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Symbiotic bacteria associated with ascidian vanadium accumulation identified by 16S rRNA amplicon sequencing

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ABSTRACT

Ascidians belonging to Phlebobranchia accumulate vanadium to an extraordinary degree (≤ 350 mM). Vanadium levels are strictly regulated and vary among ascidian species; thus, they represent well-suited models for studies on vanadium accumulation. No comprehensive study on metal accumulation and reduction in marine organisms in relation to their symbiotic bacterial communities has been published. Therefore, we performed comparative 16S rRNA amplicon sequence analyses on samples from three tissues (branchial sac, intestine, and intestinal lumen) involved in vanadium absorption, isolated from two vanadium-rich (*Ascidia ahodori* and *Ascidia sydneiensis samea*) and one vanadium-poor species (*Styela plicata*). For each sample, the abundance of every bacteria and an abundance value normalized to their abundance in seawater were calculated and compared. Two bacterial genera, *Pseudomonas* and *Ralstonia*, were extremely abundant in the branchial sacs of vanadium-rich ascidians. Two bacterial genera, *Treponema* and *Borrelia*, were abundant and enriched in the intestinal content of vanadium-rich ascidians. The results suggest that specific selective forces maintain the bacterial population in the three ascidian tissues examined, which contribute to successful vanadium accumulation. This study furthers the understanding of the relationship between bacterial communities and metal accumulation in marine life.

1. Introduction

Ascidians, also known as sea squirts or tunicates, provide an excellent model system to study the absorption of vanadium because some species accumulate high levels of vanadium ions in their blood cells (Michibata and Ueki, 2012; Ueki and Michibata, 2011; Ueki et al., 2014). The concentration of vanadium in seawater is ~35 nM (Cole et al., 1983; Collier, 1984) but ascidians of the suborder Phlebobranchia have been reported to contain levels as high as 350 mM, 10^7-fold higher than seawater (Michibata et al., 1991). Vanadium ions are absorbed from seawater in a $+5$ state, reduced to a $+4$ state in ascidian tissues, and stored in a $+3$ state in a type of blood cell called vanadocytes. Vanadocytes are specialized cells that contain one or more large acidic vacuoles, which store vanadium in a soluble, $+3$ state (Hirata and Michibata, 1991; Michibata et al., 1991; Ueki et al., 2002). Vanadium levels are strictly regulated in ascidian tissues and vary among different phylogenetic groups (Michibata et al., 1986, 1991). Thus, ascidians represent well suited models for molecular studies on metal absorption.

Radioautographic analysis of *Ascidia ceratodes* and *Ciona intestinalis* incubated with $^{48}V^{+5}$ revealed that vanadium is contained primarily in the ovary, gut wall, eggs, and branchial basket (Goldberg et al., 1951). For ascidians to extract vanadium ions in a $+5$ state directly from the seawater, a high-affinity vanadium ion transporter is necessary. As the structure of vanadium in a $+5$ state (VO_4^{3-}) resembles that of a phosphate anion (PO_4^{3-}), phosphate family transporters were considered likely involved in vanadium transportation as well. Preliminary studies suggest that there are eight possible phosphate/vanadium transporters in the *C. intestinalis* genome and that at least one of these eight is able to transport vanadium in a $+5$ state (Ueki et al., unpublished data). However, its affinity for vanadium is too low to uptake vanadium directly from seawater. Thus, we hypothesized that mutualistic bacteria might enable the absorption of vanadium through the epithelium of the branchial sac and intestine, which are the interfaces to the seawater and the intestinal microenvironment, respectively.

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Our recent study on *Ascidia sydneiensis samea*, a vanadium-rich ascidian, indicated that the vanadium concentration in the mixture of planktonic and bacterial contents from the intestinal lumen, *i.e.* intestinal content, can reach 0.67 mM, about 20,000-fold higher than seawater (Romaidi and Ueki, 2016). Thus, the intestinal lumen is vanadium-rich and we postulated that intestinal cells absorb vanadium (as V^{+5} or V^{+4}) from this microenvironment. Toward testing this hypothesis, we successfully isolated nine vanadium-accumulating bacterial strains from the intestinal content of *A. sydneiensis samea*; these were classified into two genera (*Vibrio* and *Shewanella*) based on their partial 16S rRNA sequences. These strains are thought to facilitate vanadium sequestration by increasing the concentration of vanadium in the intestinal lumen (Romaidi and Ueki, 2016). However, that study only identified bacterial strains that could be cultured on a limited variety of media.

Several studies have investigated the importance of vanadium accumulation and reduction by bacteria (Antipov et al., 1998; Carpentier et al., 2003, 2005; Lyalkova and Yurkova, 1992; Marwijk et al., 2009; Ortiz-Bernad et al., 2004; Zhang et al., 2014). For example, Lyalkova and Yurkova revealed that *Pseudomonas* strains isolated from a metal slag and an ascidian can reduce vanadium in a + 5 state into a + 4 state. Antipov et al. reported that *Pseudomonas isachenkovii* isolated from the intestine of an ascidian could resist vanadium toxicity and use vanadate as the electron acceptor during anaerobic respiration. Carpentier et al. reported that *Shewanella oneidensis* was also capable of growing in the presence of vanadate as the sole electron acceptor and reduced vanadium in a + 5 state to a + 4 state. However, as did our study mentioned above, these studies only identified and analyzed bacterial strains that could be cultured. Therefore, we sought to perform a more comprehensive study on the role of bacteria in metal accumulation and reduction.

Symbiotic bacterial populations affect the physiological condition of their animal hosts and contribute to the absorption of nutrients. Though metal ions are one of micronutrients and many of metal elements are essential to life, little is known about the bacterial contribution to metal absorption. Existing studies are limited to the digestion of dietary fibers or production of vitamins. Additionally, there are many reports of bacterial metal absorption and utilization but very few published on these processes in a symbiotic context.

Gut bacterial communities in marine invertebrates have been examined by Harris et al. using first- (Harris, 1993) and next-generation DNA sequencing techniques (McFall-Ngai et al., 2013). Next-generation sequencing (NGS) enables metagenomic or metatranscriptomic analyses that can pinpoint the genetic underpinnings of biological functions. In ascidians, a comprehensive analysis of the intestinal microbial diversity in a solitary ascidian *C. intestinalis* was achieved using NGS (Dishaw et al., 2014b); this is discussed later in relation to gut immunity (Dishaw et al., 2014a). Similar approaches have also been used to profile the ascidian tunic, which is their outermost layer, well-known for its unique microbial community. Many comprehensive studies have used NGS to determine microbial diversity, including the tunic and biofilm of the colonial ascidian *Lissoclinum patella* (Behrendt et al., 2012), tunic of the solitary ascidian *C. intestinalis* (Blasiak et al., 2014), and the tunics of 25 species of ascidians from the Great Barrier Reef (Erwin et al., 2013). An even more comprehensive analysis of the tunic using NGS has revealed the within-species conservation of microbiome rather than geographical conservation (Cahill et al., 2016). Although these across-species studies suggest that the species-specific bacterial population can be correlated with the production of chemicals specific to each species of ascidian (Tianero et al., 2014), to date, none have focused on the metal accumulation and reduction activity of the symbiotic bacteria.

In this study, we obtained metagenomic DNA from two organs (branchial sac and intestine) and the intestinal content of two vanadium-rich ascidians (*A. ahodori* and *A. sydneiensis samea*) and a vanadium-poor ascidian (*S. plicata*). We employed NGS to generate 16 s

rRNA amplicon sequence data from these samples and then compared the microbial diversity of the three examined areas across all three species. We also determined the relationship between the bacterial populations and vanadium concentration in these samples. Furthermore, we identified several bacterial families/genera/species that were abundant and enriched in the branchial sac and intestine of vanadium-rich ascidians.

2. Materials and methods

2.1. Biological materials

Adult ascidians (*A. ahodori*, *A. sydneiensis samea,* and *S. plicata*) were collected from the Kojima Port, Okayama, Japan (34°26′36.3" N 133°48′43.5" E). They were cultivated in an aquarium with flowing natural seawater for over one month at Marine Biological Laboratory, Hiroshima, Japan (34°21′55.3" N 133°12′55.9" E) prior to experimentation and were fed regularly with a mixture of diatoms. Almost all of the blood from every individual organism (~0.5–3 mL) was extracted and diluted with Ca^{2+} and Mg^{2+}-free artificial seawater (460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 6 mM $NaHCO_3$, 5 mM HEPES, and 5 mM EDTA, pH 7.0). The blood cells were then isolated by centrifugation at $300 \times g$ for 10 min at 4 °C. Giant cells stratified on top of the other blood cells in *A. sydneiensis samea*; these huge cells are uniquely found in *A. sydneiensis samea* (Michibata et al., 1991). Because they occupy ~90% of the volume of the cell pellet but do not accumulate vanadium and can greatly affect vanadium measurement, they were removed with a pipette and only the remaining cells were used for future experiments. The branchial sac (BR) and intestine (IN) were excised with scissors and tweezers. Intestinal contents were aspirated with a pipette. Samples for DNA extraction were frozen at −80 °C until use. Seawater samples were obtained by filtering natural seawater from the Marine Biological Laboratory, Hiroshima, Japan through a 5-μm filter (Advantec Tokyo, Ltd., Japan). From this method, plankton were collected in the filter with their accompanying bacteria. The plankton samples as well as associating bacteria (here we refer as SW), which were trapped by the branchial sac and moved to the intestine as food for ascidians and can represent the base population, were used to extract metagenomic DNA.

2.2. Metal measurement

For vanadium measurements, the samples were dried overnight at 65 °C and vanadium was extracted using 1 N nitric acid also overnight at 65 °C. After centrifugation at $10,000 \times g$ for 10 min, the vanadium concentration of the supernatant was determined using atomic absorption spectrophotometry (AA-220Z, Agilent, USA). A vanadium standard solution was purchased from Fujifilm Wako Pure Chemical, Ltd., Japan.

2.3. Library construction and sequencing of 16S ribosomal DNA amplicons

DNA was extracted from each ascidian and SW sample (Supplementary Table S1) using a method based on two publications (Kim et al., 2013; Morita et al., 2007). Samples were suspended in 1 mL of TE buffer with 150 μL of lysozyme (100 mg/mL) and incubated for one hour at 37 °C using a Thermo Block Rotator (SNP-24B, Nisshin Rika, Ltd., Japan). Fifteen microliter of achromopeptidase (200 U/mL) was added to the sample solution and the ensuing reaction was performed at 37 °C for 30 min. One hundred microliter of 10% SDS and 15 μL of Puregene Proteinase K (Qiagen, Netherlands) were added to the sample solution followed by incubations at 55 °C for one hour. DNA samples were extracted using phenol and chloroform in 5PRIME Phase Lock Gel tubes (Quantabio, MA, USA), precipitated, and resuspended in 100 μL of TE. The quality and integrity of the resulting genomic DNA were examined using an Agilent 2100 Bioanalyzer (Agilent). 16S ribosomal DNA fragments (~459 bp) were amplified with Amplicon

(forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-<u>CCTACGGGNGGCWGCAG</u>-3′, reverse primer: 5'-GTCTCGTGGGCTCG-GAGATGTGTATAAGAGACAG<u>GACTACHVGGGTATCTAATCC</u>-3′). The 3′ underlined parts of these primers correspond to 16S rRNA sequences, while 5′ overhang sequences are artificial sequences to be used in the following analytical process. Multiplex analysis using a MiSeq DNA sequencer (Illumina, Inc., CA, USA) provided paired-end reads (300 bp × 2). The paired-end reads were overlapped to assemble the V3–V4 region of the 16S ribosomal DNA. The MiSeq Reporter (Illumina) was used to automatically assign taxonomic classifications by comparing the read sequences to an Illumina-curated version of the Greengenes 16S rRNA gene database using a Bayesian method. Because Miseq system uses a non-patterned flow cell, the probability of index hopping is expected to be very low (< 0.1%) in 16S rRNA sequence amplicon sequencing (http://jimngsnotes.blogspot.com/2017/04/index-hopping-problem-with-illumina.html), no special step was included to remove chimeras in this workflow. 16S ribosomal DNA amplicon sequence data have been deposited in the DDBJ BioProject database with accession number DRA005735 at https://www.ddbj.nig.ac.jp/dra/index-e.html.

2.4. Alpha and beta diversity analyses

In order to calculate the alpha (within sample) and beta (between sample) diversities, each assembled sequence was rarefied to a maximum of 150,000 reads. The matrix of samples and the occurrence of each genus or species were formatted into operational taxonomic unit (OTU) tables. Alpha diversity was calculated with QIIME, an open-source bioinformatics pipeline, using the Chao1 algorithm (Caporaso et al., 2010). The OTU tables were subsampled and rarefaction curves were drawn using QIIME and the same algorithm. Beta diversity was determined by computing the Bray-Curtis dissimilarity (Bray and Curtis, 1957) between samples in QIIME. Sample clusters were generated with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm also using QIIME. A Bootstrap confidence value for each branch of the master phylogenic tree was calculated by subsampling 300 genera and species from each OTU table 100 times and clustering each subset of data using the UPGMA method.

2.5. Draft genome sequencing of nine vanadium-accumulating bacteria

Four *Shewanella* and five *Vibrio* strains (V-RA-1 to 5 and S-RA-6 to 9) were cultured in standard media overnight at 20 °C as previously described (Romaidi and Ueki, 2016). Cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C. DNA was extracted from each sample with a Nucleospin Tissue DNA Extraction Kit (Takara Bio Inc., Japan). Shotgun libraries were constructed using the TruSeq DNA Sample Preparation Kit v2 (Illumina). In addition, mate-pair libraries were constructed with five different insert sizes (1 kb, 2 kb, 4 kb, 8 kb, and 12 kb) using the Mate Pair Library Prep Kit (Illumina) according to the manufacturer's protocol. Multiplex analysis with Illumina's Hiseq 2500 Rapid Run DNA sequencer generated paired-end reads (250 bp × 2) from each library. About 3.6 Gbp of shotgun sequence data and about 14 Gbp of mate-pair sequence data were obtained on average for each strain. Shotgun sequence data were assembled with the DiscovarDeNovo software (Weisenfeld et al., 2014) and scaffolding was achieved using the SSPACE software (Boetzer et al., 2011). The average number of scaffolds was 294.6 and the average scaffold size was 35,167 bp. 16S rRNA sequences were searched with RNAmmer (Lagesen et al., 2007). For the several strains that did not yield reliably assembled data, the Platanus software was employed to assemble the sequence data and hand-selected putative 16S rRNA sequences with BLASTN. Assembled sequence data for 16S rRNA sequences are deposited in DDBJ database with accession numbers LC416553–LC416561.

2.6. Statistical analyses

Measurement of vanadium concentration was conducted in biological triplicate and the averages for each measurement of each sample are shown with their standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to compare alpha diversities. Statistical significance was verified using Fisher's least significant difference (LSD) test.

3. Results

3.1. 16S rRNA amplicon sequencing analysis

The purpose of this study is to perform a comprehensive study of symbiotic bacterial communities involved in metal accumulation and reduction in the ascidians. We chose three ascidian species, two of which belonged to the genus *Ascidia* (*A. ahodori* and *A. sydneiensis samea*) which is known to accumulate high levels of vanadium. Another species is *Styela plicata* which does not accumulate vanadium at high level.

Three samples, branchial sac (BR) and intestine (IN) and intestinal content (IC) were collected aseptically from each individual (n = 3) of the three ascidian species. The BR and IN samples were mainly composed of cells obtained from the ascidian tissue itself, although some bacterial cells likely remained attached to the ascidian tissue; the IC samples were mainly composed of plankton and bacterial cells. Hereafter these three samples (BR, IN, and IC) are referred to as 'tissues.' Additionally, planktonic material filtered from flowing natural seawater (Here we refer as SW; n = 3) was analyzed in parallel to reflect the external marine conditions. We used 5 μm filter to collect planktons as well as associating bacteria, which are trapped by the branchial sac and moved to the intestine, to represent the base population as food for ascidians. Thus, we analyzed 30 samples in total.

DNA was extracted from all samples and libraries were constructed and sequenced (Supplementary Table S1). The ascidian samples yielded 10,264,474 total reads, of which 9,805,516 (95.53%) passed the quality test. One of the IN DNA samples from *S. plicata* (ID #27) yielded 10-fold more data than its counterparts, while a BR DNA sample from *S. plicata* (ID #25) yielded only 888 high-quality sequences. The three SW samples yielded 901,471 total reads, of which 838,293 (92.00%) passed the quality test.

The rate for the identification of species is generally lower than those for higher taxonomic levels. The data for classification rates are summarized in a Supplementary Table S2. The average classification rates in ascidian tissues from kingdom to genus levels were between 90.56% and 99.76%. The average classification rate in ascidian tissues for species was 72.69%. Although this value is relatively low, we here describe the results equally to the higher levels.

Toward comparing the bacterial diversity, within sample (alpha) and between sample (beta) diversity indices were calculated at the genus and species levels. Aside from ID #25, the lowest read count was 156,583 (BR DNA from *A. sydneiensis samea*, ID #04). A single rarefied OTU table for each genus and species was used for both alpha and beta diversity analyses.

Alpha diversity was calculated for each DNA sample (Fig. 1). ANOVA was performed to compare alpha diversities among tissues and ascidian species, and statistical significance was verified using LSD test. The overall diversity profiles were similar between genus and species levels. The alpha diversities calculated from the BR and IN samples were similar among ascidian species. The alpha diversities from the BR samples were significantly smaller than those from the SW samples at both the genus and species level. The alpha diversities from the IN samples were significantly smaller than those from the IC and SW samples.

The deviation of BR in *S. plicata* is large because we included the data from ID #25 (outlier with only 888 reads). The alpha diversity
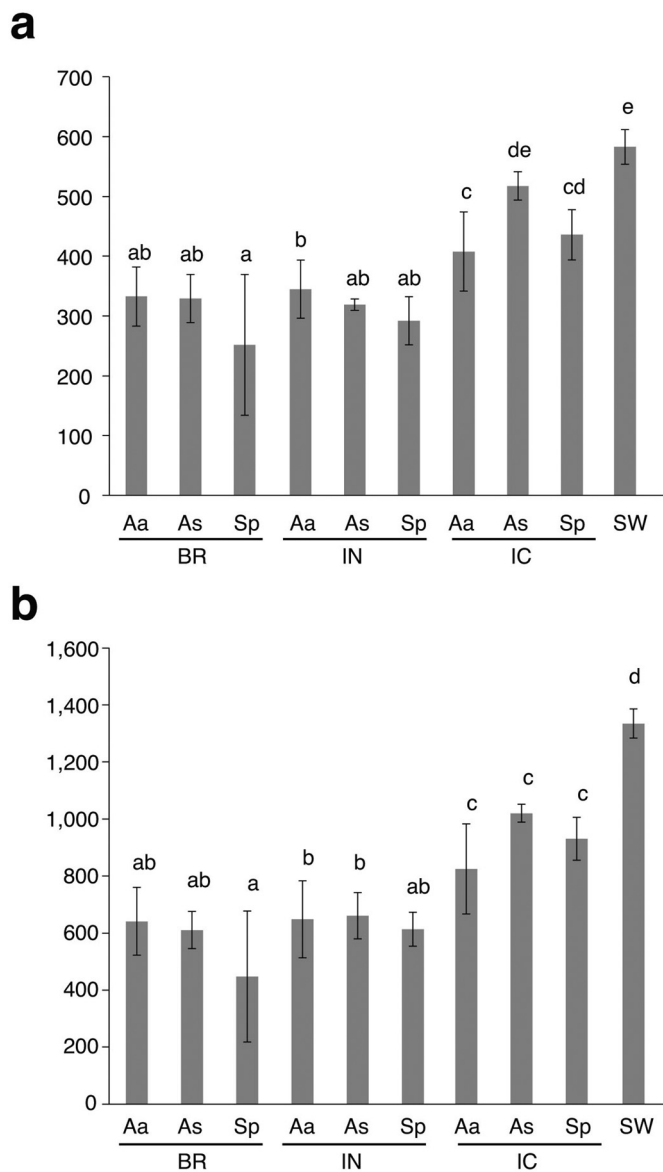
a

b

Fig. 1. Mean alpha diversity of bacteria in branchial sac and intestine are similar among ascidian species but smaller than those of the intestinal contents and seawater. Y-axis indicates mean alpha diversity values for each tissue in each species calculated at the generic (a) and species (b) levels. Aa: *Ascidia ahodori*, As: *Ascidia sydneiensis samea*, Sp: *Styela plicata*. BR: Branchial sac, IN: Intestine, IC: Intestinal content, SW: seawater. Data are presented as means ± S.D. (*n* = 3). For each sample, different letters indicate a significant difference of mean alpha diversity of bacteria in each ascidian's tissue and natural seawater at the level of *P* < 0.05.

indices of ID #25 were 116.9 (genera) and 182.5 (species). Thus, we excluded this sample from further analyses and proceeded with the other two (ID #07 and ID #16) to represent the BR of *S. plicata*.

Beta diversity is the measure of similarity between two communities (Benz, 1988; Benz et al., 1985; Nikaido and Rosenberg, 1983). From this determination, when two bacterial communities are composed of similar sets of bacterial taxa, they are clustered in the same clade. The IN samples from all three species clustered in a single clade, as did the BR samples from the two vanadium-rich species (*A. ahodori* and *A. sydneiensis samea*) (Fig. 2). This clustering was supported 100% by Bootstrap confidence analysis at both the genus and species levels. No clear distinction was found between the BR and IN bacterial communities of each clade, suggesting that the bacterial communities associated with these tissues in the two vanadium-rich species experience similar selective forces. The two BR samples from vanadium-poor *S. plicata*, (#07 and #16) were clustered in an independent clade with a Bootstrap confidence value of 100%.

The IC samples clustered into three species-specific clades and were distinguishable from the BR and IN samples. The three IC replicate samples from *A. ahodori* were divided into two clades but the associated Bootstrap confidence values were small (58–59% and 40–53% at the genus and species levels respectively). These data suggest that the IC samples somehow diverged among the individuals in each ascidian species. The SW samples comprised a single cluster at both the genus and species levels and there were no significant relation to any of the tissue samples.

3.2. Vanadium levels

We determined the vanadium concentration of each sample, as well as of the blood cells from all nine individuals (Fig. 3). The concentration of vanadium in the blood cells from *A. ahodori* was 12.42 mM, ~25% of previously reported values (Ueki et al., 2003). The concentration of vanadium in the blood cells from *A. ascidia sydneiensis samea* was 5.40 mM, ~50% of reported values (Samino et al., 2012). These results imply that current culture conditions are not optimal and require improvement. However, the variation in each species was small and, therefore, these specimens were used to predict the relationship between vanadium level and bacterial flora.

3.3. Bacterial populations

The average abundance for each bacterial phylum was calculated for each ascidian species and for all tissues (Supplementary Fig. S1 and Supplementary Table S3). Bacterial populations on the BR and IN were dominated by Proteobacteria, which accounted for > 95% of all bacteria associated with the two vanadium-rich *Ascidia* species, while in *Styela,* Proteobacteria represented only 84% of the total population. In the IC, the relative abundance of Proteobacteria ranged from 37 to 59%. Three phyla commonly dominated the IC samples: Cyanobacteria (11–28%), Bacteroidetes (1–30%), and Firmicutes (4–6%).

Second, the average abundance for each bacterial family was calculated for each ascidian species and tissue (Supplementary Fig. S2 and Supplementary Table S4). Bacterial populations on the BR of the two vanadium-rich *Ascidia* species were Pseudomonadaceae-dominant (70% and 69%). The relative abundance of this family was low in *S. plicata* (6%), indicative of a relationship between these bacteria and vanadium accumulation. The second most prevalent family, Oxalobacteraceae, displayed a similar tendency (18% and 21% in each of the *Ascidia* species, 2% in *S. plicata*). On the other hand, Oceanospirillaceae and Aerococcaceae were predominant on the BR in *S. plicata* (68% and 11% respectively) and represented < 1% of the bacteria on the BR in the two vanadium-rich *Ascidia* species. Pseudomonaceae and Oxalobacteraceae dominated the IN of all three ascidian species. The four families (Vibrionaceae, Desulfobulbaceae, Campylobacteraceae, and Pseudoalteromonadaceae) were relatively abundant in the IC of the two vanadium-rich *Ascidia* species (5.6–10.5% in *A. ahodori* and 0.26–1.28% in *A. sydneiensis samea*) but not of the *S. plicata* (0.05–1.05%).

Third, the MiSeq Reporter output was analyzed at the genus (Fig. 4 and Supplementary Table S5) and species (Fig. 5 and Supplementary Table S6) levels. The average abundance of each bacterial genus was calculated for each species of ascidian and its tissues. The *Pseudomonas* and *Ralstonia* genera were abundant in both the BR and IN of the two vanadium-rich species and dominant in the IC of all three ascidian species. At the species level, the predominant bacteria in the BR and IN of the two vanadium-rich *Ascidia* were *Pseudomonas brenneri* and *Ralstonia pickettii*. The predominant genus in the BR of *S. plicata* was *Amphritea* (68%), mostly *Amphritea atlantica*, which belongs to the Oceanospirillaceae family.
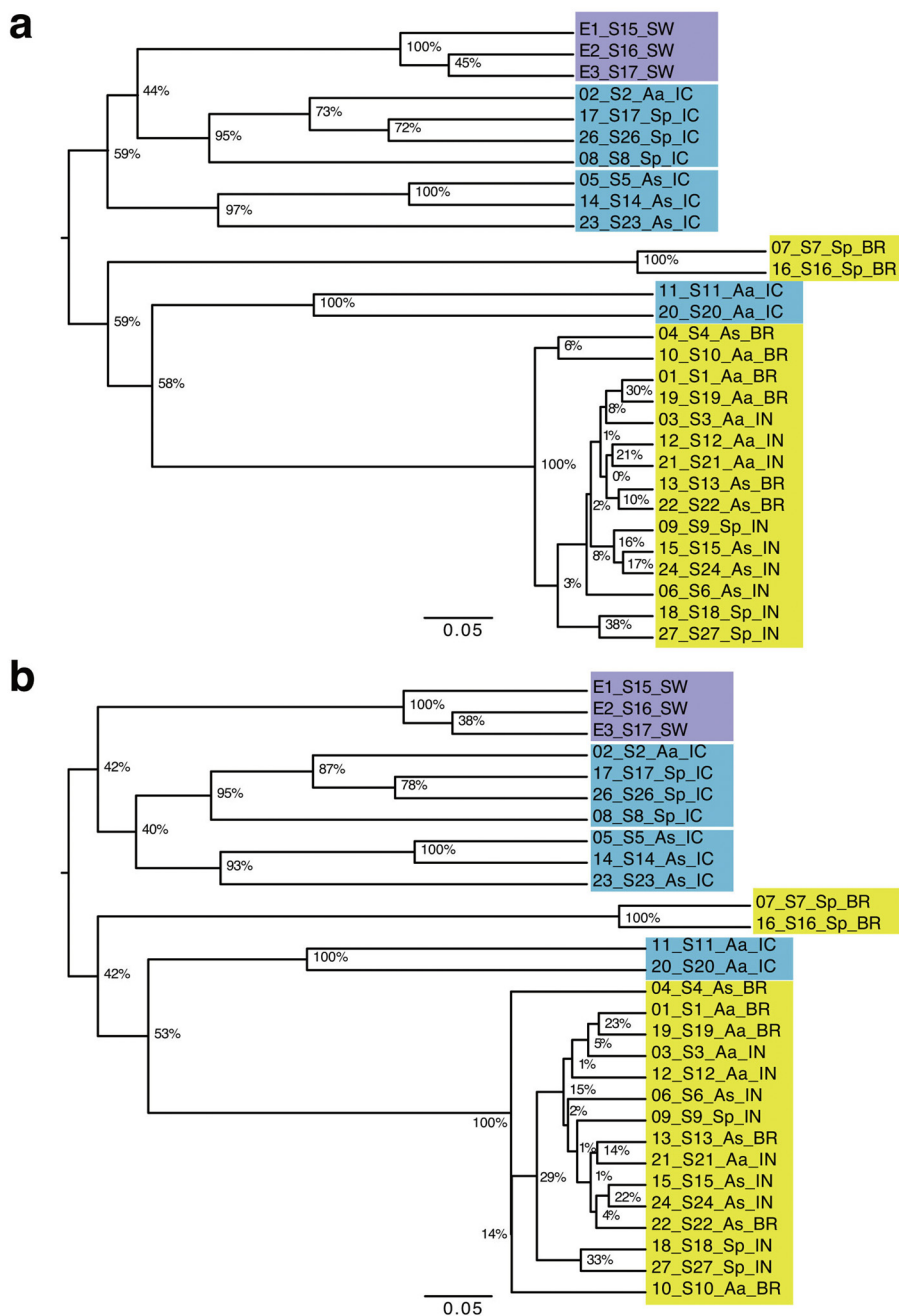
Fig. 2. Beta diversity of bacteria in branchial sac and intestine are not distinguishable but significantly different from that in the intestinal contents. A beta diversity was calculated among all samples at the generic (a) and species (b) levels, and clustering employed the UPGMA algorithm. *Aa*: *Ascidia ahodori*, *As*: *Ascidia sydneiensis samea*, *Sp*: *Styela plicata*. BR: Branchial sac, IN: Intestine, IC: Intestinal content, SW: seawater. Bootstrap values are shown in percentages for 100 times repeats.

3.4. Tissue-specific relationship between bacterial abundance and vanadium concentration

It follows that symbiotic bacteria whose function is necessary or beneficial for vanadium accumulation become abundant in vanadium-rich but not vanadium-poor species, though some may be more abundant irrespective of vanadium accumulation. Because the sample number for each tissue from each species was small ($n = 2$ or 3), we did not calculate the correlation factor between vanadium concentrations and abundance of each bacteria but, instead, identified bacteria whose abundance in vanadium-rich ascidians was higher than that in vanadium-poor species. We also calculated an abundance enhancement value for each bacterial taxon in each tissue by dividing by its

abundance in the SW samples. From this calculation, an 'abundance value' > 100% indicates that the taxon in question was symbiotically associated with ascidians and is denoted as 'enriched.' We assumed that the abundance value for symbiotic bacteria was higher than SW in each tissue sample from the vanadium-rich ascidian species. The results are summarized in Supplementary Tables S7–S12.

At the genus level, 45 genera were identified in one or more tissues from the two vanadium-rich ascidians but not from the vanadium-poor ascidian. Here we categorized each bacterium as 'major' when the abundance was higher than 1%. In the BR, the abundance of 28 genera was determined specific to the two vanadium-rich ascidians (Supplementary Table S7). The two major genera (*Pseudomonas* and *Ralstonia*) met this criterion. *Pseudomonas* was highly enriched in the
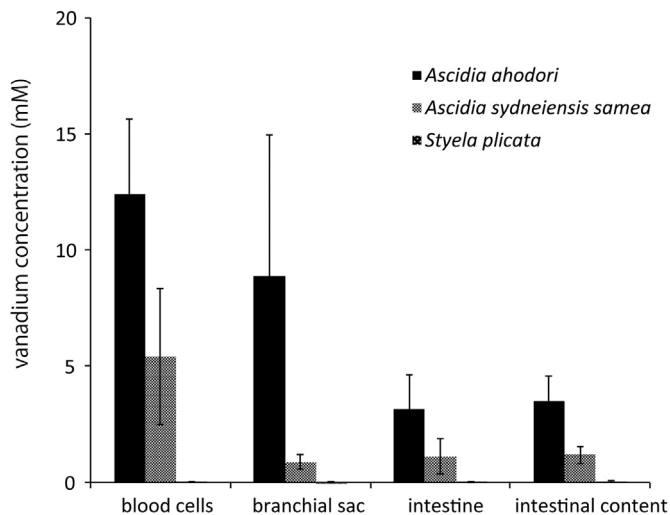
Fig. 3. Vanadium concentrations are determined in all samples. Vanadium concentrations (mM) are presented as means ± S.D. (n = 3). Vanadium concentration in all four tissues in the vanadium-poor ascidian species *Styela plicata* are very small and hardly visible in this graph.

BR from the two vanadium-rich ascidians (1134% and 1074%) but not in *S. plicata* (95%). While *Ralstonia* was also highly enriched in the BR of the two vanadium-rich ascidians (1807% and 1954%), it was also enriched in in *S. plicata* (185%). In the IN, three genera (*Ochrobactrum, Borrelia,* and *Treponema*) were found to be abundant in the vanadium-rich ascidians but not in the vanadium-poor ascidian (Supplementary Table S8). One of them, *Borrelia,* was enriched in both vanadium-rich ascidians but not in the vanadium-poor ascidian. In the IC, 18 genera were abundant in the two vanadium-rich ascidians but not in the vanadium-poor ascidian (Supplementary Table S9). Among them, two genera (*Treponema* and *Borrelia*) were considered to be major in *A. ahodori* and enriched in both vanadium-rich ascidians but not in the vanadium-poor one. Another genus, *Vibrio,* was notable because it was major in both vanadium-rich ascidians and had a very high abundance value in IC in all three species (659%, 633%, and 103% respectively).

At the species level, 92 species were found to be abundant in one or more of the tissues from the two vanadium-rich ascidians but not the vanadium-poor ascidian. Some species names could not be clearly determined but were included as dominant taxa. Here we also categorized each bacterium as 'major' when its abundance was higher than 1%. In the BR, 65 species were found to be abundant in the two vanadium-rich ascidians but not the vanadium-poor one (Supplementary Table S10). Seven species were major in *A. ahodori*, including four *Pseudomonas* and two *Ralstonia* species. Among them, *P. brenneri* and *P. colliera* were highly enriched in the BR from the two vanadium-rich ascidians (764–1018%) but not from *S. plicata* (70–74%). The other five species were relatively enriched in the BR from both vanadium-rich ascidians (1173–3714%) and from *S. plicata* (143–318%). In the IN, five species were found to be abundant in vanadium-rich ascidians but not in the vanadium-poor one (Supplementary Table S11). One of these, *Borrelia* sp., was enriched in both vanadium-rich ascidians but not in the vanadium-poor ascidian. In the IC, among the 28 species that were abundant in the two vanadium-rich ascidians but not in the vanadium-poor ascidian (Supplementary Table S12), three species were major in *A. ahodori*.

3.5. Presence of previously identified vanadium-accumulating bacteria

In our previous study, five *Vibrio* and four *Shewanella* strains were isolated from the IC of vanadium-accumulating *A. sydneiensis samea* (Romaidi and Ueki, 2016). Two of these strains, V-RA-4 and S-RA-6, accumulated vanadium at a higher rate than the other three. Partial 16S rRNA sequence analysis for V-RA-4 and S-RA-6 indicated that these two strains were closely related to *Vibrio tasmaniensis* and *Shewanella kaireitica* respectively.

Here we sought to determine the abundance of these strains using the read data from the16S rRNA amplicon sequencing analyses of this study. Since the previously sequenced region (Romaidi and Ueki, 2016) differed from the present 16S rRNA analysis using the MiSeq Reporter, the data had to be generated anew. Using random shotgun genomic analysis, we first confirmed that the genomic sequences covered the complete 16S ribosomal RNA sequences. Because the purpose of the sequencing was solely to reveal 16S rRNA sequences, the genomic sequence data was not fully assembled in this study. Comparisons between previous PCR sequence data (Romaidi and Ueki, 2016) and the present shotgun sequence data indicated that the reproducibility for each strain ranged from 92.17%–100%, except for V-RA-5 (88.7%), which was the lowest and requires further confirmation.

Comparisons among the four *Shewanella* strains suggested that S-RA-6, S-RA-8, and S-RA-9 form one clade and S-RA-7 belonged in another. The comparison of the four *Vibrio* strains revealed that V-RA-1 and V-RA-2 comprise one clade and V-RA-3 and V-RA-4 another.

In order to apply the MiSeq Reporter method, we isolated the V3–V4 region of each DNA sequence and used them to probe the Greengenes database. The V3–V4 region of S-RA-6 was most closely related to otu_3466 and otu_3472, which were classified as *Shewanella kaireiae* and *S. piezotolerans* respectively in the Greengenes database. These two species were not found in any ascidian tissue samples in the present analysis. On the other hand, the V3–V4 region of V-RA-4 was most closely related to otu_3981 and otu_4005, both of which are classified as *Vibrio* sp. respectively in the Greengenes database.

4. Discussion

In this study, we sought to determine the relationship between the bacterial community and vanadium accumulation in ascidians. We performed comparative 16S rRNA amplicon sequence analysis on two vanadium-rich ascidian species (*A. ahodori* and *A. sydneiensis samea*) and a vanadium-poor species (*S. plicata*). The diversity and relative abundance value of the bacterial populations were systematically analyzed in each tissue.

Several reports pointed out that abundance estimation by 16S rRNA amplicon sequencing is affected by DNA extraction protocols, PCR biases and copy numbers (Poretsky et al., 2014; Rubin et al., 2014; Sun et al., 2015; Větrovský and Baldrian, 2013). To avoid biases on abundance estimation, we solely used our present data to screen for bacteria whose abundance in vanadium-rich ascidians was higher than in the vanadium-poor species. Here we discuss the results of the three tissues examined in this study (BR, IN, and IC respectively). Although the average classification rate for bacterial species in ascidian tissues was relatively lower than those for higher taxonomic levels, we here discuss them equally.

First, in the BR, at the genus level, *Pseudomonas* and *Ralstonia* were abundant and their abundance in vanadium-rich ascidians was higher than in the vanadium-poor ascidian. *Pseudomonas,* one of the most commonly reported bacterial genera in the IN of marine invertebrates, was also prominent in this study. This study revealed that *Pseudomonas* was predominant in the bacterial community in the BR of vanadium-rich ascidians. At the species level, *Pseudomonas brenneri* was abundant in the BR. *Pseudomonas moraviensis* and *Pseudomonas* sp. showed similar tendencies. In the abundance value calculation, *P. brenneri* showed high population values in the BR of the two vanadium-rich ascidians but was not enriched in the vanadium-poor ascidian. *P. collierea* also showed similar tendencies. *P. brenneri* was first isolated from French natural mineral water (Baïda et al., 2001). It is a fluorescent bacteria that produces a fluorescent pigment (pyoverdin) in King's B medium and secretes several enzymes, including catalase and cytochrome oxidase, but the relevance of these properties is not yet well-understood. A sub-
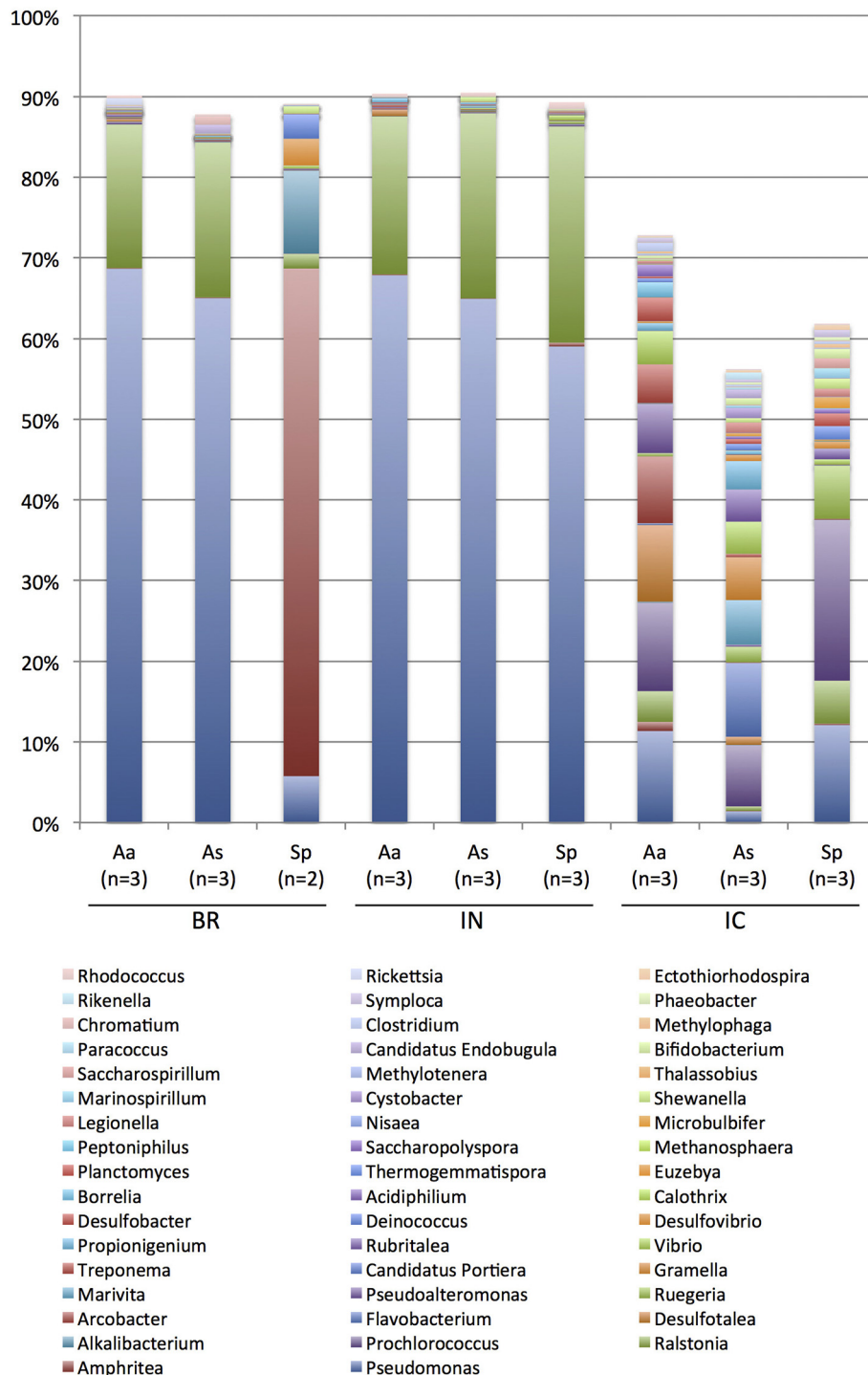
Fig. 4. Two genera, *Pseudomonas* and *Ralstonia*, were abundant in branchial sac of the two vanadium-rich ascidian species. Abundance of the top 50 bacterial genera in the 16S rRNA sequencing analysis are plotted. Original data is given as Supplementary Table S5. Aa: *Ascidia ahodori*, As: *Ascidia sydneiensis samea*, Sp: *Styela plicata*. BR: Branchial sac, IN: Intestine, IC: Intestinal content.

species of *P. moraviensis* was recently identified as an endophyte of the hyper-accumulator *Stanleya pinnata*, which is capable of efficiently reducing selenite to elemental selenium (Staicu et al., 2015). Draft genomes of the two strains of *P. moraviensis* have been published (Hunter et al., 2014; Miller et al., 2016), one of which contains a native mercury resistance plasmid. *Ralstonia* is a relatively new genus of Proteobacteria, previously included in the genus *Pseudomonas*. We could not find any reports linking *Ralstonia* and metal accumulation.

The BR is the organ that captures plankton, using a sheet of mucus secreted by the endostyle. The mucus traps bacteria and transfers them to the stomach. Given that symbiotic bacteria are present and maintained in the ascidian BR, a stable relationship between branchial cells and bacteria must exist. Otherwise, a higher affinity between specific bacteria and the mucus could underlie this symbiotic phenomenon. We can imagine two potential functional roles for the symbiotic bacteria of the BR. They could stably attach to the BR, accumulate vanadium, and hand it to branchial cells; alternatively, they could interact transiently with the BR, accumulate vanadium, and then transition to the intestinal
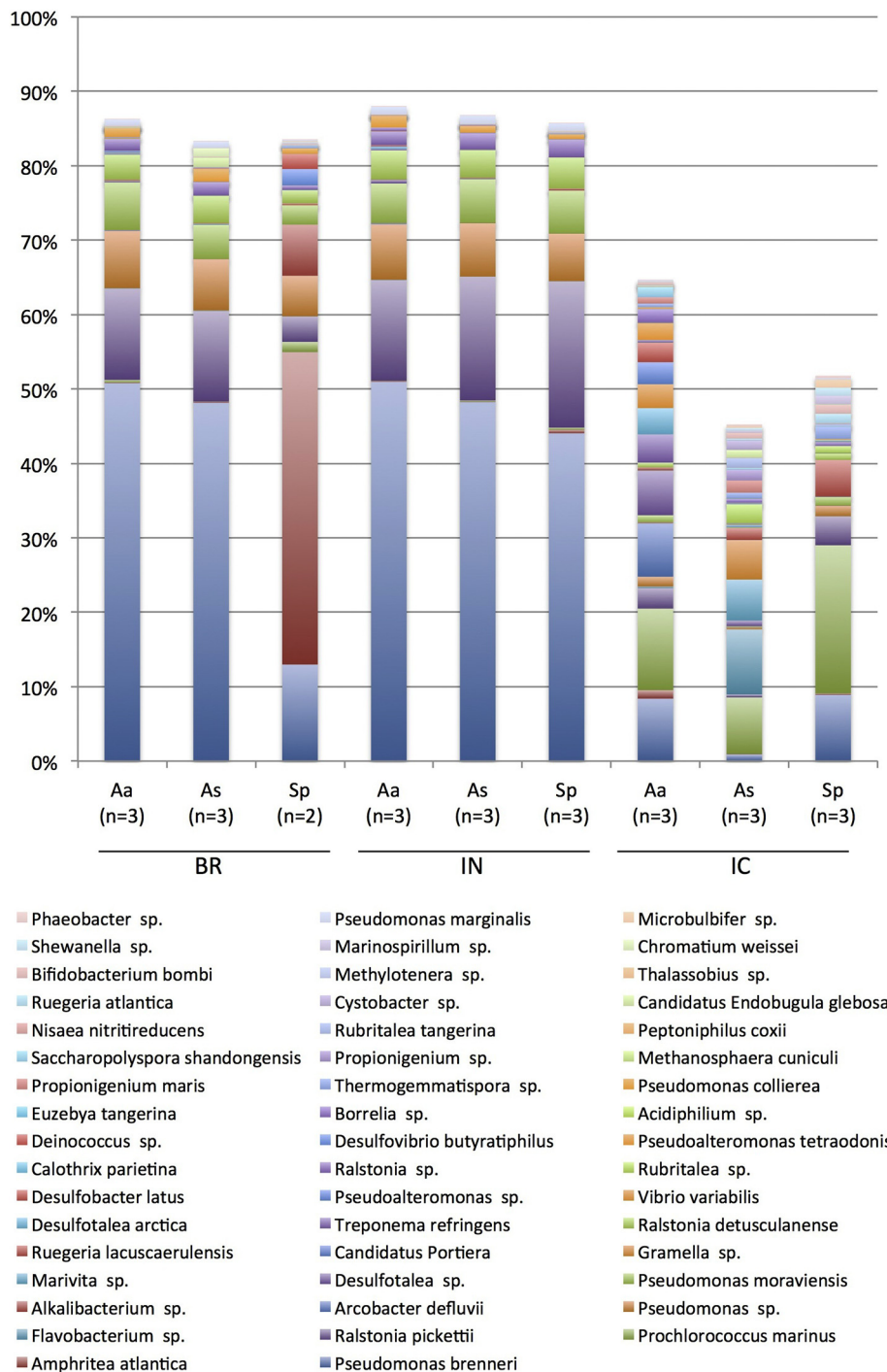
Fig. 5. Two species, *Pseudomonas brenneri* and *Ralstonia pickettii*, were abundant in branchial sac of the two vanadium-rich ascidian species. Abundance of the top 50 bacterial species in the 16S rRNA sequencing analysis are plotted. Original data is given as Supplementary Table S6. Aa: *Ascidia ahodori*, As: *Ascidia sydneiensis samea*, Sp: *Styela plicata*. BR: Branchial sac, IN: Intestine, IC: Intestinal content.

lumen together with the mucus.

Second, among the three genera abundant in the IN from vanadium-rich ascidians but not the vanadium-poor ascidian, only one genus (*Borrelia*) was enriched in all three ascidians. At the species level, one (*Borrelia* sp.) was enriched in both vanadium-rich ascidians and another (*Treponema refringens*) was enriched only in *A. ahodori*. Most published reports of *Borrelia* and *Treponema* focus on their pathogenicity and pro-inflammatory properties. Several publications on metal homeostasis and metal tolerance in *Borrelia* exist in the literature. *Treponema* are mostly found in the oral cavity and intestinal lumen of humans and

other mammals where they can be pathogenic and parasitic; no reports of this genus in marine animals have been found to date.

The sampling method for the IN used in this study included a simple wash to remove most (but not all) of the free bacteria from the IC. Nevertheless, the bacterial diversity of the IN and IC were found to be significantly different. Thus, we expect that these bacterial taxa likely firmly attach to the surface of the IN or may exist within intestinal cells as symbionts. Such stable symbionts could assist intestinal cells to acquire vanadium from the intestinal lumen.

Third, in the IC, two genera (*Treponema* and *Borrelia*) were found to

be major in *A. ahodori* and enriched in both vanadium-rich ascidians but not in the vanadium-poor ascidian. Although the bacterial populations of the IN and IC were different overall, these two exist in both tissues as major genera and are, therefore, likely involved in vanadium accumulation.

Two genera of interest in the IC were *Vibrio* and *Shewanella*. We tried to reveal the abundance of the *Vibrio* strains previously reported as a vanadium-accumulating strain from the IC of *A. sydneiensis samea* (Romaidi and Ueki, 2016) in relation to the present study, but they were not clearly assigned at species level. On the other hand, we could assign one of *Shewanella* strains S-RA-6 that we reported as vanadium-accumulating bacteria from the IC of *A. sydneiensis samea* (Romaidi and Ueki, 2016) as closely related to two OTUs for *S. kaireiae* and *S. piezotolerans*. They were not found in any ascidian tissue samples in the present analysis, but was identified by a functional screening and could contribute for vanadium accumulation and reduction. This could be an example of a 'rare biosphere' that contributes to important biological functions, as previously suggested by Pester et al. (2010)). It is of special interest that, as described in the Introduction, several *Shewanella* species have been reported to reduce vanadium in +5 state to a +4 state. The *Shewanella* strain S-RA-6 could contribute to the reduction of vanadium in IC.

The IC used in this study included semisolid materials from the lumen of the IN. Our analysis suggests that the IC does not contain a mere mixture of plankton and bacteria passing through the intestinal lumen but, instead, specific selective forces maintain this unique bacterial diversity. In this study, we did not consider the anteroposterior position when collecting the SW samples. Since the composition of the bacterial community in the IC may vary along the anteroposterior axis, future studies should consider the localization of specific bacteria and the cooperation of bacteria and intestinal cells in vanadium uptake along the anteroposterior axis.

Feeding conditions have been shown to greatly affect bacterial populations (Dishaw et al., 2014b; López-Legentil et al., 2016). In this study, we used ascidians fed with the same few species of diatoms in a regulated aquarium. However, the circulating natural SW contains many different types of plankton. We believe the environment experienced by the ascidians used in this study is reflective of their natural conditions, though further comparative studies on this matter may be necessary.

5. Conclusion

In summary, this study identified abundant and enriched bacteria taxa from the BR, IN, and IC of ascidians that are involved in vanadium uptake. These bacteria seem to stably associate with ascidian tissues and assist with vanadium accumulation by creating a specialized microenvironment in ascidian tissues. Further studies on the distribution of each bacterium *in vivo* and sequestration studies *in vitro* will provide a better functional understanding of the symbiotic relationship between bacterial communities and their aquatic hosts.

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Conflict of interests

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.margen.2018.10.006.

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