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

A. Rodriguez Jimenez¹,², E. Dechamps¹, A. Giaux¹, L. Goetghebuer³, M. Bauwens³, P. Willenz³,⁴, S. Flahaut⁵, M.S. Laport⁶ and I.F. George¹,³

1 Ecology of Aquatic Systems, Université Libre de Bruxelles, Bruxelles, Belgique
2 Ecological and Evolutionary Genomics, Université Libre de Bruxelles, Bruxelles, Belgique
3 Marine Biology, Université Libre de Bruxelles, Bruxelles, Belgique
4 Taxonomy & Phylogeny, Royal Belgian Institute of Natural Sciences, Bruxelles, Belgique
5 Applied Microbiology, Université Libre de Bruxelles, Bruxelles, Belgique
6 Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

**Keywords**
- antibiotics, antimicrobials, environmental, resistance, staphylococci.

**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 nonsusceptible 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 the Study:** This study was the first to explore the antibacterial potential of cultivable 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 nonsusceptible and methicillin-resistant ones, against which new antibiotics are urgently needed.

**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 SCCmec (*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*
Sponge antibiotic-producing bacteria

A.R. Jimenez et al.

(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).

Although resistance to most antibiotic 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 antibiotic 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 (Taconelli 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 micro-organisms (Thomas et al. 2010; Indraningrat et al. 2016). In fact, these micro-organisms are considered as part of the sponge holobiont, that is, 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). 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 2017). Previous studies have reported that percentages of culturable strains 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; Guteleben 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).

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; Imhof 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 multidrug-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°36’000E), located in Nord-Pas-de-Calais, France, on 20 October 2013. A specimen of H. perlevis was collected at low tide on the rocks of the beach of Audresselles (50°49’24” N–1°36’000E) located in Nord-Pas-de-Calais, France, on 15 April 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.

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, Sparks, MD, USA), Reasoner’s 2A Broth (R2, Melford, Ipswich, UK) and Marine Broth (M; Difco BD, Sparks, MD, USA) during 2 and 8 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 on 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⁻¹ 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.

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

<table>
<thead>
<tr>
<th>Code</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>20-fold diluted Difco Marine Broth* (1.87 g), 20 ml sponge extract</td>
</tr>
<tr>
<td>b</td>
<td>100 ml sponge extract</td>
</tr>
<tr>
<td>c</td>
<td>Twofold diluted Difco Marine Broth* (18.7 g)</td>
</tr>
<tr>
<td>d</td>
<td>20-fold diluted Difco Marine Broth* (1.87 g)</td>
</tr>
<tr>
<td>e</td>
<td>no Difco Marine Broth, no sponge extract</td>
</tr>
</tbody>
</table>

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

*Compared to the manufacturer’s instructions.

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, Madison, WI, USA) and 20 pmol of each universal primer, 27F (5’-GAGTTTGTACMTGGCT-CAG-3’) and 1492R (5’-TACCCTTGTAGGTACCTTAC-GACTT-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, Hilden, Germany) 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 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 ver. 2.0.4 (Yu 2020) and ape ver. 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 Plahaut, 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 ver. 6.0 (2017) on Mueller-Hinton Agar (Merck,
Darmstadt, Germany). The S. aureus ATCC 29213 strain was used as quality control following EUCAST (Matuschek et al. 2014). The following antimicrobial discs (Bio-rad, Marnes-la-Coquette, France) were used: amikacin (30 µg), cefotixin (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 (1-25–23-75 µg). Susceptibility to teicoplanin and vancomycin (Sigma-Aldrich, St. Louis, MO, USA) was tested following the microdilution method for minimal inhibitory concentration determination in Mueller-Hinton Broth (Merck) with a concentration range from 64 to 0-25 µg ml{}^{-1}. The results were interpreted using WHONET ver. 20.12.8 (Stelling and O’Brien 2016) with the EUCAST Clinical Breakpoint Tables v.11-0 (2020) as reference. Multidrug resistance (MDR) was defined as acquired nonsusceptibility to at least one drug out of three or more antimicrobial categories (Margassery 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 cefotixin resistance), 15 vancomycin non-susceptible strains and six strains resistant to both compounds (383a, 399, 400, 403, 498a and MR25). A heatmap with dendrograms (Fig. S1) was plotted on R ver. 3.6.3 using the ComplexHeatmap package ver. 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 millilitres of stationary-phase cultures (about 10^9 CFU) of each bacterial isolate were spotted onto agar plates and incubated at 25°C until the colony diameter reached 5–8 mm (24–48 h). The bacteria were then killed by exposure to chloroform (VEL, Leuven) vapour 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).

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 (Fig. 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 (Fig. 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 Vibrionaceae (16 isolates, 35-5%), followed by Paracoccus (six isolates, 13-3%). A total of 13 different genera were recovered using dilute media (Fig. 1).

In total, 91 isolates were recovered from H. perlevis specimens using eight nutritional media (Fig. 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).
Table 2 Phylogenetic affiliation of sponge bacterial isolates with 16S rRNA gene sequence identity to the Reference RNA database (RefSeq, NCBI) below 97%

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Phylum*</th>
<th>Genus*</th>
<th>Closest 16S rRNA gene relative†</th>
<th>% identity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>2d</td>
<td>Actinobacteria</td>
<td>Nocardioides iriomotensis</td>
<td>Nocardioides iriomotensis strain IR27-53</td>
<td>95.78</td>
</tr>
<tr>
<td>11d</td>
<td>Nocardioides iriomotensis</td>
<td>Enterococcus Enterococcus faecalis strain ATCC 19433</td>
<td>Enterococcus faecalis strain ATCC 19433</td>
<td>96.67</td>
</tr>
<tr>
<td>12d</td>
<td>Nocardioides iriomotensis</td>
<td>Enterococcus Enterococcus faecalis strain ATCC 19433</td>
<td>Enterococcus faecalis strain ATCC 19433</td>
<td>95.78</td>
</tr>
<tr>
<td>11M</td>
<td>Firmicutes</td>
<td>Enterococcus Enterococcus faecalis strain ATCC 19433</td>
<td>Enterococcus faecalis strain ATCC 19433</td>
<td>96.42</td>
</tr>
<tr>
<td>15B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis subsp. cremoris strain NBRC 100676</td>
<td>95.76</td>
</tr>
<tr>
<td>37B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>94.41</td>
</tr>
<tr>
<td>24B</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.14</td>
</tr>
<tr>
<td>23R</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>95.02</td>
</tr>
<tr>
<td>19M2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>34R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>97.79</td>
</tr>
<tr>
<td>13B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.79</td>
</tr>
<tr>
<td>18B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>21B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>31B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>34B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>27R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>211R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>17R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>13M</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>32M2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>11B2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>11B</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>128</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>34R</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>36R</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>23R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>26R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
<tr>
<td>31R2</td>
<td>Firmicutes</td>
<td>Lactococcus</td>
<td>Lactococcus lactis strain NBRC 100933</td>
<td>96.24</td>
</tr>
</tbody>
</table>

*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.

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 (Fig. 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.

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 (Fig. S1).

Sixty-one of these strains (76-3%) inhibited at least one hospital-isolated *S. aureus* strain (Fig. 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.
Sponge antibiotic-producing bacteria

A.R. Jimenez et al.

Citrobacter
Enterobacteriaceae
Buttiauxella
Morganella
Proteus
Vibrio

Pseudoalteromonas
Aeromonas
Oceanimonas

Pseudomonas

Enterococcus
Vagococcus

Bacillus
Paenibacillus
Lysinibacillus

Lactococcus

Nocardioidaceae
Rhodococcus
Curtobacterium
Aquimarina
Dokdonia
Marinobacter
Paracoccus
Ruegeria
Paracoccus
Paracoccus
Denitrabaculum
Marinomonas
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 nonsusceptible 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 (Fig. 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 (Fig. S2). Our results (Fig. 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).

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%) (Fig. 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 glyco-glycerolipids (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 \textit{S. aureus} (70-2\%) than against \textit{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 \textit{S. aureus} and \textit{P. aeruginosa} strains, exhibiting a broader spectrum of antibacterial action (Fig. 3). All isolates affiliated to the genus \textit{Denitrobacterium} and most of those affiliated to \textit{Vibrio} exhibited such antibacterial activity. Some isolates of other genera, such as \textit{Pseudomonas}, \textit{Enterococcus}, \textit{Lactococcus} or \textit{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.

The percentage (70-2\%) of sponge isolates inhibiting the growth of antimicrobial-susceptible \textit{S. aureus} ATCC 29213 is also remarkable. A review of the literature on the antibacterial activity of sponge bacteria against \textit{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 \textit{S. aureus} was found (74\%), but it exclusively focused on \textit{Pseudovibrio} isolates (O’ Halloran et al. 2011).

Activity of the isolates was therefore further assessed against a collection of 36 \textit{S. aureus} strains isolated from hospital infections. To the best of our knowledge, this study uses the largest panel of antibiotic-resistant \textit{S. aureus}. 

\begin{itemize}
  \item Figure 3 Antibacterial activity of sponge bacterial isolates against the reference strains \textit{Staphylococcus aureus} ATCC 29213 (black bar), \textit{Pseudomonas aeruginosa} ATCC 27853 (grey bar) and both (lined bar) represented by the percentage of active isolates of each genus.
\end{itemize}
Figure 4 Antibacterial activity of marine sponge isolates against a collection of 36 MDR Staphylococcus 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.
Sponge antibiotic-producing bacteria

A.R. Jimenez et al.

S. aureus clinical strains to determine sponge-bacteria inhibitory activities. As observed in Fig. 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 (Fig. 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 analysing 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 Halichondria 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 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 (Plemner 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.

Acknowledgements

ARJ benefited from a PhD grant from the Fonds de la Recherche dans l’Industrie et l’Agriculture (FRIA) of the Fonds de la Recherche Scientifique (FNRS). The authors thank Science Without Borders, a CNPq Program for the post doctorate scholarship to MSL.

Conflict of Interest

No conflict of interest declared.

Authors contribution

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 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.

References


Sponge antibiotic-producing bacteria


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Phylogenetic affiliation, GenBank Accession Number, and activity against reference strains of bacterial isolates obtained from the sponges *H. panicea* and *H. perlevis*.

**Figure S1.** Susceptibility profiles of the 37 *Staphylococcus aureus* strains tested in this study.

**Figure S2.** Taxonomic distribution, at the phylum level, of the *H. panicea* and *H. perlevis* bacterial microbiomes.