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Bacteria Isolated From the Antarctic Sponge *Iophon* sp. Reveals Mechanisms of Symbiosis in *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia*

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Antarctic sponges harbor a diverse range of microorganisms that perform unique metabolic functions for nutrient cycles. Understanding how microorganisms establish functional sponge–microbe interactions in the Antarctic marine ecosystem provides clues about the success of these ancient animals in this realm. Here, we use a culture-dependent approach and genome sequencing to investigate the molecular determinants that promote a dual lifestyle in three bacterial genera *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia*. Phylogenomic analyses showed that four sponge-associated isolates represent putative novel bacterial species within the *Sporosarcina* and *Nesterenkonia* genera and that the fifth bacterial isolate corresponds to *Cellulophaga algicola*. We inferred that isolated sponge-associated bacteria inhabit similarly marine sponges and also seawater. Comparative genomics revealed that these sponge-associated bacteria are enriched in symbiotic lifestyle-related genes. Specific adaptations related to the cold Antarctic environment are features of the bacterial strains isolated here. Furthermore, we showed evidence that the vitamin B5 synthesis-related gene, *pan*E from *Nesterenkonia* E16_7 and E16_10, was laterally transferred within Actinobacteria members. Together, these findings indicate that the genomes of sponge-associated strains differ from other related genomes based on mechanisms that may contribute to the life in association with sponges and the extreme conditions of the Antarctic environment.

Keywords: Antarctic sponges, symbiotic lifestyles, sponge microbiome, Antarctic ecosystem, sponge-associated bacteria

INTRODUCTION

The sponge holobiont- or marine sponge-microorganism assemblage is considered one of the most basal and complex symbiotic relationships and proposed as one of the principal contributors to microbial diversity and functionality across global ocean ecosystems (Ruby, 2008; Pita et al., 2016; Thomas et al., 2016). The sponge holobiont is composed of a permanent, very dense, and diverse microbial community, dominated by the bacterial domain

(Hentschel et al., 2006; Taylor et al., 2007). The sponge-associated bacteria participate actively in various fundamental metabolic processes including metabolizing sponge waste compounds, nutrient cycling (carbon, nitrogen, phosphorus, and sulfur), and production of secondary metabolites as chemical defensive molecules (Unson et al., 1994; Fan et al., 2012; Zhang et al., 2015).

Symbiotic functions are essential in the maintenance process of the host–microorganism relationship, which ultimately affects the health of the sponge holobiont. These functions, in turn, affect the health of the ecosystems (Pita et al., 2018). The establishment of the functional sponge–microbe interaction depends, in part, on a series of molecular determinants that mediate the symbiotic lifestyle adaptation (Gao et al., 2014; Tian et al., 2014, 2016 Burgsdorf et al., 2015; Zhang et al., 2019). These symbiotic signatures include eukaryotic-like protein domains (ELPs), clustered regularly interspaced short palindromic repeats (CRISPR), restriction-modification (R-M), and toxin–antitoxin (T-A) systems, which are involved in avoiding the digestion by the sponge host and defending against incoming foreign DNA (Magnuson, 2007; Nguyen et al., 2014; Horn et al., 2016; Díez-Vives et al., 2017; Slaby et al., 2017; Jahn et al., 2019). Fundamental insights into the specific strategies that promote the symbiotic lifestyle have been provided by genome studies from sponge symbionts (Slaby et al., 2017; Tian et al., 2017). The genome from *Candidatus Synechococcus spongiarum* revealed singular differences from free-living Cyanobacteria including the following: genome streamlining, lack of amino acid biosynthesis genes and the partial loss of DNA repair mechanisms, antioxidant enzymes, and photosynthetic complex genes (Gao et al., 2014; Burgsdorf et al., 2015). In the case of the sponge symbiont *Candidatus* Poribacteria, evidence of symbiotic lifestyle adaptation was inferred based on the loss of the flagellar structure, the presence of exclusive adhesion-related proteins, and a broad set of carbohydrate-degrading enzymes (Siegl et al., 2011; Zhang et al., 2019).

The study of sponge–microorganism symbiotic relationship is particularly relevant in Antarctica, where sponges are pivotal members of the benthic system with a high abundance and species-level endemism (McClintock et al., 2005; Downey et al., 2012). Antarctic sponge microbiomes are diverse, host specific, temporally stable, and different from tropical and temperate sponges (Webster et al., 2004; Rodríguez-Marconi et al., 2015; Savoca et al., 2019; Steinert et al., 2019). Functional insights of the Antarctic sponge-associated bacteria revealed that these bacteria produce antibacterial compounds that could control microbial populations inside the sponges (Papaleo et al., 2012). These compounds include a broad range of antibacterial compounds and xenobiotics (Steinert et al., 2019). More recently, the metagenomic analysis of two Antarctic sponge microbiomes showed the potential for nutrient cycling (carbon, nitrogen, sulfur, and phosphorus) and a high versatility of autotrophic carbon fixation (Moreno-Pino et al., 2020). Furthermore, enrichment of genes related to transposons, phages, CRISPR, T-A, and R-M systems was detected in these metagenomes, supporting the notion of genomic adaptations to symbiotic lifestyle in Antarctic sponges (Moreno-Pino et al., 2020).

Here, we describe molecular determinants of the Antarctic sponge microbiome interactions of five bacterial strains isolated from the Antarctic sponge *Iophon* sp. using a culture-dependent approach and genome sequencing. This study characterizes the mechanisms that promote a dual lifestyle in bacterial members belonging to the *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia* genera. We aim to understand the genomic adaptations of these bacteria to live in association with the Antarctic sponges and whether their genomes present differences to the free-living counterparts.

MATERIALS AND METHODS

Bacterial Cultures and Physiological Characterization

Seventeen bacterial strains were originally isolated from the Antarctic sponge *Iophon* sp. (class Demospongiae). The sponge sample was obtained in January 2014 and collected from Fildes Bay, King George Island, Antarctica (62° 12′ 11″ S, 58° 55′ 15″ W) at a depth of 5 m. For bacterial cultures and isolation, triplicate pieces (1 cm^3) from the inner structure of the sponge tissue were cut with a sterile scalpel blade and were rinsed three times with sterilized seawater. The sponge tissues were homogenized, and the obtained extracts were plated in Marine Agar 2216 (BD-Difco) and incubated in the dark at 4°C for 30 days. Bacterial growth was examined weekly, and the bacterial colonies were isolated and transferred to Marine Broth 2216 (BD-Difco). Pure isolates were cryopreserved in Marine Broth supplemented with 10% glycerol at −80°C. The bacterial strains selected for genome sequencing included in this study were chosen based on their preliminary taxonomic assignation and the availability of reference genomes for comparative genomic analyses.

Strains E16_2, E16_3, E16_7, E16_8, and E16_10 were initially characterized by Gram staining, according to Dussault (1955). Enzymatic activities from these strains were tested with the API-ZYM Test System (bioMérieux) at 21°C for 3 days.

Whole-Genome Sequencing and Bioinformatic Analysis

Genomic DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma-Aldrich). DNA was used to prepare a sequencing library with NEBNext dsDNA Fragmentase kit (New England Biolabs, Ipswich, MA, United States) and quantified using a standard qPCR assay with a Library Quant Kit Illumina (Kapa). Libraries were sequenced using the Illumina Miseq platform with 2 × 150 cycles using the Miseq kit v.2. In order to improve sequence coverage of the strain E16_10, it was resequenced with a TruSeq Nano DNA LT Kit and using the Illumina HiSeq 4000 with 2 × 100 cycles. All raw whole-genome sequencing reads were filtered using PrinSeq (Schmieder and Edwards, 2011) (version 0.20.4). Adapters were removed with Cutadapt (version 1.10; -q 30) (Martin, 2011; Schmieder and Edwards, 2011). High-quality reads were assembled using SPAdes

(version 3.10.1) with default parameters (Bankevich et al., 2012). Genome assembly of strain E16_10 was improved by performing genome scaffolding, combining the previously generated Miseq and Hiseq contigs with MeDuSa, using default parameters (Bosi et al., 2015). ORFs were predicted for all contigs >500 bp using Prodigal (version 2.6.3) (Hyatt et al., 2010). Functional annotation was done using eggNOG mapper v.1 against the eggNOG v.4.5 (Huerta-Cepas et al., 2017; see **Table 1** for details).

Taxonomic classification of strains was performed using the full-length 16S rRNA gene sequences detected with barrnap version 0.7[1], and RNAmmer 1.2 Server (Lagesen et al., 2007). Closely 16S rRNA gene sequences were found using BLASTn against NCBI-nr database (August 2019), with a cutoff of *E*-value $< 10^{-5}$, in Geneious software version R10.2. The retrieved 10 best hits and sequences of neighboring representative lineages were aligned using MUSCLE (version 3.5) (Edgar, 2004), with 999 iterations, in Geneious version R10.2. The alignment was manually edited and used to construct Maximum Likelihood (ML) phylogenies. ML trees were generated using the best-fit models (K2 + GAMMA for *Cellulophaga* and *Sporosarcina,* and TN93+ GAMMA for *Nesterenkonia*) and were computed with 500 bootstrap replicates in MEGA7 (Kumar et al., 2016).

Comparative Genomic Analyses

Fragment Recruitment

To estimate the abundance of genomes related to the bacterial strains E16_2, E16_3, E16_7, E16_8, and E16_10 in marine sponge microbiomes and planktonic communities, we performed a fragment recruitment analysis using 23 publicly available metagenomic datasets. These metagenomes included microbiomes associated with Antarctic sponges (n = 3), microbiomes from tropical and temperate sponges (n = 11), and marine microbial communities (n = 9), including one sample from surrounding seawater from the Antarctic sponges (see **Supplementary Table 1** for details).

Metagenomic datasets were filtered based on a $Q > 30$ quality threshold using Prinseq (Schmieder and Edwards, 2011). Non-ribosomal reads were obtained using SortMeRNA (Kopylova et al., 2012), fragmented and filtered applying a previously published script (Plominsky et al., 2018) to obtain high-quality sequences within 50–100 bp. These were randomly subsampled according to the sample with the lowest number of reads across metagenomes (i.e., *Iophon* sp.). The recruitment of metagenomic sequences was performed using the genome of the bacterial strains from the Antarctic sponges as reference with FR-HIT (Niu et al., 2011) with a cutoff of 90% identity and 75% coverage. To further evaluate the relative abundance of genera associated with the five isolates obtained from Antarctic sponges, we performed a protein sequence classification of the metagenomes mentioned above. Non-ribosomal sequences were randomly subsampled according to the lowest reads across samples (*Iophon* sp.). The subsampled sequences were classified with Kaiju with default settings, aligning them against the NCBI BLAST nr +euk database (version 2019-06-25) (Menzel et al., 2016).

Pangenome Analysis

Pangenome analysis was performed by comparing 73 genomes from the *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia* genera using the workflow implemented in the Anvi'o package[2]. Twenty-one percent of these genomes were complete, 16% were in the scaffold level, and 46% in the contig level (NCBI Sequence Read Archive version March 2019). We included 44 genomes from *Cellulophaga* spp., 17 genomes from *Sporosarcina* spp., and 12 genomes from *Nesterenkonia* spp. (see **Supplementary Table 2** for details). Genomes were transformed to the Anvi'o format and filtered by contig length >500 bp (anvi-script-reformat-fasta; -l 500). Protein clustering was performed using BLAST and Markov Clustering (MCL) (van Dongen and Abreu-Goodger, 2012) algorithm with an inflation value of 0.1 (anvi-pan-genome; -minbit 0.5 –mcl-inflation 0.1 –use-ncbi-blast). Average nucleotide identity (ANI) was computed using BLAST (anvi-compute-ani; –method ANIb). Pangenomes were analyzed by clustering the genomes depending on whether the strains were host associated or free living.

Core and accessory genes obtained from the pangenome analysis were classified according to the following parameters. For core genes: maximum number of genes from each genome, 1; maximum functional homogeneity index, 0.9; minimum geometric homogeneity index, 1, minimum functional homogeneity index, 0.9; and maximum functional homogeneity index, 1. For accessory genes, only genes that were present in the host-associated strains were selected.

To test for significant differences in COG abundances between sponge-associated and free-living and between host-associated and free-living genomes, we used a two-tailed Wilcoxon rank-sum test in R environment using rstatix and STAMP 2.0.9 (Parks et al., 2014). COGs with a p-value < 0.05 were considered enriched. Analysis of similitude (ANOSIM) was used to test for significant differences between host-associated and free-living genomes using Primer 6 (PRIMER-E Ltd.).

Phylogenomics

The phylogenomic analysis was performed using the core genes. For this, 756, 355, and 190 core proteins for *Cellulophaga* spp., *Sporosarcina* spp., and *Nesterenkonia* spp., respectively, were concatenated and aligned using MUSCLE (version 3.5) (Edgar, 2004) with 999 iterations, and ambiguous positions were removed using trimAl v1.3 (-automated 1) (Capella-Gutiérrez et al., 2009). ML trees were performed using RAxML version 8 (Stamatakis, 2014), with 1,000 bootstrap replicates under the best-fit model for the three genera (LG + GAMMA + F). A multilocus sequence analysis (MLSA) based on 23 single-copy genes (SCG) shared between all genomes analyzed was performed to support the phylogenomic inferences (**Supplementary Table 3**; Wu et al., 2013). SCG were concatenated, aligned using MUSCLE (version 3.5) (Edgar, 2004) with 999 iterations in Geneious

[1]http://www.vicbioinformatics.com/software.barrnap.shtml

[2]http://merenlab.org/2016/11/08/pangenomics-v2

software version R10.2, and trimmed using trimAl v1.3 (Capella-Gutiérrez et al., 2009). ML trees were performed using RAxML (Stamatakis, 2014), with 1,000 bootstrap replicates, under the best-fit model (for *Nesterenkonia*, LG + GAMMA + F and for *Sporosarcina* and *Cellulophaga*, DAYHOFF + F).

Horizontal Gene Transfer and Synteny Analyses

Genomic islands (GIs) were identified in the genomes of *Sporosarcina* sp. E16_3 and E16_8, *Cellulophaga* E16_2, and *Nesterenkonia* E16_7 and E16_10 using IslandViewer 4 with default settings (Bertelli et al., 2017). IslandViewer 4 integrates a comprehensive approach to predict GIs using four different methods: IslandPick, IslandPath-DIMOB, SIGI-HMM, and Islander.

The genomic GC content (%) from the GIs identified in *Nesterenkonia* E16_7 and E16_10 was computed with Geneious software version R10.2. Finally, the GI containing the ketopantoate reductase coding gene (*pan*E) was analyzed to confirm horizontal gene transfer (HGT) events using phylogenetic and comparative genomic analyses. The closest relatives of *pan*E gene were determined using bidirectional BLASTp against the NCBI-nr database (August 2019), retrieving the 50 non-redundant best hits. The *panE* genes were aligned using MUSCLE (version 3.5) with 999 iterations in Geneious software version R10.2, and ambiguous positions were removed using trimAl v1.3 (-automated 1) (Edgar, 2004; Capella-Gutiérrez et al., 2009). ML tree was performed using RAxML (Stamatakis, 2014), with 1,000 bootstrap replicates, under the LG + GAMMA + F model. Phylogenetic analysis of five neighboring genes to *panE* was also performed using 13–20 non-redundant best hits as described before. Additionally, to explore the synteny of *pan*E-related sequences in E16_7 and E16_10 strains, and *Nesterenkonia* sp. AN1 (NZ_JEMO00000000), and *Nesterenkonia aurantiaca* DSM27373 (NZ_SOAN00000000), contigs containing ORFs related to panE were extracted using Geneious version R10.2 and were compared with BLASTn using Trebol[3].

RESULTS

Phylogenetic analyses using the full-length 16S rRNA gene sequences of the five bacterial strains isolated from the Antarctic sponge *Iophon* sp. showed that strains E16_3 and E16_8 form a strongly supported monophyletic group with *Sporosarcina globispora* and *Sporosarcina psychrophila* (**Supplementary Figure 1A**). A whole-genome comparison showed that these sponge-associated strains shared 92% of ANI between them and below 89% of ANI to all *Sporosarcina* genomes (**Supplementary Figure 2A**). Furthermore, core and SCG showed a separation between the genomes of *Sporosarcina* sp. E16_3 and E16_8, and *S. psychrophila*, suggesting a species divergence between them (**Figure 1A** and **Supplementary Figure 3A**).

For the strain E16_2, the 16S rRNA gene displayed a high similarity with *Cellulophaga algicola*, *Cellulophaga pacifica*, and *Cellulophaga baltica*, which formed a well-supported monophyletic clade (**Supplementary Table 4** and **Supplementary Figure 1B**). *Cellulophaga* sp. E16_2 displayed ANI values from 74 to 98% among *Cellulophaga* genomes (**Supplementary Figure 2B**). The close relationship between *Cellulophaga* sp. E16_2 and *C. algicola* DSM 14237 was confirmed with SCG (**Figure 1B** and **Supplementary Figure 3B**). These results indicate a close phylogenetic relationship between *Cellulophaga* E16_2 and *C. algicola* DSM 14237.

The 16S rRNA gene of strains E16_7 and E16_10 showed a high similarity with *Nesterenkonia* sp. AN1, *Nesterenkonia aurantiaca*, and *Nesterenkonia lutea* (**Supplementary Table 4**). ML tree showed that the strains E16_7 and E16_10 cluster together with *Nesterenkonia* sp. AN1 and *N. aurantiaca* (**Supplementary Figure 1C**). *Nesterenkonia* E16_7 and E16_10 showed 100% of ANI between them, and below 93% when

[3]https://inf.imo-chile.cl/software/trebol.html

TABLE 1 | Summary of the genomic features of the Antarctic sponge-associated isolates.

	Sporosarcina* sp. strain *E16_3	***Sporosarcina* sp. strain *E16_8***	***Cellulophaga* sp. strain *E16_2***	***Nesterenkonia* sp. strain *E16_7***	***Nesterenkonia* sp. strain *E16_10***
Genome size	4,018,218	4,383,297	4,945,754	3,295,803	3,296,753
GC content (%)	40.8	40.7	33.5	67.3	67.3
No. of predicted genes	3,843	4,254	4,351	3,078	3,066
No. of CDS	3,790	4,178	4,308	3,025	3,014
No. of tRNA loci	40	61	36	47	46
No. of rRNA loci (5S)	5	6	1	1	1
No. of rRNA loci (16S)	2	3	1	1	1
No. of rRNA loci (23S)	1	1	1	1	1
No. ncRNAs	5	5	4	3	3
CRISPR Arrays	0	0	1	0	0
Completeness (%)	98.68	98.68	99.50	98.88	98.42
Contamination (%)	2.21	2.54	1.48	1.44	1.44
Heterogeneity (%)	0	0	0	0	0

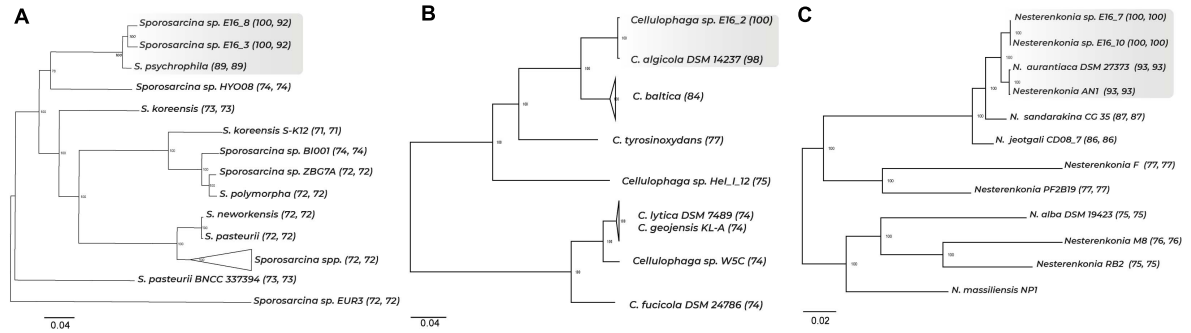

FIGURE 1 | Phylogenomic tree of core genes and ANI among representative *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia* genomes. A total of 756, 355, and 190 core genes were used to perform the phylogenomic tree *for Sporosarcina*, *Cellulophaga*, and *Nesterenkonia*. ANI values are shown in parenthesis. In **(A)**, first and second ANI values correspond to comparison with *Sporosarcina* E16_8 and E16_3, respectively; in **(B)**, ANI value corresponds to comparison with *Cellulophaga* E16_2, and in **(C)**, first and second ANI values correspond to comparison with *Nesterenkonia* E16_7 and E16_10, respectively.

compared with all *Nesterenkonia* genomes (**Supplementary Figure 2C**). Phylogenomic trees showed a clear separation between the sponge-associated strains and Antarctic strains *Nesterenkonia* sp. AN1 and *N. aurantiaca* (**Figure 1C** and **Supplementary Figure 3C**).

Altogether, these results indicate that some strains isolated from the Antarctic sponge *Iophon* sp. correspond to new species within two different phyla, i.e., Firmicutes and Actinobacteria. *Sporosarcina* sp. E16_3 and E16_8 represent two putative novel species. *Nesterenkonia* sp. E16_7 and E16_10 correspond to a single putative novel species, while the fifth strain (E16_2) corresponds to *Cellulophaga algicola*, a member of the phylum Bacteroidetes.

A general enzymatic characterization of these five strains reflected their taxonomic separation. The strains share the presence of proteases (valine arylamidase, leucine arylamidase) and a phosphohydrolase. Phosphatases were unevenly distributed between the sponge-associated strains (**Supplementary Figure 4** and **Supplementary Table 5**).

Distribution of the Sponge-Associated Bacterial Genomes in Host-Associated Microbiomes and Seawater

Fragment recruitment against 23 metagenomes showed that three of the five strains isolated have a higher abundance in seawater than in sponge microbiomes (**Figure 2A**). The relative abundance of *Sporosarcina* E16_3 and E16_8 was higher in the Antarctic sponge microbiomes *Myxilla* sp. and *Leucetta antarctica* and in seawater from Antarctica (Fildes Bay, King George Island, and Cooperation Sea), the Arctic, and the British Columbia coast. The genome of *Cellulophaga* E16_2 was most abundant in the Antarctic seawater and the Northern Pacific Ocean. However, we found the highest abundance of *Nesterenkonia* E16_10 and E16_7 in the non-Antarctic sponges *Sarcotragus foetidus*, *Aplysina aerophoba*, and *Petrosia ficiformis* (**Figure 2A**).

The taxonomic classification of the 23 metagenomic sequences revealed similar results, showing that *Sporosarcina*, *Nesterenkonia*, and *Cellulophaga* were in equal abundance in sponge microbiome and seawater samples (**Figure 2B**). Altogether, our results indicate that these sponge-associated bacteria inhabit marine sponges and their surrounding water, suggesting metabolic adaptations to thrive in both environments.

Genes Related to the Symbiotic Lifestyle in the Antarctic Sponge Bacterial Strains

To explore the genomic adaptations that allow these bacteria to live in association with marine invertebrates in an extremely cold environment, we performed comparative genomic analyses focused on the genomes from host-associated bacteria. Summary of genomic features of sponge-associated strains is shown in **Table 1**. Among all genomes of *Sporosarcina* spp., *Cellulophaga* spp., and *Nesterenkonia* spp., we generated a total of 9,632, 3,312, and 4,483 protein clusters, and we predicted 2,595, 1,481, and 1,701 COGs, respectively.

Symbiosis-related genes were widespread among the analyzed genomes (**Supplementary Table 6**). ANOSIM according to lifestyle indicates that host-associated and free-living groups were similar although with some differences (R: 0.2). Enriched functions in host-associated genomes were T-A and R-M systems, transposases, and ELPs (p-value < 0.05) (**Figure 3** and **Supplementary Table 7**), as well as transporter-encoding genes (osmotic response, efflux of threonine/homoserine lactone, and arsenic detoxification system) (**Supplementary Figure 5**). Genes involved in cold and osmotic stress response were widely distributed in the genomes analyzed (**Supplementary Table 8**). However, COGs related to Na+/H+ antiporter, universal stress protein family, and binding-protein-dependent transport system, were significantly enriched in the bacteria isolated from Antarctica in comparison with the strains isolated from other environments (**Supplementary Figure 6** and **Supplementary Table 9**).

The genomes of *Sporosarcina* sp. PTS2304, *Sporosarcina* sp. P1, *Sporosarcina* sp. E16_8, *Nesterenkonia* E16_7, *Nesterenkonia* E16_10, *N. massiliensis*, and *N. jeotgali* CD08_7 were enriched in COGs related to transposases. The genomes of *Cellulophaga* sp. E16_2, *C. algicola* DSM14237, and *C. lytica* CL8139 in proteins

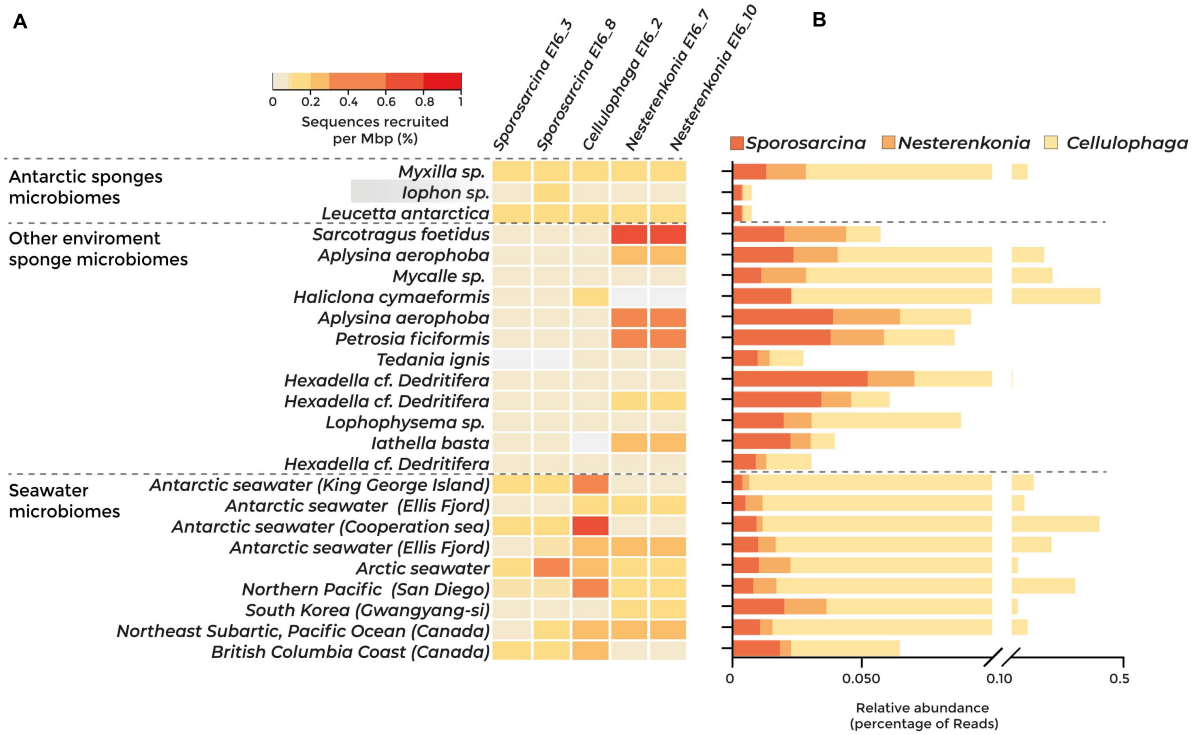

FIGURE 2 | Abundance of the sponge-associated bacterial genomes among sponge and seawater microbiomes, determined by metagenomic fragment recruitment and taxonomic classification of protein sequences. Dashed lines separate Antarctic sponge microbiomes, sponge microbiomes from other environments, and seawater microbiomes. Highlighted in gray is the metagenome from the Antarctic sponge host of the isolated strains. **(A)** Heatmap of fragment recruitment analysis. The scale bar indicates the percentage of recruited sequences per megabase pair. **(B)** Abundance of sequences classified at the protein level belonging to *Sporosarcina*, *Cellulophaga*, and *Nesterenkonia* genera.

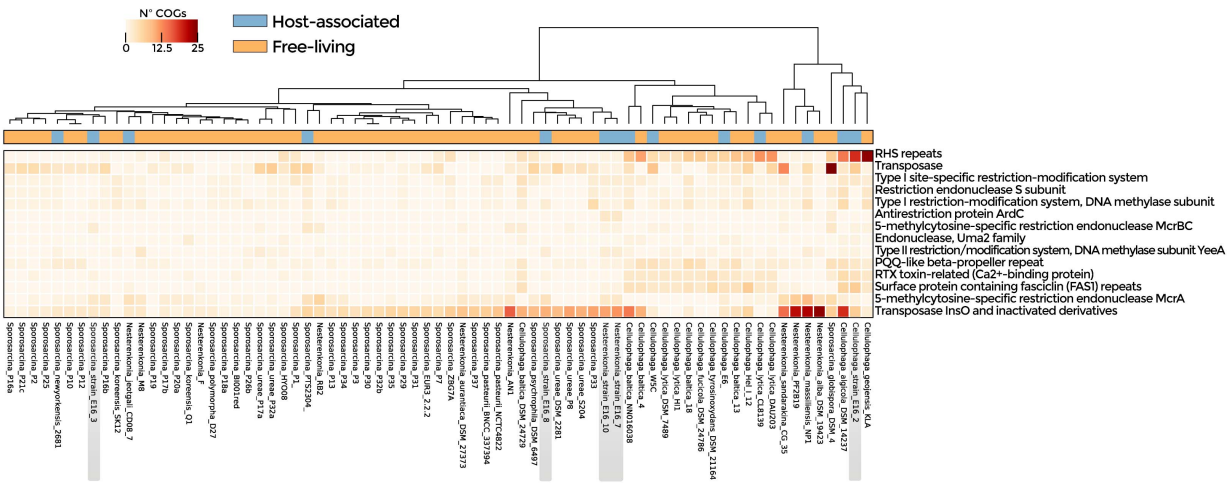

FIGURE 3 | Symbiotic lifestyle functions detected in the genomes of *Nesterenkonia*, *Sporosarcina*, and *Cellulophaga*. COGs related to CRISPR, R-M, and T-A systems, transposases, and ELPs are shown. Heatmap represents the abundance of statistically significant COGs in host-associated and free-living genomes (p-value < 0.05).

with rearrangement hotspot RHS repeats, members of the ELPs. *Sporosarcina* E16_3 and E16_8 shared three COGs related to carbohydrate degradation, virulence, and tRNA modifications (**Figure 4A** and **Supplementary Table 10**).

Accessory COGs for *Sporosarcina* E16_3 were related to DNA recombination, degradation of sialic acids, and chitin. Specifically, accessory COGs related to neuraminidase and endo-β-*N*-acetylglucosaminidase suggest the potential for the

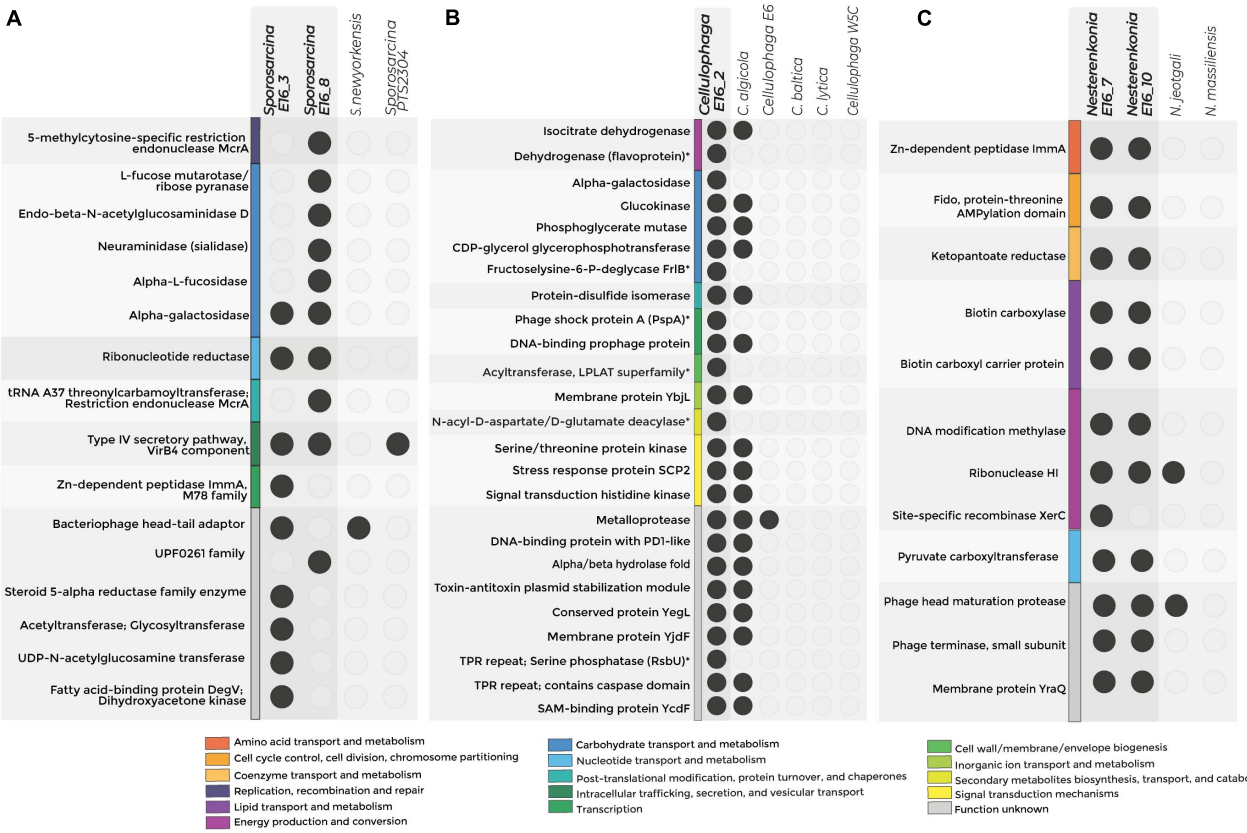

FIGURE 4 | Exclusive COGs detected in the genome of host-associated bacteria. The figure shows the exclusive COG for *Sporosarcina* **(A)**, *Cellulophaga* **(B)**, and *Nesterenkonia* **(C)**. Colors represent categories of exclusive COGs, and empty circles denote the absence of the COG in the genome. *p-value < 0.05—functions without statistical support.

digestion of structural polymers of sponge tissue (**Figure 4A** and **Supplementary Tables 10,11**).

For *Sporosarcina* E16_8, accessory COGs were related to cell wall biosynthesis and extracellular fatty acid incorporation into phospholipids. Specifically, UDP-*N*-acetylglucosamine enolpyruvyl transferase and the fatty acid kinase's structural components, the fatty acid-binding protein, and dihydroxyacetone kinase were detected exclusively in *Sporosarcina* E16_8 (**Figure 4A**).

The Antarctic strains *Cellulophaga* E16_2 and *C. algicola* DSM 14237 shared COGs potentially involved in environmental adaptations to extremely cold conditions, e.g., correct formation of disulfide bonds for protein folding and tellurite resistance, as well as COGs linked to the symbiotic lifestyle, e.g., T-A system and protein-containing tetratricopeptide (TPR) repeats. From the seven accessory COGs that the genome from *Cellulophaga* sp. E16_2 contains, three were related to d-galactose degradation and environmental stressor response by the sigma factor (serine phosphatase RsbU) and phage shock protein (Psp) (**Supplementary Table 10** and **Figure 4B**).

The pangenome analysis of the sponge-associated *Nesterenkonia* E16_7 and E16_10 revealed that their genomes shared COGs related to the T-A system (Zn-dependent peptidase ImmA), Mobile Genetic Elements (Phage head maturation protease and phage terminase), and pantothenate (vitamin B5) synthesis (**Figure 4C** and **Supplementary Tables 10,11**).

In all *Sporosarcina* genomes analyzed, the *pan*BCDE cluster is complete (**Supplementary Table 12**). This cluster codes for the vitamin B5 synthesis pathway, which is absent in sponges. *pan*BCDE cluster was complete in 26% of the available genomes of *Cellulophaga*. However, this cluster was exclusively found in the Antarctic sponge-associated *Nesterenkonia* strains (**Supplementary Figure 7** and **Supplementary Table 13**).

Genes coding for the enzymes biotin carboxyl carrier protein of acetyl-CoA carboxylase (*pyc*A), biotin carboxylase subunit of acetyl coenzyme A carboxylase (*pyc*B), and pyruvate carboxylase (*pyc*C) involved in the central anaplerotic step in the TCA cycle were also detected exclusively in both sponge-associated *Nesterenkonia* strain (**Figure 4C**). Altogether, all the functions exclusively found in the Antarctic sponge-associated bacteria's genomes could be related to genomic adaptations to cold environments and the host-associated lifestyle in the form of virulence factor as well as degradation of skeletal components of the sponges.

Horizontal Gene Transfer Related to a Symbiotic Lifestyle

We found 20 and nine GIs in the genomes of *Cellulophaga* E16_3 and E16_2, respectively, 20 GIs in *Sporosarcina* E16_8 and 34 in the sponge-associated *Nesterenkonia* strains (**Supplementary Table 14**). The presence of GIs indicated putative horizontal transfer events of the *pan*E gene in the genome of the sponge-associated *Nesterenkonia* strains. The genomic region containing the *pan*E gene represents a GI of 8,497 bp, characterized by a codon usage bias variation and a lower GC percentage than the GC-content for the whole genomic region (57.7% GC). This region is also characterized by a loose in the synteny in the genome region where the GI is present (**Figure 5A**).

Phylogenetic analysis of *pan*E-deduced protein from *Nesterenkonia* E16_7 and E16_10 revealed a relationship with *pan*E of the Actinobacteria *Corynebacterium massiliense* (member of the family Corynebacteriaceae), *Arthrobacter* sp., and *Glutamicibacter nicotianae* (members of the family Micrococcaceae) (**Figure 5B** and **Supplementary Table 15**). The adjacent genes to the *pan*E gene in the genomes of *Nesterenkonia* E16_7 and E16_10 show a close relationship with members belonging to the order Corynebacteriales (*Rhodococcus* sp., and *Corynebacterium* sp.), and Micrococcales (*Arthrobacter* sp., *Kocuria* sp., *Mycobacterium* sp., and *Micrococcus* sp.) (**Supplementary Figure 8**), indicating that most likely this complete gene cluster has undergone horizontal gene transfer within Actinobacteria members.

DISCUSSION

The sponge holobiont corresponds to one of the most complex symbiotic units, characterized by a huge microbial diversity that is considered higher than in planktonic communities globally (Thomas et al., 2016). This intimate host–microorganisms relationship is supported by a series of genomic adaptations to the symbiotic lifestyle that are still not fully understood. The whole-genome analysis presented here allows us to interrogate the genomic adaptations to the symbiotic lifestyle acquired by five Antarctic sponge-associated bacteria belonging to the genera *Cellulophaga*, *Sporosarcina*, and *Nesterenkonia*.

We conclude that sponge-associated *Sporosarcina* E16_3 and E16_8 and *Nesterenkonia* E16_7 and E16_10 may correspond to previously undescribed species. We follow multiple approaches to strengthen the analyzed isolates' taxonomic classification, including ANI analysis and phylogenomics using core genes and SCG. Based on the conventional threshold of $\geq$95 ANI used for species delineation, the sponge-associated *Sporosarcina* and *Nesterenkonia* strains showed identity values below the species-level cutoff threshold (Konstantinidis et al., 2017). ANI is a widely accepted criterion for bacterial species classification purposes (Konstantinidis and Tiedje, 2005; Yarza et al., 2014; Konstantinidis et al., 2017; Jain et al., 2018) and has been helpful for the classification of sponge symbionts as *Ca.* Poribacteria (Kamke et al., 2013), *Ca. Synechococcus spongiarum* (Gao et al., 2014), *Pseudovibrio* sp. POLY-S9 (Alex and Antunes, 2015), and *Shewanella* sp. OPT22 (Alex and Antunes, 2019). Phylogenomic analysis further supports that sponge-associated *Sporosarcina* and *Nesterenkonia* are putative novel species. However, it is important to note that all genome-based taxonomy approaches are strongly affected by genome availability in public databases, which also applies to our study. Thus, efforts to increase whole-genome sequencing of sponge-associated bacteria would be relevant to expand our taxonomic observations described here.

Notably, we observe that the five bacterial strains isolated from the Antarctic sponge *Iophon* sp. grouped in a separated phylogenetic cluster consisting exclusively of Antarctic strains. The phylogenetic separation between Antarctic and non-Antarctic clades could be associated with environmental adaptation, as observed in several bacterial members, including *Arthrobacter*, *Pseudoalteromonas*, *Nesterenkonia*, and *Paenibacillus darwinianus* (Dsouza et al., 2014, 2015; Aliyu et al., 2016). Although the underlying molecular mechanisms involved in cold adaptation in bacteria are still not fully understood, these bacteria display specialized metabolism to survive under extremely cold conditions, like heat-shock proteins, cold-active enzymes, extracellular polymeric substances, and specialized metabolites production (Pearce, 2017; Caruso et al., 2018a,b; Millán-Aguiñaga et al., 2019).

Fragment recruitment indicated that the relative abundance of the Antarctic sponge-associated strains is relatively low in *Iophon* compared with other sponges and seawater. This could be due to a low intrinsic microbial abundance in their tissues or due to the technical impairments of cultivability of marine bacteria and sponge-associated bacteria (Hardoim et al., 2014; Esteves et al., 2016). A common categorization for sponge microbiomes is between high microbial abundance (HMA) and low microbial abundance (LMA) and refers to the abundance and diversity of bacterial cells inside the sponges (Hentschel et al., 2003). This dichotomy has profound implications for sponge physiology, as HMA sponges tend to have higher pumping rates and higher nutrient exchanges (Weisz et al., 2008; Ribes et al., 2012). The HMA and LMA dichotomy has not been thoroughly studied in the sponge species *Iophon* sp.; however, *Iophon methanophila* from asphalt seeps can be considered both LMA and HMA, due to its low diversity but high abundance of associated microorganisms (Rubin-Blum et al., 2019).

Here, we show that the bacterial strains characterized contain genes relevant to the host–microorganisms relationship under cold conditions. Between them, we detect the gene coding for fatty acid-binding protein and dihydroxyacetone kinase, in *Sporosarcina* E16_8. These enzymes participate in fatty acid modification of bacterial cell membranes which mediate the adaptation to growth in cold temperatures, as well described in *S. globispora* (Yoon et al., 2001; Diomandé et al., 2015). However, fatty acid synthesis is an energy-demanding process, their exogenous uptake and assimilation would be particularly relevant for optimizing cellular metabolic processes. The same was described in the gram-positive pathogen *Staphylococcus aureus*, which incorporates host-origin unsaturated fatty acids using the fatty acid kinase (Parsons et al., 2014). This process allows *S. aureus* to lead energetic metabolism to virulence factor production (Parsons et al., 2014).

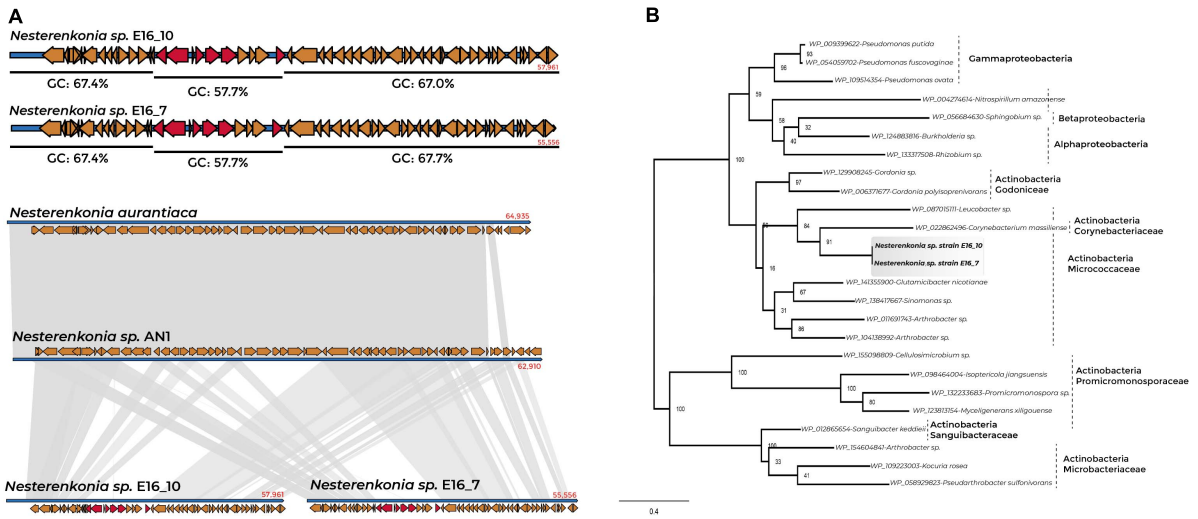

FIGURE 5 | Synteny conservation and phylogenetic tree of *pan*E gene for sponge-associated *Nesterenkonia* strains and Actinobacteria members. **(A)** %GC content of the contig containing the *pan*E gene of *Nesterenkonia* E16_7 and E16_10 and genomic organization between sponge-associated *Nesterenkonia*, *N. aurantiaca*, and *Nesterenkonia* AN1. **(B)** Phylogenetic tree of *pan*E genes of *Nesterenkonia* E16_7 and E16_10 and Actinobacteria members.

Another example is the general stress response sigma factor B, which modulates the expression of antibiotic resistance and cold stress genes (Price et al., 2001). The factor sigma factor B requires the serine phosphatase, RsbU, to release the anti-sigma factor RsbW and respond to stressors (Pané-Farré et al., 2009). Our results show that RsbU is present exclusively in the genome of *Cellulophaga* E16_2, which makes us speculate that sigma factor B-regulator would be key to respond to cold-conditions as has been proposed previously in the genome of other Antarctic bacterial strains (Aliyu et al., 2016). However, we do not rule out that RsbU could also promote the establishment of the sponge–microorganism relationship based on their role in regulating antibiotic resistance genes (Price et al., 2001). Antibiotic production in the sponge holobiont is an important process in the control of microbial pathogens and their resistance has been described as an adaptive mechanism to survive in the sponge microbiome (Phelan et al., 2011; Mangano et al., 2013; Versluis et al., 2016; Almeida et al., 2019). Thus, we can suppose that sigma B-regulator can mediate antibiotic resistance in *Cellulophaga*, as has been proposed for the pathogenic bacteria *S. aureus* and *Listeria monocytogenes* (Price et al., 2001; Kumar et al., 2014). Future studies, including transcriptomic analysis, would help clarify the role these genes play in establishing the host–microorganism relationship under cold conditions.

Furthermore, we demonstrate that the genomes from the sponge-associated strains described here have molecular determinants related to their host-associated lifestyle. These determinants include ELPs, CRISPR, and the R-M and T-A systems, which are typically enriched and highly active in sponge symbiont genomes (Slaby et al., 2017). Another accessory function detected in the establishment of microorganisms in the sponge is the degradation of structural biopolymers of sponge tissue. It has been detected in diverse pathogens of invertebrates and also in the sponge-symbiont *Poribacteria* (Kamke et al., 2013; Podell et al., 2019). The demosponge skeleton is composed of polysaccharides, such as chitin, and mucopolysaccharides in their cell surface (Brunner et al., 2009; Ehrlich et al., 2018). Accordingly, we found that the genomes of the sponge-associated *Sporosarcina* species are enriched in chitinases and neuraminidases, supporting their sponge-associated lifestyle.

Finally, we show that sponge-associated *Nesterenkonia* possesses all the genes responsible for vitamin B5 synthesis. Pantothenate (vitamin B5) forms the core of coenzyme A and acyl carrier protein, being crucial in energy and fatty acid metabolism (Leonardi and Jackowski, 2007). In contrast to animals, some sponge–symbionts can perform *de novo* synthesis of B vitamins such as thiamine (vitamin B1), riboflavin (vitamin B2), pantothenate (vitamin B5), pyridoxine (vitamin B6), biotin (vitamin B7), and cobalamin (vitamin B12) to likely provide to the sponge their vitamin requirements (Engelberts et al., 2020). Moreover, our results strongly suggest that sponge-associated *Nesterenkonia* E16_7 and E16_10 acquired the gene encoding the ketopantoate reductase within Actinobacteria members by HGT. It has been demonstrated that HGT within sponge microbiomes plays a relevant role in the metabolic versatility of sponge-associated microorganisms (Gauthier et al., 2016). Thus, microorganisms within the Antarctic sponge likely perform *de novo* pantothenate synthesis as a genomic adaptation to inhabit the sponge holobiont.

CONCLUSION

Our genomic analysis of five bacterial strains belonging to *Cellulophaga*, *Sporosarcina*, and *Nesterenkonia* reveals that their genomes contain unique genomic adaptations to life associated with the Antarctic sponges. The sponge-associated bacteria

isolated differ with respect to their free-living counterparts by the presence of exclusive genes related to cold environment adaptation and host-associated lifestyle. These genomic features include fatty acids incorporation from host origin, antibiotic resistance, and degradation of sponge structural biopolymers. Furthermore, HGT in sponge-associated *Nesterenkonia* strains suggests a putative role of *pan*E gene in the symbiotic lifestyle in the cold Antarctic waters. Overall, genomic adaptations described in the five strains suggest a high metabolic versatility that may be involved in the capability to inhabit the sponge tissue and live in extremely cold seawater environments.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article **/Supplementary Material**.

AUTHOR CONTRIBUTIONS

MM-P and NT conceived the experiments and drafted the manuscript. MM-P, GP-P, and SR-M conducted the experiments. MM-P analyzed the data. MM-P, NT, JU, and JV interpreted the data. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.660779/full#supplementary-material

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