Insights into the cultured bacterial fraction of corals

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Abstract

Bacteria associated with coral hosts are diverse and abundant, with recent studies suggesting involvement of these symbionts in host resilience to anthropogenic stress. Despite the putative importance of bacteria, the work dedicated to culturing coral-associated bacteria has received little attention. Combining published and unpublished data, here we report a comprehensive overview of the diversity and function of culturable, coral-associated bacteria. A total of 3055 isolates from 52 studies were considered by our meta-survey. Of these, 1045 had full length 16S rRNA gene sequences, spanning 138 formally described and 12 putatively novel bacterial genera across the Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla. We performed comparative genomic analysis using the available genomes of 74 strains and identified potential signatures of beneficial bacterial-coral symbioses among them. Our analysis revealed >400 biosynthetic gene clusters that underlie the biosynthesis of antioxidant, antimicrobial, cytotoxic, and other secondary metabolites. Moreover, we uncovered genomic features - not previously described for coral-bacterial symbioses - involved in host colonization and host-symbiont recognition, antiviral defence mechanisms, and/or integrated metabolic interactions, which we suggest as novel targets for the screening of coral probiotics. Our results highlight the importance of bacterial cultures to elucidate coral holobiont functioning, and guide the selection of probiotic candidates to promote coral resilience and improve reef restoration efforts.

KEY WORDS: symbiosis, holobiont, metaorganism, cultured microorganisms, coral, probiotics, beneficial microbes

Introduction

In recent years, the concept of the metaorganism or 'holobiont' has become a cornerstone of biology [1], although the view of the holobiont as the unit of selection is a matter of current debate [2]. Notwithstanding, the term metaorganism has gained momentum in modern studies of complex host-microbe interactions, as it defines the association formed by a host organism and its microbiome [2,3,4]. Scleractinian corals are an excellent example of such an association, as they build reefs through close symbiotic interactions between the host modular animal, its endosymbiotic dinoflagellates (Symbiodiniaceae) and an array of other microbial partners

including bacteria, archaea and fungi [1]. The bacterial taxa associated with corals can vary between coral species and geographical origin, though often there are patterns in the community structure that link microbial and coral taxa [5,6]. The majority of recent studies exploring the importance of coral-associated microbes have focused on the use of cultivation-independent approaches, based on 16S rRNA gene amplicon sequencing [7] and, more recently, shotgun metagenomics [8]. Such methods are central in identifying what bacteria are associated with corals and how their metabolic and functional potential contribute to holobiont health and response to environmental conditions [7,9 10]. However, the bacterial metabolic pathways that interact with the host and respond to environmental changes are often best understood using culture-based approaches [11]. This is particularly relevant because metagenomic information gives insights into potential functional traits and other cellular traits only, and often environmental changes have pleiotropic effects on holobiont physiology that are impossible to grasp using metagenomics alone [12,13,14,15].

Inherently, culture-based approaches retrieve only a small fraction of the total bacterial diversity within any given environment, a phenomenon known as the "great plate anomaly" [16,17,18]. Often however, it is not a case of being 'unculturable', but of not yet knowing the range of conditions needed to culture these microorganisms. Cultivating host-associated microorganisms can be challenging, as their nutrient requirements and cross-feeding networks are often unknown [19]. In addition, many environmental microorganisms grow very slowly (in contrast to clinical isolates), and are not adapted or capable of growing on commonly used nutrient-rich media [20,21]. To counter this, at least to some degree, recent studies have implemented novel and alternative culture-based methods to retrieve a higher proportion of the bacterial diversity in any given sample [19,22], and these approaches have also been applied to corals [23,24,25].

Tissue compartmentalization and organismal complexity are thought to underlie high bacterial diversity in corals [26,27,28]. The diverse coral bacteriome plays an integral role in the balance between health and disease of the coral holobiont [29] and represents a valuable source of biotechnological products [30]. [31] was perhaps the first to actively isolate bacteria from coral, recovering strains from the skeletal regions of *Porites lobata* followed by [32] who reported on bacteria isolated from mucus of *Porites astreoides* and two soft coral species. Microbial mediated diseases have also been well documented as driving declines in reef health,

especially throughout the Caribbean for example [33]. This has fostered a great interest in understanding coral disease causative agents, stimulating cultivation efforts of coral-associated bacteria [34,35,36]. For example, [37] isolated a bacterium that caused bleaching of the coral *Oculina patagonica*, and many subsequent studies have implicated vibrios in coral disease causation [38,39,40]. Many of these focused on targeted isolation and conducted reinfection studies to satisfy Koch's postulates (reviewed in [41]).

Similarly, growing evidence underlines the key role compounds produced by bacteria have on host health[42,43,29,44,45]. For instance,[46] was among the first to demonstrate that mucus-associated bacteria from healthy colonies inhibit the growth of potential pathogens, like the vibrios mentioned above. Subsequent studies revealed high antimicrobial activity among culturable coral-associated bacteria, with up to 25% of the isolates producing antimicrobial compounds [47]. [48] showed a strong link between observed antibiotic activity in well diffusion assays and existence of polyketide synthase (PKS) and/or non-ribosomal peptide synthetase (NRPS) genes in the bacterial isolates. More recently, [13] identified the antimicrobial compound tropodithietic acid (TDA) to be produced by the coral-associated bacterium *Pseudovibrio* sp.. In fact, interestingly, *Pseudovibrio* species harbour several biosynthetic gene clusters for the synthesis of bioactive compounds [49,50].

These studies reinforce the notion that the isolation of bacteria from corals is a vital part of coral science. They are invaluable for assessing the virulence of potential pathogens, and for applying classical clinical approaches to elucidate disease aetiology [51]. Beneficial traits that bacteria may provide to coral holobiont functioning can also only be proven using pure bacterial cultures [14,8]. Perhaps most importantly, bacteria isolated from corals can be used as probiotics to facilitate host health [52,53]. Such approaches have been proposed to promote coral resilience in the face of environmental stress. For example, [44] showed that application of these 'beneficial microorganisms for corals' (or BMCs) increases the resilience of the coral to temperature stress and pathogen challenge.

Here, we sought to centralize and curate the current cultured fraction of coral bacteria by combining published data with unpublished collections from around the world. We explore relationships between the bacteria acquired and the host origin and the media utilized for growth. A total of 74 genomes of cultured coral bacteria, 36 of which are available in public

databases and 38 of which are presented in this study for the first time, were investigated to infer potential genetic signatures that may facilitate a host-associated lifestyle. Alternative ways and improvements for the isolation of bacterial groups not yet recovered from corals (including targeting specific obligate symbionts) are also discussed. This study provides the most comprehensive synthesis of the cultured bacterial fraction of the coral holobiont to date.

Methodology

Literature search and data curation

The National Center for Biotechnology Information (NCBI) was searched for publicly available 16S rRNA gene sequences of cultured coral-associated bacteria. This was supplemented with culture collections from laboratories around the world through a public invitation to researchers working in this field. The initial invite started through social media (Twitter), then developed by word of mouth. In total, we were able to obtain bacterial isolates originating from 84 coral species (representing tropical, temperate, and cold-water habitats), and representatives from all major oceans (**Figure 1**; supplementary material, Supplementary **Tables 1**).

Phylogenetic analysis and tree generation

In total, we were able to collate 3055 individual isolates which had a part of the 16S rRNA gene sequenced (**See Supplementary Table 1 for details**). Sequences with less than 500 base pairs (bp), longer than 1600 bp, or containing more than 1 ambiguity were removed. Screen-seqs (mothur version.1.42.0) was used to remove poorly aligned sequences and filter-seqs was used to remove positions without sequence information [54]. This left 1045 isolates with near full-length 16S rRNA gene sequences. These were then aligned using the SILVA 138.1 database as a reference [55], and the clear-cut command was used within mothur to generate a phylogenetic tree. The tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model. A phylogenetic tree of coral hosts was also generated using the Taxonomy Common Tree tool of NCBI [56]. Species names were added manually to create a tree file. Tree features were optimized using iTOL v4 [57].

Taxonomic composition of bacterial isolates by medium

Bacterial strains listed in **Supplementary Table 1** were sorted by isolation medium and subsequently grouped at phylum, order, and genus levels according to the current SILVA (138.1) taxonomy[55]. Stacked column graphs, showing relative abundances of the cultivated taxa were created thereafter. At the genus level, all groups representing less than 1% of the total pool in each medium were included in a group labelled "others".

Genome analysis

The integrated Microbial Genomes and Microbiomes database (IMG; https://img.jgi.doe.gov/) from the Joint Genome Institute (DOE-JGI), and the assembly database from NCBI (https://www.ncbi.nlm.nih.gov/assembly) were searched for publicly available genomes from cultured coral bacteria in February 2020. Thirty-six bacterial genome assemblies (21 from scleractinian coral and 15 from octocoral associates) were downloaded from NCBI and included in this analysis. A further 38 bacterial genomes from scleractinian corals were uploaded as part of this manuscript (Project Accession Nos. GS0145871, and PRJNA343499). The annotation of general genomic features such as genome size, GC-content, and number of coding sequences (CDSs) was performed for all 74 genomes with the RAST server [58] (**see Supplementary Table 2**). Protein families (Pfams) were predicted with the online-server WebMGA (default settings) [59] and using amino-acid sequence files obtained from RAST. The resulting individual Pfam annotation files were then joined using a customized script in R and the resulting count tables were Hellinger-transformed for multivariate analyses. Dissimilarity between genomes based on the Pfam profiles were then calculated using the Bray-Curtis index and data clustered using a principal coordinate analysis (PCoA) and plotted in Eigenvalue scale (i.e. scaling of each axis using the square root of the Eigenvalue) using PAST v3.25[60]. PERMANOVAs (permutational multivariate analysis of variance) were performed with 999 permutations to test for overall differences in the Bray-Curtis dissimilarity matrix between Pfam profiles of bacterial genomes from different taxonomic classes. Five groups (classes) were used: Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Cytophagia, and Flavobacteriia. A separate PERMANOVA analysis of Bray-Curtis dissimilarities calculated for the 11 available *Vibrio* genomes was then performed in order to highlight differences between strains identified as

potentially pathogenic (n = 5, group 1) and those apparently non-pathogenic (n = 6, group 2) (identification of pathogenicity from available literature – see references). Finally, AntiSMASH version 5.0 [61] was used with default parameters to identify biosynthetic gene clusters (BGCs) in all genomes.

Results

Phylogenetic analysis of culturable coral-associated bacteria

Published and unpublished datasets were interrogated, identifying 3055 cultured coral-associated bacteria, for which 1045 high-quality full-length 16S rRNA gene sequences were available (**Figure 2a**). This collection demonstrates that many bacterial genera (138) can be cultured from corals. While most isolates belong to the phylum Proteobacteria (72% of those cultured), strains from Firmicutes (14%), Actinobacteria (10%), and Bacteroidetes (5%) were also recovered. The genera *Ruegeria*, *Photobacterium, Pseudomonas, Pseudoalteromonas, Vibrio, Pseudovibrio, and Alteromonas* were commonly isolated across studies (**Figure 2 and 3**; see **Supplementary Tables 1, 3 and 4**). Of 43 genera identified as likely beneficial microbes (proposed in current literature see references in **Supplementary Table 4**), 25 (58%) have been cultured and are represented in this collection (**Supplementary Table 4**). Most of the isolates that have been cultured from diseased corals belong to the phylum Proteobacteria, specifically to the family Vibrionaceae. However, it should be noted that many of the studies reporting these focused on a targeted approach to isolate these bacteria. Among the isolates from the Proteobacteria phylum, 25.5% were associated with diseased coral colonies, as were 7.4% of the isolates belonging to the phylum Bacteroidetes. Firmicutes and Actinobacteria had the lowest percentages, with 5.0% and 0.7%, respectively. Although the majority of the isolates were matched with other representatives on GenBank, 12 were highly divergent with low identify to known isolates suggesting that they may be novel genera.

Taxonomic composition of bacterial isolates by culture medium

The taxonomic patterns of the cultured bacterial strains at phylum, order, and genus level, vary according to the type of medium used to isolate them **(Figure 4)**. Marine agar (MA) (including its diluted versions) was the most commonly utilised medium across studies, and produced 715 unique isolates collectively. Bacterial isolates belonging to the families

Vibrionaceae, Alteromonadaceae, Pseudoalteromonadaceae, Rhodobacteraceae, Flavobacteriaceae, and Micrococcaceae could all be isolated from MA from a diverse set of coral species. The next most productive non selective medium, glycerol artificial seawater agar (GASWA), produced 572 isolates, while a variety of 'custom' media from different laboratories produced 523 isolates. Interestingly, this latter collection of media i.e. the custom variants (along with blood agar specifically), favoured the retrieval of Firmicutes and Actinobacteria species at the expense of Proteobacteria representatives. In contrast, media commonly deployed to sample a wider bacterial diversity, such as marine agar, favoured the growth of several Proteobacteria species, which in turn is usually affiliated with diverse clades within the Alphaproteobacteria and Gammaproteobacteria classes (**Figure 2 and 4**). Curiously, use of Thiosulfate-citrate-bile salts-sucrose medium (TCBS) supported the growth of manifold bacterial lineages across the four phyla documented in this study, despite its presumed selectivity for *Vibrio* species.

As mentioned, the majority of the isolates (72%) belonged to the Proteobacteria. Indeed, members of this phylum could be retrieved from nearly all cultivation media and conditions examined in our survey – in higher or lower numbers, according to the design and scope of the study (**Figure 2**). Bacteria belonging to the phyla Firmicutes, Bacteroidetes, and Actinobacteria, also appeared to be cultured on most media, though in lower numbers (**Figure 4a**). Orange Serum Agar seemed to be selective for Actinobacteria (**Supplementary Table 1**). The media MA, R2A, and minimal basal agar shared a very similar pattern at the order level, with all yielding similar proportions of members from the orders Vibrionales, Rhodobacterales, Pseudomonadales, Flavobacteriales and Actinomycetales (**Fig. 4**). Likewise, LB, blood agar, and the 'custom' media shared similar proportion patterns, which included the orders Vibrionales, Pseudomonadales, Bacillales, Alteromonadales, and Actinomycetales (**Figure 4b**). At the genus level, no immediate patterns seemed to be shared among the media (**Figure 4c**). The highest number of unique isolates identified to genus level was obtained from MA (115), followed by custom media (55), minimal basal media (48), and GASWA (47) (**Supplementary Table 1**). However, when dividing the number of different genera by the total number of isolates in each medium, the normalized ratios show that nutrient agar (0.64), followed by DMSP-enriched media (0.54) and R2A (0.4), supported the growth of higher bacterial diversity. Conversely, lowest bacterial diversities were found on TCBS (0.04), Nfb

(0.04), and GASWA (0.08). The normalized ratios for each medium (considering all the isolates analysed here) can be found in **Supplementary Table 1**.

Functional genomics of coral bacterial isolates

A total of 74 cultured coral associated bacteria had full or draft genomes available (36 genomes were accessible as of February 2020, with a further 38 genomes now available from this study **(Supplementary Table 2).** The genome sizes ranged from 2.71 Mb in *Erythrobacter* sp. A06_0 (associated with the scleractinian coral *Acropora humilis*) with only 2669 coding sequences (CDSs), to 7.28 Mb in *Labrenzia alba* EL143 (associated with the octocoral *Eunicella labiata*) with 4551 CDSs (**Supplementary Table 2**). The mean and median genome size was 4.77 Mb and 4.71 Mb, respectively. The average GC-content of these genomes was 52.99%, with the lowest GC-content (32.9%) found in *Aquimarina megaterium* strain EL33 (isolated from *E. labiata*), and the highest GC-content (71.4%) found in *Janibacter corallicola* strain NBRC 107790 (from *Acropora gemmifera*).

Multivariate analysis, based on protein family (Pfam) profiles (**Figure 5a**), unsurprisingly showed that the genomes grouped mostly according to their (class-level) taxonomic affiliations (PERMANOVA, $F = 11.55$, $P = 0.0001$). Exceptions were the two Actinobacteria and the two Bacteroidetes genomes, which clustered with four Alphaproteobacteria genomes of the order Sphingomonadales and Caulobacterales and the *Luteimonas sp.* JM171 (Gammaproteobacteria) genome, respectively. However, this is likely a reflection of the very low number of genomes available from coral-associated Actinobacteria and Bacteroidetes rather than a significant functional overlap between the two phyla. Interestingly, a PERMANOVA analysis performed on the Pfam profiles of the Vibrionales genomes revealed that the five *Vibrio* genomes from known pathogens were significantly different from all non-pathogenic Vibrionaceae strains ($P = 0.0006$, df = 1, $F = 1.829$).

Functions that potentially have a role in host-microbial interactions, such as proteins containing Eukaryotic-like domains involved in host-symbiont recognition [62,63], secretion systems important for host colonization, and biosynthetic gene clusters encoding for secondary metabolites were investigated across the isolates (**Figure 5b**). The *Endozoicomonas* strains G2_1, G2_2 and Acr-14 had the highest number of ankyrin repeats (> 789), and high

numbers of WD40 repeats (between 37-116). All Alteromonadales strains (including the *Pseudoalteromonas* BMCs), had high numbers of tetratricopeptide (>250) and WD40 (29-142) repeats. The strain with the overall highest number of Eukaryotic-like repeat protein related entries (1367 repeats) was *Endozoicomonas* sp. strain G2_01 from *Acropora cytherea*, closely followed by the octocoral associate *Aquimarina megaterium* EL33 (class Flavobacteria) (1208 repeats). By contrast, ankyrin repeats were absent or only present in low numbers in all the *Vibrio* strains. *Endozoicomonas montiporae* strain EL-33 displayed the highest number of domains related to antiviral defence mechanisms, such as CRISPR proteins and endonucleases, which are known to be enriched in the microbiomes of marine sponges [63,64]. Further, 49 out of the 74 genomes assessed, harboured the TauD (PF02668) gene. TauD is involved in the degradation of host-derived taurine into sulfide which is then assimilated into microbial biomass [65,66,67]. An elevated number of TauD encoding CDSs was found in the two BMC strains *Cobetia marina* BMC6 and *Halomonas tateanensis* BMC7, both isolated from *Pocillopora damicornis.* Further, several isolates (*N* = 11) of the Rhodobacteraceae family (Alphaproteobacteria) contained CDSs involved in dimethylsulfoniopropionate (DMSP) degradation, potentially contributing to sulphur cycling in corals.

Among secretion systems, type II (T2SS), III (T3SS), IV (T4SS), and VI (T6SS), known to be involved in host colonization e.g.[68], horizontal gene transfer e.g. [69], or interbacterial antagonism and/or virulence e.g. [70], dominated the genomes of coral-associated bacteria. We found a high number of entries related to T2SS in the Gammaproteobacteria associates, particularly in the *Endozoicomonas* and *Vibrio* genomes (see [71] for roles of the T2SS in symbiosis and pathogenicity). The Vibrionales genomes were further characterised by an elevated number of T6SS-related Pfam domains, whereby the five pathogenic *Vibrio* strains encoded a significantly higher number of T6SS domains (mean=27 T6SS domains in CDSs) than the six non-pathogenic *Vibrio* strains (mean=10 T6SS domains in CDSs; Man-Whitney U-test, $p = 0.0126$).

We also assessed the secondary metabolite coding potential in the 74 genomes. AntiSMASH v.5.0 detected a total of 416 biosynthetic gene clusters (BGCs) across all genomes, whereby the number of BGCs varied substantially between strains, from zero BGCs in *Endozoicomonas montiporae* CL-33 to 12 BGCs in *Pseudoalteromonas luteoviolacea* HI1 (**Figure 6**). Bacteriocin clusters (*N* = 75), found in 81% of the strains, were the most frequently detected BGCs,

followed by homoserine lactone (*N* = 62; in 43% of strains), non-ribosomal peptide synthetase (NRPS; *N* = 59; in 51% of strains), beta-lactone (*N* = 46; in 53% of strains), terpene (*N* = 34; in 38% of strains), ectoine (*N* = 28; in 35% of strains), and siderophore (*N* = 25; in 28% of strains) clusters. The relatively large group of coral-associated Rhodobacteraceae genomes analysed in this study presented a consistently rich BGC profile, characterised by the presence of bacteriocin, homoserine lactone, and NRPS-T1PKS clusters, while siderophore clusters were typically absent in this group. Siderophores were typically found in the *Vibrio* genomes of this study as well as in three of the four *Endozoicomonas* genomes. Characteristic for all *Pseudoalteromonas* genomes, including the BMC strains, was the presence of aryl polyene clusters, a compound class functionally related to antioxidative carotenoids [72]. The absence of known BGC in the genome of *E. montiporae* CL-33 is an unusual outcome, as for example the closely related strains in the Oceanospirillales order usually display > 4 BGCs (**Figure 6**). The *E. montiporae* CL33 genome is complete (100% completeness, 0.9% contamination, 95.5% quality; 1 contig); hence, low assembly quality - which sometimes compromises the identification of large BGCs - does not explain the lack of BGCs in this genome.

Discussion

Here we show that many coral-associated bacteria (n = 3055), can be isolated using a variety of medium and culture conditions. 138 of these isolates (recruited from 52 studies) have been formally described and at least 12 are putatively novel bacteria genera. It is promising that such extensive phylogenetic diversity can be captured from a limited number of culture media employed in the examined studies. Additional diversity is therefore likely to be captured through the design of alternative cultivation procedures that may improve our capacity to "cultivate the as-yet-uncultured''. Testimony to this is the observation that most of the strains assigned to the Firmicutes phylum in our metanalysis were obtained almost exclusively from the various "custom media" utilised by different labs and blood agar alone, illustrating how diversification in cultivation design can widen the phylogenetic spectrum of the organisms domesticated in such endeavours. In this regard, we anticipate that broader phylogenetic diversity will be gained within the culturable fraction if gradients in aerophilic conditions, temperature, and other physicochemical parameters are attempted along with innovative, less invasive techniques to extract microbial cells from the host matrix. Intriguingly, the richness of bacterial phyla uncovered in this study corresponds to the phyla more often

reported to dominate bacterial communities in corals by cultivation-independent studies[9], namely Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, yet how diversity at lower taxonomic ranks within each phylum is captured remains to be determined .Another exciting challenge ahead of us, is the unveiling of host-microbe and microbe-microbe molecular interdependence networks (e.g. cross-kingdom signalling and cross-feeding cascades). Such knowledge would ultimately enable laboratory captivation of so-far "unculturable", coral-specific or enriched lineages. Microbial-host and microbe-microbe interactions rely on several functions that are often found in host-associated and free-living bacteria [73,74,49,75]. Hence, having pure cultures of coral-associated bacteria can help to identify the genomic features that could underpin the interaction with the host and its microbiome and lead to their experimental validation.

Although one of the initial aims of this study was to ascertain the percentage of culturable bacteria from any given coral species, it was deemed too speculative to report the findings due to variation in culture effort across the various studies. Indeed, this highlights the paucity of studies dedicated to determine exactly this, and there is an urgent need for such mechanistic projects deploying multiple culture media and conditions to comprehensively sample bacterial associates from a single or few host species to be undertaken (see [76,77,25] for examples of such studies on sponges and corals, respectively). Collectively, studies designed as such, hold promise in illuminating our view of the cultivability of coral bacterial communities in a straightforward manner, and in solidly delineating the "cultivability gap" that is yet to be bridged in future experiments.

That said, having a catalogue of cultures as presented here (and one which will hopefully be ever-expanding) means we are, from now on, in a position to increase our understanding of host-symbiotic relationships. The ability to describe, understand, and culture specific symbionts from any given organism (like corals) also opens up the potential to utilise them as pro-biotics to restore degraded habitats [44,52]. In addition, such a resource increases the possibility of identifying novel compounds of biotechnological interest[78]. This seems particularly relevant in the case of coral-microbe symbioses, which are known to rank as one of the most prolific sources of bioactive molecules in the oceans [79].

A search in public databases (NCBI) found that, despite the 1045 cultured coral-associated bacterial sequences with full-length 16S rRNA gene sequences, only 36 had genomes available as of February 2020. Clearly, a systematic effort to disclose the genomic features of coral-associated bacteria is needed in order to better understand the holobiont ecology and identify potentially beneficial microbes. As part of this study, we were able to add a further 38 to this tally **(Supplementary Table 2).** Even with this addition, the number of publicly available coral-associated bacterial genomes remains scant and it is recognised that to more fully understand the roles of the cultivable fraction of coral bacteria, a thorough characterization of the species kept in culture, including genome sequencing, needs to be fostered alongside experimental biology and manipulative approaches. Moreover, a large collection of coral-associated genomes could also help to identify specific traits that are needed to thrive in the various niches within the hosts or point to those bacteria which offer a specific benefit to their host.

All of the available genomes were screened for an array of functions potentially important in establishing and maintaining interactions between bacterial symbionts and their marine invertebrate hosts. Overall, the *Endozoicomonas* and *Pseudoalteromonas* strains displayed high numbers of eukaryotic-like protein-encoding genes essential for host-symbiont recognition in well-studied systems such as marine sponges [80,81,63]. The strain with the second highest number of eukaryotic-like repeat protein related entries (1208 CDSs, after *Endozoicomonas* sp. G2_1 with 1367 CDSs), was the octocoral-associate *Aquimarina sp.* EL33 (class Flavobacteria). In the current culture collection, 15 additional *Aquimarina* isolates are reported, from the scleractinian corals *Porites lutea*, *Pocillopora acuta*, *Stylophora pistillata, Acropora millepora*, *A. tenuis* and the octocoral *E. labiata*. Retrieving the genomes from these candidates will allow us to further explore these emerging patterns in greater detail. For example, a recent comparative genomics survey of host-associated and free-living *Aquimarina* species revealed complex secondary metabolite biosynthesis and poly-carbohydrate degradation capacities [82], but further investigation into their mechanisms of interactions with corals is warranted.

Only eight *Endozoicomonas* isolates (five of them type species) have so far been cultured (according to our collated information). These are from the octocorals *Eunicea fusca* and *Plexaura* sp. and the scleractinian corals *Montipora aequituberculata*, *Acropora cytherea*, *A.*

hemprichii and *Acropora.* sp. To date, only four of these (two from this study), have had their genomes sequenced (all from scleractinian corals) [14,83]. This is surprising given that numerous studies found that this genus is highly abundant in the healthy coral holobiont (e.g. reviewed in[29,84]. Future cultivation efforts should therefore be directed towards the Endozoicomonadaceae family, in order to increase the representation of their taxonomic and functional diversity in culture collections. In this regard, this study finds evidence that supplementing culture media with DMSP is an approach worth investing in future attempts to cultivate coral-associated *Endozoicomonas*. The metabolic data obtained from the comparative analysis of these four strains can be used, for example, to drive the selection of specific nutrients and conditions required to culture this particular genus of coral symbionts. Furthermore, there are 55 cultured *Pseudoalteromonas* strains in our collection which should also be explored regarding their symbiotic properties and their functional gene content (only 6 genomes currently available). Similar to *Endozoicomonas*, *Pseudoalteromonas* species are also frequent members of coral-associated microbiomes [29]. A number of *Pseudoalteromonas* have been shown to harbour high antimicrobial activity and many of these bacteria are isolated from coral mucus, lending support to the protective role the surface mucus layer has for the host and its importance in the coral holobiont's defense - against gram-positive coral pathogens in particular [85]. Indeed, five of the six *Pseudoalteromonas* (where genomes are available), were shown to be effective BMCs when corals were challenged with the coral pathogen *Vibrio coralliilyticus* [44].

Having genomes available from the potential pathogens also allows for greater insight into coral biology, especially when interested in ascertaining pathogenicity-related traits [86,87]. For example, from the 11 *Vibrio* species where genomic data was available, we were able to show functional separation (based on Pfam profiles) of known pathogenic and non-pathogenic strains. This was further accompanied by a significantly higher abundance of CDSs encoding for the Type VI secretion system, important for virulence in the pathogenic strains [70]. Prevalence of siderophore-encoding genes was also noted in the Vibrionaceae strains, suggesting that these bacteria likely gain competitive advantages through efficient and extensive iron acquisition, which is a trait often seen in opportunistic and pathogenic bacteria [88,89]. Hypothetically, the selection of beneficial microbes that are also good siderophore producers could add to the biological control of these pathogens. Indeed, two proposed BMC

strains *Cobetia marina* BMC6 and *Halomonas taenensis* BMC7, harbour such siderophore clusters on their genomes and so did three of the four *Endozoicomonas* strains. However, the five *Pseudoalteromonas* BMC strains and the *Endozoicomonas montiporae* CL-33, had contrasting low numbers of BGCs, possibly indicating a reduced investment into secondary metabolism. Indeed, the low number of BGCs in these *Pseudoalteromonas* strains is in contrast to the established prevalence of biologically active compounds in many marine host-associated *Pseudoalteromonas* strains [90]. In part, this may reflect a limitation of the software utilised to detect genes for all secondary metabolites, as genes for common metabolites (such as for the production of the antibiotic marinocin and those that produce tetrabromopyrrole coral larval settlement cues by *Psuedoalteromonas* [91,92]), were not picked up. These bioinformatic limitations emphasize the importance of having bacterial cultures for the elucidation of the chemical ecology underpinning coral holobiont functioning.

Broader functional traits can also be ascertained from looking at the complete picture of isolates with annotated genomes. For example, 66% (49 out of 74) harboured the TauD gene, which is involved in taurine utilization [93]. Two proposed BMCs, the *Cobetia marina* BMC7 and *Halomonas taeanensis* BMC7, revealed the highest copy number of TauD CDSs (severn and eight, respectively), while others range between one and five TauD copies. Taurine is an organo-sulphur compound widely present in animal tissues, and recent research has shown that obligate symbionts of sponges have enriched copies of taurine catabolism genes and taurine transporters in comparison with free-living bacteria [63,67,66]. The widespread capability of the isolates studied here to potentially utilize host-derived taurine, could guide the formulation of novel, taurine-containing cultivation media in the attempt to captivate coral symbionts, particularly from the important, yet underrepresented order Oceanospirillales (TauD was consistently present in all Oceanospirillales genomes (*N*=8) analysed here). The ubiquitous occurrence of bacteriocin clusters among the genomes is another example of broad scale trends which we have identified in our genome meta-analysis. These may confer the specific culturable symbionts with particular competitive capacities towards closely related taxa in highly dense microbiomes, as is commonly identified across corals and sponges [94,95]. Moreover, the widespread presence of NRPS and beta-lactone clusters hint towards broad-spectrum antimicrobial and cytotoxic capabilities in multiple associates. It also corroborates the hypothesis that these marine metaorganisms are promising sources of novel

bioactive compounds, representing targets for bioprospection[79]. Many strains also possess homoserine lactone encoding BGCs indicative for sophisticated, cell-density dependent chemical communication mechanisms. Antioxidant activities are likely conferred by the presence of aryl polyene BGCs in the genomes [96]. These pigment type compounds, functionally related to carotenoids, characterised most of the proposed BMC strains. Furthermore, several coral-associated bacteria of different taxonomic origins are seemingly well equipped to handle osmotic stress as revealed by the occurrence of ectoine and N-acetylglutaminylglutamine amide (NAGGN) encoding genes. Therefore, there is a need to continue the effort in culturing coral-associated bacteria to explore new biosynthetic potentials, both for bioprospecting purposes and for better understanding the chemical ecology of the metaorganism.

Identifying likely candidates for symbiosis is one challenge; but, once these are confirmed and characterised, the need to understand how the host establishes symbiosis and retains the relationship will also be critical. However, this is a two-way street. Current research in sponges has revealed that bacteria expressing the ankyrin genes avoid phagocytosis by sponge amoebocytes, thus becoming residents of the sponge microbiome by evading the host's immune system [64]. The evolutionary forces shaping the symbiosis are even trickier here, as bacteriophages encode for ankyrin biosynthesis in their genomes and might transfer this information across different community members [64]. Further, as ankyrin repeats are enriched in the microbial metagenomes of healthy corals [8], a similar pattern of symbiosis establishment would be expected for corals.

To conclude, here we have highlighted that diverse coral-associated bacteria are already cultured, although these are often scattered across collections and rarely collated into one easily accessible location. Further, only a few of these have had their genomes sequenced. In spite of the lack of genomes we were able to identify a number of genetic features that seem to be enriched in these coral bacterial associates. These include the production of broad-spectrum antimicrobial, antioxidant, and cytotoxic capabilities, high abundance of ankyrin repeat entries, tetratricopeptide, and WD40 repeats, and taurine degradation genes. We have also observed a reduced investment into secondary metabolism, as a feature, in a number of coral bacterial associates. That said, this can only be quantitatively assessed in a robust manner if we could compare metagenome profiles from corals vs. other environments,

such as sediments and seawater in a comprehensive fashion (several samples with replication etc). Such metagenome-based analyses should be complemented by (large scale) marker gene surveys and/or visualization techniques to determine the nature and holobiont site of bacterial association, in particular since any metaorganism (configuration) is specific to a time and place and not static given the temporal ('fluidic') nature of host-microbial interactions [97]. The statistical power, with only the few representative genomes available from cultures (as in this study), is therefore not going to be the most reliable to generate concrete conclusions, so we should take these results more qualitatively and with caution. This is especially so, as many of the cultivable fraction may not even be the dominant members of the coral microbiome.

We end by highlighting the importance and need for a global initiative, to create an online catalogue of genomic and physiological features of cultured coral-associated bacteria. Combining the use of these genomic insights with innovative culturing techniques [30], aimed at improving the collection of coral-associated bacterial isolates will see this field of coral biology serge forward. Such an initiative should likely start with those microbes which have their complete genomes sequenced. This study pioneers the organization of this global collection, as part of the efforts from the Beneficial Microbes for Marine Organisms network (BMMO), through a public invitation to researchers working in this field. As a result, we have here provided a list of all the cultured bacteria from all types of corals that are currently available in public databases, plus isolates that were kept in collections from all the labs that have attended our invitation (**Supplementary Table 1** and available now, open access via isolates.reefgenomics.org). Now other researchers can access this virtual collection and/or contact specific labs for collaborations or solicitations of specific microbial strains.

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Legends to Figures

Figure 1. Overview of the data used and generated in this manuscript. Sampling sites of the coral species used as isolation sources (a). Data summary recovered from the publications and accession numbers available in data banks (b). Overview of the analyses performed in the current manuscript using the available isolates.

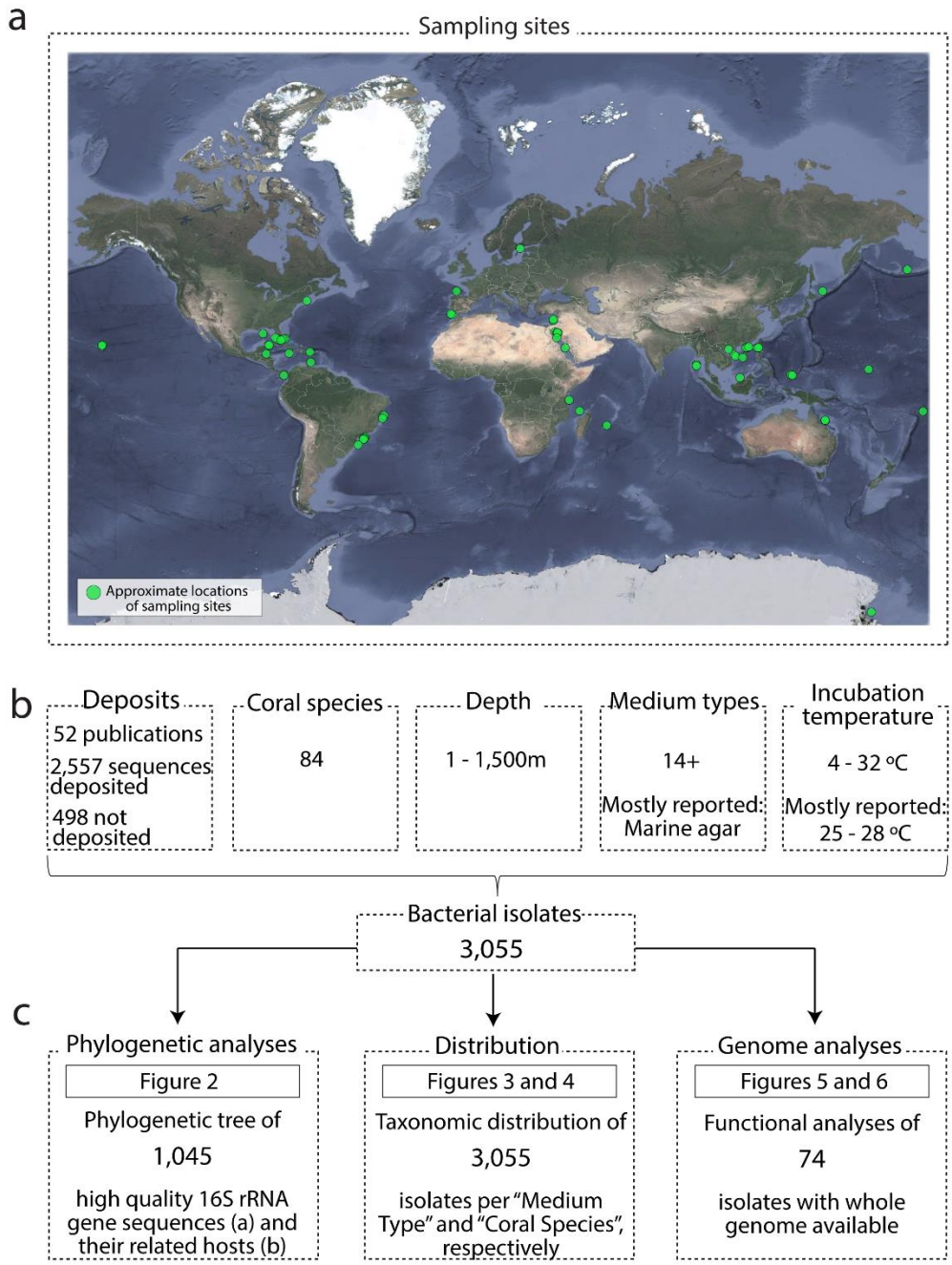


Figure 2. Phylogenetic trees of bacterial strains and coral species. (A) 16S rRNA gene-based phylogenetic inference of 1,045 coral-associated bacterial isolates, plus eight type-strains representing the species *Vibrio alginolyticus, Vibrio bivalvicida, Pseudoalteromonas aestuariivivens, Pseudomonas guariconensis, Massilia namucuonensis, Vibrionimonas magnilacihabitans, Mycetocola tolaasinivorans,* and *Bacillus subtilis*. The outer ring groups, by colour, the medium used to isolate the strains and the inner ring labels the coral genera used as sources for bacterial isolation. **(B)** Phylogenetic tree of the species of corals used in this study produced via https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). The label colours used to identify the genera are linked to the inner ring of Figure 1A.

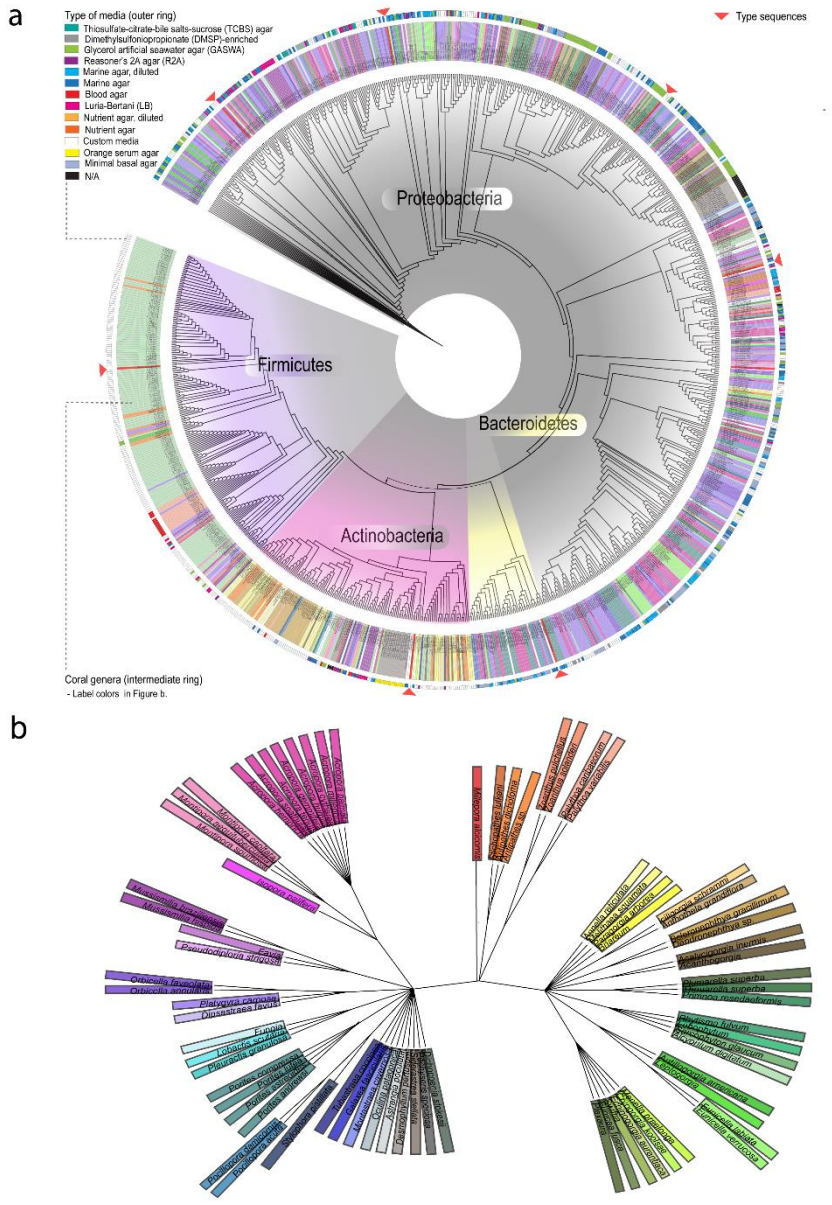


Figure 3. Phylum (A), order (B) and genus (C) -level profiles of coral-associated bacteria isolated from each coral species. Taxa (i.e. orders and genera) representing less than 1% of the total percentage of isolates were pulled together and classified as “others”.

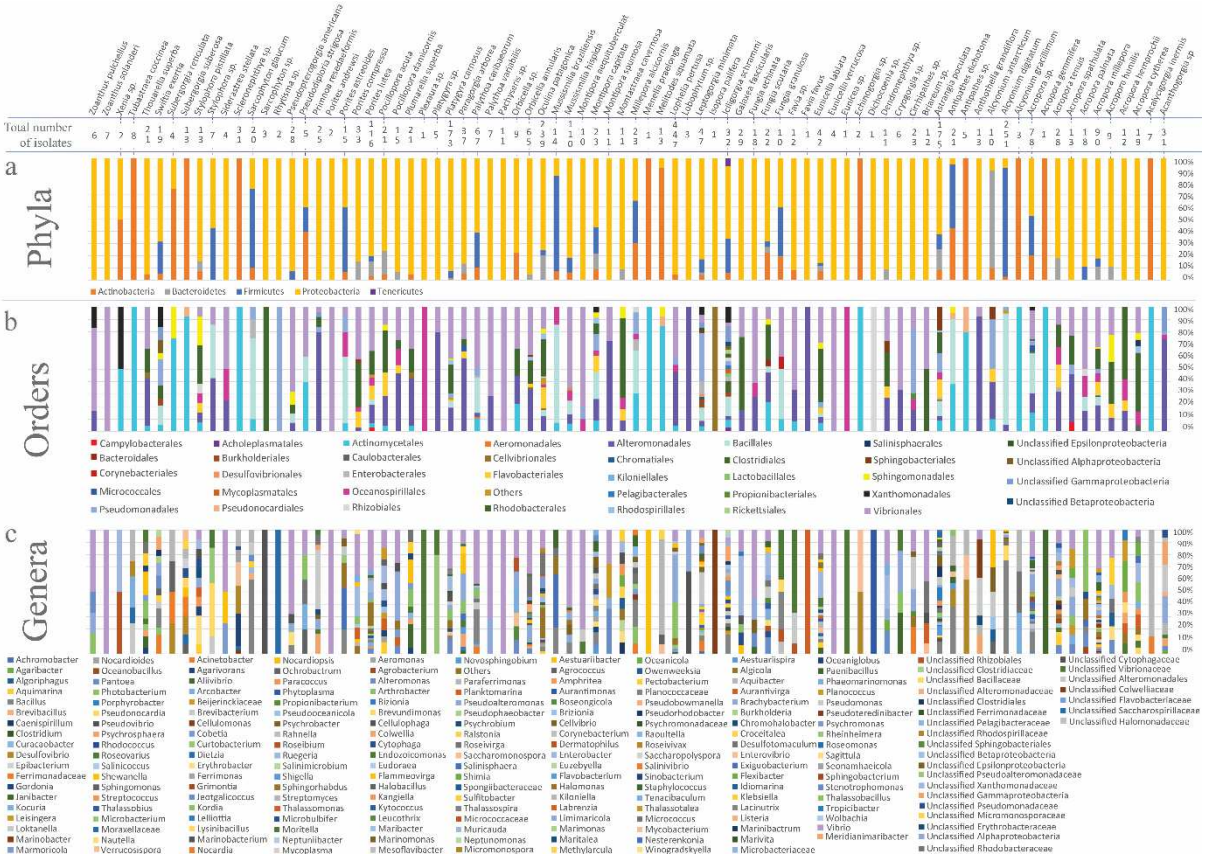


Figure 4. Phylum (A), order (B,) and genus (C) -level profiles of coral-associated bacteria isolated from each type of culture medium. Taxa (i.e. orders and genera) representing less than 1% of the total percentage of isolates were pulled together and classified as “others”.

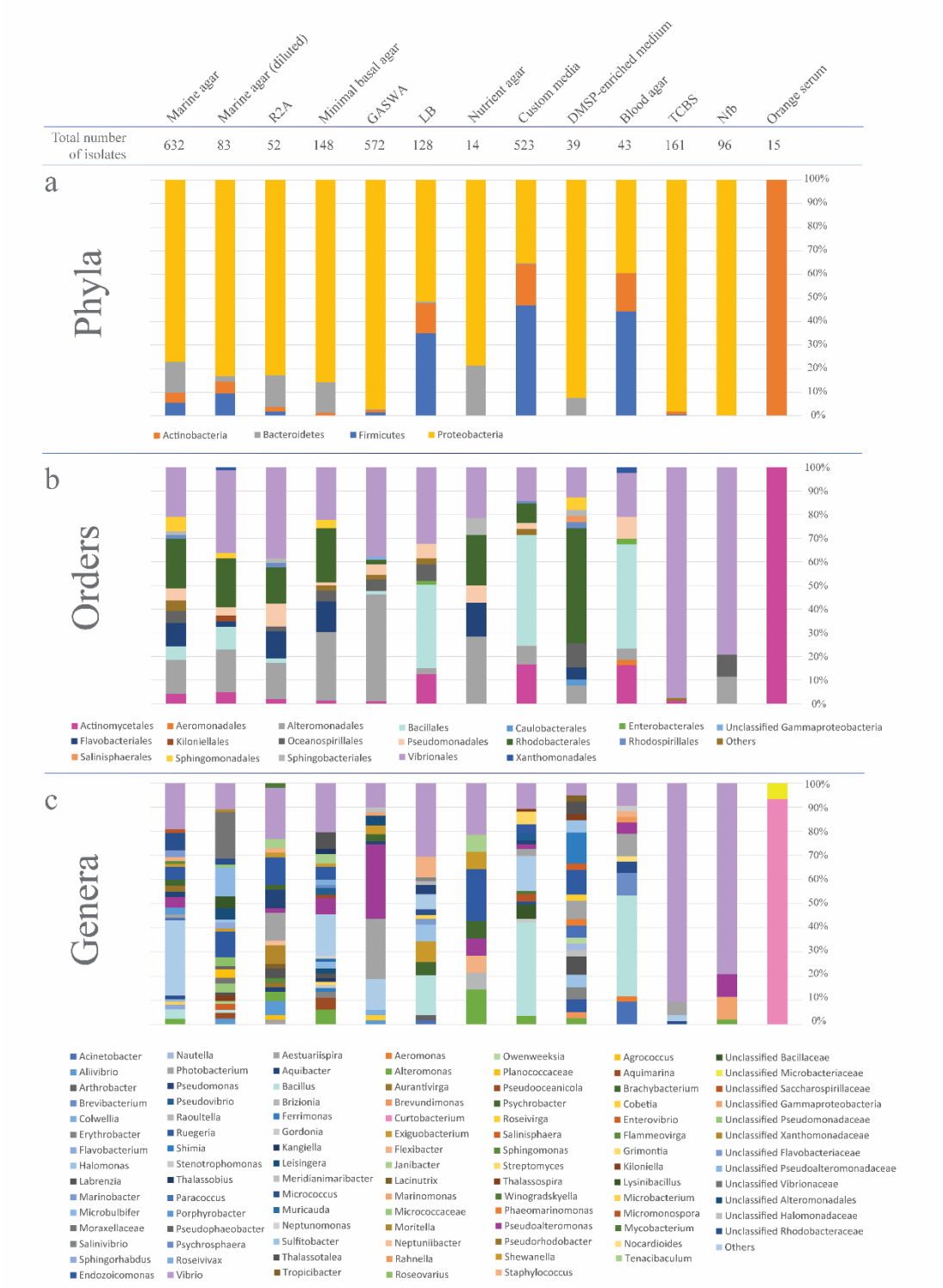


Figure 5. Functional analysis of 74 genomes of cultured coral bacteria according to their protein family (Pfam) profiles. Principal coordinates analysis (PCoA) was performed on the Pfam profiles using the Bray-Curtis similarity matrix calculated from Hellinger-transformed abundance data (A). The ordination is shown in Eigenvalue-scale. Symbol shapes indicate the taxonomic class of each genome and the host origin (filled symbols – Scleractinian corals; open symbols - Octocorals). In addition, BMC bacteria are highlighted in cyan blue while typical coral pathogens are highlighted in dark red. Isolate numbers (as in panel B) are given next to each symbol. The number of CDSs assigned to Pfam entries related to Eukaryotic-like proteins "ELPs" (i.e. ankyrin-, tetratricopeptide-, WD40- and leucine-rich repeats) and other features involved in host-microbe interactions are highlighted in the table below (B). The colour code from dark blue to dark red reflects an increase in the number of CDSs related to each function. ELPs, CRISPR proteins, endonucleases, transposases and secretion systems were each represented by more than one Pfam entry across the dataset. The CDS counts of these functionally belonging Pfams were summed. The number of Pfams that contributed to each function were as follows: ankyrin repeats – 5 Pfam entries; tetratricopeptide repeats - 21 Pfam entries; WD40 repeats – 6 Pfam entries; leucine-rich repeats – 8 Pfam entries ; CRISPR proteins – 21 Pfam entries; endonucleases – 42 Pfam entries; transposases – 37 Pfam entries; T2SS – 17 Pfam entries; T3SS – 19 Pfam entries; T4SS – 15 Pfam entries; T6SS – 18 Pfam entries. In the case of taurine and dimethylsulfoniopropionate (DMSP) catabolism only one Pfam entry (PF02668.16 and PF16867.5) was found, respectively.

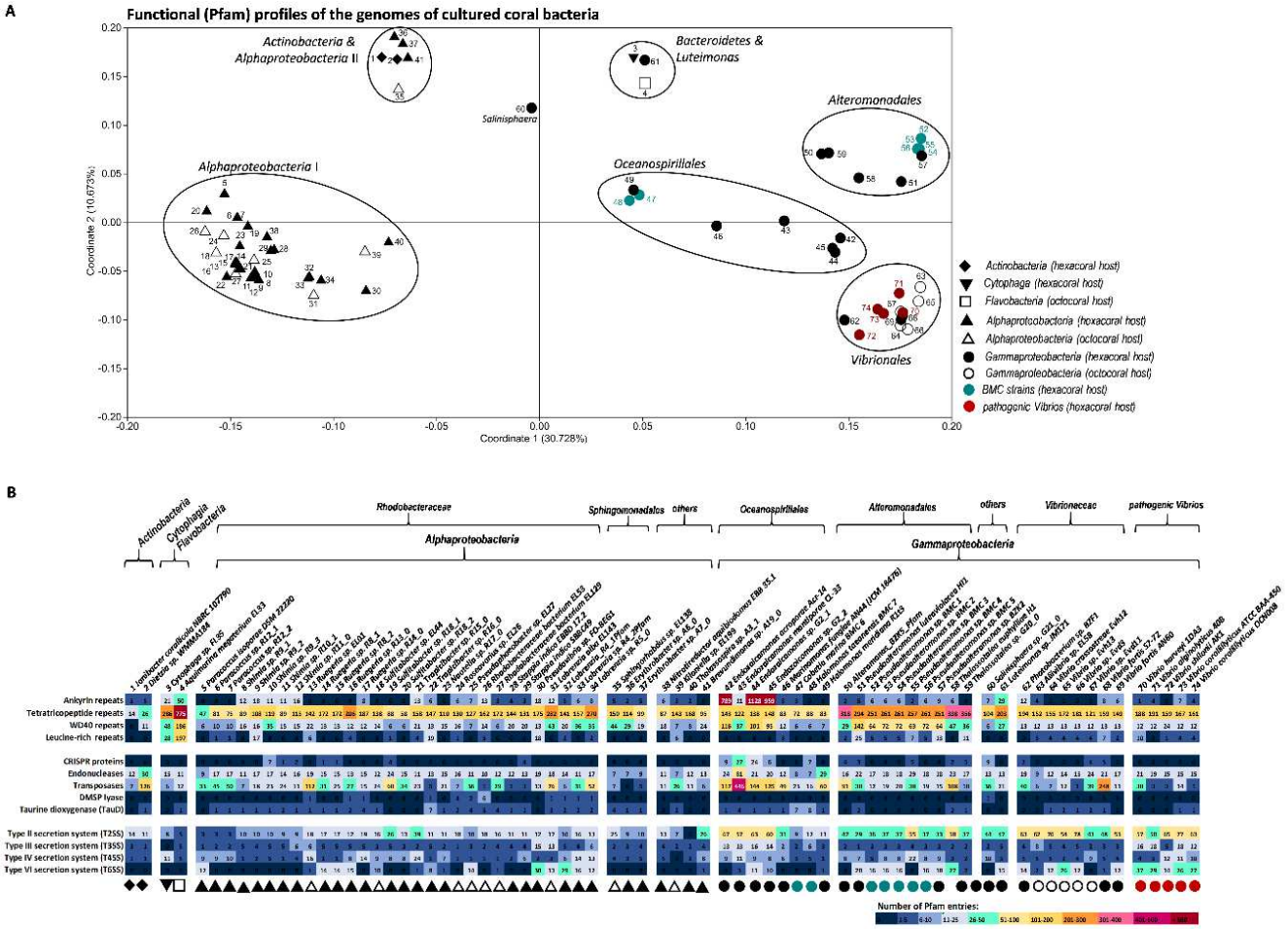
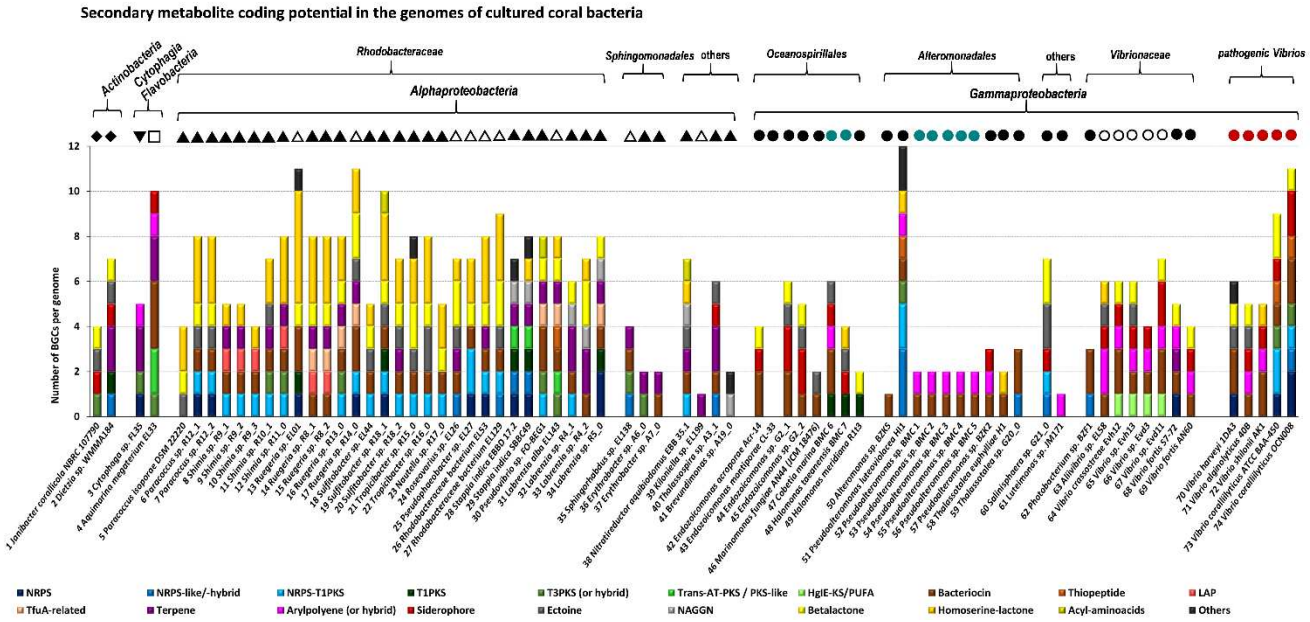

A
Functional (Pfam) profiles of the genomes of cultured coral bacteria
Actinobacteria & Alphaproteobacteria II
Bacteroidetes & Luteimonas
Salinisphaera
Alteromonadales
Alphaproteobacteria I
Oceanospirillales
Vibrionales
Coordinate 2 (10.673%)
Coordinate 1 (30.728%)
Actinobacteria (hexacoral host)
Cytophaga (hexacoral host)
Flavobacteria (octocoral host)
Alphaproteobacteria (hexacoral host)
Alphaproteobacteria (octocoral host)
Gammaproteobacteria (hexacoral host)
Gammaproteobacteria (octocoral host)
BMC strains (hexacoral host)
pathogenic Vibrios (hexacoral host)
B
Actinobacteria
Cytophagia
Flavobacteria
Rhodobacteraceae
Sphingomonadales
others
Oceanospirillales
Alteromonadales
others
Vibrionaceae
pathogenic Vibrios
Alphaproteobacteria
Gammaproteobacteria
Ankyrin repeats
Tetratricopeptide repeats
WD40 repeats
Leucine-rich repeats
CRISPR proteins
Endonucleases
Transposases
DMSP lyase
Taurine dioxygenase (TauD)
Type II secretion system (T2SS)
Type III secretion system (T3SS)
Type IV secretion system (T4SS)
Type VI secretion system (T6SS)
Number of Pfam entries:

Figure 6. Distribution of biosynthetic gene clusters (BGCs) across 74 genomes of cultured coral bacteria. BGC counts per compound class were obtained using antiSMASH v.5.0 with default settings (and all extra features on). NAGGN - N-acetylglutaminylglutamine amide; LAP - linear azol(in)e-containing peptide; hglE-KS- heterocyst glycolipid synthase-like PKS; PUFA-polyunsaturated fatty acids; NRPS - non-ribosomal peptide synthetase cluster; PKS – polyketide synthase cluster; TfuA-related - TfuA-related ribosomal peptides. The category "others" comprises rare BGCs that had each less than three entries across the dataset (among those were furan, ladderane-hybrid, phosphonate, polybrominated diphenyl ethers, lassopeptide, lanthipeptide and butyrolactone BGCs). Symbol shapes above bars indicate the taxonomic class and the host origin of each genome (as in Figure 5).



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Supplementary material

Species of coral used to isolate bacteria

Acropora spathulata, A. tenuis, A. millepora, A. cytherea, A. humilis, A. hemprochii, A. gemmifera, A. palmata, Astrangia poculata, Alcyonium digitatum, Antipathes sp., Antipathes dichotoma, Acalycigorgia inermis, Alcyonium antarcticum, Anthothella grandiflora, Acanthogorgia sp.,

Briareum sp.,

Cirrhipathe lutkeni, Cryogorgia koolsae,

Dendronephtheya sp.,

Eunicella labiata, E. verrucosa,

Favia favus, Fungia granulosa, Fungia echinata, Fungia scutaria,

Galaxea fascicularis,

Iciligorgia schrammi, Isopora palifera,

Montipora spumosa, Montipora capitata, Montipora aequituberculata, Mussismilia hispida, Mussismilia braziliensis, Millepora alcicornis, Montastrea cavernosa, Menella praelonga, Melitodes squamata,

Orbicella faveolata, Orbicella annularis, Oculina patagonica,

Pocilopora damicornis, Pocilopora acuta, Pachyseris speciose, Porites lutea, Porites astreoides, Porites compressa, Porites andrewsi, Pseudodiploria strigosa, Palythoa caribaeorum, Palythoa variabilis, Platygyra sp., Platygyra carnosus, Platygra caribaeorum, Plexaura sp., Pseudopterogorgia americana, Paragorgia arborea, Plumarella superba, Primnoa resdaeformis,

Rhytisma fulvum,

Stylophora pistillata, Siderastrea stellate, Siderastrea siderea, Sarcophyton glaucum, Scleronephthya sp., Sarcophyton sp., Swiftia exertia, Subergorgia suberosa,

Thouarella superba, Tubastraea coccinea,

Leptogorgia minimata, Lobophytum sp., Lophelia pertusa,

Xenia sp.,

Zoanthis solanderi, Zoanhus pulchellus.

Figures

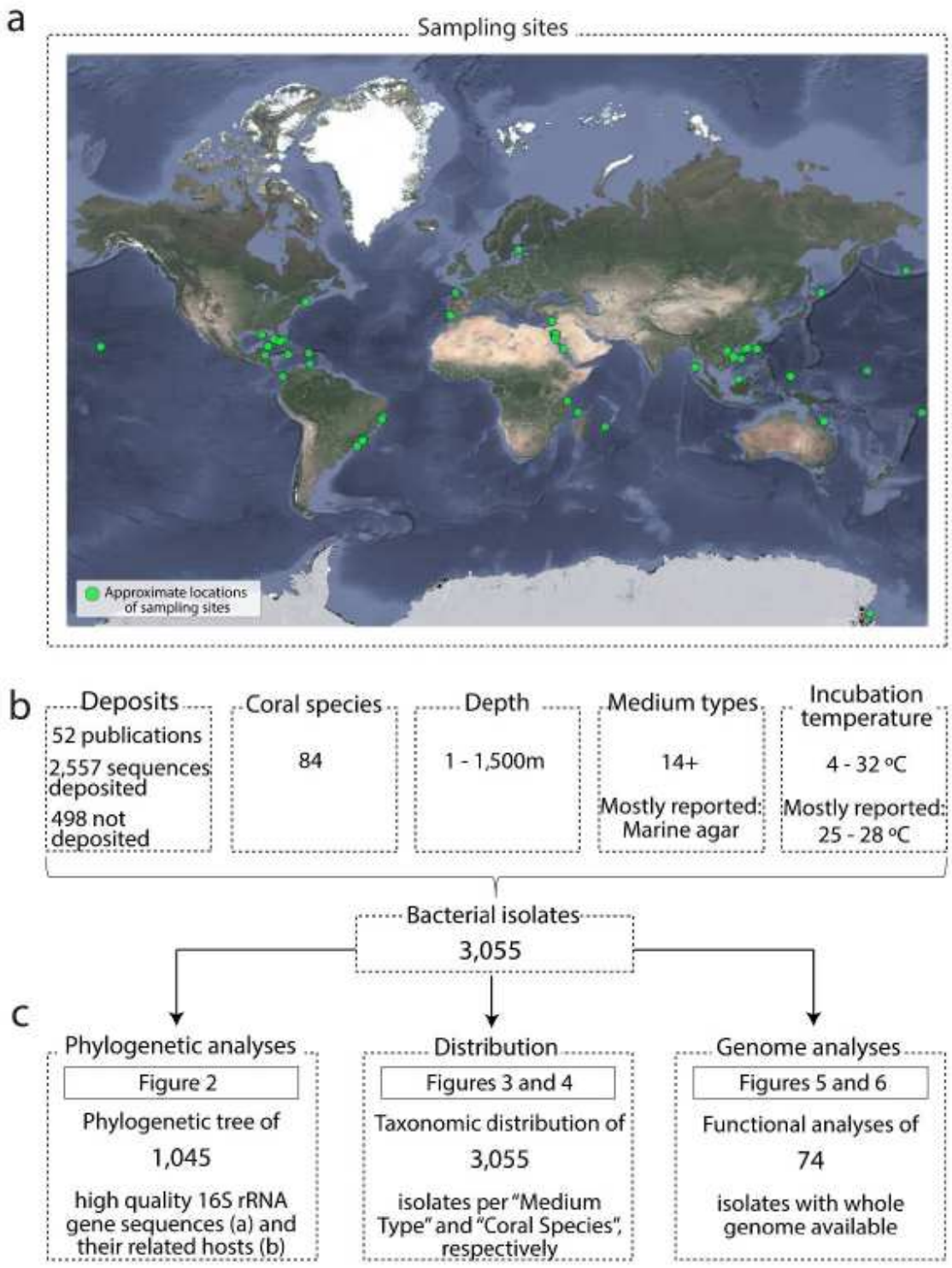

Figure 1

Overview of the data used and generated in this manuscript. Sampling sites of the coral species used as isolation sources (a). Data summary recovered from the publications and accession numbers available in data banks (b). Overview of the analyses performed in the current manuscript using the available

isolates. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

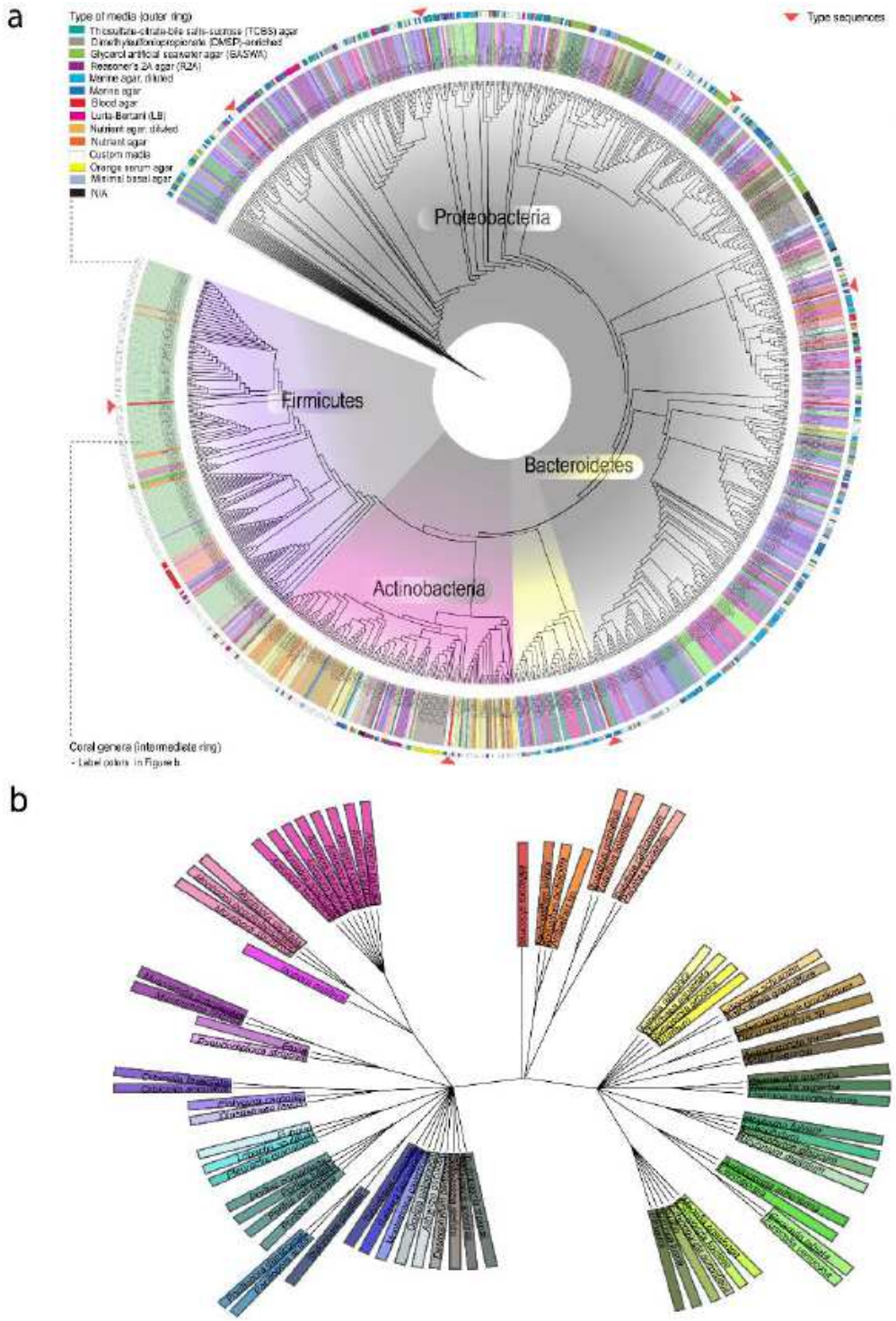

Figure 2

Phylogenetic trees of bacterial strains and coral species. (A) 16S rRNA gene-based phylogenetic inference of 1,045 coral-associated bacterial isolates, plus eight type-strains representing the species Vibrio alginolyticus, Vibrio bivalvicida, Pseudoalteromonas aestuariivivens, Pseudomonas guariconensis, Massilia namucuonensis, Vibrionimonas magnilacihabitans, Mycetocola tolaasinivorans, and Bacillus subtilis. The outer ring groups, by colour, the medium used to isolate the strains and the inner ring labels the coral genera used as sources for bacterial isolation. (B) Phylogenetic tree of the species of corals used in this study produced via https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). The label colours used to identify the genera are linked to the inner ring of Figure 1A.

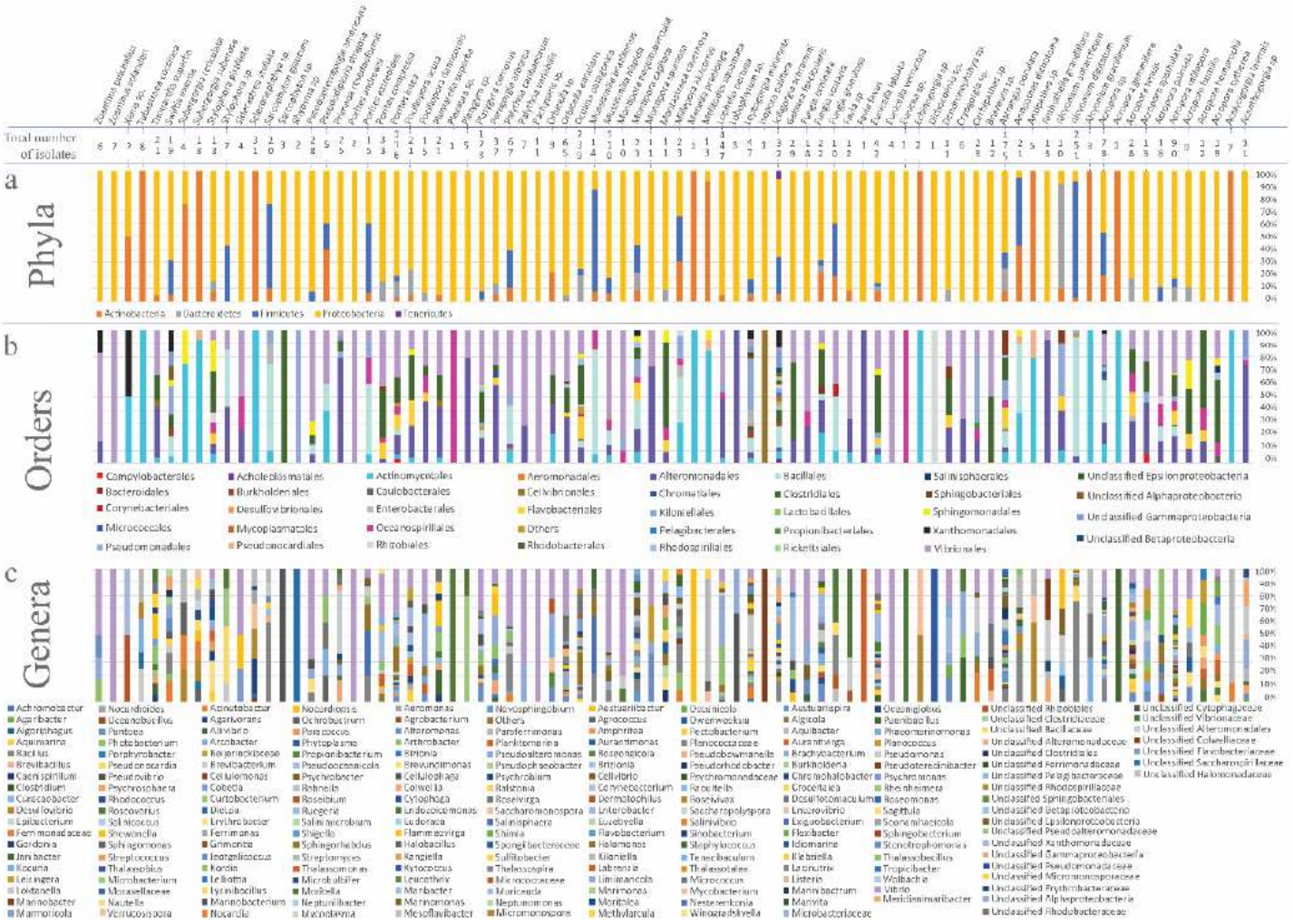

Figure 3

Phylum (A), order (B) and genus (C) -level profiles of coral-associated bacteria isolated from each coral species. Taxa (i.e. orders and genera) representing less than 1% of the total percentage of isolates were pulled together and classified as “others”.

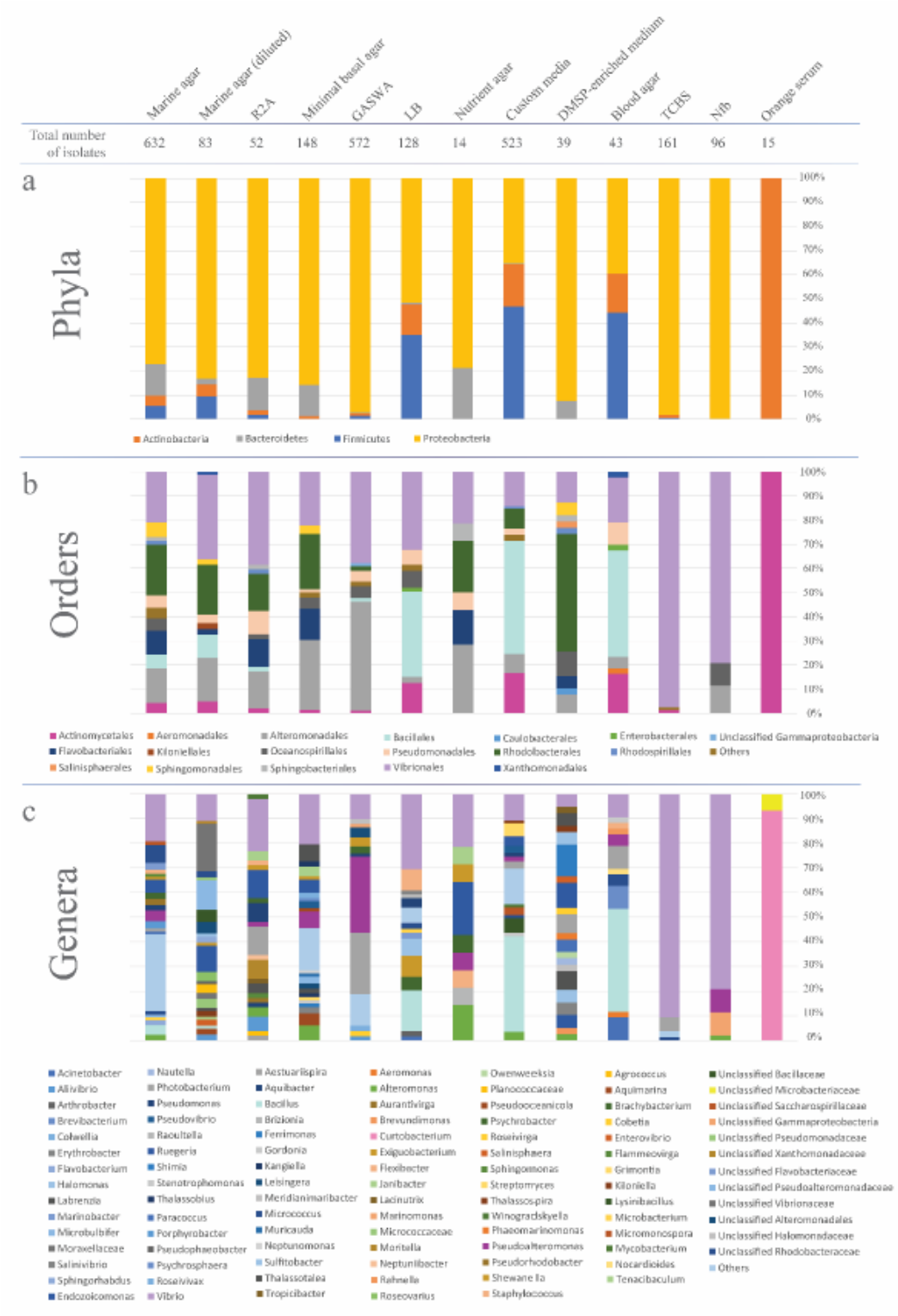

Figure 4

Phylum (A), order (B,) and genus (C) -level profiles of coral-associated bacteria isolated from each type of culture medium. Taxa (i.e. orders and genera) representing less than 1% of the total percentage of isolates were pulled together and classified as “others”.

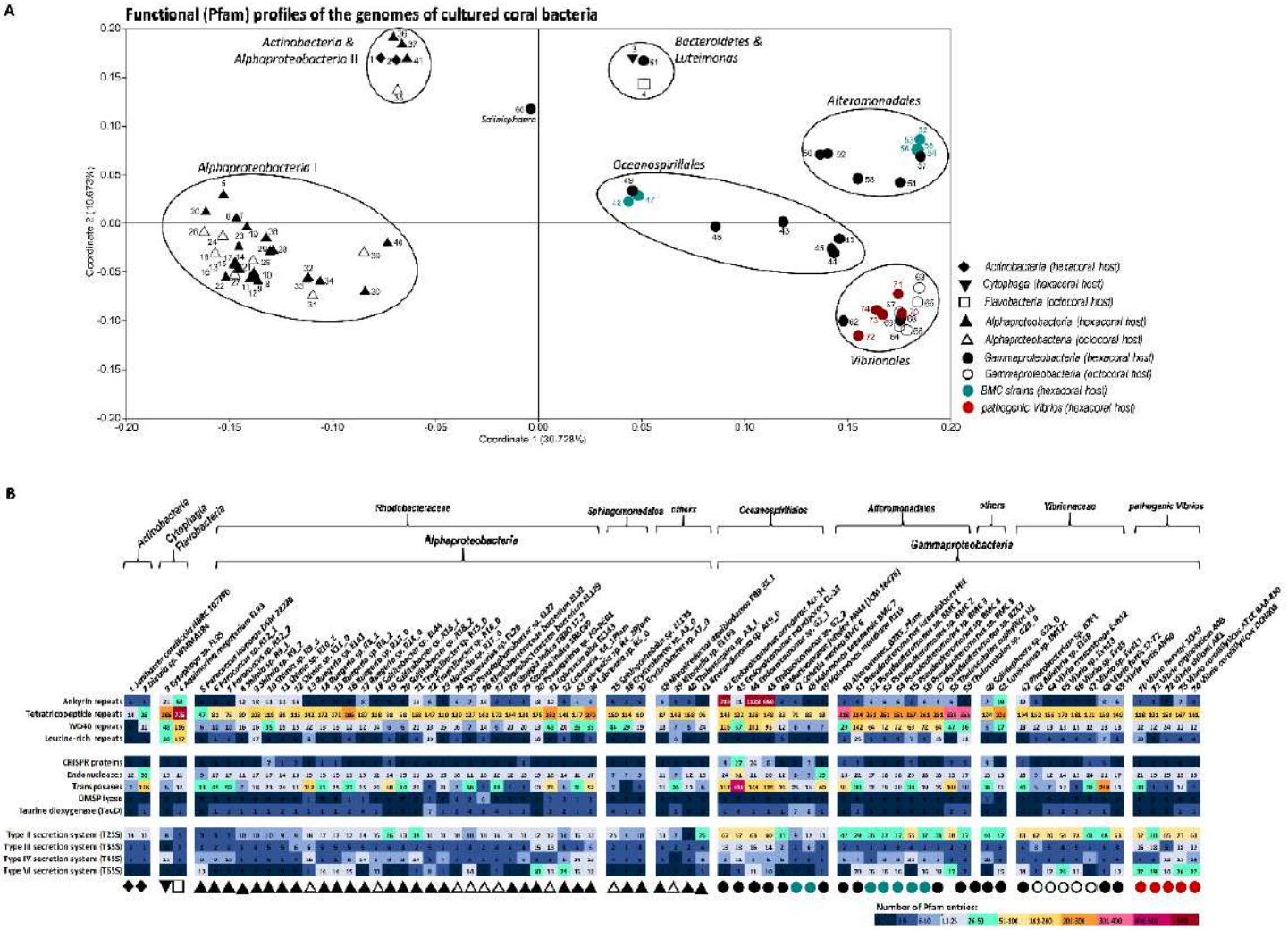

Figure 5

Functional analysis of 74 genomes of cultured coral bacteria according to their protein family (Pfam) profiles. Principal coordinates analysis (PCoA) was performed on the Pfam profiles using the Bray-Curtis similarity matrix calculated from Hellinger-transformed abundance data (A). The ordination is shown in Eigenvalue-scale. Symbol shapes indicate the taxonomic class of each genome and the host origin (filled symbols – Scleractinian corals; open symbols - Octocorals). In addition, BMC bacteria are highlighted in cyan blue while typical coral pathogens are highlighted in dark red. Isolate numbers (as in panel B) are given next to each symbol. The number of CDSs assigned to Pfam entries related to Eukaryotic-like proteins “ELPs” (i.e. ankyrin-, tetratricopeptide-, WD40- and leucine-rich repeats) and other features involved in host-microbe interactions are highlighted in the table below (B). The colour code from dark blue to dark red reflects an increase in the number of CDSs related to each function. ELPs, CRISPR proteins, endonucleases, transposases and secretion systems were each represented by more than one Pfam entry across the dataset. The CDS counts of these functionally belonging Pfams were summed. The number of Pfams that contributed to each function were as follows: ankyrin repeats – 5 Pfam entries; tetratricopeptide repeats - 21 Pfam entries; WD40 repeats – 6 Pfam entries; leucine-rich repeats –

8 Pfam entries ; CRISPR proteins – 21 Pfam entries; endonucleases – 42 Pfam entries; transposases – 37 Pfam entries; T2SS – 17 Pfam entries; T3SS – 19 Pfam entries; T4SS – 15 Pfam entries; T6SS – 18 Pfam entries. In the case of taurine and dimethylsulfoniopropionate (DMSP) catabolism only one Pfam entry (PF02668.16 and PF16867.5) was found, respectively.

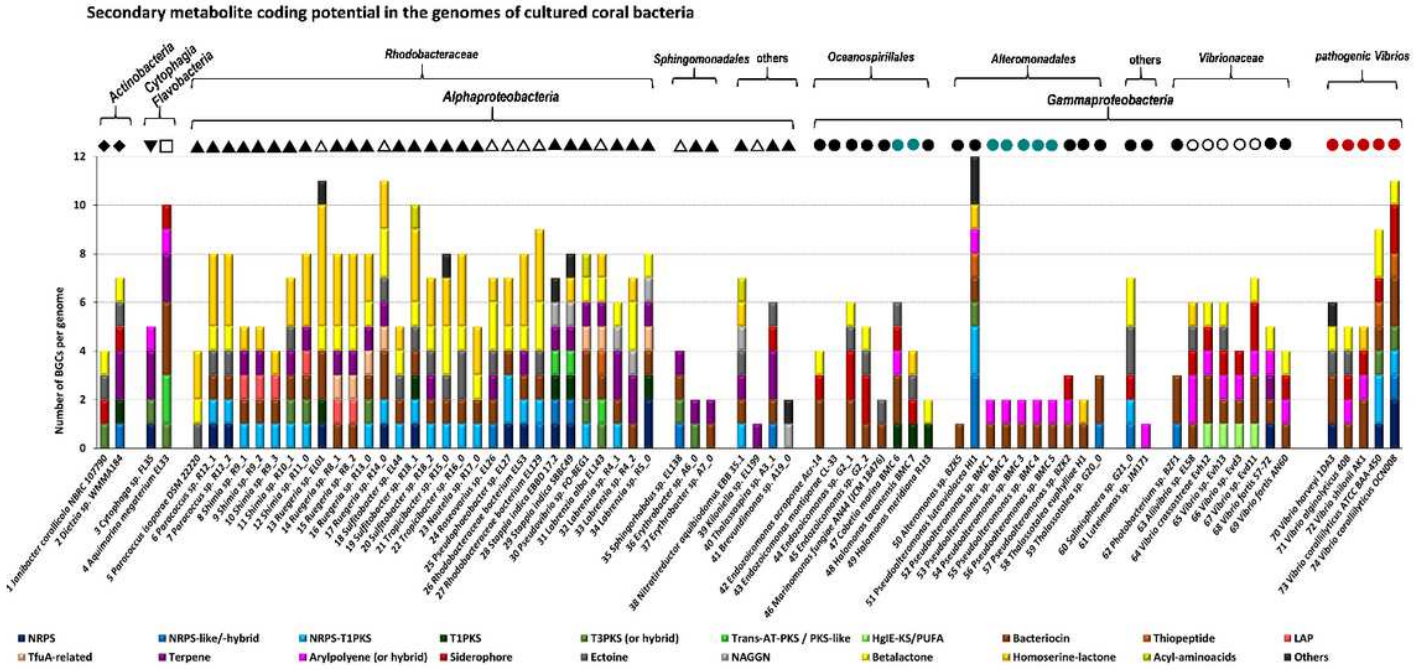

Figure 6

Distribution of biosynthetic gene clusters (BGCs) across 74 genomes of cultured coral bacteria. BGC counts per compound class were obtained using antiSMASH v.5.0 with default settings (and all extra features on). NAGGN - N-acetylglutaminylglutamine amide; LAP - linear azol(in)e-containing peptide; hglE-KS- heterocyst glycolipid synthase-like PKS; PUFA- polyunsaturated fatty acids; NRPS - non-ribosomal peptide synthetase cluster; PKS – polyketide synthase cluster; TfuA-related - TfuA-related ribosomal peptides. The category "others" comprises rare BGCs that had each less than three entries across the dataset (among those were furan, ladderane-hybrid, phosphonate, polybrominated diphenyl ethers, lassopeptide, lanthipeptide and butyrolactone BGCs). Symbol shapes above bars indicate the taxonomic class and the host origin of each genome (as in Figure 5).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SuuplementaryTable3.xlsx
- SupplementaryTable4.xlsx