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Diversity, composition and potential roles of sedimentary microbial communities in different coastal substrates around subtropical Okinawa Island, Japan

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Abstract

Background Marine benthic prokaryotic communities play crucial roles in material recycling within coastal environments, including coral reefs. Coastal sedimentary microbiomes are particularly important as potential reservoirs of symbiotic, beneficial, and pathogenic bacteria in coral reef environments, and therefore presumably play a core role in local ecosystem functioning. However, there is a lack of studies comparing different environments with multiple sites on the island scale, particularly studies focusing on prokaryotic communities, as previous investigations have focused mainly on a single site or on specific environmental conditions. In our study, we collected coastal sediments from seven sites around Okinawa Island, Japan, including three different benthic types; sandy bottoms, seagrass meadows, and hard substratum with living scleractinian corals. We then used metabarcoding to identify prokaryotic compositions and estimate enzymes encoded by genes to infer their functions.

Results The results showed that the three substrata had significantly different prokaryotic compositions. Seagrass meadow sites exhibited significantly higher prokaryotic alpha-diversity compared to sandy bottom sites. ANCOM analysis revealed that multiple bacterial orders were differentially abundant within each substratum. At coral reef sites, putative disease- and thermal stress-related opportunistic bacteria such as *Rhodobacterales*, *Verrucomicrobiales*, and *Cytophagales* were comparatively abundant, while seagrass meadow sites abundantly harbored *Desulfobacterales*, *Steroidobacterales* and *Chromatiales*, which are common bacterial orders in seagrass meadows. According to our gene-coded enzyme analyses the numbers of differentially abundant enzymes were highest in coral reef sites. Notably, superoxide dismutase, an important enzyme for anti-oxidative stress in coral tissue, was abundant at coral sites. Our results provide a list of prokaryotes to look into in each substrate, and further emphasize the importance of considering the microbiome, especially when focusing on environmental conservation.

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Conclusion Our findings prove that prokaryotic metabarcoding is capable of capturing compositional differences and the diversity of microbial communities in three different environments. Furthermore, several taxa were suggested to be differentially more abundant in specific environments, and gene-coded enzymic compositions also showed possible differences in ecological functions. Further study, in combination with field observations and temporal sampling, is key to achieving a better understanding of the interactions between the local microbiome and the surrounding benthic community.

Keywords Coral reefs, Seagrass meadows, Sandy bottoms, Prokaryotic community, Bioindicator

Background

Microbiomes play crucial roles in global biochemical cycles, influencing essential compounds like phosphate, sulfate, nitrate, carbon, and metals in marine habitats [1, 2]. Recent advancements in technologies, such as high throughput sequencers, allow researchers to better investigate marine microbial communities in various habitats and understand their responses to environmental factors with high resolution [2–4]. Exploring microbial compositions in the environment is not only essential for the study of biodiversity and for understanding species' interactions and aggregations at local scales [5, 6], but is also useful for assessing the general health of environments, as microbes are highly sensitive to marine environmental pollution [7–10]. Environmental assessments using microbial communities as proxies have explored the biological impact of anthropogenic activities, for example of mariculture [11–13]. Sedimentary prokaryotes have been shown to potentially mitigate anthropogenic contamination in the sediment, such as from heavy metals, polyaromatic hydrocarbons (PAHs), petroleum, and excess nutrients [14–17]. However, in the long term, intense environmental disturbances can lead to the lack of recovery of prokaryotic communities or their functions [18, 19].

In coral reef ecosystems, the interactions between benthic organisms and their associated prokaryotes play critical and fundamental roles in maintaining the overall health of the entire ecosystem [20–25]. These interactions have become increasingly important as coastal coral reefs face numerous threats such as nutrient loading [26, 27], land reclamation [28], ocean acidification [29–31], coral disease outbreaks [32–34], outbreaks of various organisms [35–38], and rising sea surface temperatures [39–41]. These global scale disturbances have made coral reefs at risk to “phase-shifts” [42], or large scale substitution of benthic structures from a coral-dominated state to being dominated by other organisms (e.g. macroalgae [43–46], sponges [47, 48], soft corals [49, 50], zoantharians [51, 52], etc.) or even to primarily abiotic substrate (e.g. rocky substrate) as suggested by Bellwood *et al.* [42]. To prevent such drastic changes, and to facilitate coral recovery from damage, environmental microbiome-based methodologies are likely to be critical [53]. However, while there have been many studies on scleractinian

coral-associated prokaryotes [54–58], sponge-associated prokaryotes [5, 59–66], seawater prokaryotes [4, 67, 68], and potentially free-living Symbiodiniaceae [69–72], research on sedimentary prokaryotes in different coral reef habitats remains generally quite limited (e.g., Dong *et al.* [73]).

Scleractinian corals are known to exude substantial amount of mucus, which can introduce their body surface microbes and/or trapped microbes into the water column and sediments, and this coral mucus may potentially influence the surrounding sedimentary prokaryotic community [74]. Sedimentary prokaryotic communities are known to be more diverse and/or more similar to coral-associated communities than to those found in seawater [5, 75–78]. Carlos *et al.* [75] suggested that sediments might serve as potential coral pathogen reservoirs. Indeed, resuspended sedimentary prokaryotes can migrate onto the surface of coral, and the coral's mucus layer can provide a means for prokaryotes to hitch a ride via mucus shed from the coral's body surface [76]. Monitoring prokaryotic compositions in sediments, therefore, can be a key task in tracking coral health at the local level. Likewise, in seagrass meadows, which are vital for carbon sequestration (also known as “blue carbon” [79, 80]), the rhizosphere microbiome plays a crucial role in maintaining meadow health, involving essential biochemical pathways such as nitrogen fixation, sulfate reduction, sulfide oxidation, and urea turnover [81–83]. Alterations in sedimentary chemical conditions, such as hypoxia and/or enriched sulfide, often combined with other stressors such as high/low salinities or temperatures, can lead to large-scale die-offs of seagrass [84–86], which also affect prokaryote composition [83, 87, 88]. Prokaryotic communities have been shown to have the ability to recover relatively quickly after such disturbances due to their composition plasticity [19, 89, 90]. However, the recovery of organisms that rely on important biochemical pathways provided by prokaryotes may take longer.

Taking all of these fact into consideration, monitoring sedimentary microbial communities in coral reef ecosystems is of great importance, especially when comparing multiple environments with different communities (e.g., scleractinian corals, seagrass, sandy bottoms) within a small geographic range that could be connected via local currents and tidal fluctuations. In this context,

despite its small size, subtropical Okinawa Island, Japan is an excellent natural laboratory for such studies as it has different marine environments with varying levels of anthropogenic impacts. In this study, we characterized the bacterial taxa of different marine habitats, namely coral-abundant sites, seagrass-abundant sites, and sandy bottom sites using sediment samples, and then consider which protist taxa could be appropriate bioindicators for future monitoring of coral reefs environmental health. We further assess the potential functional diversity (also known as “enzymic capabilities”) of each community.

Materials and methods

Sample collection

Sediment samples were collected from seven different sites around Okinawa Island; Sunabe, Kayou, Kin, Manza, Odo, Sesoko, and Uruma (see Fig. 1), from November 10 to December 25, 2020. Detailed descriptions of each sampling site are as follows.

Sunabe; near a well-populated and reclaimed land area, also with heavy coastal armoring and frequent visitors. Relatively close to the reef edge, presence of small river mouth and military base.

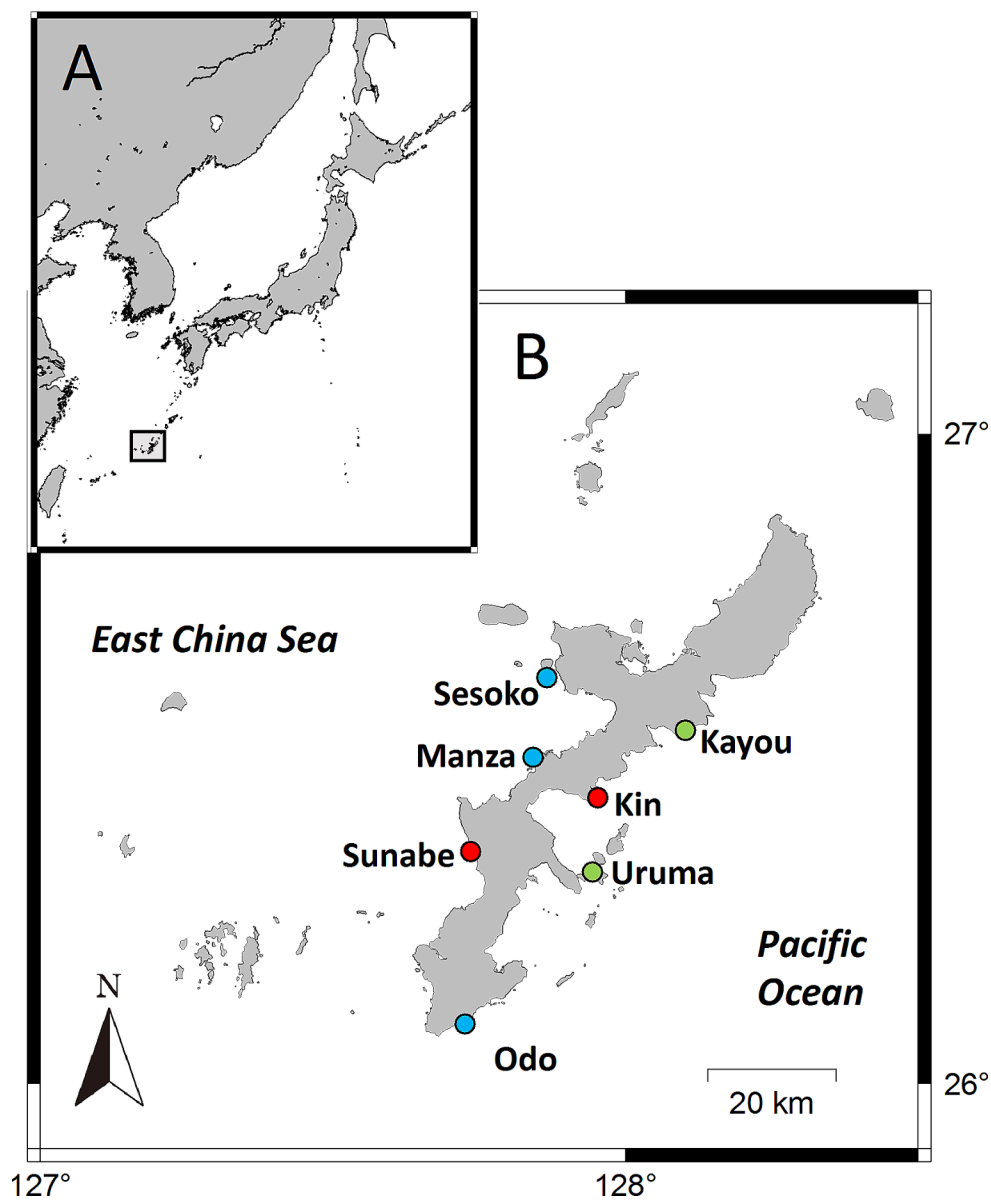


Fig. 1 Maps showing sites investigated in the present research. (A): Okinawa Islands in the Japanese Archipelago. (B): Okinawa Island in detail. Each dot represents a study site, and color indicates substratum. Blue = coral reef, red = sandy bottom, green = seagrass meadow sites, respectively

Kin; less populated natural beach with relatively more agriculture nearby. Large river mouth and mangrove forests within a kilometer.

Kayou; pristine site at a sparsely populated area with a small river mouth nearby. Zosteraceae, Hydrocharitaceae and Cymodoceaceae sp. growing abundantly.

Uruma; right next to an artificially constructed, reclaimed land causeway (“Kaichu-Doro Causeway”). No clear reef edge but some rocky substrates exist. Zosteraceae, Hydrocharitaceae and Cymodoceaceae sp. form assemblages.

Sesoko; near the southern edge of Sesoko Island, with relatively pristine environment, with small reef edge. Scleractinian corals abundant; *Acropora* spp. being most abundant. This is the only site which has soft corals nearby among our sampling sites.

Manza; the most natural site amongst our examined coral reef sites, with sharp and steep reef edge right in front of the sampling site. Scleractinian corals abundant, with *Montipora* spp. most abundant.

Odo; southernmost site with a large tide pool and beach nearby. Groundwater upwelling in front of the sampling site, as well as large civil engineering excavation ongoing roughly three hundred meters away on top of a hill nearby. Scleractinian corals abundant, *Montipora* spp. most abundant.

Sediment collection was done manually while snorkeling by scooping the sediment surface (<0.5 cm) using 15 mL Falcon® tubes (ThermoFisher Scientific, Waltham, MA, USA). At each site, 20 sediment samples were collected, except at Sunabe where only 15 samples were collected due to rough sea surface conditions. Among these sites, Manza, Sesoko, and Odo were characterized by hard reefs with living scleractinian corals, and we hereafter refer to them as “coral reef” sites. Uruma and Kayou were characterized by abundant seagrass, and are referred to as “seagrass meadow” sites, whereas Sunabe and Kin were characterized by fine sand, and are referred to as “sandy bottom” sites in this study. The number of sampling sites was determined based on the available resources and the need to ensure comprehensive analyses. Under such conditions, considering the high biodiversity and our research priorities, we focused more on coral reef sites than on the other two ecosystems.

In coral reef sites, sediments were sampled at small distances (approximately <1 m) from live scleractinian corals, and in seagrass meadows, sediments were sampled adjacent to seagrass while taking care to not include seagrass. Depths and GPS coordinates were recorded for all samples, except for one sample from Kayou (Ky_20) due to GPS failure (see Supplementary Table S1). After collection, sediment samples were immediately placed on dry ice, and kept frozen until further investigation.

DNA extraction, amplification and sequencing

Each sample was split into two (equal) subsamples and was subjected to the following steps. Whole microbial genomic DNA was extracted using DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocols except for pre-heating at 65°C for 10 min right before vortexing, as well as reducing the final elution volume to 75 µl. Yields of the extracted DNA were measured using a Qubit 4 Fluorometer (ThermoFisher Scientific) following the manufacturer’s protocol and were subsequently diluted to 1 ng/µl. DNA was then used as template for targeting the V3-V4 regions of the prokaryotic 16 S rRNA gene using MightyAmp™ DNA Polymerase Ver.2 (Takara Bio Inc., Kusatsu, Japan) following the manufacturer’s protocol, with the universal primer set Pro341F/Pro805R [91]. After the first PCR and purification, indexing PCR was performed using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc.). All detailed amplification methods are available in supplementary materials. Sequencing libraries of 135 samples were adjusted to be 4 nM each and were pooled into two amplicon pools for next generation sequencing. The prepared amplicon pools were mixed with the Phix Control V3 (Illumina, San Diego, CA, USA) conditioned to be up to 30% of total volume, and then loaded into Miseq Reagent Kit V3 (600 cycles, Illumina). Finally, the samples were sequenced on Illumina Miseq (Illumina) using a 301 bp paired-end chemistry by two individual runs. All raw sequences are available online (see data availability sections for details).

Bioinformatics

The demultiplexed paired-end sequence reads were analyzed in the QIIME2 v2021.11.0 [92] framework with several plug-in programs (All codes executed for this step is available in Additional file 1). Primers and the middle adaptor sequences were trimmed with Cutadapt v3.5 [93] (via q2-cutadapt) respectively from the 5’-end and 3’-end after applying a maximum of ten iterations. In case the primer sequences were not found or the trimmed length of sequences was less than 100 bp, reads were disposed. Quality control including quality filtering, denoising, correcting reading errors, merging paired-end reads and removal of non-biological sequences (including chimera) were performed by DADA2 v1.18.0 [94] (via q2-dada2) with the below custom values. For denoising, the maximum expected error values, ‘maxEE’ [95], were set to 2 for forward and 5 for reverse reads, respectively. The truncate length at 3’-end of both reads were independently decided by the base position, represented as a phred score of less than 20 at the first quartile of the total reads. After quality control, a count table of representative sequences was generated with dereplication of the same amplicon sequence variant (ASV). To standardize

ASV richness of each sample biased by different numbers of reads, resampling, so called ‘coverage-based rarefaction’ [96], was performed in all samples equalizing the slope of the saturation curve to the least saturated sample. After the rarefaction, copy number correction was employed to justify the number of reads considering the copy numbers using the open-source software PICRUSt2 [97], as well as Integrated Microbial Genomes (IMG) as a reference database [98]. As well, Enzyme Commission numbers and abundance of each enzyme that is coded by bacteria detected in this research were obtained from IMG (all codes executed for copy number correction and obtaining Enzyme Commission numbers are available in Additional file 2). Taxonomic assignment to ASV was conducted with pre-trained Naive Bayes classifier [99] referring to the 16S ribosomal RNA sequence database SILVA v138.1 [100, 101], and then operational taxonomic units (OTUs) were determined against each ASV using scikit-learn [102], a machine-learning classifier plugin in Qiime2. ASVs assigned to mitochondria, chloroplasts, eukaryotes, or to none of the domains were removed from the dataset to focus on the target microbiome. The OTU count table obtained through these processes was used in subsequent community analyses. It should be noted that there are some bacterial taxa for which taxonomic classification is still under discussion; in this study we based our discussion on information from SILVA v138.1.

Distance among samples was calculated under Bray-Curtis configuration using “vegan” package [103], and then Unweighted Pair-group Method with Arithmetic Means (UPGMA) clustering was performed under “average” configuration using “pvclust” package [104] in R 4.1.2 [105]. We then conducted silhouette analysis to evaluate how many groups the samples could be divided into, and used these groups (i.e., “sandy bottom”, “seagrass meadow” and “coral reef”) to perform further analyses.

The Shannon diversity indices of the prokaryotic community in each sample were calculated with “vegan” package [103]. The Kruskal-Wallis test in “kruskal.test” function in “stats” package [105] was employed to confirm statistical significance of calculated Shannon indices among benthic components, in cooperation with “pairwise.wilcox.test” in the same package. Further, non-metric multidimensional scaling (nMDS) was performed using “metaMDS” function in “vegan” package [103]. To test how group similarity was affected by factors, we conducted PERMANOVA using “adonis2” function in “vegan” package [103] with setting benthic substrate types (“coral reef”, “sandy bottom”, and “seagrass meadow”) as the factor. Then we assessed which pairs were significantly different using “pairwise.adonis” function in “pairwiseAdonis” package [106] with 999 times permutation and Bonferroni correction settings.

Bacterial taxa that were differentially abundant in coral, sand or seagrass were examined using “ancombc2” command in “ANCOMBC” package [107]. For interpretation of results, bacterial orders for which names did not follow nomenclature or were not assigned in the SILVA database (e.g. “HOC36”, NB1-j”, “KD4.96” etc.) were excluded. Linear and non-linear correlations between bacterial orders were tested using “secom_linear” and “secom_dist” commands in “ANCOMBC” package [107]. All R codes executed and metadata used are available in Additional files 3 and 4, respectively.

Enzymic composition analysis

Using estimated enzymic abundance based on the genes coded by the prokaryotic community obtained, the Shannon diversity index was calculated with “vegan” package [103] in R 4.1.2 [105]. The ANOVA test was employed to confirm statistical significance of calculated Shannon Indices among benthic components using “aov” and “TukeyHSD” functions in “stats” package [105]. Further, we analysed the enzymes differentially abundant in specific substrates using the “ancombc2” command in the “ANCOMBC” package [107].

Results

Number of OTUs and the most abundant bacterial phyla

After sequence processing, 4,675 OTUs from 83,138 ASVs were obtained, and then applied in further analyses (in total 3,417,550 contigs, average of 25,315 contigs/sample; detailed information in Supplementary Table S2). The 4,675 OTUs were assigned into the following taxonomic ranks in SILVA: 79 phyla (Supplementary Table S3); 214 classes (Supplementary Table S4); 522 orders (Supplementary Table S5); 880 families (Supplementary Table S6); 1871 genera (Supplementary Table S7). Also original count table used for analyses are all available in Additional files 5 to 11. The prokaryotic community had only a few domain Archaea (0.77% of total counts), while domain Bacteria was predominant (99.23% of total counts). The most predominant phylum was *Proteobacteria* for all three benthic types (33.5% for corals, 33.2% for sandy bottoms and 37.0% for seagrass, respectively, Fig. 2, Table S8). The second- to fifth-most predominant phyla of coral reef sites were *Bacteroidota*, *Cyanobacteria*, *Actinobacteriota* and *Verrucomicrobiota* (15.7%; 14.0%; 11.0%, 5.5%, respectively). Sandy bottom and seagrass meadow sites were similar in terms of bacterial composition, sharing the second- to fourth-most predominant taxa in the same order; *Actinobacteria* (15.3% and 13.9%, respectively), *Bacteroidota* (11.5% and 12.5%) and *Acidobacteriota* (8.2% and 6.4%, respectively) with a difference in the fifth-most abundant phylum, namely *Chloroflexi* for sandy bottoms (=5.3%) and *Desulfobacterota* for seagrass meadows (=5.9%).

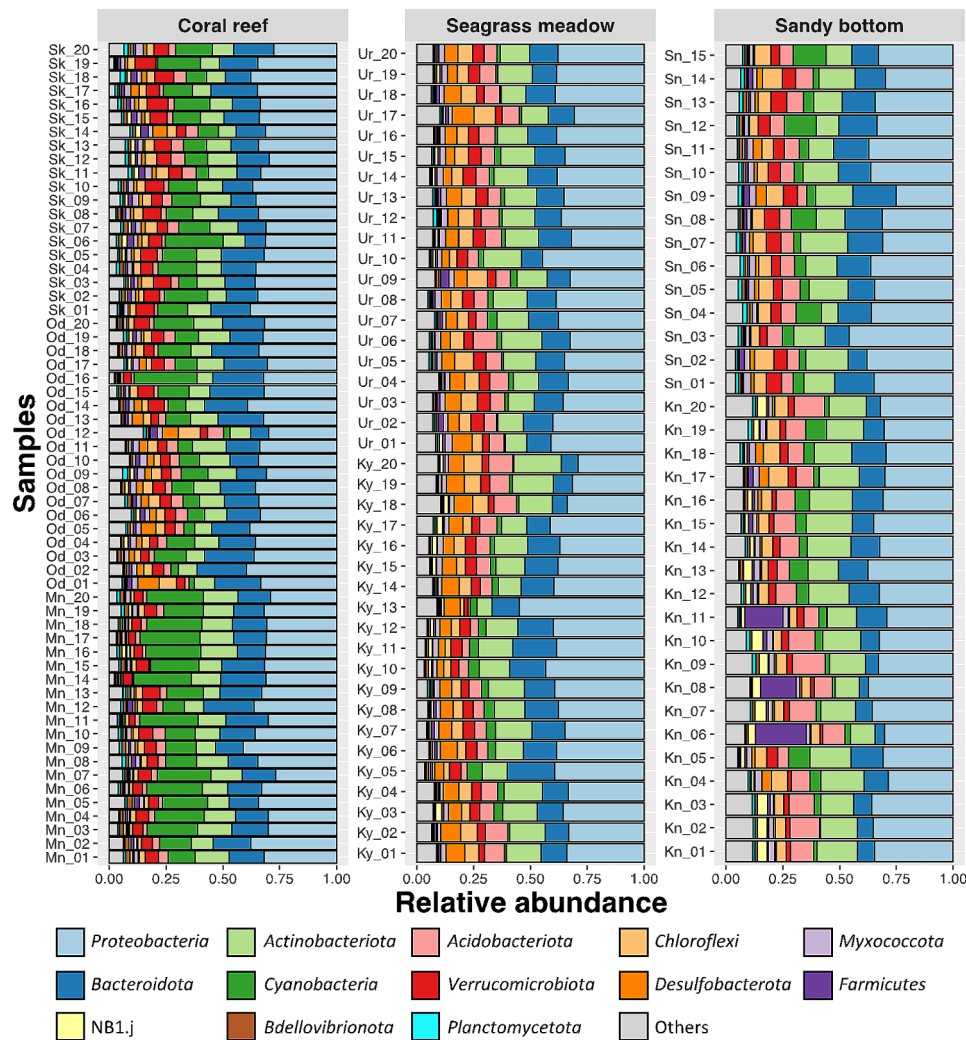


Fig. 2 Bar plot showing the top 13 phyla in each benthic type. Different colors show different bacterial phyla. Columns show samples from coral reefs, seagrass meadows, and sandy bottom sites, from left to right. Color bar widths indicate relative abundance of each taxon

Structure of bacterial community

The hierarchical clustering (UPGMA) plot showed three major branches, composed of (i) Manza and Sesoko, (ii) Sunabe and Odo, and (iii) Uruma and Kayou clustered with more distant Kin (Supplementary Figure S1). The AU (Approximately Unbiased) p-values and BP (Bootstrap Probability) were low to moderate (ranging from 67 to 89%, and from 35 to 57% for AU and BP, respectively) for all branches except for the branch formed by Uruma and Kayou (AU=99%, BP=97%).

The result of the silhouette analysis based on OTU level data showed that the samples could be divided into three groups; sandy bottoms, seagrass meadows, and hard rocky substratum with living scleractinian corals (Supplementary table S9), and we subsequently used these categories in this research. In our results, most of the samples were assigned to their supposed groups (“sandy bottom” group (Kin and Sunabe), =29/35 (82%); “seagrass

meadow” group (Kayou and Uruma), 40/40 (=100%); and “coral reef” group (Manza, Sesoko, and Odo), 45/60 (=75%) (Supplementary tables S10).

The nMDS plot based on the Bray-Curtis dissimilarity indices result illustrates these three different groupings (Fig. 3).

Prokaryotic richness and diversity

The highest value of prokaryotic Shannon diversity indices was observed at coral reef sites, whereas the lowest was found at sandy bottom sites (Supplementary Table S11). There was no significant difference in Shannon index between coral reef sites and sandy bottom sites or between coral reef sites and seagrass meadow sites (Fig. 4, Supplementary Table S12). However, seagrass meadow sites had significantly higher prokaryotic diversity when compared to sandy bottom sites ($p < 0.01$). When prokaryotic Shannon indices were compared among

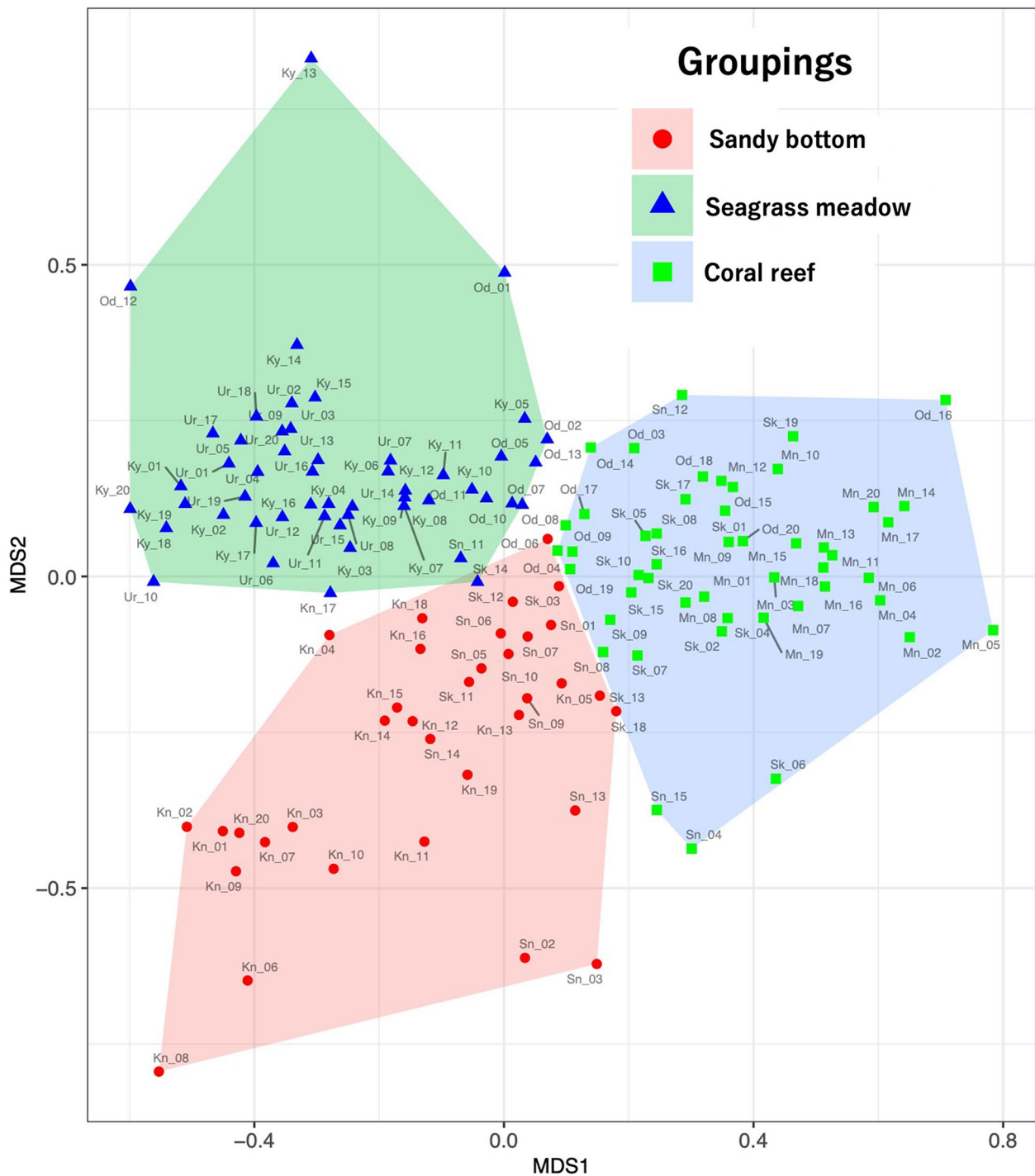


Fig. 3 nMDS plot of Bray-Curtis dissimilarity index of samples. Each circle, triangle or square represents a different sample. Sites have been abbreviated as follows: Kn: Kin, Sn: Sunabe, Ky: Kayou, Ur: Uruma, Sk: Sesoko, Mn: Manza, Od: Odo. Different colored mesh indicates groupings based on silhouette analysis results, mostly corresponding to red = sandy bottoms, blue = coral reefs, green = seagrass meadows

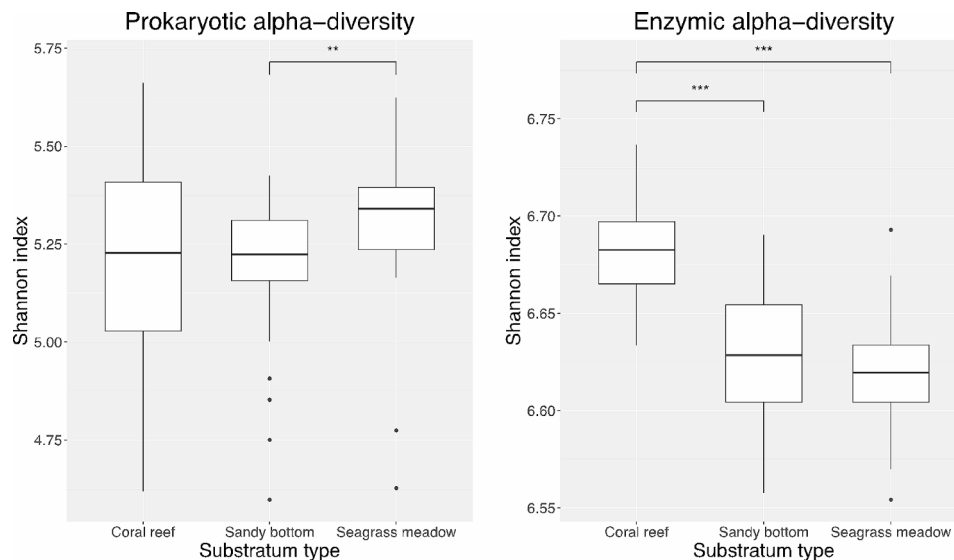


Fig. 4 Box plots of prokaryotic (left) and enzymic (right) Shannon diversity indices among different types of substrata. Asterisks show statistical significance (***) $p < 0.005$

Table 1 Result of PERMANOVA analysis to test if bacterial composition between substrata are significantly different

Comparison	Df	Sums Of Squares	F.Model	R2	p.value	p.adjusted
Sandy bottom vs. Coral reef	1	0.6886399	33.26826	0.2634729	0.001	0.003
Seagrass meadow vs. Coral reef	1	0.8596397	47.87419	0.3281882	0.001	0.003
Sandy bottom vs. Seagrass meadow	1	0.2832032	14.14899	0.162354	0.001	0.003

individual sites, Uruma showed significantly higher diversity compared to Kin ($p < 0.01$), Kayou ($p < 0.01$), and Sunabe ($p < 0.05$) (Supplementary Figure S2).

Similarity and dissimilarity among coral reef, seagrass meadow and sandy bottom sites

The result of PERMANOVA showed that all three groups had significantly different bacterial compositions ($p < 0.001$, Table 1). Further, ANCOM analysis of the top 10% of the most abundant orders indicated that specific bacterial taxa were differentially abundant in each benthic substratum (Fig. 5). Orders *Flavobacteriales*, *Rhodobacterales*, *Verrucomicrobiales*, *Cytophagales*, *Enterobacterales*, and *Eurycoccales* were exclusively abundant in coral reef sites. *Kiloniellales* and *Entomoplasmatales* were prominent only in sandy bottom sites, and *Steroidobacterales*, *Desulfobacterales*, *Chromatiales*, and *Thiotrichales* were distinctly abundant only in seagrass meadow sites. *Actinomarinales* and *Thermoanaerobaculales* were distinctly less abundant in coral reef sites, *Opitutales*, *Bacteroidales*, and *Sphingomonadales* were

less abundant in sandy bottom sites, and *Dadabacteriales* was distinctly less abundant in seagrass meadow sites.

Correlation between bacterial orders of each substratum

Most correlations of the top ten most abundant bacterial orders with a correlation coefficient exceeding 0.7 were detected by both Pearson and Distance methods ($n = 16$), whereas a few were detected by only Pearson's method ($n = 5$, Fig. 6).

In coral reef sites, the abundance of *Actinomarinales* had a negative correlation with *Cyanobacteriales*, while showing positive correlations with the abundances of *Desulfobacterales* and *Thermoanaerobaculales*.

Secondly, in sandy bottom sites, *Thermoanaerobaculales* exhibited a positive correlation with *Actinomarinales*, as seen in coral reefs. However, *Thermoanaerobaculales* showed negative correlations with *Verrucomicrobiales*, *Rhodobacterales* and *Flavobacteriales*. Additionally, *Rhodobacterales* and *Verrucomicrobiales* had positive correlations, while *Flavobacteriales* and *Rhizobiales* showed negative correlations. Furthermore, *Actinomarinales* was

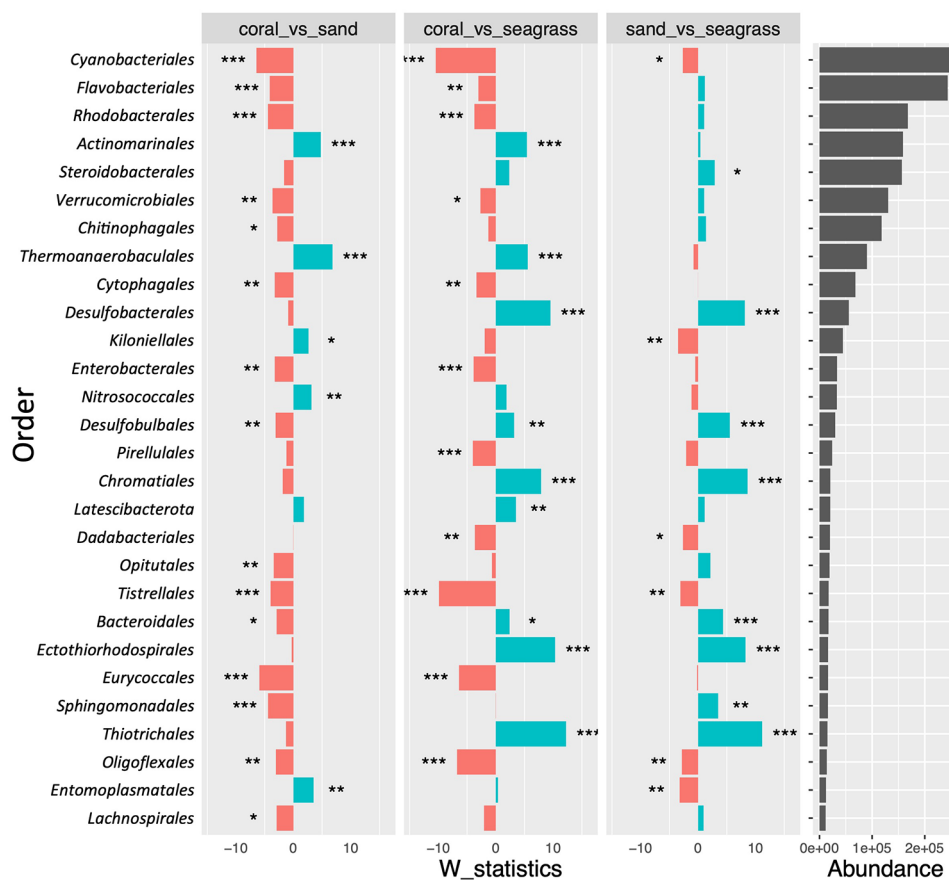


Fig. 5 Combined graph of the results obtained with ANCOM analysis (left to middle) and the total absolute abundance (right) of each bacterial order. For the ANCOM analysis, compared substratum types are shown at the top of each column. “W_statistics” stands for a log fold change value divided by standard error for effect size correction, and indicates to what type of substratum each bacterial order was differentially abundant. Asterisks show statistical significances (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

positively correlated with *Rhizobiales*, and negatively correlated with *Rhodobacteriales* and *Verrucomicrobiales*.

Thirdly, in seagrass meadow sites, *Desulfobacteriales* and *Microtrichales* had negative correlations while *Actinomarinales* and *Thermoanaerobaculales* showed a positive correlation. As well, positive correlations between *Caldilineales* and *Thermoanaerobaculales* as well as between *Actinomarinales* and *Caldilineales* were detected by only Pearson’s method.

Enzymic capability estimation and analysis

In this study, a total of 2431 enzymes were estimated to be coded in the prokaryotic metagenome based on the IMG database (Supplementary Table S13, also original count table is available in Additional files 12 to 15). The Shannon diversity of enzymes was significantly higher in coral reef sites compared to the other two benthic types (Supplementary Table S14). When comparing individual sites, Kin showed significantly lower diversity compared to all other sites except for Kayou. Similarly, the diversity indices of Kayou and Uruma were significantly lower

than those at Manza, Odo, Sesoko, and Sunabe (Supplementary Figure S2).

Among the top 5% of the most abundant enzymes in all samples, the ANCOM test revealed that 20 enzymes were significantly and exclusively abundant in coral reef sites, namely site-specific DNA-methyltransferase (adenine-specific) (EC 2.1.1.72), Cysteine desulfurase (EC 2.8.1.7), Ribonucleoside-diphosphate reductase (EC 1.17.4.1), Thioredoxin-disulfide (EC 1.8.1.9), C-terminal processing peptidase (EC 3.4.21.102), 23 S rRNA pseudouridine1911/1915/1917 synthase reductase (EC 5.4.99.23), Dihydroorotase (EC 3.5.2.3), N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28), Endopeptidase Clp (EC 3.4.21.92), 8-oxo-dGTP diphosphatase (EC 3.6.1.55), Protein-serine/threonine phosphatase (EC 3.1.3.16), Superoxide dismutase (EC 1.15.1.1), Mannose-1-phosphate guanylyltransferase (EC 2.7.7.13), Arsenate reductase (glutathione/glutaredoxin) (EC 1.20.4.1), 3-oxoacyl-[acyl-carrier-protein] reductase (EC 1.1.1.100), Threonine ammonia-lyase (EC 4.3.1.19), XTP/dITP diphosphatase (EC 3.6.1.66), Phosphoserine phosphatase (EC 3.1.3.3), Protein-glutamate methylesterase

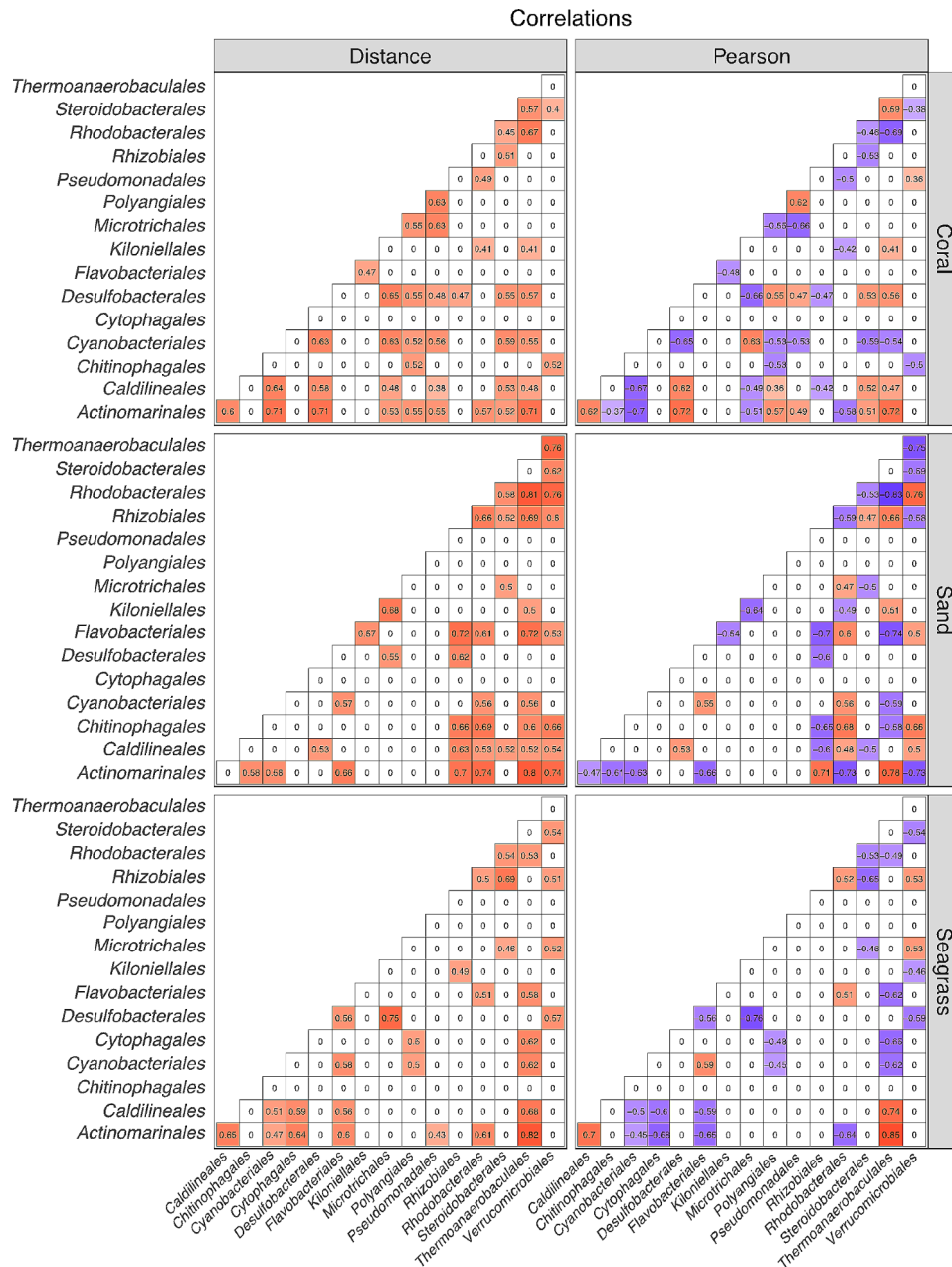


Fig. 6 Combined graph showing the results of correlation among bacterial orders. From the top to bottom, rows show coral reef sites, sandy bottom sites, and seagrass meadow sites, respectively. Left column is based on the distance method, while right column is based on Pearson’s coefficient of correlation method, where warm colors mean positive correlation, and cold colors mean negative correlation

(EC 3.1.1.61), and GTP cyclohydrolase I (EC 3.5.4.16) in descending manner (Fig. 7). On the other hand, nine enzymes were more abundant in seagrass meadow sites, namely Histidine kinase (EC 2.7.13.3), Peptidylprolyl isomerase (EC 5.2.1.8), H⁺-transporting two-sector ATPase (EC 3.6.3.14), Dihydrolipoyl dehydrogenase (EC 1.8.1.4), Glutamate synthase (NADPH) (EC 1.4.1.13), Glutamate synthase (NADH)(EC 1.4.1.14), Type I site-specific deoxyribonuclease (EC 3.1.21.3), Citrate (Si)-synthase (EC 2.3.3.1), and Asparagine synthase

(glutamine-hydrolysing) (EC 6.3.5.4) in the same manner as above. No enzymes were exclusively abundant in sandy bottom sites.

Discussion

While prokaryotic compositions in seawater and on the surfaces of benthic organisms have been extensively studied [e.g. 56, 58, 68], there is limited research available on sedimentary prokaryotes from different marine environments. In this study, we investigated the



Fig. 7 Combined graph of the results obtained with the ANCOM analysis (left to middle) and the total absolute abundance (right) of each coded enzyme by prokaryotes. For the ANCOM analysis, compared substratum types are shown at the top of each column. “W_statistics” stands for a log fold change value divided by standard error for effect size correction and indicates to what type of substratum each bacterial order was differentially abundant. Asterisks show statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

prokaryotic compositions in marine sediments from Okinawa Island and sought candidate prokaryotes as bio-indicators to be used in future monitoring of coral reef ecosystem health. As a result, we revealed that different habitats have significantly different prokaryotic communities, and revealed bacterial taxa that characterize each environment that can potentially be candidates for

environmental monitoring using metabarcoding methods. These approaches may help in preventing serious damage to corals caused by heat stress or diseases.

Dominant taxa and diversity indices

Among three substrata, the top four most abundant phyla were shared except for *Cyanobacteria*, which only

appeared in coral reef sites, and *Acidobacteriota* which appeared in seagrass meadows and sandy bottom sites. The fifth-most abundant phylum was different among substrata, namely *Verrucomicrobiota* at coral reefs, *Chloroflexi* at sandy bottom sites, and *Desulfobacterota* in seagrass meadows. In all benthic types, *Proteobacteria* was the most dominant phylum, consistent with many previous studies focusing on various marine environmental samples such as shallow to deep sediments and water [108–113]. *Cyanobacteria* are suggested to be prominent in coral mucus when exposed to heat stress [21], thus its abundance only in coral reef sites may indicate such stress, although further investigations of the coral mucus microbiome and bleaching conditions are needed to verify this hypothesis. *Chloroflexi* has been reported to be abundant in marine sediments compared to seawater not only in coral reefs but worldwide, especially at anoxic sediments [114]. Therefore, our results may indicate that sandy bottom sites where *Chloroflexi* was abundant may have been experiencing deoxygenation, possibly due to the lower abundance of grazers when compared to coral reefs and seagrass meadows, as similarly observed in Okinawan sea cucumbers [115], as such organisms can increase O₂ penetration depth in the sediment layer by plowing sediments via their feeding activities [116]. *Desulfobacterota* is a strict anaerobe, with chemoor-ganotrophic, chemolithoheterotrophic or chemolithoau-totrophic bacteria, found across a wide range of aquatic environments [117]. This phylum is often predominant in coastal deep layer sediments where hypoxic conditions can occur [112, 118, 119]. It is also known that seagrass meadows occasionally become hypoxic possibly due to abnormally high temperatures [120], however water temperatures in November to December, when we conducted sampling for this study, were lower than summer maximum temperatures. Still, further investigations combined with continuous temperature measurements around Okinawa Island should assist to better understand the relationship of *Desulfobacterota*'s abundance and hypoxic environments caused by global warming.

The significantly lower alpha diversity at sandy bottom sites when compared to seagrass meadow sites may reflect the low surface complexity of sandy environments. Seagrass is known to have a unique bacterial community associated with its leaves, roots and rhizosphere [121–123], and these bacteria may contribute to the ecological function in seagrass meadow sites. While corals are also known to harbor symbiotic bacteria on their surfaces or inner skeletons [58, 76, 124, 125], there was no significant difference in diversity index between coral reef and sandy bottom. This could be attributed to the substantial variation in bacterial community diversity known to occur in corals [126, 127]. In fact, the second lowest and highest Shannon diversity indices in this study were

obtained from Manza, one of the coral-rich sites (Shannon index=4.62 and 5.66, respectively).

Microbial composition and differentially abundant prokaryotic orders in each substratum

The PERMANOVA analysis revealed the prokaryotic composition is significantly different among the three benthic types. This is the first report rigidly uncovering such distinct differences among different benthic components in coral reefs on a geographically small scale. Interestingly, both coral reef and sandy bottom sites formed individual groups despite individual sites in each group are largely distanced. This result suggests benthic type-specific prokaryotic compositions exist around Okinawa Island, which may have acclimated to their respective conditions over a long period of time. However, it is important to consider that human-caused impacts can also affect the local prokaryotic community in coral reefs [22], and thus what we observe here in each benthic type might be a result of anthropogenic disturbances. Prokaryotic communities are known to be highly plastic and capable of recovering relatively quickly after disturbances. Therefore, continuous monitoring of the prokaryotic components at each site is crucial to understand and track any changes that may occur over time, and such data should provide valuable insights into the resilience and stability of coral reef ecosystems in response to environmental changes and disturbances.

At sandy bottom sites, there were only two bacterial orders, *Kiloniellales* and *Entomoplasmatales*, that were differentially abundant exclusively. Although these bacterial orders have been reported from marine sediments in a few studies [128, 129], the ecological feature and environmental preference of these bacteria remain largely unknown. One fact that is shared between these orders is that they are both known to be symbiotic to other organisms. For example, *Entomoplasmatales* is a well-known symbiotic bacteria found the guts of crustaceans (e.g., [130, 131]), and has also been reported as a major bacterial symbiont of marine lake jellyfish *Mastiguas* sp. [132]. *Kiloniellales* is a marine-restricted bacterial order [133], that is commonly found as a symbiotic bacteria among cnidarians (jellyfish; *Tripedalia* cf. *cystophora* [132], *Gonionemus vertens*; [134], hydrozoan *Millepora platyphylla* but not found in scleractinians *Porites lobata* or *Pocillopora meandrina*; [127]); also known from juvenile sea star *Mithrodia clavigera* [135]). Therefore, the biased abundance of these bacterial orders in sandy bottom sites might reflect the high population density of such host organisms. However, comprehensive population density and species aggregation information of these taxa around Okinawa Island is currently lacking, as known for some other taxa (e.g., zoantharians [136], giant clams [137], and holothurians [115]).

There were four bacterial orders differentially abundant in seagrass meadow. In the order *Steroidobacterales*, the genus *Woeseia* predominantly occupied 99.8% of entire order in this research. *Woeseia* was originally described within *Chromatiales* by Du et al. [138] as a salt-dependent and facultative anaerobic bacteria, and *Chromatiales* is also one of the differentially abundant bacterial orders in seagrass meadows in this study. *Woeseia* is a globally distributed and ubiquitous marine bacteria with chemorganoheterotrophy and facultative chemolithoautotrophy based on inorganic sulfur and hydrogen compounds, often found in marine sediments [139, 140]. This genus is also a major symbiotic rhizosphere bacteria of seagrasses, such as *Caulerpa* and *Cymodocea* [110], as well as *Zostera* [141], seepweed *Suaeda* and common reed *Phragmites* [142], and the mangrove tree *Kandelia candel* [143]. *Desulfobacterales*, a sulfate-reducing and strictly anaerobic bacteria, is known to be abundant in coastal deep layer sediments, and was also differentially abundant in seagrass meadows. Seagrass meadows and mangrove habitats often have low oxygen concentrations [144–147]. *Desulfobacterales* is also thought to be a major player of nitrogen cycling, involved in an estimated average of 12% of the entire process in mangrove ecosystems [148]. Nie et al. [148] also suggested that the high abundance of nitrate reducing bacteria such as *Desulfobacterales* may contribute to the prevention of excessive nitrate-nitrogen accumulation, which could harm the local ecosystem in combination with some other bacterial orders such as *Chromatiales*, which was abundant in the current research. *Chromatiales* is known to be a symbiont of the sponge *Stylissa carteri* [149], and is also abundant in saltmarsh sediments covered by cordgrass *Spartina alterniflora* along with *Thiotrichales* [150].

Of potential importance, there are reports that some of these bacteria are related to prevalent environmental pollution. For instance, *Woeseia* is known to increase with iron and arsenic increases in seagrass meadows [151], and *Desulfobacterales* is influenced by heavy metal pollution in mangrove forests [152]. *Desulfobacterales* has also been reported to be prominent at both natural (e.g., estuarine [153]) and artificially eutrophicated sites (prawn mariculture pond [154] and salmon mariculture area [155]). *Chromatiales* has been suggested to be elevated by environmental stress (i.e., unseasonal heavy rainfall) in the seagrass *Halophila ovalis* when compared to healthy individuals [156]. Eutrophication in seagrass meadows is suggested to stimulate an unfavorable surge of heterotrophic bacteria, that occasionally can result in large-scale die-off events due to elevated sulfide concentrations in sediments [157]. Therefore, these bacteria can potentially be used as bioindicators of such disturbances, and further research combined with direct detection of

biotic and abiotic, as well as natural and anthropogenic cues may assist to track local environmental stress better.

Our examined coral reef sites had six bacterial orders that were significantly and exclusively abundant. Order *Rhodobacterales* is a facultative anaerobic bacteria, known to be a primary surface colonizer in temperate coastal waters [158]. In tropical coastal areas, *Rhodobacterales* are known to be abundant on some coral species' outer surfaces (e.g. *Porites* [127]), especially in aged surface mucus [76]. This order has been proposed to be prevalent in stressed or diseased coral individuals (pathogenic *Vibrio* infection [159], stony coral tissue loss disease [160], heat stress due to El Niño [161]). Notably, it has been implied that many of the bacteria within *Rhodobacterales* are capable of detecting and degrading dimethylsulphoniopropionate (DMSP) [162–164], which is mainly produced by Symbiodiniaceae dinoflagellates symbiotic with coral [165]. Considering the fact that heat-stressed coral colonies express five-fold higher DMSP concentrations than healthy colonies [166], Rosales et al. [167] proposed that DMSP could chemoattract some kinds of bacteria and therefore is the cause of the prominence of *Rhodobacterales* in stressed coral colonies. *Flavobacteriales* was also abundant in coral reef sites, and this bacteria is known to encode diverse polymer-degrading enzymes, indicating their capacity to utilize a wide range of carbon sources [168]. This order has been suggested to be plentiful in coral mucus and tissue [167], being especially pronounced before and while host corals are exposed to environmental stress [169], such as disease infections [167]. Furthermore, it has been suggested that *Flavobacteriales* encodes similar functional genes as *Rhodobacterales*, although their expressions are substantially different [170]. *Verrucomicrobiales*, an order belongs to the phylum *Verrucomicrobiota* that has been reported as almost ubiquitous in marine habitats including coastal sediments (reported as *Verrucomicrobia* [171]), is also known to be abundant in coral mucus, and potentially pathogenic to corals [76]. *Verrucomicrobiales* increases in corals infected with black band disease [172] and also with growth anomalies [173]. *Enterobacterales* is a Gram-negative, rod shaped and facultative anaerobic bacteria [174], which occasionally has been reported to associate with aquatic organisms such as freshwater fish [175] and marine sponges [176]. This order has been suggested to be pronounced in bleached *Acropora digitifera* [177], although the genus *Vibrio*, a representative pathogenic taxon within this order, was not abundant in this research. *Cytophagales* is an order that prefers habitat where organic materials are abundant, and is capable of producing flexirubin-type pigments [178]. *Cytophagales* are often found in freshwater, and are also relatively common in coastal waters [178], and have been suggested to be a major decomposer of plant litter in marine wet

land in China [179]. In tropical coral reefs, *Cytophagales* has been known to be an opportunistic bacteria that was observed to become more abundant on the body surface of the coral *Montastraea cavernosa* after infection of pathogenic *Vibrio* bacteria, presumably due to a decrease in predatory bacteria *Halobacteriovorax* [159]. Although *Halobacteriovorax* has been reported from marine sediments [180, 181], it was barely detected in this research. *Cytophagales* is also known to become more abundant once a coral host is subjected to continuous heat stress, e.g. by El Niño [161]. *Euryococcales* is a photosynthetic cyanobacterial order, which has been previously reported from coastal sediments [155]. However, information about this order, such as its preferred habitat(s) or nutrient requirement(s), is still limited and more studies are needed to better interpret the meaning of their abundance in coral reef sites. Meanwhile, coral symbiotic bacteria, represented by family *Endozoicomonaceae* [24, 76, 77, 182–184] and a few other taxa (i.e. *Oxalobacteraceae* [76], *Alteromonadales* and *Simkaniaceae* [184, 185], *Oceanospirillales* [58]), were comparatively rare in the current research.

Overall, the current results may reflect high levels of environmental stressors such as heat stress, coral disease, and pathogenic bacteria in coral reef sites around Okinawa Island. Bacterial interactions between coral-ambient sediment and coral-ambient seawater have been reported in healthy [58, 76, 186] and diseased corals [187], and it has been suggested that coastal surface sediments serve as a possible pathogen reservoirs or “seed-banks” as proposed by Carlos et al. [75]. Although we did not conduct direct sampling of coral tissue or mucus, considering several putative stress-related bacterial orders (e.g., *Flavobacteriales* and *Verrucomicrobiales*) were relatively more abundant in this research compared to coral reef sediments in previous research (Supplementary Table S15), there is an urgent need to monitor coastal sedimentary microbial communities to track stress-related prokaryote demography around Okinawa. Simultaneously, there is yet only a few examples of research on coastal sedimentary microbiomes, especially from the western Pacific, making it harder to compare our results to previous research. Therefore, to take advantage of metabarcoding methods as environmental monitoring measures, it would be very helpful to create a local catalogue of conspicuous prokaryotic taxa depending on substratum type.

Correlation between bacterial orders at each environment

There were several correlations observed among bacterial orders in this research. However, due to the lack of essential information to interpret these relationships, such as habitat preferences, secondary metabolites and/or biochemical capabilities, many of these relationships remain

unknown at this stage. Thanks to the recent technological advancements, further comparative details between some microbial groups could be provided; for example, the negative correlation observed between *Actinomarinales* and *Cyanobacteriales* at coral sites. Recently, López-Pérez et al. [188] explored the genome of *Actinomarinales*, a photoheterotrophic bacterial order, and revealed that it encodes cyanophycinase, enabling *Actinomarinales* to use cyanophycin as a source of arginine, which is one of the amino acids that they are auxotrophic for. Cyanophycin is an amino acid polymer produced by most *Cyanobacteria* that has arginine as a branched side chain and aspartic acid as its backbone [189]. Cyanophycin is predicted to work as a temporary storage of nitrogen, specifically accumulating fixed nitrogen at night and utilizing it during the day to uncouple nitrogen assimilation and photosynthesis, and hence cyanophycin is important in these species' survival strategies [190].

Based on these facts, the negative correlations observed here might indicate the following scenario: increases of *Actinomarinales* raise its dependency on cyanophycin, and subsequently *Cyanobacteria* are consumed by *Actinomarinales* as a source of arginine. *Actinomarinales* is also known to work as a module-hub among small sized free-living planktonic bacteria in eutrophic estuaries [191]. *Desulfobacteriales*, which showed a positive correlation with *Actinomarinales*, is one of the bacterial orders abundant in eutrophic environments such as estuaries [153]. Xie et al. [192] revealed that these two orders are abundant in plastic-polluted mangrove soil specifically in spring, while *Actinomarinales* become infrequent as water temperature rises. Therefore, abundant nutrient resources at coral sites or other condition (e.g. water temperatures, light intensity etc.) may lead to a positive correlation of these orders. *Actinomarinales* and *Thermoanaerobaculales* exhibited positive correlation in both coral sites and sandy bottom sites. According to our obtained data, family *Thermoanaerobaculaceae* occupied more than 90% of the entire *Thermoanaerobaculales*. *Thermoanaerobaculaceae* have been described as thermophilic, neutrophilic and chemoheterotrophic anaerobes that prefer some sugars, organic acids and proteinaceous compounds as growth substrates [193], and they have been suggested to be sulfate reducers [194, 195]. *Thermoanaerobaculaceae* is known to increase upon nitrate nitrogen contamination into the environment [196]. *Actinomarinales* is known to perform anammox (anaerobic ammonium oxidation) by reducing natural organic matter (NOM), and thus increase NOM availability [197]. Therefore, these orders might represent an increase in total nitrogen input into the environment. *Flavobacteriales*, which had a negative correlation with *Rhizobiales* and *Thermoanaerobaculales* at sandy bottom sites, was mostly occupied by a member

of family *Flavobacteriaceae* in the current data (>90%). Recent genome mining research of *Flavobacteriaceae* has revealed that they encode antimicrobial, antioxidant and cytotoxic compounds [168], and this might work to suppress *Rhizobiales* and *Thermoanaerobaculales*. To achieve a better understanding of such correlations among orders, however, more rigid information about their ecological and biochemical capabilities, as well as direct detection of abiotic variables such as salinity, dissolved oxygen, nitrate and phosphate concentrations, and pH are essentially needed.

Enzymic capability estimation

Although the actual amounts of enzymes were not investigated directly, the significantly higher enzymic diversity and the greater number ($n=20$) of enzymes that were differentially abundant in coral reef sites may be explained by coral symbiotic prokaryotes' complex enzymic reactions to various environmental stressors [198–200]. Likewise, although enzymic diversity was significantly lower than at coral rich sites, seagrass meadow sites had several enzymes exclusively abundant ($n=8$), and this could also mirror diverse biochemical pathways of microbial symbionts in this habitat [87, 201, 202]. Comparing those two substrata, sandy bottom sites had poor three-dimensional rugosity, and this may potentially have resulted in the absence of exclusively abundant enzymes.

Amongst the enzymes that were exclusively abundant in coral reef sites, cysteine desulfurase (EC 2.8.1.7) is known to be one of the most abundant and ubiquitous genes encoded in any organism's genome [203], including bacteria [204]. This enzyme is involved in the conversion of L-cysteine into L-alanine and sulfane sulfur, and this process also involves iron-sulfur cluster synthesis [205]. So far, three types of cysteine desulfurase, namely NifS, IscS, and SufS, have been identified, with all of them considered important in oxidative stress mitigation [206–208].

Another enzyme enriched in coral sites was superoxide dismutase (EC 1.15.1.1). Generally, superoxide dismutase is known to mediate superoxide compounds such as hydrogen peroxide [209], and have also been confirmed to be produced by various bacteria [210, 211]. One major and well-studied oxidative stressor within scleractinian corals are individual endosymbiotic dinoflagellate Symbiodiniaceae under heat-stressed conditions [212], and thus superoxide dismutase production capability is important for biochemical defense in coral tissue, especially under harsh condition such as high seawater temperatures during coral bleaching [213]. We must note, however, that we neither detected enzymes directly nor collected coral tissue sample directly. Still, considering the nature of microbial interactions between a coral's surface and its surrounding environment (known as “the coral

ecosphere” in [68]), our finding that coral sites have significantly higher production potential of superoxide dismutase than other substrate types needs to be followed up with more concrete future studies including metagenomic analyses or classical culturing to assess what bacteria are actually contributing to these differences.

The histidine kinase (EC 2.7.13.3) was abundant in seagrass meadow sites. In bacterial cells, histidine kinase is known as a part of membrane-anchored proteins forming the two-component regulatory system [214], and is essential for bacteria to detect and react to external stimuli by phosphorylating histidine as a signaling cue [215]. As well, this enzyme is suggested to be crucial for biofilm formation [216]. Therefore, the abundance of this enzyme may show dynamic environmental fluctuation in these sites. Coral reef sites are also presumed to have substantial environmental fluctuations, however the histidine kinase coding gene was not abundant in coral reef sites. This may indicate the ecological strategy of bacterial community in each substratum is different, and further investigation ideally employing other methods such as transcriptome analyses and enzyme assays are needed for a better understanding.

Conclusions

Our data showed that the different habitats in coral reefs have different prokaryotic communities, with characteristic taxa that are exclusively more abundant in specific environments. Although the main driver that determined community structure was not fully revealed in this study, some bacterial taxa that characterized our observed coral reef microbiome have previously been linked to environmental stressors such as heat stress, bleaching, and coral diseases. Considering the potential of benthic sediments to interact with coral surface prokaryotic communities as suggested previously, sedimentary prokaryote monitoring is extremely important to assess the potential risk of environmental degradation in coral reefs, and harmful pathogen prevalence in coral reefs. Therefore, further studies on the interactions between the microbial community and biotic (e.g., coral disease prevalences, coral-livore abundances, organic content concentrations) and/or abiotic (e.g. water temperatures, hydrodynamics, solar radiation) parameters needs to be performed. In addition, our samples were from a single time period, and therefore continued sampling and longer-term monitoring are needed to track spatio-temporal variability. For now, our data from the current study provide a preliminary reference list of prokaryotic taxa we should pay attention to in order to better forecast coral reef health with higher resolution.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40793-024-00594-1>.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9
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Supplementary Material 16

Acknowledgements

We are grateful to members of the Molecular Invertebrate Systematics and Ecology laboratory, University of the Ryukyus, especially Ms. Marilyn Carletti, Ms. Chloé Lois Fourreau, Ms. Iori Mizukami, Mr. Yusuke Iwaki, and Mr. Hiroki Nakajima for their help in sampling. Finally, Kohei Hamamoto dedicates this paper to the memory of his late beloved dog Mimimi, who supported him throughout this work.

Author contributions

The research was designed by KH, MM, AI, and JDR. Sampling was done by KH and AP. Laboratory experimentation was directed by MN, and performed by KH, AM, and KG. Sequence processing, data curation and data analyses were done by KH, MM, and AI. The original draft was written by KH, then reviewed and edited by all authors. AI and JDR supervised entire research. All authors approved the final manuscript.

Funding

This work was supported by a MEXT/JSPS KAKENHI Grants (20H00653 and 24H00784), and the on Environmentally-conscious Developments and Technologies (E-code) research laboratory at the National Institute of Advanced Industrial Science and Technology (AIST).

Data availability

All data used in this study, including codes, sampling metadata, and results of each analysis, are available in the supporting materials of this article. The raw sequences have been deposited in DDBJ with accession numbers DRR506213 - DRR506511, under bio-project ID PRJDB16769 (<https://ddbj.nig.ac.jp/resource/bioproject/PRJDB16769>). The data can also be accessed via NCBI at <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJDB16769>.

Declarations

Ethical approval

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 25 January 2024 / Accepted: 8 July 2024

Published online: 30 July 2024

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