicrobial Activities from the Marine Sponge Haliclona simulans Collected from Irish Waters. *Mar Biotechnol*. **11**, 384–396.

Laport, M.S. (2017) Isolating Bacteria from Sponges: Why and How? *Curr Pharm Biotechnol*. **18**, 1224–1236.

Laport, M.S., Bauwens, M., de Oliveira Nunes, S., Willenz, P., George, I. and Muricy, G. (2017) Culturable bacterial communities associated to Brazilian Oscarella species (Porifera: Homoscleromorpha) and their antagonistic interactions. *Antonie Van Leeuwenhoek*. **110**, 489–499.

Laport, M.S., Pinheiro, U. and Rachid, C.T.C. da C. (2019) Freshwater Sponge Tubella variabilis Presents Richer Microbiota Than Marine Sponge Species. *Front Microbiol*. **10**.

Laport, M.S., Santos, O.C.S. and Muricy, G. (2009) Marine Sponges: Potential Sources of New Antimicrobial Drugs. *Curr Pharm Biotechnol*. 21.

Lavy, A., Keren, R., Haber, M., Schwartz, I. and Ilan, M. (2014) Implementing sponge physiological and genomic information to enhance the diversity of its culturable associated bacteria. *FEMS Microbiol Ecol*. **87**, 486–502.

Lee, N.-K., Han, E.J., Han, K.J. and Paik, H.-D. (2013) Antimicrobial Effect of Bacteriocin KU24 Produced by Lactococcus lactis KU24 against Methicillin-Resistant Staphylococcus aureus. *J Food Sci*. **78**, M465–M469.

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schäberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R.,

This article is protected by copyright. All rights reserved

Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C. and Lewis, K. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature*. **517**, 455–459.

Loh, T.-L. and Pawlik, J.R. (2014) Chemical defenses and resource trade-offs structure sponge communities on Caribbean coral reefs. *Proc Natl Acad Sci*. **111**, 4151–4156.

Margassery, L.M., Kennedy, J., O'Gara, F., Dobson, A.D. and Morrissey, J.P. (2012) Diversity and antibacterial activity of bacteria isolated from the coastal marine sponges Amphilectus fucorum and Eurypon major: Diversity and antibacterial activity of bacteria. *Lett Appl Microbiol*. **55**, 2–8.

Marinho, P.R., Moreira, A.P.B., Pellegrino, F.L.P.C., Muricy, G., Bastos, M. do C. de F., Santos, K.R.N. dos, Giambiagi-deMarval, M. and Laport, M.S. (2009) Marine Pseudomonas putida: a potential source of antimicrobial substances against antibiotic-resistant bacteria. *Mem Inst Oswaldo Cruz*. **104**, 678–682.

Matobole, R., van Zyl, L., Parker-Nance, S., Davies-Coleman, M. and Trindade, M. (2017) Antibacterial Activities of Bacteria Isolated from the Marine Sponges Isodictya compressa and Higginsia bidentifera Collected from Algoa Bay, South Africa. *Mar Drugs*. **15**, 47.

Matuschek, E., Brown, D.F.J. and Kahlmeter, G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect*. **20**, O255–O266.

O' Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O' Gara, F. and Dobson, A.D.W. (2011) Diversity and antimicrobial activity of Pseudovibrio spp. from Irish marine sponges: Pseudovibrio spp. with antimicrobial activity. *J Appl Microbiol*. **110**, 1495–1508.

Okuda, K., Zendo, T., Sugimoto, S., Iwase, T., Tajima, A., Yamada, S., Sonomoto, K. and Mizunoe, Y. (2013) Effects of Bacteriocins on Methicillin-Resistant Staphylococcus aureus Biofilm. *Antimicrob Agents Chemother*. **57**, 5572–5579.

Pang, Z., Raudonis, R., Glick, B.R., Lin, T.-J. and Cheng, Z. (2019) Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol Adv*. **37**, 177–192.

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Papaleo, M.C., Fondi, M., Maida, I., Perrin, E., Lo Giudice, A., Michaud, L., Mangano, S., Bartolucci, G., Romoli, R. and Fani, R. (2012) Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against Burkholderia cepacia complex bacteria. *Biotechnol Adv*. **30**, 272–293.

Paradis, E. and Schliep, K. (2019) ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*. **35**, 526–528.

Perovic, S., Wichels, A., Schütt, C., Gerdts, G., Pahler, S., Steffen, R. and Müller, W.E.G. (1998) Neuroactive compounds produced by bacteria from the marine sponge Halichondria panicea: activation of the neuronal NMDA receptor. *Environ Toxicol Pharmacol*. **6**, 125–133.

Pita, L., Rix, L., Slaby, B.M., Franke, A. and Hentschel, U. (2018) The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome*. **6**.

Pollitt, E.J.G., Szkuta, P.T., Burns, N. and Foster, S.J. (2018) Staphylococcus aureus infection dynamics. *PLOS Pathog*. **14**, e1007112.

Purrello, S.M., Garau, J., Giamarellos, E., Mazzei, T., Pea, F., Soriano, A. and Stefani, S. (2016) Methicillin-resistant Staphylococcus aureus infections: A review of the currently available treatment options. *J Glob Antimicrob Resist*. **7**, 178–186.

Rajasabapathy, R., Ghadi, S.C., Manikandan, B., Mohandass, C., Surendran, A., Dastager, S.G., Meena, R.M. and James, R.A. (2020) Antimicrobial profiling of coral reef and sponge associated bacteria from southeast coast of India. *Microb Pathog*. **141**, 103972.

Santos, J.D., Vitorino, I., De la Cruz, M., Díaz, C., Cautain, B., Annang, F., Pérez-Moreno, G., Gonzalez Martinez, I., Tormo, J.R., Martín, J.M., Urbatzka, R., Vicente, F.M. and Lage, O.M. (2019) Bioactivities and Extract Dereplication of Actinomycetales Isolated From Marine Sponges. *Front Microbiol*. **10**.

Santos, O.C.S., Pontes, P.V.M.L., Santos, J.F.M., Muricy, G., Giambiagi-deMarval, M. and Laport, M.S. (2010) Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Res Microbiol*. **161**, 604–612.

This article is protected by copyright. All rights reserved

Schneemann, I., Nagel, K., Kajahn, I., Labes, A., Wiese, J. and Imhoff, J.F. (2010) Comprehensive Investigation of Marine Actinobacteria Associated with the Sponge Halichondria panicea. *Appl Environ Microbiol*. **76**, 3702–3714.

Sipkema, D., Schippers, K., Maalcke, W.J., Yang, Y., Salim, S. and Blanch, H.W. (2011) Multiple Approaches To Enhance the Cultivability of Bacteria Associated with the Marine Sponge *Haliclona* (*gellius*) sp. *Appl Environ Microbiol*. **77**, 2130–2140.

Stefani, S., Campanile, F., Santagati, M., Mezzatesta, M.L., Cafiso, V. and Pacini, G. (2015) Insights and clinical perspectives of daptomycin resistance in Staphylococcus aureus: A review of the available evidence. *Int J Antimicrob Agents*. **46**, 278–289.

Stelling, J. and O'Brien, T.F. (2016) WHONET: Software for Surveillance of Infecting Microbes and Their Resistance to Antimicrobial Agents. In *Molecular Microbiology*. pp.692–706. John Wiley & Sons, Ltd.

Stokes, J.M., Yang, K., Swanson, K., Jin, W., Cubillos-Ruiz, A., Donghia, N.M., MacNair, C.R., French, S., Carfrae, L.A., Bloom-Ackermann, Z., Tran, V.M., Chiappino-Pepe, A., Badran, A.H., Andrews, I.W., Chory, E.J., Church, G.M., Brown, E.D., Jaakkola, T.S., Barzilay, R. and Collins, J.J. (2020) A Deep Learning Approach to Antibiotic Discovery. *Cell*. **180**, 688-702.e13.

Tacconelli, E. and Magrini, N. (2017) Global Priority List of Antibiotic-Resistant Bacteria to Fuide Research, Discovery, and Development of New Antibiotics. *World Health Organ*. 7.

Thomas, T., Moitinho-Silva, L., Lurgi, M., Björk, J.R., Easson, C., Astudillo-García, C., Olson, J.B., Erwin, P.M., López-Legentil, S., Luter, H., Chaves-Fonnegra, A., Costa, R., Schupp, P.J., Steindler, L., Erpenbeck, D., Gilbert, J., Knight, R., Ackermann, G., Victor Lopez, J., Taylor, M.W., Thacker, R.W., Montoya, J.M., Hentschel, U. and Webster, N.S. (2016) Diversity, structure and convergent evolution of the global sponge microbiome. *Nat Commun*. **7**, 11870.

Thomas, T.R.A., Kavlekar, D.P. and LokaBharathi, P.A. (2010) Marine Drugs from Sponge-Microbe Association—A Review. *Mar Drugs*. **8**, 1417–1468.

Toth, G. and Lindeborg, M. (2008) Water-soluble compounds from the breadcrumb sponge Halichondria panicea deter attachment of the barnacle Balanus improvisus. *Mar Ecol Prog Ser*. **354**, 125–132.

This article is protected by copyright. All rights reserved

Trianto, A., Nirwani, N., Susanti, O., Maesaroh, D.S. and Radjasa, O.K. (2019) The bioactivity of bacterium and fungi living associate with the sponge Reniera sp. against multidrug-resistant Staphylococcus aureus and Escherichia coli. *Biodiversitas J Biol Divers*. **20**.

Turner, N.A., Sharma-Kuinkel, B.K., Maskarinec, S.A., Eichenberger, E.M., Shah, P.P., Carugati, M., Holland, T.L. and Fowler, V.G. (2019) Methicillin-resistant Staphylococcus aureus: an overview of basic and clinical research. *Nat Rev Microbiol*. **17**, 203–218.

Turner, T.L. (2020) *The marine sponge* Hymeniacidon perlevis *is a globally-distributed invasive species* (preprint). Zoology.

Versluis, D., McPherson, K., van Passel, M.W.J., Smidt, H. and Sipkema, D. (2017) Recovery of Previously Uncultured Bacterial Genera from Three Mediterranean Sponges. *Mar Biotechnol*. **19**, 454–468.

Wang, H., Butt, L., Rooks, P., Khan, F., Allen, M.J. and Ali, S.T. (2016) Characterisation of algicidal bacterial exometabolites against the lipid-accumulating diatom Skeletonema sp. *Algal Res*. **13**, 1–6.

Webster, N.S. (2007) Sponge disease: a global threat? *Environ Microbiol*. **9**, 1363–1375.

Webster, N.S. and Hill, R.T. (2001) The culturable microbial community of the Great Barrier Reef sponge Rhopaloeides odorabile is dominated by an a-Proteobacterium. *Mar Biol*. 843–851.

Webster, N.S., Wilson, K.J., Blackall, L.L. and Hill, R.T. (2001) Phylogenetic Diversity of Bacteria Associated with the Marine Sponge Rhopaloeides odorabile. *APPL Env MICROBIOL*. **67**, 11.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. **173**, 697–703.

Wicke, C., Hüners, M., Wray, V., Nimtz, M., Bilitewski, U. and Lang, S. (2000) Production and Structure Elucidation of Glycoglycerolipids from a Marine Sponge-Associated *Microbacterium* Species. *J Nat Prod*. **63**, 621–626.

Wohlleben, W., Mast, Y., Stegmann, E. and Ziemert, N. (2016) Antibiotic drug discovery. *Microb Biotechnol*. **9**, 541–548.

Xi, L., Ruan, J. and Huang, Y. (2012) Diversity and Biosynthetic Potential of Culturable Actinomycetes Associated with Marine Sponges in the China Seas. *Int J Mol Sci*. **13**, 5917–5932.

Accepted Article

This article is protected by copyright. All rights reserved

Yu, G. (2020) Using ggtree to Visualize Data on Tree-Like Structures. *Curr Protoc Bioinforma*. **69**.

Zeng, Z., Zhao, J., Ke, C. and Wang, D. (2013) Antimicrobial activities of novel cultivable bacteria isolated from marine sponge Tedania anhelans. *Chin J Oceanol Limnol*. **31**, 581–590.

Zhang, W., Zhang, F., Li, Z., Miao, X., Meng, Q. and Zhang, X. (2009) Investigation of bacteria with polyketide synthase genes and antimicrobial activity isolated from South China Sea sponges. *J Appl Microbiol*. **107**, 567–575.

Zheng, L., Chen, H., Han, X., Lin, W. and Yan, X. (2005) Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge Hymeniacidon perleve. *World J Microbiol Biotechnol*. **21**, 201–206.

Authors contribution statement

ARJ, LG and IG performed sampling and PW identified the sponges. ARJ, ED, AG, LG and MSL carried out the experiments. SF provided the collection of clinical *S. aureus* isolates and BSL-2 lab to cultivate them. ARJ analysed the data and wrote the manuscript with the assistance of IG. All authors revised the manuscript.

Figure 1. Maximum-likelihood phylogenetic tree of 16S rRNA fragments of the sponge-associated isolates. The tree tip symbols detail the host sponge species (▲, *Hymeniacidon perlevis* and ●, *Halichondria panicea*) and isolation medium (■, Brain Heart Infusion; ■, Marine; ■, Reasoner's 2; ■, a; ■, b; ■, c; ■, d and ■, e, formulated media detailed in Table 1). Genus-level grouping and labelling is represented on the right side of the tree.

Figure 2. Bacterial genera isolated from the sponge *Hymeniacidon perlevis* with different isolation media. The size of each bubble represents the number of isolates (smallest representing one and biggest representing nine) belonging to the corresponding genus and isolated on the corresponding medium. Commercial media: BHI, Brain Heart Infusion Agar; M, Marine Agar; R2, Reasoner's 2 Agar. For the composition of formulated dilute media "a" to "e", please refer to Table 1.

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Figure 3. Antibacterial activity of sponge bacterial isolates against the reference strains *Staphylococcus aureus* ATCC 29213 (black bar), *Pseudomonas aeruginosa* ATCC 27853 (grey bar) and both (lined bar) represented by the percentage of active isolates of each genus.

Figure 4. Antibacterial activity of marine sponge isolates against a collection of 36 MDR *S. aureus* strains. Only isolates that were active against *S. aureus* ATCC 29213 (reference strain, last column) were tested against MDR *S. aureus* strains. In the heatmap, a white square represents the absence of inhibition of a given isolate (row) against a given test strain (column) and a black one represents the presence of inhibition.

Table 1. Dilute culture media used for isolation of sponge-associated bacterial strains.

Code	Composition
a	20-fold diluted Difco Marine Broth* (1.87g), 20mL sponge extract
b	100mL sponge extract
c	2-fold diluted Difco Marine Broth* (18.7g)
d	20-fold diluted Difco Marine Broth* (1.87g)
e	no Difco Marine Broth, no sponge extract

* compared to the manufacturer's instructions

Volume is adjusted to 1L with natural sea water and 15g agar (VWR) are added into each medium (a to e).

Table 2. Phylogenetic affiliation of sponge bacterial isolates with 16S rRNA gene sequence identity to the Reference RNA database (RefSeq, NCBI) below 97%.

Isolate	Phylum *	Genus *	Closest 16S rRNA gene relative †	% identity ‡
2d	*Actinobacteria*		*Nocardioides iriomotensis* strain IR27-S3	95.78
11d			*Nocardioides iriomotensis* strain IR27-S3	95.99
12d			*Nocardioides iriomotensis* strain IR27-S3	96.43
11M	*Firmicutes*	*Enterococcus*	*Enterococcus faecalis* strain ATCC 19433	96.67
15B2			*Enterococcus faecalis* strain ATCC 19433	95.78

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37B2			*Enterococcus faecalis* strain ATCC 19433	96.42
24B		*Lactococcus*	*Lactococcus lactis subsp. cremoris* strain NBRC 100676	95.76
23R			*Lactococcus lactis* strain NBRC 100933	94.41
25R2			*Lactococcus lactis* strain NBRC 100933	96.14
19M2		*Lysinibacillus*	*Lysinibacillus macroides* strain LMG 18474	95.02
34R2	*Proteobacteria*	*Aeromonas*	*Aeromonas media* strain RM	96.24
13B2		*Citrobacter*	*Citrobacter freundii* ATCC 8090 = MTCC 1658	94.97
18B2			*Citrobacter gillenii* strain CDC 4693-86	95.37
21B2			*Citrobacter gillenii* strain CDC 4693-86	96.42
31B2			*Citrobacter gillenii* strain CDC 4693-86	96.22
32B2			*Citrobacter gillenii* strain CDC 4693-86	95.80
34B2			*Citrobacter gillenii* strain CDC 4693-86	96.43
27R2			*Citrobacter gillenii* strain CDC 4693-86	95.38
211R2		*Enterobacteriaceae spp.*	*Lelliottia amnigena* strain JCM1237	96.79
17R2		*Morganella*	*Morganella morganii* strain NBRC 3848	96.87
13M		*Oceanimonas*	*Oceanimonas smirnovii* strain 31-13	96.70
32M2			*Oceanimonas doudoroffii* strain NBRC 103032	92.84
11B2		*Proteus*	*Proteus terrae* strain N5/687	95.38
11B		*Pseudomonas*	*Pseudomonas qingdaonensis* strain JJ3	94.24
12B			*Pseudomonas oryzihabitans* strain L-1	96.03
34R			*Pseudomonas qingdaonensis* strain JJ3	92.63
36R			*Pseudomonas putida* strain NBRC 14164	95.00
23R2			*Pseudomonas qingdaonensis* strain JJ3	96.25
26R2			*Pseudomonas qingdaonensis* strain JJ3	96.19
31R2			*Pseudomonas qingdaonensis* strain JJ3	96.77

* Based on the SILVA SINA alignment service of the ARB-Silva database (http://www.arb-silva.de/aligner/)

† Determined using the Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology (NCBI) with the Reference RNA sequences (refseq_rna) database

‡ Percent sequence identity between query sequences and closest matches

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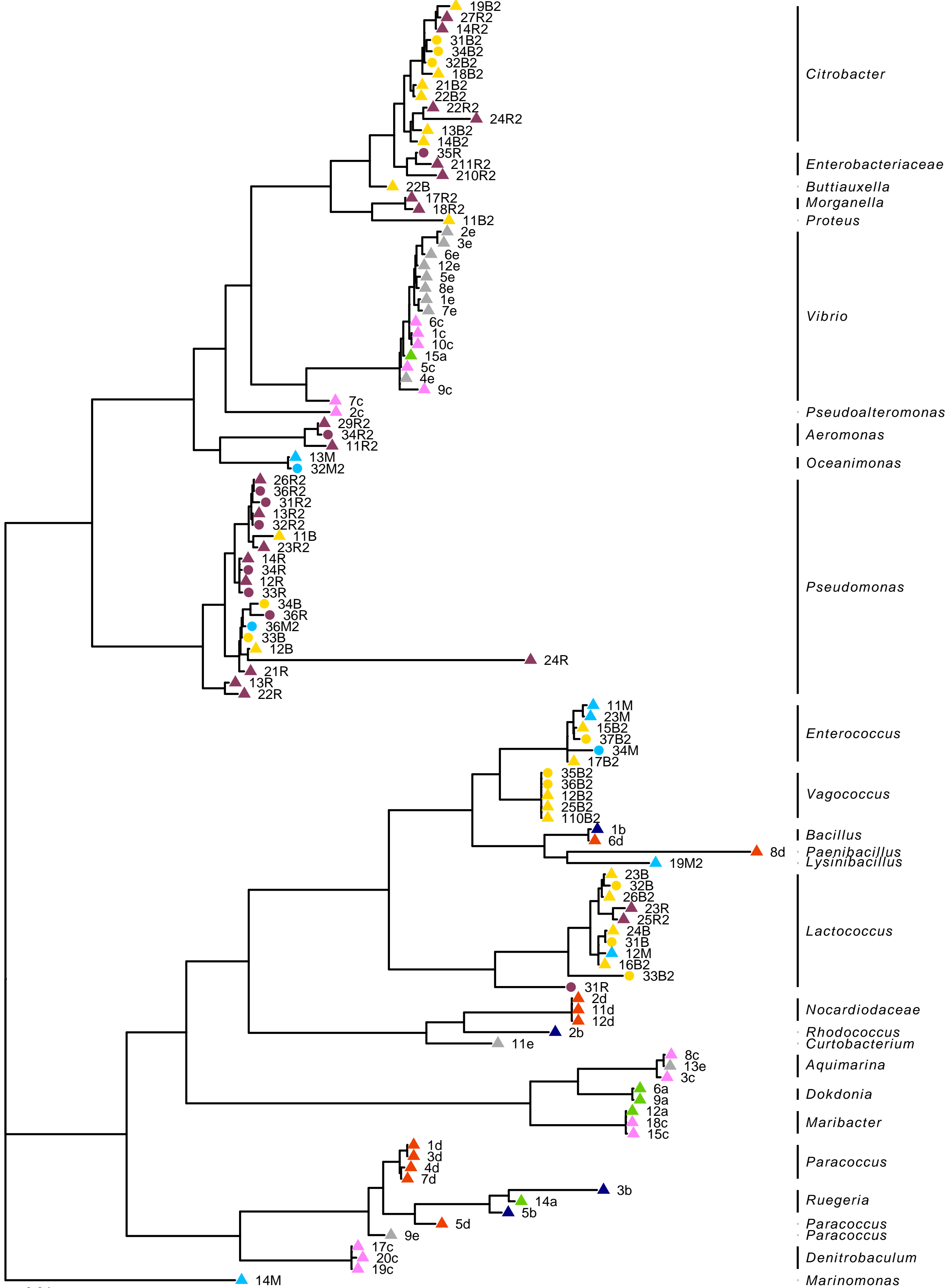
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35R
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Bacillus
Paenibacillus
Lysinibacillus
Lactococcus
Nocardiodaceae
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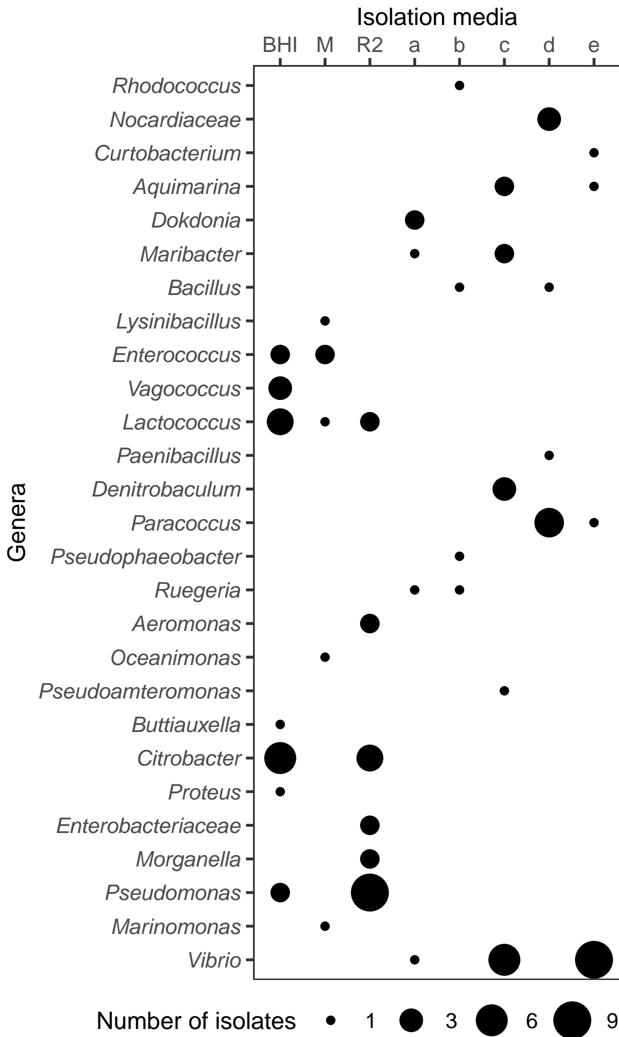
Isolation media
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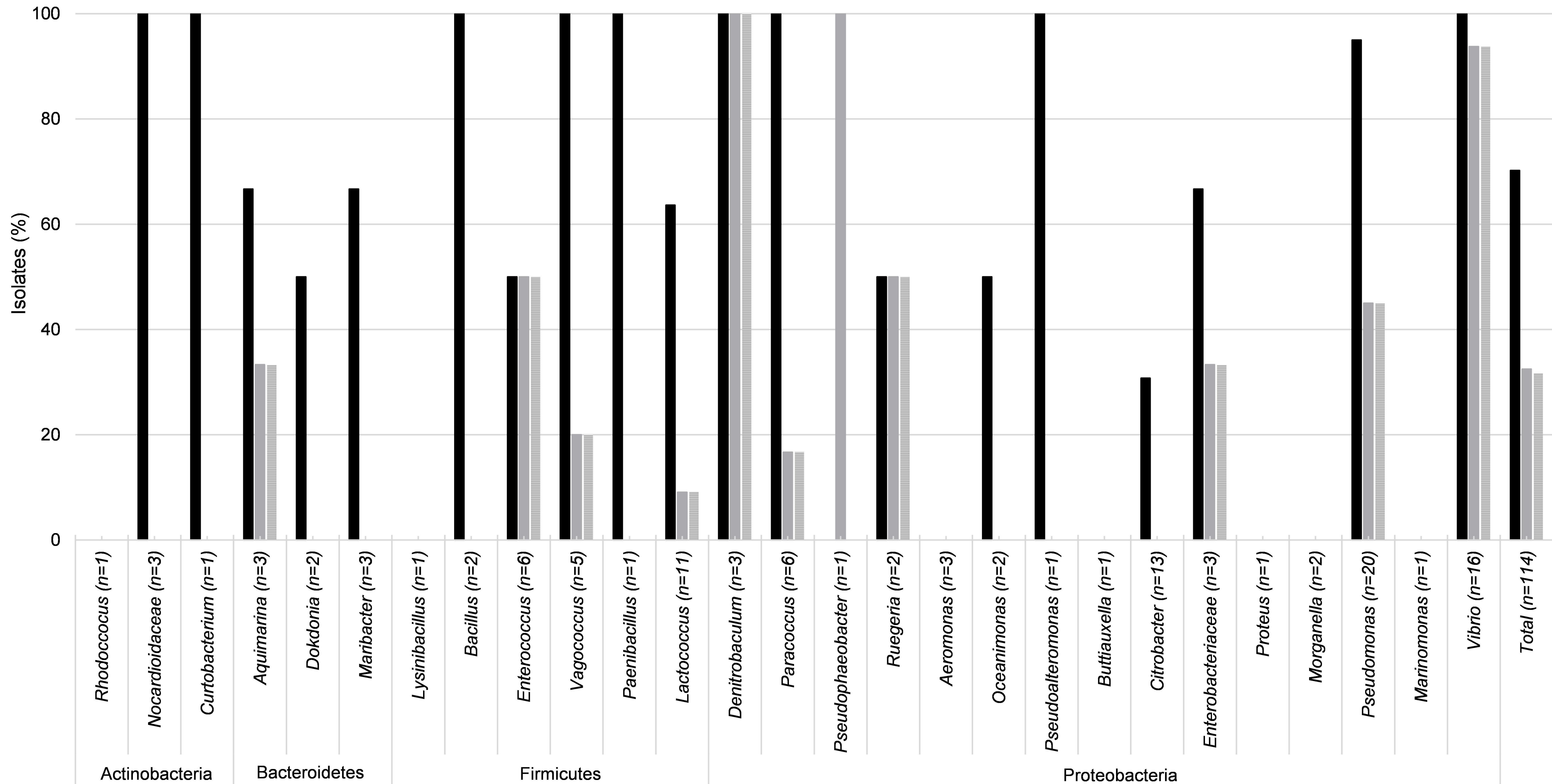
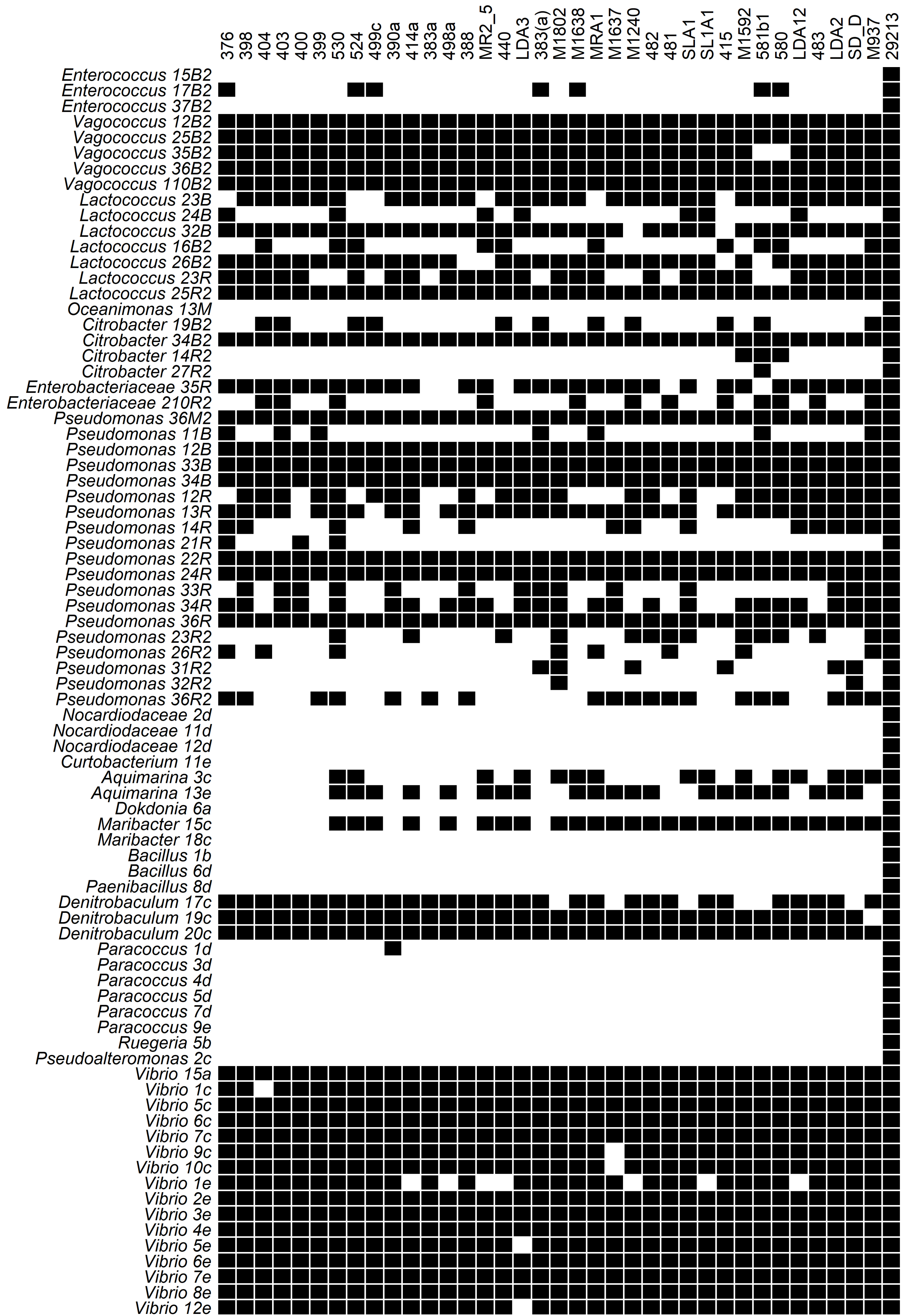
Nocardiaceae
Curtobacterium
Aquimarina
Dokdonia
Maribacter
Bacillus
Lysinibacillus
Enterococcus
Vagococcus
Lactococcus
Paenibacillus
Denitrobaculum
Paracoccus
Pseudophaeobacter
Ruegeria
Aeromonas
Oceanimonas
Pseudoamteromonas
Buttiauxella
Citrobacter
Proteus
Enterobacteriaceae
Morganella
Pseudomonas
Marinomonas
Vibrio
Number of isolates
1
3
6
9



Isolates (%)
0
20
40
60
80
100
Rhodococcus (n=1)
Nocardioidaceae (n=3)
Curtobacterium (n=1)
Aquimarina (n=3)
Dokdonia (n=2)
Maribacter (n=3)
Lysinibacillus (n=1)
Bacillus (n=2)
Enterococcus (n=6)
Vagococcus (n=5)
Paenibacillus (n=1)
Lactococcus (n=11)
Denitrobaculum (n=3)
Paracoccus (n=6)
Pseudophaeobacter (n=1)
Ruegeria (n=2)
Aeromonas (n=3)
Oceanimonas (n=2)
Pseudoalteromonas (n=1)
Buttiauxella (n=1)
Citrobacter (n=13)
Enterobacteriaceae (n=3)
Proteus (n=1)
Morganella (n=2)
Pseudomonas (n=20)
Marinomonas (n=1)
Vibrio (n=16)
Total (n=114)
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria



S. aureus strains
376
398
404
403
400
399
530
524
499c
390a
414a
383a
498a
388
MR2_5
440
LDA3
383(a)
M1802
M1638
MRA1
M1637
M1240
482
481
SLA1
SL1A1
415
M1592
581b1
580
LDA12
483
LDA2
SD_D
M937
29213
Sponge isolates
Enterococcus 15B2
Enterococcus 17B2
Enterococcus 37B2
Vagococcus 12B2
Vagococcus 25B2
Vagococcus 35B2
Vagococcus 36B2
Vagococcus 110B2
Lactococcus 23B
Lactococcus 24B
Lactococcus 32B
Lactococcus 16B2
Lactococcus 26B2
Lactococcus 23R
Lactococcus 25R2
Oceanimonas 13M
Citrobacter 19B2
Citrobacter 34B2
Citrobacter 14R2
Citrobacter 27R2
Enterobacteriaceae 35R
Enterobacteriaceae 210R2
Pseudomonas 36M2
Pseudomonas 11B
Pseudomonas 12B
Pseudomonas 33B
Pseudomonas 34B
Pseudomonas 12R
Pseudomonas 13R
Pseudomonas 14R
Pseudomonas 21R
Pseudomonas 22R
Pseudomonas 24R
Pseudomonas 33R
Pseudomonas 34R
Pseudomonas 36R
Pseudomonas 23R2
Pseudomonas 26R2
Pseudomonas 31R2
Pseudomonas 32R2
Pseudomonas 36R2
Nocardiodaceae 2d
Nocardiodaceae 11d
Nocardiodaceae 12d
Curtobacterium 11e
Aquimarina 3c
Aquimarina 13e
Dokdonia 6a
Maribacter 15c
Maribacter 18c
Bacillus 1b
Bacillus 6d
Paenibacillus 8d
Denitrobaculum 17c
Denitrobaculum 19c
Denitrobaculum 20c
Paracoccus 1d
Paracoccus 3d
Paracoccus 4d
Paracoccus 5d
Paracoccus 7d
Paracoccus 9e
Ruegeria 5b
Pseudoalteromonas 2c
Vibrio 15a
Vibrio 1c
Vibrio 5c
Vibrio 6c
Vibrio 7c
Vibrio 9c
Vibrio 10c
Vibrio 1e
Vibrio 2e
Vibrio 3e
Vibrio 4e
Vibrio 5e
Vibrio 6e
Vibrio 7e
Vibrio 8e
Vibrio 12e
Antibacterial activity
Negative
Positive