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Abstract

Global antimicrobial resistance is a health crisis that can change the face of modern medicine. Exploring diverse natural habitats for bacterially-derived novel antimicrobial compounds has historically been a successful strategy. The deep-sea presents an exciting opportunity for the cultivation of taxonomically novel organisms and exploring potentially chemically novel spaces. In this study, the draft genomes of 12 bacteria previously isolated from the deep-sea sponges *Phenomena carpenteri* and *Hertwigia* sp. are investigated for the diversity of specialized secondary metabolites. In addition, early data support the production of antibacterial inhibitory substances produced from a number of these strains, including activity against clinically relevant pathogens *Acinetobacter baumannii, Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Draft whole-genomes are presented of 12 deep-sea isolates, which include four potentially novel strains: *Psychrobacter* sp. PP-21, *Streptomyces* sp. DK15, *Dietzia* sp. PP-33, and *Micrococcus* sp. M4NT. Across the 12 draft genomes, 138 biosynthetic gene clusters were detected, of which over half displayed less than 50% similarity to known BGCs, suggesting that these genomes present an exciting opportunity to elucidate novel secondary metabolites. Exploring bacterial isolates belonging to the phylum Actinomycetota, Pseudomonadota and Bacillota from understudied deep-sea sponges provided opportunities to search for new chemical diversity of interest to those working in antibiotic discovery.

Introduction

In the year 2019 there was an estimated 4.95 million deaths associated with bacterial antimicrobial resistance (AMR) (Murray *et al.* 2022), and it is predicted that by 2050 there will be 10 million annual deaths (O'Neill, 2014). As such, there needs to be considerable emphasis on mobilizing global initiatives to tackle AMR. One strategy for addressing this global health crisis is the discovery and development of new antimicrobials. Marine microbes isolated from invertebrates are well recognized as a promising source of novel pharmaceuticals, including those with antimicrobial properties. Marine sponges, in particular, are prolific sources of novel chemistry (Piel 2004). Microbes from all domains of life (Bacteria, Archaea, Eukarya and Viral) are known to associate with sponges (Li *et al.* 2016; Pascelli *et al.* 2020). Sponges form intimate associations with symbionts and associated bacteria, which contributes to holobiont metabolism. Functions include nutrient cycling, vitamin production and defence compound production (Pita *et al.* 2018). Marine sponge research has intensified over the past decade due to the potential of sponges and their associated microbes to produce secondary metabolites relevant to industry and medicine (Piel 2004; Anjum *et al.* 2016). In addition, secondary metabolites from shallow water sponges have found uses in medicine, including compounds with antimicrobial, anti-inflammatory or anti-tumour activity, muscle relaxants and cardiovascular agents, to name a few (Anjum *et al.* 2016; He *et al.* 2017).

Marine invertebrates are important reservoirs of natural products (NPs); a metareview of the trends in NP discovery stated a 6.5% increase in the number reported from marine sponges (Porifera) in the past decade (Calado *et al.* 2022). Deep-sea sponges are increasingly being shown to have NP potential similar to that of their shallow-water counterparts, although their recovery from the deep-sea is more difficult meaning they remain less explored than shallow-water sponges (Borchert et al., 2016; Borchert, Knobloch, et al., 2017; Borchert, Selvin, et al., 2017). The deep-sea sponges themselves are a source of novel chemistry, as demonstrated by the identification of an entire family of peptides with proposed anti-fouling activity, the Barrettides, from *Geodia barretti* (Steffen *et al.* 2021). However, it is the microbial symbionts and associated microbes that have garnered the greatest attention so far. The cultured bacteria associated with deep-sea sponges have been reported to be diverse and display a range of antibacterial activity against clinically relevant bacterial pathogens (Xin *et al.* 2011; Xu *et al.* 2018; Williams *et al.* 2020; Back *et al.* 2021). The majority of the bacterial isolates reported belong to the phylum Actinomycetota and include

members of the following genera: *Streptomyces, Dietzia, Rhodococcus*, *Salinispora*, in addition to a novel *Micromonospora* strain (Back *et al.* 2021).

More than 70% of antibiotics approved for clinical use are derived from species within the phylum Actinomycetota (Newman, Cragg and Snader 2003; Newman and Cragg 2012; Seipke 2015; Qin *et al.* 2017), with those belonging to the genus *Streptomyces* possessing genomes that have an abundance and diversity of specialist metabolites that illustrates their incredible therapeutic potential (Seipke 2015; Gavriilidou *et al.* 2022). While less studied than terrestrial species, marine Actinomycetes are a source of novel antimicrobials and are frequently associated with, and cultivable from, marine sponges (Bull and Stach 2007; Marinho *et al.* 2009; Xin *et al.* 2011; Abdelmohsen *et al.* 2014).

The Actinomycetota represent a small fraction of previously reported deep-sea sponge microbiota, while members of the Pseudomonadota are present in high relative abundance (Steinert *et al.* 2020). Despite this low relative abundance, they are often reported in cultivation efforts (Xin *et al.* 2011; Xu *et al.* 2018; Williams *et al.* 2020). While not as historically prolific producers of antibiotics, Pseudomonadota are abundant members of the marine sponge microbiomes (Steinert *et al.* 2020), and likewise are valuable sources of marine-derived antimicrobial peptides (*see review* (Desriac *et al.* 2013).

Novel chemistry with potential relevance to pharmaceuticals appears to be present in sponge microbiomes. A survey of three species of deep-sea demosponges (*Inflatella pellicula*, *Stelletta normani*, and *Poecillastra compressa*) sequencing the subunits of polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) demonstrated the genomic diversity of specialized secondary metabolite (SM) genes present within the deep-sea sponge microbiota (Borchert *et al.* 2016). Elsewhere, culture-independent strategies have been applied successfully in identifying antimicrobials from bacteria recovered from the deep-water species *Lissodendoryx diversichela* and *I. pellicula*. Whole-genome sequencing of 13 sponge-associated *Streptomyces* spp. revealed that two deep-sea strains were enriched in gene clusters encoding NRPS with an overall high abundance of Biosynthetic Gene Clusters (BGCs), while assay-based approaches were used to identify activity against clinically relevant pathogens (Romano *et al.* 2018). More importantly, these BGCs showed little to no homology with those previously reported.

Hexactinellida is a class of marine sponges typically found in deep waters >200 m, with only 5 known shallow-water species (Leys, Mackie and Reiswig 2007), and they

represent 7% of all described members of the phylum Porifera (Hooper and van Soest 2002; Van Soest *et al.* 2012). Compared to other classes of sponges (i.e., Demospongiae), hexactinellid sponges are only occasionally included in microbial studies (Mangano *et al.* 2008; Xin *et al.* 2011; Borchert *et al.* 2017a; Steinert *et al.* 2020). An early indication of a sponge-class specific microbiome may indicate that the hexactinellids are a promising unexplored taxonomic space for antibiotic NP discovery (Koch *et al.* 2021). Antibiotic discovery from deep-sea sponges is still in its early days, and there remains a large selection of taxonomically distinct deep-sea sponges to be explored for this purpose. Already, there is much promise, with novel genomic and chemical diversity reported. In this study, our aims were to sequence and investigate the whole genomes of 12 bacterial strains recovered from two deep-sea sponges from a previous investigation (Koch *et al.* 2021), in addition to generating preliminary data on the range of antimicrobial substances produced by these isolates.

Materials and Methods

Sample collection

Deep-sea sponges were collected from the North Atlantic west of the UK and Ireland as part of the NERC funded Deep Links project research cruise JC136 (2016), and the Sensitive Ecosystem Assessment and ROV Exploration of Reef (SeaRover) RH17001 (2017) and CE19015 (2019) research cruises, jointly funded by the Irish Government and EU (Fig. 1). Samples were collected by Remotely Operated Vehicles (ROVs). Sponges were rinsed with local surface seawater, photographed, placed in ziplock bags, and frozen at -20°C for the remainder of the cruise. Sponges were transported from the research vessel to the University of Galway, Ireland for temporary storage at -20°C. Sponges were finally transported on dry ice to the University of Plymouth for storage at -80°C.
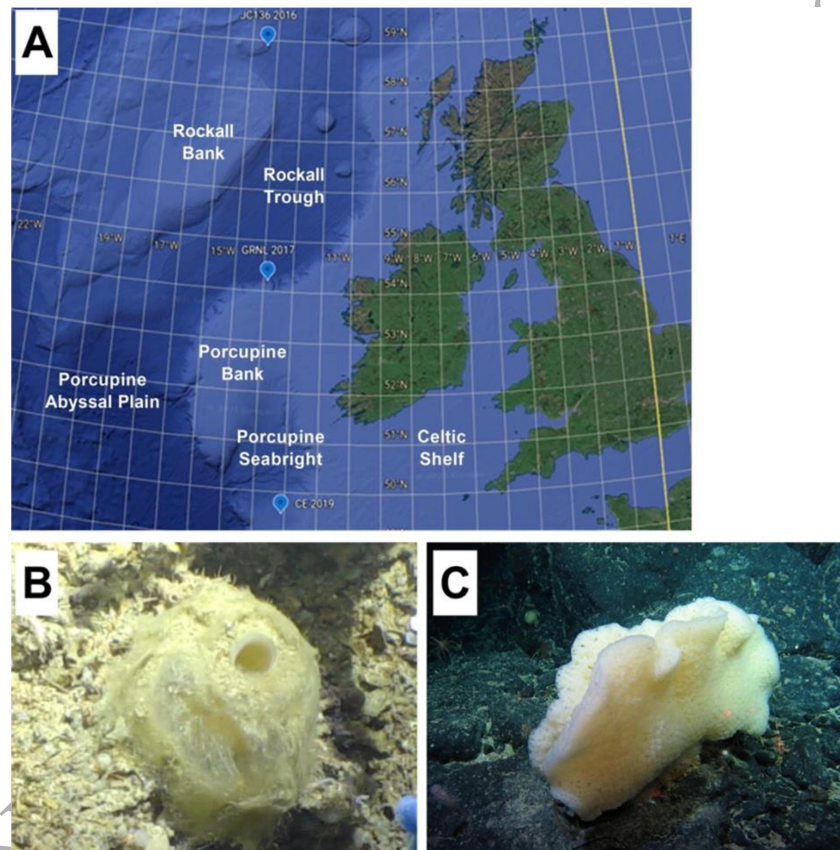

Figure 1. Map of sampling sites and sponges used for bacterial cultivation. (A) Map of deep-sea sampling points for sponges. Blue marker points from top to bottom, sampling sites for Deep Links JC136, SeaRovers RH17001 and SeaRovers CE19015. *In situ* images of deep-sea sponges, (B) *P. carpenteri* JC136_125 (Deep Links), and (C) *Hertwigia* sp.

GRNL_81 (SeaRovers RH17001). Two red laser dots visible in (C) illustrate a 10 cm distance.

Sponges were identified from the analysis of internal and external morphological features (i.e., body shape, type, size, and arrangement of spicules) following the Systema Porifera taxonomic key (Hooper and van Soest 2002). Sponges used in this study collected on the JC136 (2016) and CE19015 (2019) cruises were identified as *Pheronema carpenteri* (order Amphidiscoside, family Pheronematidae) (Table 1, Fig. 1B). While sponges GNRL_81 and GNRL_82 (2017) were provisionally identified as associated with the sponge *Hertwigia falcifera* (order Lyssacinosida, family Euplectellidae) (Fig 1C).

Table 1. Summary of hexactinellid sampling metadata and taxonomy used in cultivation efforts. Name prefix refers to the research vessel during which the sponges were collected: CE, RV Celtic Explorer (CE19015), Republic of Ireland; GRNL, Irish Lights Vessel Granuaile RH17001, Republic of Ireland; JC, RSS James Cook (JC136), United Kingdom.

Sample ID	Sponge identity	Sampling Date	Depth (m)	Coordinates (lat, long)	Cruise report
JC136_125	*P. carpenteri*	16/06/2016	1,051	58.85, -13.39	(Howell *et al.*, 2016)
JC136_134	*P. carpenteri*	16/06/2016	1,054	58.85, -13.39	
JC136_135	*P. carpenteri*	16/06/2016	1,051	58.85, -13.39	
CE_015_09	*P. carpenteri*	13/08/2019	1,209	49.53, -12.09	(O'Sullivan *et al.*, 2019)
CE_015_10	*P. carpenteri*	13/08/2019	1,209	49.53, -12.09	
CE_015_27	*P. carpenteri*	15/08/2019	1,103	50.98, -13.68	
CE_015_29	*P. carpenteri*	15/08/2019	1,103	50.98, -13.68	
GRNL_81	*Hertwigia* sp.	21/07/2017	2,227	54.18, -12.84	(O'Sullivan *et al.*, 2017)
GRNL_82	*Hertwigia* sp.	21/07/2017	2,175	54.18, -12.84	

Draft whole genome sequence generation for sponge-associated bacteria

Recovery of sponge-associated bacteria

The bacterial cultures utilised for the purpose of this study were recovered during a previous study by Koch *et al.*, (2021). This included the cultivation of bacterial isolates in pressurized chambers (5Bar), and utilised various isolation media. In summary, sponge tissue was cut from the sponge body disregarding the outer 1 cm of tissue. Individual tissue segments (approx. 10 g wet weight) were homogenized and transferred to 50 ml centrifuge tubes (Fisher Scientific). Large and un-degradable (i.e. glass spicules) debris was allowed to

settle for 5 minutes and remaining suspended homogenate was transferred to a new 50ml centrifuge tube. Homogenised tissue was centrifuged at 4,696 x *g* for 20 minutes and supernatant was discarded. The resulting pellet was resuspended in 2 ml sterile PBS and 100 µl aliquots used to inoculate agar plates. Bacterial isolates were passaged until visibly pure cultures were achieved. Pure cultures were preserved with Pro-Lab Diagnostics™ Microbank™ Bacterial and Fungal Preservation System tubes for long-term storage at -80°C. A summary of the cultivation condition used for the recovery of each strain is detailed in Supplementary Table S1.

Illumina whole-genome sequencing and assembly by MicrobesNG

DNA extraction and sequencing was outsourced and performed by Microbial Genomics Ltd. (MicrobesNG, UK; https://microbesng.com/). Strains were revived from cryopreservation beads and streaked out on Reasoner's 2 agar (R2a; Oxoid, UK) plates (Reasoner and Geldreich 1985) and incubated for 5 days at 15ºC. Cultures were passaged three times to ensure no contamination was present. Cells were harvested from a streak plate and suspended in a tube with cryoperservative (Microbank™, Pro-Lab Diagnostics UK, United Kingdom) provided by MicrobesNG. Preserved cells were sent to the company where the DNA extraction was performed, as described below.

Five to forty microlitres of the bacterial suspension were lysed with 120 µL of Triss-EDTA buffer containing lysozyme (final concentration 0.1 mg/mL) and RNase A (ITW Reagents, Barcelona, Spain) (final concentration 0.1 mg/mL), incubated for 25 min at 37°C. Proteinase K (VWR Chemicals, Ohio, USA) (final concentration 0.1mg/mL) and SDS (Sigma-Aldrich, Missouri, USA) (final concentration 0.5% v/v) were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer (Qiagen, Germany). DNA was quantified with the Quant-iT dsDNA HS kit (ThermoFisher Scientific) assay in an Eppendorf AF2200 plate reader (Eppendorf UK Ltd, United Kingdom).

Illumina 2x250 bp paired-end sequencing and genome assembly were performed by MicrobesNG. Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA), further details regarding their protocols can be found on the company website. Reads were adapter trimmed using Trimmomatic 0.30 (REF) with a sliding window quality cutoff of Q15. MicrobesNG identified the closest available reference genome with Kraken2 (Wood and Salzberg 2014). Reads were mapped to reference genome with

BWA-mem (Li 2013), assessing the quality of the data. *De novo* assembly of the reads was performed with SPAdes (Bankevich *et al.* 2012), and reads were mapped to *de novo* assembly with BWA-mem. Genomes were annotated with Prokka (Seemann 2014) and variants were detected with VarScan (Koboldt *et al.* 2009, 2012).

Phylogenetic analysis

Bacterial genomes were taxonomically classified and novelty determined by Average Nucleotide Identity by BLAST+ (ANIb) performed on the JWSpecies server (http://jspecies.ribohost.com/jspeciesws/), in addition to digital DNA-DNA hybridization with the Genome-to-Genome Distance Calculator (DDGC) (Meier-Kolthoff *et al.* 2013; Meier-Kolthoff and Göker 2019). These two tests mimic digital DNA-DNA Hybridisation (dDDH). An ANIb value below 95% indicates that two strains belong to different species, while for DDGC it is below 70%. The Type Strain Genome Server (TYGS) was used to generate a phylogenetic tree of sponge-associated bacteria, utilizing-genome BLAST distance phylogeny. Phylogeny was created by the TYGS, and the resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME v. 2.1.6.1 including subtree pruning and regrafting (SPR) postprocessing (Lefort, Desper and Gascuel 2015). Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint (Farris 1972).

Using the NCBI database, the 16S rRNA gene sequences for cultured sponge bacteria from nine previous studies were retrieved (Lafi, Garson and Fuerst 2005; Kim and Fuerst 2006; Jiang *et al.* 2007; Mangano *et al.* 2008; Zhang *et al.* 2008; Sipkema *et al.* 2011; Xin *et al.* 2011; Bibi *et al.* 2020; Koch *et al.* 2021) (Supplementary Table S2). These nine studies were selected for analysis as they contained (i) the full taxonomy of the sponges utilised for bacterial isolation, (ii) the studies identified the bacterial strain by sequencing the 16S rRNA gene, and (ii) finally these nine studies includes ones that did and did not perform antibiotic screening of the recovered strains. Following a manual curation of the retrieved sequences, highly similar sequences from the same study were removed by clustering nucleotide sequences using CD-HIT at 98% sequence identity (Li and Godzik 2006). Multiple sequence alignments were performed with MAFFT v. 7.407 using a standard iterative refinement method (Katoh *et al.* 2002; Yamada, Tomii and Katoh 2016). Phylogeny was inferred by Maximum Likelihood implementation in RAxML v. 8 using GTR+GAMMA approximation model (Stamatakis 2006; Kozlov *et al.* 2019), and RAxML halted bootstrapping

automatically. All trees were visualised and metadata added using the interactive Tree Of Life v. 6 (iTOL; Letunic and Bork, 2007, 2019), with final adjustments made using Inkscape v. 1.

Predicting of biosynthetic gene clusters (BGCs)

BGCs were predicted by uploading genomes to the antiSMASH v.6 online portal (Medema *et al.* 2011; Blin *et al.* 2019) with relaxed strictness and all extra features. Each BGC was classified based on its similarity to known BGCs in the antiSMASH database, according to the classification described by Benaud and colleagues (2021). In summary; there are five classification levels: 1) 'Analogous' BGCs, identical to known BGC in both number and placement of genes, and individual genes exhibited an average of >80% sequence identity; 2) High Similarity BGCs, clusters with 61%-100% of genes similar to known BGC, includes all core biosynthetic gene clusters and an average gene sequence identity >50%; 3) 'Moderate Similarity' BGCs, between 11%-87% gene similarity to known cluster, and BGCs contained additional or unmatched core biosynthetic genes; 4) 'Low Similarity' BGCs, cluster share <20% of genes with known cluster and an absence of all core biosynthetic genes, with individual genes having >40% sequence identity; 5) 'Nil Similarity' BGCs, clusters with no significant similarity to any BGC in the antiSMASH database. To facilitate predicting antibacterial biological activity from BGCs, DeepBGC v. 0.1.29 was utilized (Hannigan *et al.* 2019). The tool is capable of predicting BGCs and product class and activity of detected BGCs using Random Forest classifier. DeepBGC scores were filtered as follows: deepbgc score > 50, cluster_type score > 50, activity score > 50, number of domains > 5, number biodomains > 1, number of proteins > 1, as previously described (Hrab *et al.* 2021). The .GBK and .JSON files generated by DeepBGC were uploaded onto antiSMASH for visualisation.

Detection of antibacterial activity

Bacterial cultures with putative antibacterial activity based on *in silico* BGC analysis (as described above) were screened (*in vitro*) using a soft-agar overlay technique (Williams *et al.* 2020). Briefly, six solid media were prepared with 1.8% (w/v) agar including R2A, Marine agar (MA), Mueller Hinton 2 agar (MHA), International Streptomyces Project 2 agar (ISP2) and Actinomycetes Isolation agar (ACT). A single colony of each isolate was then streak-plated onto the centre of the agar plate creating a smear of bacterial cells between 5-10 mm in diameter. The cultures were then incubated aerobically at 15°C under atmospheric pressure for 5-10 days until a solid mass of cells was observed. Thereafter, the cultures were then overlaid with a 0.7 % (w/v) soft-agar inoculum containing a pathogen culture at a concentration of 0.1 OD/mL at Abs $_{600\,nm}$, and incubated overnight at 37°C. Lastly, the plates were inspected for zones of clearance and antibacterial activity was quantified by subtracting the size of the colony (mm) from the size of the halo (mm). The panel of bacterial pathogens utilised was: The isolates were screened against a panel of *ESKAPE* pathogens including *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* 10418, *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104, *Micrococcus luteus* NCTC 2665, *Pseudomonas aeruginosa* JCM 5962, and *Staphylococcus aureus* ST20190863.

Results

Taxonomy and novelty of sponge-associated bacteria

The draft whole genomes of 12 sponge-associated bacteria were assembled using data acquired from Illumina sequencing platform achieving approximately 30X coverage (Table S1). The draft genomes are assembled into contigs, with M4NT being the least fragmented genome with 29 contigs, and strain PP-21 the most fragmented comprising 186 contigs. Despite the fragmentation, all the genomes displayed greater than 99% completion, and all but the two *Streptomyces* strains (ACT2-R2a and DK15) displayed less than 1% contamination as measured by CheckM (Table 2).

There were six strains belonging to the phylum Actinomycetota, four Pseudomonadota and two Bacillota (Table 2, multi-locus phylogeny Fig. S1 to S3). The closest relative of sponge-associated bacterial strains was identified as *Streptomyces fulvissimus* for ACT2-R2a; *Streptomyces lavendulae* for DK15; *Microbacterium oxydans* for PC-227; *Brevundimonas vesicularis* for PC206-O; *Psychrobacter pacificensis* for PP-21; *Pseudomonas xanthomarina* for PP-22; *Bacillus pumilus* for PC-24; *Bacillus altitudinis* for Ph1628; *Dietzia psychralcaliphila* for PP-33; *Stenotrophomonas rhizophila* for RG-453; *Micromonospora tulbaghiae* for M1TU; and *Micrococcus yunnanensis* for M4NT.

Of the 12 strains, five were identified as potentially novel species based on values for both ANIb and dDDH below the threshold to be the same species as the closest relative (Table 2, Fig. S4). These genomes were: *Microbacterium* sp. strain PC-227; *Psychrobacter* sp. strain PP-21; *Dietzia* sp. strain PP-33; *Streptomyces* sp. DK15; *Micrococcus* strain M4NT, and *Stenotrophomonas rhizophila* strain RG-453. Multi-locus phylogeny for individual organisms can be found in Fig. S5 to S9. The amplification and sequencing of the 16S rRNA gene for these strains from previous work did not indicate these were potentially novel species (Koch *et al.* 2021).

Table 2. Taxonomic identification of isolated bacteria strains and identity to close relatives. (ANIb, average nucleotide identity; GGDC, Genome-Genome Distance Calculator; dDDH, digital DNA-DNA Hybridisation; diff., difference; Contam, Contamination; Comp., Completion). Detailed assembly statistics and isolation conditions for the strains can be found in Table S1.

Isolate	Assembled size (Mbp)	Contigs	N50 (kb)	L50	%GC	Comp. (%)	Contam. (%)	Phylum	Closest relative (Accession number)	ANIb (%)	GGDC DD-DH (%)	%GC diff.
M1TU	6.4	89	249.6	10	73	100	0.79	Actinomycetota	*Micromonospora tulbaghiae* DSM 45142 (GCA_900091605.1)	99.59	98.6	0.0
PC-227	3.7	45	393.5	8	69.5	99.9	0.58	Actinomycetota	*Microbacterium algeriense* G1T (GCA_008868005.1)	83.5	95.3	0.13
ACT2_R2a	8.5	105	318.5	5	71.3	99.9	1.05	Actinomycetota	*Streptomyces fulvissimus* DSM 40593 (GCA_000385945.1)	98.0	84.9	0.21
DK15	9.2	137	226.92	13	72	99.64	2.35	Actinomycetota	*Streptomyces lavendulae* NRRL B-2774 (GCA_000718155.1)	**84.6**	**39.8**	0.9
PP-33	4.2	46	399.4	4	69.6	99.4	0.59	Actinomycetota	*Dietzia psychralcaliphila* ILA-1 (GCA_003096095.1)	**86.8**	**66.8**	0.1
M4NT	2.4	29	169.6	6	73.1	98.42	0.92	Actinomycetota	*Micrococcus yunnanensis* DSM 24531 (GCA_014874165.1)	**82.1**	**50.7**	0.0
Ph1628	3.8	38	393.44	4	41	99.59	0.00	Bacillota	*Bacillus altitudinis* SGAir0031 (GCA_002443015.2)	98.22	91.9	0.5
PP-24	4.3	46	501.2	3	66.6	99.6	0.21	Bacillota	*Bacillus pumilus* LLTC96 (GCA_002998365.1)	97.46	92.9	0.1
PP-22	5.0	105	266.4	7	60.3	100.0	0.74	Pseudomonadota	*Pseudomonas xanthomarina* PX_371 (GCA_016622425.1)	95.7	81.0	0.2
RG-453	3.8	82	479.1	4.0	41.5	100.0	0.89	Pseudomonadota	*Stenotrophomonas rhizophila* DSM 14405 (GCA_003751305.1)	**91.0**	**50.6**	0.75
PC206-O	3.4	43	285.3	4.0	66.1	99.7	0.81	Pseudomonadota	*Brevundimonas vesicularis* FDAARGOS_289 (GCA_002208825.2)	99.0	80.8	0.0
PP-21	3.2	186	222.4	4.0	43.6	99.5	0.51	Pseudomonadota	*Psychrobacter pacificensis* DSM 23406 (GCA_900101915.1)	**83.1**	**53.2**	0.3

Values in bold denote results below the threshold value to the closest relative (<95% for ANIb; <75% for GGDC dDDH)

The phylogenetic diversity of the genomes investigated in this study was compared to bacterial strains cultivated from sponges described in published literature. The accession numbers of 16S rRNA amplicon sequences from nine published studies were used to this effect (Lafi, Garson and Fuerst 2005; Kim and Fuerst 2006; Jiang *et al.* 2007; Mangano *et al.* 2008; Zhang *et al.* 2008; Sipkema *et al.* 2011; Xin *et al.* 2011; Bibi *et al.* 2020; Koch *et al.* 2021). The sequences were retrieved from NCBI and after removing 16S rRNA gene sequences from the same study with a sequence identity of >98%, 329 sequences remained. This consisted of 186 sequences from studies that screened for bioactivity and 143 sequences from studies that did not (Fig. 2, Table S2). In comparing the phylogeny, it was observed that Actinomycetota (102 sponge-associated bacteria isolates from 8 studies), Pseudomonadota (94 bacteria from 7 studies) and Bacillota (66 bacteria from 7 studies) are highly represented among previously reported bacterial producers cultivated from sponge samples. Bacteriodetes had the fewest; only 8 sponge bacteria isolated from 2 studies. From the studies selected, demosponges constituted most sponges used for bacterial cultivation. This included 13 species of sponges, *Haliclona* sp. being the most frequently tested organism. There are 74 sponge-associated bacteria cultivated from hexactinellid sponges in which bioactivity was tested. No single study cultivated bacteria from hexactinellids to explore cultivable diversity, though this was extensively done for demosponges. The 74 isolates included 33 Pseudomonadota, 26 Actinomycetota, 12 Bacillota, and three Bacteroidota. There were only four distinct hexactinellid sponges tested, compared to 13 demosponges.
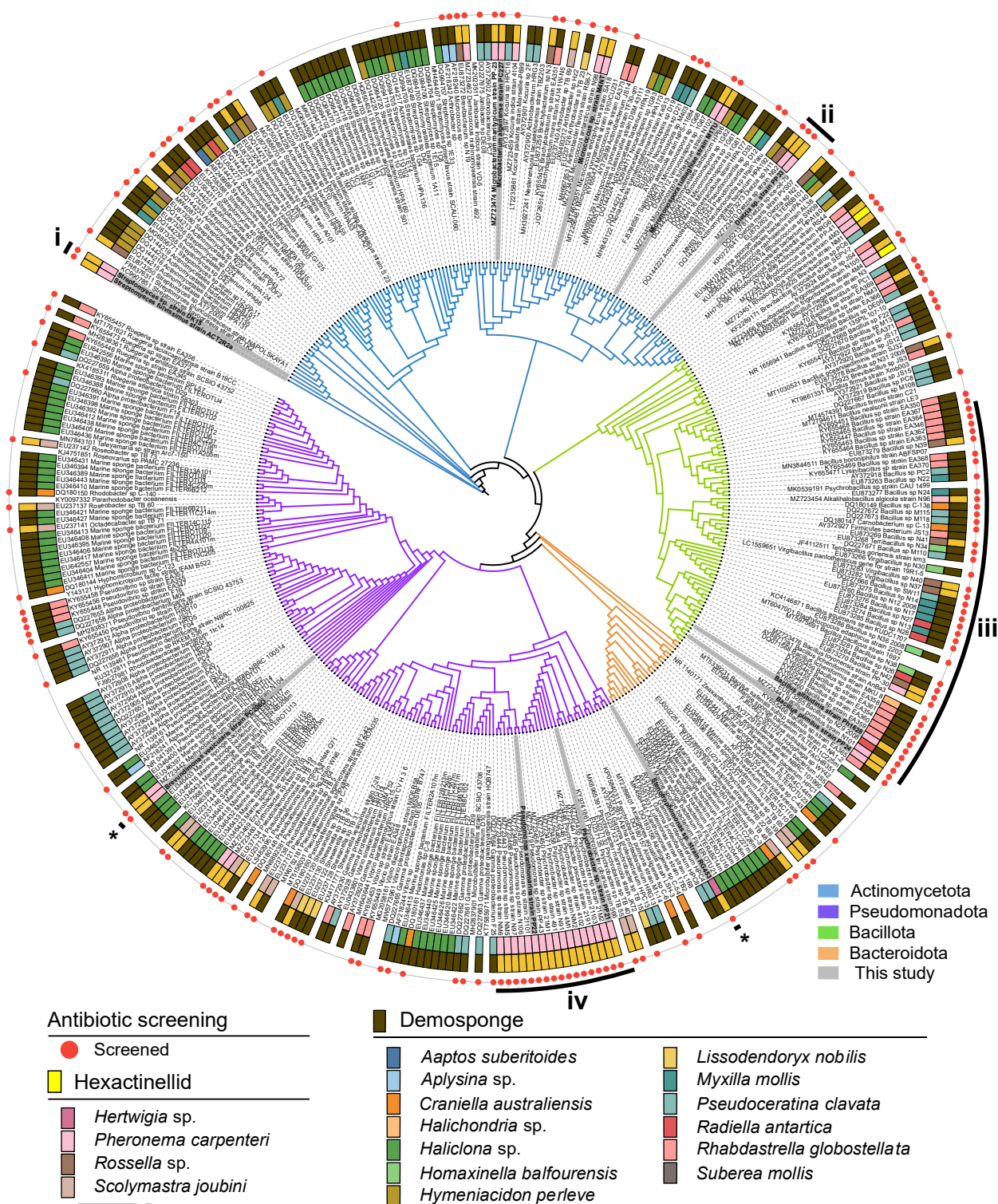
Figure 2. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences from cultivable sponge-associated bacteria showing that antibiotic producers are phylogenetically diverse across all classes and species of sponge. Cultivable sponges

presented here include reference organisms, a selection of bacteria cultivated in this study that display antimicrobial activity and those from published studies (Lafi, Garson and Fuerst 2005; Kim and Fuerst 2006; Jiang *et al.* 2007; Mangano *et al.* 2008; Zhang *et al.* 2008; Sipkema *et al.* 2011; Xin *et al.* 2011; Bibi *et al.* 2020; Koch *et al.* 2021). The outer ring represents whether organisms were screened for antibiotic activity in the published study, the middle ring the class of the sponge bacteria were cultivated, and the inner ring the taxonomy of the sponge. Regions of interest are marked numerically (i-iv). Sequence names as part of this study are labelled first with an NCBI accession number and the taxonomic classification and isolate name of the bacteria from the original study (see Supplementary Table 2). Bacterial isolates investigated in this study are labelled with their unique isolate name in bold and a grey strip.

Several features are observed from exploring the phylogenetic relationships of cultivated sponge-associated bacteria from previous studies, these are denoted in the phylogenetic tree (Fig. 2). (i). *Dietzia* sp. organisms have only been cultivated from both *P. carpenter* and *Rossella* spp. (ii). There do appear to be more Bacillota sponge bacteria recovered demonstrating antibacterial activity than those that were not screened (iii). Many of these isolates lack detailed taxonomy and are classified as just *Bacillus* sp. but are placed phylogenetically quite broadly clustering with *Psychrobacillus* sp., *Brevibacillus* sp., and *Virgibacillus* sp.

Among Pseudomonadota, a large clade of metabolically active sponge bacteria was found to include organisms closely related to *Pseudoalteronomas* sp., *Shewanella* sp. and *Vibrio* sp. (iv). This clade contains bacteria recovered from multiple species of sponges, including both sponge classes. There is a diverse clade of cultivable *Psychrobacter* sp. strains from the sponges *P. carpenteri* and *Lissodendoryx nobilis*, which includes the potentially novel *Psychrobacter* strain PP-21 presented in this study. Finally, two unique organisms have been cultivated from Hexactinellid sponges (marked with an asterisk); *Strenotrophomonas* sp. RG453 and *B. vesicularis* PC206-O. The two *Streptomyces* spp. strains investigated in this study have no close relatives among the published sequences used in this investigation

Biosynthetic gene cluster analysis of all deep-sea sponge-associated bacterial genomes

The antiSMASH webtool was used to identify putative biosynthetic genes and organise them into predictive gene clusters. The 12 draft genomes contained a total of 139

predicted BGCs across 97 contigs (Fig. 3, Supplementary Tables S3 and S4). Approximately 30% of the contigs contained multiple BGC classes, and 69.5% of BGCs shared less than 50% of genes in the cluster with homologues in the database. Terpenes were the most abundant class of BGC with 22 detected, followed by 11 Betalactones, 11 Non-ribosomal peptide synthases (NRPS), and 10 NRPS-hybrids (Supplementary Table S4). PKS-containing clusters and hybrids totalled 22, which included five Type I PKS, seven T1PKS-hybrids, three T2PKS, one T2PKS-hybrid, five T3PKS, and one T3PKS-hybrid (Table S3 and Table S4). Of the 12 strains, six contained PKS clusters, these were Actinomycetota strains ACT2-R2a, DK15, M1TU, PP-33, and the *Bacillus* spp. strains Ph1628 and PP-24.
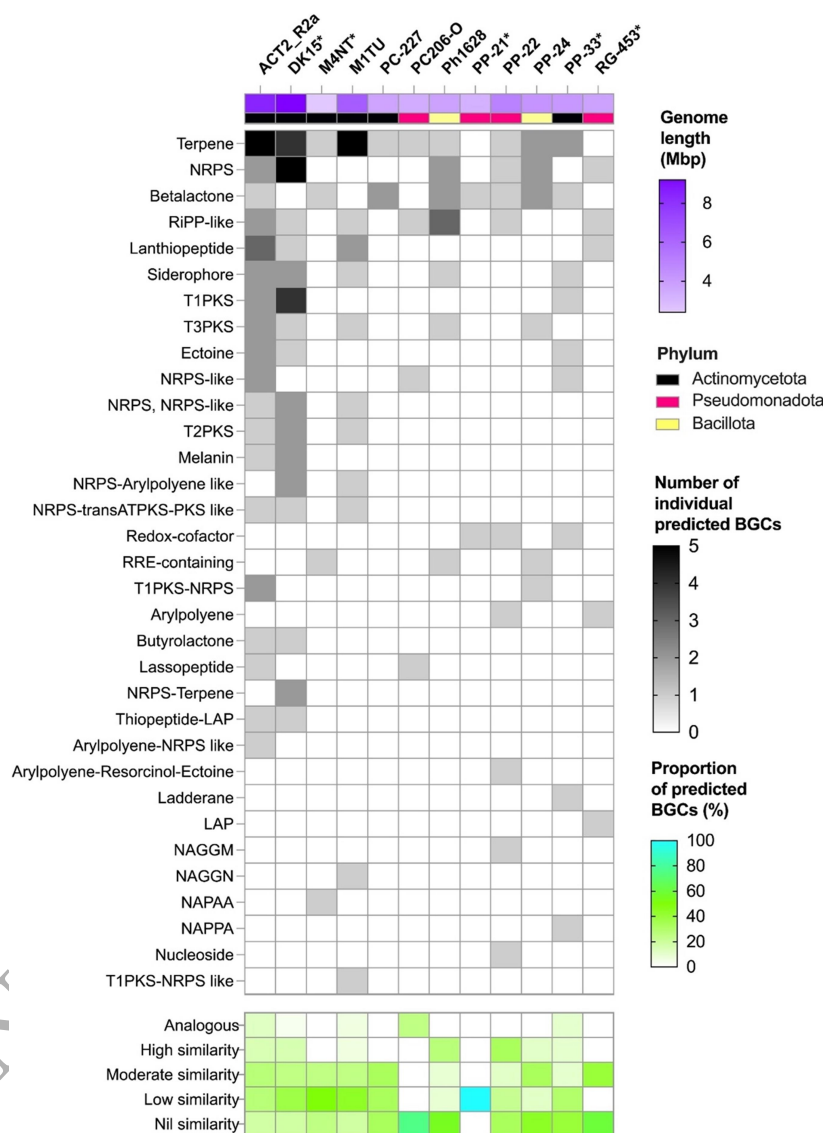
Figure 3. Predicted biosynthetic gene clusters (BGCs) from sponge-associated bacterial genomes. Summary of the predicted BGCs, and the proportion of BGCs classified based on

similarity to known BGCs. Strains are ordered from highest to lowest number of predicted BGCs going from the left to the right. For a detailed breakdown of all predicted BGCs refer to Table S2. (RiPP, ribosomally synthesized post-translationally modified; PKS, Polyketide synthase; NRPS, non-ribosomal peptide synthase; *, potentially novel species).

Analysis of the BGCs found 14 were 'Analogous', 19 were 'High similarity', 31 were 'Moderate similarity', 40 with 'Low similarity' and 43 showed 'Nil similarity' (Table S6). Strain PC-206-O had the highest proportion of BGCs with Nil similarity (75%), followed by RG-453 (60%), and then Ph1628 (54.5%). A total of 674 BGCs were detected by DeepBGC, 4.8X more than antiSMASH predicted (Fig. 4A), then after stringent filtering this reduced to 345 BGCs or 2.5X more than predicted by using antiSMASH (Fig. 4A). Most of the classified activity was antibacterial followed by unclassified (Fig. 4C). Furthermore, DeepBGC was unable to assign a class to the majority of BGCs detected (Fig. 4B).
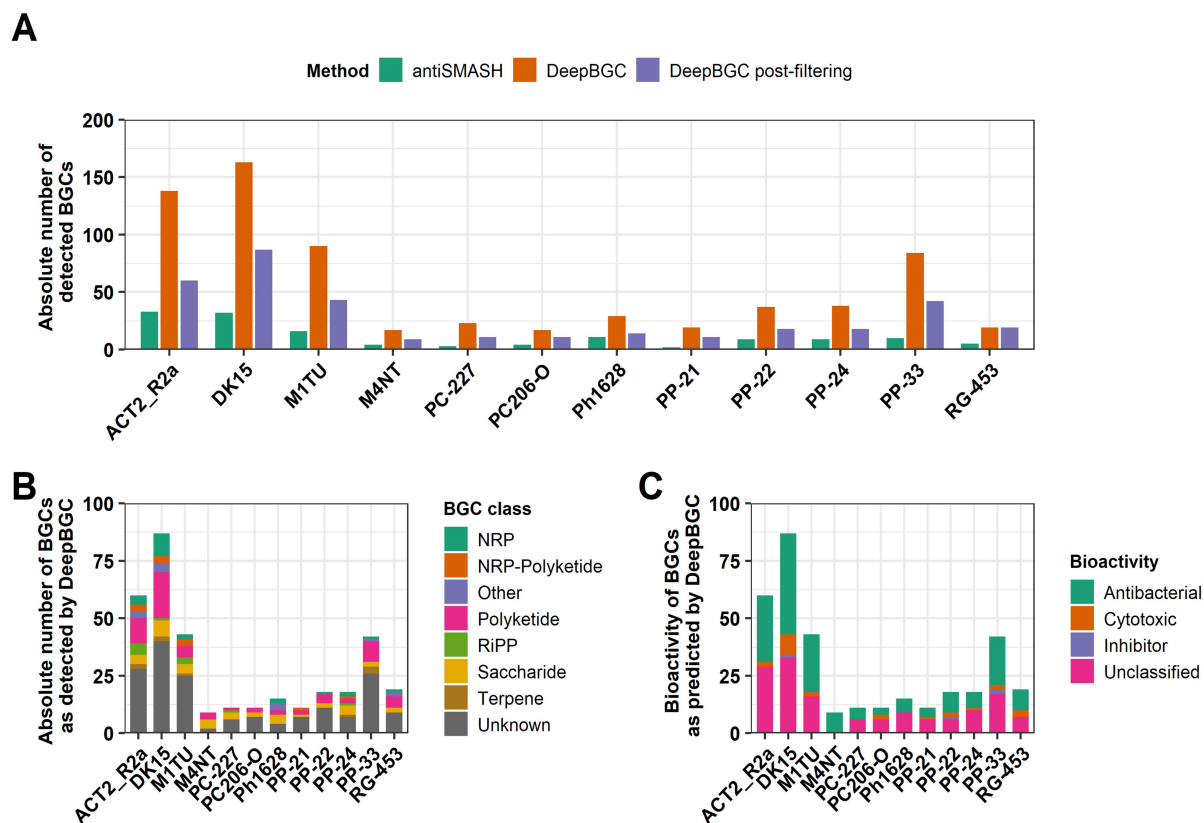
A

B

C

Figure 4. Summary of biosynthetic gene clusters (BGCs) and proposed activity as predicted by DeepBGC. (A) Comparison of BGCs as detected by the two tools utilized, including a stringent filtering step using DeepBGC to remove low-quality hits. Breakdown of the (B) classes of BGCs and (C) the predicted biological activity of proposed BGCs as detected by DeepBGC.

Possible novel deep-sea biosynthetic gene clusters

Of the 139 identified BGCs, over half had less than 50% similarity to known BGCs, this included 40 with 'low similarity' and 43 with 'nil similarity' to known BGCs (0-51%) as well as genome regions (6-35%) (Summarised in Fig. 3, Table S6, and detailed in Table S7). These BGCs may encode novel compounds for which the BGCs have not yet been characterised. The novel genomes contained high abundances of low to nil similarity BGCs, especially the Actinomycetota strains such as PP-21 and M4NT. All detected BGCs predicted for *Psychrobacter* sp. strain PP-21 were of low similarity to previously reported BGCs. Two

of the four *Micrococcus* sp. M4NT BGCs did have low similarity to known BGCs, while the remaining two had either nil or moderate similarity. The genomes of strains that were not novel with a low abundance of detected BGCs were largely classified as low to nil similarity, this includes *M. oxydans* PC227 and *B. vesicularis* PC206-O. Potentially novel Actinomycetota BGCs are of particular interest given that three of the five novel genomes presented in this study are Actinomycetota and the historic value of Actinomycetota in natural product discovery (Table 3).

Table 3. Novel deep-sea Actinomycetota biosynthetic gene clusters (BGCs) with lowest similarity to known BGCs and sequenced bacterial genome regions. Full data for all strains can be found in Table S6. (Rgn, Region; Sim., Similarity)

Strain	Rgn	Type	Most similar known cluster	Sim. (%)	Top significant ClusterBlast hit
Streptomyces sp. DK15	1.4	Terpene	monensin	5	*Streptomyces* sp. INR7
	1.5	Melanin	istamycin	4	*Streptomyces vinaceus* strain ATCC 27476
	1.8	T1PKS, hglE-KS, NRPS-like, RiPP-like	sanglifehrin A	4	*Streptomyces* sp. Tue6028
	1.10	T1PKS, NRPS-like, NRPS, transAT-PKS	caniferolide A/B/C/D	8	*Streptomyces parvulus* strain 2297
	4.1	Melanin	istamycin	2	*Streptomyces* sp. W1SF4
	4.2	T2PKS	spore pigment	8	*Streptomyces* sp. W1SF4
	6.1	Butyrolactone	lasalocid	9	*Streptomyces netropsis* strain CECT 3265
	9.1	NRPS	kirromycin	10	*Streptomyces* sp. Sge12
	20.1	T1PKS	tetronasin	3	*Streptomyces* sp. C
	26.1	Siderophore	ficellomycin	3	*Streptomyces* sp. fd1-xmd
	36.1	NRPS, NRPS-like, Terpene	malacidin A/B	12	*Streptomyces lavendulae* strain CCM 3239
	55.1	NRPS-like, Arylpolyene	RP-1776	16	*Streptomyces fodineus* strain TW1S1
Micrococcus sp. M4NT	11.1	Betalactone	microansamycin	7	*Micrococcus luteus* strain R17
	12.1	NAPAA	stenothricin	13	*Micrococcus luteus* strain O'kane
Dietzia sp. PP-33	1.1	Terpene	Polyketide:Type II + Saccharide:Hybrid/ tailoring	6	*Dietzia* sp. JS16-p6b
	2.1	NRPS-like	sisomicin	5	*Dietzia psychralcaliphila* strain ILA-1
	14.1	Ladderane	arimetamycin B/C/A	13	*Dietzia psychralcaliphila* strain ILA-1
	4.1	Betalactone	ECO-02301	7	*Tsukamurella paurometabola* DSM 20162

Detection of antibacterial activity

Isolates were screened for antibacterial activity using a soft agar overlay approach. The media selected consisted of different carbon and nitrogen sources, and included R2A, MA, ACT, MHA, and ISP2 agar. The Gram positive pathogens were most susceptible to antagonistic activity with 8 of 9 producers inhibiting *M. luteus* and 4 of 9 producers inhibiting *S. aureus* (Fig. 5). Seven of the nine producers showed anti-Gram-negative activity either towards *A. baumannii* (3/9), *E. coli* (4/9), *K. pneumoniae* (4/9) or *P. aeruginosa* (3/9). ISP2 agar produced the most positive hits (17/54), followed by MHA (10/54), ACT (8/54), and R2A (7/54). The isolate DK-15 was the most prolific producer with broad spectrum activity against Gram positive and Gram-negative pathogens. When cultured on R2A, MA, ACT, and MHA weak to moderate activity was seen against *M. luteus* and *S. aureus*. However, when cultured on ISP2 a substantial increase in Gram positive activity and an increase in spectrum of activity towards the Gram-negatives including *E. coli*, *K. pneumoniae* and *P. aeruginosa* was observed.
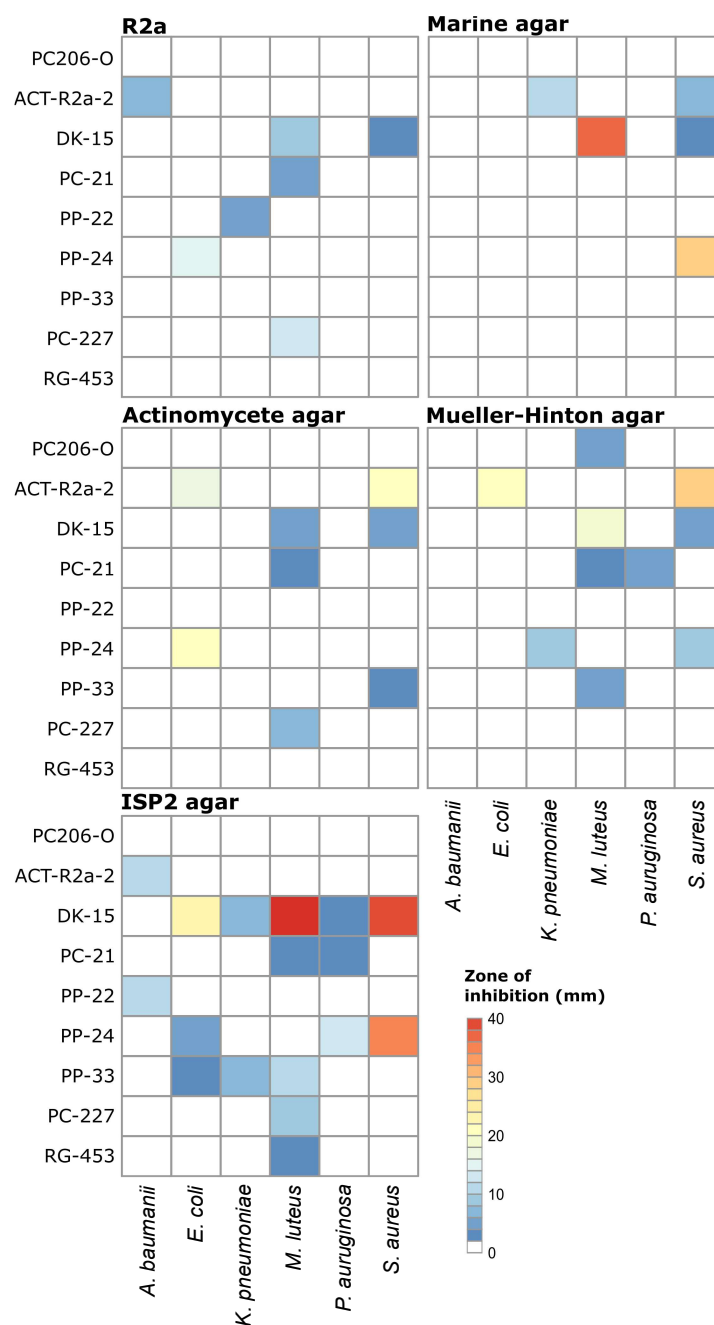
Figure 5. Evaluation of antibacterial activity of producer isolates as screened by a soft agar overlay. Isolates are listed on the y-axis, pathogens on the x-axis, and antibacterial activity is quantified by size of zone of inhibition (mm) and represented as colour shifts from white (no observed activity; 0 mm) to red (high antibacterial activity; 40 mm).

We observed a weak relationship between sponge-associated bacterial strains with a high abundance of BGCs and the number of indicators that each strain displayed antibacterial

activity against. For example, *S. fulvissimus* ACT2-R2A and *Streptomyces* sp. DK15, which both contained over 30 BGCs as detected by antiSMASH, displayed activity in all five media, with isolates cultured on ACT-R2a-2 displaying activity against all the indicator pathogens and DK15 displaying activity against all pathogens with the exception of *A. baumanii*. This relationship however was not always consistent, with the putative *Dietzia* sp. strain PP-33 containing 10 BGCs exhibiting relatively high activity only on plate assays, such as activity against both Gram-positive (*M. luteus* and *S. aureus*) and Gram-negative (*E. coli, K. pneumoniae* and *P. aeruginosa*) strains when cultivated on ISP2 media. This pattern continued with strain PP-21, which has two BGCs detected by antiSMASH, but displayed greater antibacterial activity than strain PC206-0, which had twice the number of BGCs.

Discussion

Deep-sea sponge-associated bacteria harbour a great potential for novel secondary metabolite discovery. In this study, 12 genomes were analysed and found to contain 83 biosynthetic regions with low gene similarity to known and characterised BGCs, demonstrating the value of exploring new habitats and taxa, such as deep-sea sponges, to access novel genetic and chemical spaces. Four putatively novel and eight previously described bacterial species were investigated for their potential for antibiotic production, including those from genera previously cultivated from deep-water sponges; *Dietzia* (Xin *et al.* 2011), *Psychrobacter* (Mangano *et al.* 2008), *Microbacterium* (Graça *et al.* 2013; Liu *et al.* 2019), and *Streptomyces* (Guerrero-Garzón *et al.* 2020; Williams *et al.* 2020).

Among the strains recovered in this study are previously reported producers, including *P. putida* (Marinho *et al.* 2009), *S. fulvissimus* (Guerrero-Garzón *et al.* 2020) and *B. subtillis* isolated from deep-sea corals (Liu *et al.* 2013) and marine sponges (Zhang, Zhang and Li 2009). Multiple *S. fulvissimus* strains were isolated from a freshwater sponge *Antho dichotoma* and reported to contain multiple BGCs in their whole genome as detected by antiSMASH (Guerrero-Garzón *et al.* 2020), while culture supernatant demonstrated activity against *E. coli* K12 and *Bacillus subtilis* 188.

P. xanthomarina has not, to our knowledge, previously been cultivated from sponges, but the species was first isolated from marine ascidian specimens (Romanenko *et al.* 2005), and the closely related *P. zhaodongensis* strain SST2 was recovered from the South Shetland Trench at 5,194 m deep on R2A agar supplemented with a fungicide. The strain demonstrated

activity against *A. baumannii* (Abdel-Mageed *et al.* 2020). Analysis of the draft genome of SST2 supports the presence of multiple BGCs, as detected by antiSMASH, similar observations were reported for *P. xanthomarina* strain PP-22 (Fig. 3). The bioactive potential of isolate PP-22 was confirmed by *in vitro* antibacterial screening, where it showed weak antibacterial activity against *A. baumannii* and *K. pneumoniae* (Fig. 5), this activity was only seen when cultured on ISP2 and R2A agar, respectively. Esposito and colleagues (2021) showed the impact that culture media has on the expression of antimicrobial compounds from marine bacteria. In their study, it was demonstrated that a strain of marine *Rhodococcus* cultivated in 22 different media only expressed unique antimicrobial compounds in one culture condition.

Strain PP-24, identified as *B. pumilus*, displayed activity in the OSMAC screen against only *E. coli*. *B. pumilus* strains have been isolated from various marine environments, including deep-sea corals (Liu *et al.* 2013), marine sponges (Zhang, Zhang and Li 2009), and sediments (Nithya, Devi and Karutha Pandian 2011). Bioactivity has been seen, such as anti-biofilm activity (Zhang, Zhang and Li 2009), and production of lipoamides with antimicrobial activity against bacterial and fungal pathogens (Berrue *et al.* 2009). While the bacterial isolates recovered from this study are phylogenetically diverse, many are related to those that have previously been reported from the shallow water Antarctic sponges *Rossella* spp. (Xin *et al.* 2011). This adds support to the suggested presence of a distinct hexactinellid cultivable clade (Xin *et al.* 2011), which was further supported by a 16S rRNA gene sequencing surveys (Steinert *et al.* 2020). More cultivation efforts need to occur on multiple species of hexactinellid sponges to confirm these early observations.

Observing that many recovered bacteria with activity belong to the phylum Actinomycetota, culturing strategies more targeted towards Actinomycetota recovery would be beneficial when aiming to cultivate bioactive bacteria from *P. carpenteri* and *Hertwigia* sp. This could include heat treatment used to select endospore-forming isolates (Matobole *et al.* 2017). Actinomycetota are historically regarded as prolific producers and are frequently the focus of studies intending to screen sponge bacteria cultures (Jiang *et al.* 2007; Hameş-Kocabaş *et al.* 2012; Pham *et al.* 2016). However, in the phylogenetic analysis of previously cultured sponge bacteria with and without detected antibacterial activity, it does become apparent that across the three phyla there is an abundance of cultivable sponge-associated bacteria with antimicrobial activity. Pseudomonadota and Bacillota were also widely present

among the bioactive isolates across the literature, which could be overlooked in an Actinomycetota-only focused culturing strategy.

Bias towards both spore-forming Bacillota and Actinomycetota should be expected since spores may easily survive sampling, storage and transport, in addition to growing on various standardised media. It can be challenging to compare the phylogenetic diversity of cultivable sponge-associated bacteria since media, sponge species, and cultivation conditions will affect the isolates recovered (Sipkema *et al.* 2011). Bacterial isolates from the nine studies utilised in the phylogenetic investigation of sponge-associated bacterial producers, were recovered on a wide range of isolation media with each publication utilising different media (Supplementary Table 2). In those nine studies, there are numerous isolation media utilised, such as Sipkema *et al.* (2011) utilising nineteen, while Xin *et al.* (2011) utilised 10 different isolation media. Overall, this phylogenetic tree does represent a broad overview of the possible bacterial taxa that can cultivated from both demosponges and hexactinellids.

It is important to state that many of the organisms reported in the phylogenetic analysis of cultivable sponge bacteria were not screened due to the research goals of the original publications. Furthermore, the publications that did screen for antibiotic production did not identify by 16S rRNA gene sequencing the bacterial isolates that tested negative in screening efforts. There could be valuable undetected antibiotic production or novel taxa among those unidentified, that initially did not test positive in screening efforts.

The most prolific producer in this study was *Streptomyces* sp. DK-15, which belongs to the Phylum Actinomycetota with its closest neighbour being *Streptomyces lavendulae* NRRL B-2774 (Table 2). However, according to our multi-locus phylogeny analyses, strain DK-15 was predicted to be a novel species with ANIb and dDDH scores of 84.6 and 39.8 %, respectively (Table 2). *S. lavendulae* is well known for its ability to produce lavendamycin that has anti-tumour and antibiotic properties, actinomycin C2 and other antimicrobial metabolites (2(3H)Furanone, 5acetyldihydro; 1,4 Dioxane, 2,5 dione, 3,6 dimethyl; hexanoicacid, 2phenylethylester; 2,4 Dimethyl3pentanol acetate; 2,5 piperazinedione, 3,6 bis(2methylpropyl); & ergotaman), which have broad spectrum anti-Gram positive and Gram-negative activity, anticancer, antifungal, and antioxidant activities (Balitz *et al.* 1982; Kumar *et al.* 2014). Novel species often have new or uncharacterised chemical analogues of known compounds that could have increased biological activity, a wider spectrum of activity, or new mechanisms of action (Rahman *et al.* 2010; Piddock 2015; Hobson, Chan and Wright

2021; Schneider *et al.* 2021). The genome of DK-15 was also mined for bioactive potential by searching for BGCs and assessing their novelty (Table 3 and Fig. 4).

Using the genome mining tool DeepBGC, it was seen that DK-15 returned the highest absolute number of BGCs detected; the most BGC classes including NRP, NRP-polyketide, 'other', polyketide, RiPP, saccharide, terpene and 'unknown'; and the highest percentage of predicted bioactive BGCs with putative antibacterial activity (Fig. 4). The genome mining tool antiSMASH predicted that DK-15 has potential novel BGCs using the thresholds set by Benaud *et al.* (2021) with the most similar known clusters able to produce antimicrobial compounds including monesin, istamycin, caniferolide, lasalocid, kirromycin and tetronasin (Table 3). In this study, we assessed if the putative BGC and bioactivity data correlated with DK-15 *in vitro* antibacterial activity (Fig. 5). DK-15 showed a wide spectrum of antibacterial activity against Gram positive and Gram-negative pathogens with most potent activity when cultured on ISP2 media. The *in-silico* predictions alongside the preliminary screening results, as well as the strain novelty, are good rationale for further investigations of this strain where a combined genomics-metabolomics approach is proposed to chemically dereplicate, isolate and elucidate active compounds.

Data presented here supports the observations made that sampling the rarely cultivated taxa from dominant phyla such as Actinomycetota, Pseudomonadota and Bacillota recovered from deep-sea sponges can lead to the identification of novel chemical diversity. This was certainly observed with abundance of BGCs with low similarity from the novel genomes (i.e., PP-33, RG-453, M4NT, PP-21 and DK-15) and even in the smaller genomes presented in this study (PC2-6-O).

Data Availability Statement

Bacterial whole-genome assemblies have been deposited at DDBJ/ENA/GenBank under the BioProject PRJNA852787. Individual genomes assemblies can be found with the following BioSample accessions **(**SAMN29351843-SAMN2935185**3)**: *Micromonospora tulbaghiae* M1TU, SAMN29351843; *Microbacterium algeriense* PC-227, SAMN29351844; *Streptomyces fulvissimus* Act-R2a-2, SAMN29351845; *Streptomyces* sp. DK15, SAMN29351846; *Dietzia* sp. PP-33, SAMN29351847; *Micrococcus* sp. M4NT, SAMN29351848; *Bacillus altitudinis* Ph1628, SAMN29351849; *Bacillus pumilus* PP-24, SAMN29351850; *Pseudomonas xanthomarina* PP-22, SAMN2935185; *Stenotrophomonas*

sp. RG-453, SAMN29351852; *Brevundimonas vesicularis* PC206-O, SAMN29351853; and *Psychrobacter* sp. PP-21, SAMN29351854.

Author contributions

Study conceptualization devised, planned, conducted all authors. Sponges were collected by K.L. Howell. Data curation, analysis and visualizations were carried out by P.J. Hesketh-Best, M.J. Koch, and G.G. January. Writing of original draft was performed by P.J. Hesketh-Best, M.J. Koch, and G.G. January, and all authors contributed to the reviewing and editing of the manuscript. Supervision was provided by M. Upton, K.L. Howell and P.J. Warburton.

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