広島大学学位請求論文

Bioaccumulation of Vanadium by Vanadium-Resistant Bacteria Isolated from the Intestine of *Ascidia sydneiensis samea*

（スジキレボヤの腸から単離したバナジウム耐性細菌によるバナジウム濃縮）

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Romaidi

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主論文

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I. General Introduction

Ascidians, also known as sea squirts or tunicates, can accumulate a high level of vanadium ions in blood cells (Ueki and Michibata 2011; Michibata 2012; Ueki et al. 2014). As an example, *Ascidia gemmata* has been reported to accumulate 350 mM vanadium, which is 10^7-fold higher than the vanadium concentration in seawater (Michibata et al. 1991). Vanadium ions are absorbed from natural seawater in a +5 state; are reduced to a +4 state through the branchial sac, intestine, and blood plasma; and are stored in a +3 state in vanadocytes. Several genes and proteins involved in this accumulation and reduction have been identified (Kanda et al. 1997; Ueki et al. 2003a, 2007; Yamaguchi et al. 2004; Kawakami et al. 2006; Yoshinaga et al. 2007) and the application of genetically modified bacteria that express ascidians' vanadium-binding proteins for bioaccumulation of heavy metals has been examined (Ueki et al. 2003b; Samino et al. 2012).

Vanadium is one of most abundant transition metals with an average concentration of approximately 100 mg/kg (Taylor and van Staden 1994), widely exists in the Earth's crust and is extensively employed in modern industry including metallurgy and petroleum refining (Myers et al. 2004; Zhang et al. 2014). Vanadium was also regarded as one of the essential elements, and has been used in dietary supplements and therapy for diabetic illness (French and Jones 1992; Thompson and Orvig 2001; Aureliano 2009). However, the presence of vanadium at intracellular concentration above several micromolar will becomes toxic to most organisms, which causes mutations and induces alterations of many important metabolic functions (Domingo 1996; Ghosh et al. 2014).

Now days, the discharge of vanadium and vanadium compounds into water body have caused seriously environmental problem. Several approaches was developed for the treatment of vanadium-containing waste water like electrochemical treatment, precipitation,

ion exchange, evaporation, reverse osmosis, adsorption on activated coal and later biological treatment (Mack et al. 2007; Gadd 2009; Chojnacka 2010). Among these techniques, biological treatment (biosorption or bioaccumulation) is one of the common and cost-effective method to recover or eliminate vanadium and heavy metal ions from waste water treatment (Ghazvini and Mashkani 2009; Zhang et al. 2014; Huang et al. 2014; Ueki 2016). Due to highest ability of ascidian to accumulate vanadium, several researchers also used solitary ascidian animal to remediate vanadium and other heavy metal toxicities from water environment (Jaffar et al. 2015). However, the use of animal to remediate heavy metals is less effective since the animal spends much space and difficult to maintain.

One of the possibilities for effective bioremediation or bioaccumulation technology is by using microorganism. Microorganisms have been viewed as one of the best way to deal with environmental pollution because they have ability to survive, grow and reproduce even in the harsh or extreme environment (Ghazvini and Mashkani 2009; Kamika and Momba 2014; Zhang et al. 2014). In vanadium recovery, microorganisms are reported to cope with vanadate V(V) either by accumulating it or reducing it to a less toxic tetravalent vanadyl form (Lyalikova and Yurkova 1992; Antipov et al. 1998; Antipov et al. 2000; Carpentier et al. 2003; Ortiz-Bernad et al. 2004; Carpentier et al. 2005; van Marwijk et al. 2009).

The intestinal organ of an ascidian is tough to be first location to contact with outer environment and absorbs vanadium ions. Intestinal organ of vanadium-rich ascidian, *Ascidia gemmata*, accumulated 11.9 mM of vanadium ions before stored in highest concentration in blood cell (Samino et al. 2012). Intestinal organ also harbors several types of bacteria as reported by Dishaw et al. (2014) that bacterial communities isolated from the gut of an ascidian, *Ciona intestinalis*, obtained from three disparate geographic locations exhibited

striking similarity in the abundance of operational taxonomic units (OTUs), consistent with the selection of a core community by the gut ecosystem, in which Proteobacteria (80%) were the predominant gut bacteria. In soil worm *Eisenia foetida*, host-bacterial interaction in intestinal organ could increase the ability of intestinal bacteria to accumulate heavy metals such as mercury (Kaschak et al. 2014), and for longtime interaction in such microenvironment it might lead the resistance of intestinal bacteria to heavy metal (Silver 1996).

The host-bacterial interaction in ascidian by which intestinal bacteria resist to vanadium was firstly reported by Russian researchers that successfully isolated several bacterial strains of genus *Pseudomonas* from the intestine of ascidian that could resist the toxicity of vanadate up to 6 g/L (Lyalikova and Yurkova 1992; Antipov et al. 2000). The later researchers reported *Shewanella oneidensis* that is also capable of growth in the presence of vanadate as the sole electron acceptor and reduced vanadate V(V) to vanadyl V(IV) ions (Carpentier et al. 2003; Carpentier et al. 2005).

From those findings discussed above, I expected that isolating bacteria from intestinal microenvironment of vanadium-rich ascidian *Ascidia sydneiensis samea* will result in the candidate of intestinal bacterial strains which are highly resistant to vanadium and could be used for decontaminating vanadium and other heavy metals toxicity. On other hand, I hypothesize that intestinal bacteria might contribute to vanadium distribution in ascidian by indirect mechanism. The possible contribution is that intestinal bacteria accumulate V(V), reduce it to V(IV) ions and transport it by phosphate and other metal transporters to intestinal lumen before finally is reduced it to more simple form and store in vanadocyte.

My goal in the present study was to isolate vanadium-resistant bacteria from the intestine of the vanadium-rich ascidian *A. sydneiensis samea*, which is commonly found in

Japan and can accumulate vanadium at 12.9 mM at its blood cells (Michibata 1991), and determine whether these bacterial strains could accumulate vanadium ions. Sub-cellular localization analysis was also performed to determine whether vanadium accumulation could take place in or outside bacterial cells. I also determined the effects of pH on vanadium accumulation by vanadium-accumulating bacteria exposed to 500 μM vanadium-containing NaCl medium to increase the understanding of the applications of vanadium-resistant bacterial strains for decontaminating vanadium-containing wastewater at any pH. I also examined the ability of vanadium-resistant bacterial strains in accumulating several heavy metals ions, because in the previous studies the vanadium-binding protein was able to absorb heavy metal ions other than vanadium (Ueki et al. 2003b; Samino et al. 2012), and it should lead to development of a superior metal accumulator that could be widely used to remediate effluents contaminated with metals.

In this study, I successfully isolated nine strain of vanadium-resistant bacteria from the intestine of *A. sydneiensis samea*. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that five strains of bacterial strains belong to the genus *Vibrio* and four to genus *Shewanella*. Preliminary screening for each bacterial strain in accumulating V (IV) and V(V) revealed that strains V-RA-4 and S-RA-6 were capable to accumulate vanadium higher than that of the other strain when they were cultured with initial concentration of 200- and 500-μM vanadium. In assay using 500-μM vanadium-containing media with different pHs was also found that vanadium accumulation by strains V-RA-4 and S-RA-6 decreased with the increasing of pH where the maximum absorption was achieved in pH 3, and these two bacterial strains exhibited mostly intracellular accumulation of vanadium. In addition, nine vanadium resistant bacterial strains are capable to accumulate either copper or cobalt ions but

neither molybdate nor nickel ions. These bacterial strains can be applied to protocols for bioremediation of vanadium and heavy metal toxicity and they could be also used to support my hypothesis on the contribution of intestinal bacteria in the extraordinary system of vanadium accumulation and reduction by ascidian animal.

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II. Bioaccumulation of vanadium by vanadium-resistant bacteria isolated from the intestine of *Ascidia sydneiensis samea*

Summary

Isolation of naturally occurring bacterial strains from metal-rich environments has gained popularity due to the growing need for bioremediation technologies. In this study, we found that the vanadium concentration in the intestine of the vanadium-rich ascidian *Ascidia sydneiensis samea* could reach 0.67 mM, and thus we isolated vanadium-resistant bacteria from the intestinal contents and determined the ability of each bacterial strain to accumulate vanadium and other heavy metals. Nine strains of vanadium-resistant bacteria were successfully isolated, of which two strains, V-RA-4 and S-RA-6, accumulated vanadium at a higher rate than did the other strains. The maximum vanadium absorption by these bacteria was achieved at pH 3, and intracellular accumulation was the predominant mechanism. Each strain strongly accumulated copper and cobalt ions, but accumulation of nickel and molybdate ions was relatively low. These bacterial strains can be applied to protocols for bioremediation of vanadium and heavy metal toxicity.

Introduction

Ascidians, also known as sea squirts or tunicates, can accumulate a high level of vanadium ions in blood cells (Ueki and Michibata 2011; Michibata 2012; Ueki et al. 2014). As an example, *Ascidia gemmata* has been reported to accumulate 350 mM vanadium, which is 10^7-fold higher than the vanadium concentration in seawater (Michibata et al. 1991). Vanadium ions are absorbed from natural seawater in a +5 state; are reduced to a +4 state through the branchial sac, intestine, and blood plasma; and are stored in a +3 state in vanadocytes. Several genes and proteins involved in this accumulation and reduction have been identified by our group in all organs (Kanda et al. 1997; Ueki et al. 2003a, 2007; Yamaguchi et al. 2004; Kawakami et al. 2006; Yoshinaga et al. 2007) and the application of genetically modified bacteria that express ascidians' vanadium-binding proteins for bioaccumulation of heavy metals has been examined (Ueki et al. 2003b; Samino et al. 2012).

The intestinal organ is internally exposed to natural seawater and harbors several types of bacteria. The presence of gut microbes in aquatic invertebrates has been investigated in Crustacea (Li et al. 2007; Rungrassamee et al. 2014), Mollusca (Simon and McQuaid 1999; Tanaka et al. 2004), and Echinodermata (Thorsen 1999; da Silva et al. 2006). *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Aeromona*s, and *Shewanella* are the most commonly reported bacteria in the intestine of these marine invertebrates. In ascidians, Dishaw et al. (2014) reported that bacterial communities isolated from the gut of *Ciona intestinalis* found in three disparate geographic locations exhibited striking similarity in the abundance of operational taxonomic units (OTUs), consistent with the selection of a core community by the gut ecosystem, in which Proteobacteria (80%) were the predominant gut bacteria.

The ingestion of food is the dominant function of the gut micro-ecosystem, and several types of close interactions between aquatic invertebrates and their gut bacterial community have been described by Harris (1993). Other types of interactions include nutrient absorption, immune response, epithelial development (Brune and Friedrich 2000; Hooper et al. 2001; Rungrassamee et al. 2014) and pathogenic interactions (Jayasree et al. 2006; Li et al. 2007). Another important type of host-bacterial interaction is the ability of intestinal bacteria to accumulate heavy metals such as mercury (Kaschak et al. 2014), and intestinal bacteria are thought to be the first organisms affected by heavy metal discharge into the environment, which results in an increase in metal-resistant bacteria in the microenvironment (Silver 1996). This interaction could also lead to the development of heavy metal resistance and accumulation in gut bacteria.

Several studies have investigated the importance of vanadium accumulation and reduction by bacteria (Antipov et al. 1998, 2000; Carpentier et al. 2003, 2005; Ortiz-Bernad et al. 2004; van Marwijk et al. 2009; Zhang et al. 2014). Antipov et al. (2000) reported that *Pseudomonas isachenkovii* isolated from the intestine of an ascidian exposed to 6 g/L vanadate could resist vanadium toxicity and use vanadate as an electron acceptor during anaerobic respiration. This study also identified vanadium-binding proteins related to the +4 oxidation state, and distribution of vanadium ions in special swells on the surface of cell membranes. Carpentier et al. (2003, 2005) reported that *Shewanella oneidensis* was also capable of growth in the presence of vanadate as the sole electron acceptor and reduced vanadate V(V) to vanadyl V(IV) ions.

The goal of this study was to isolate vanadium-resistant bacteria from the intestine of the vanadium-rich ascidian *A. sydneiensis samea* and determine whether these bacterial

strains could accumulate vanadium ions. Sub-cellular localization analysis was also performed to determine whether vanadium accumulation could take place in or outside bacterial cells. We also determined the effects of pH on vanadium accumulation by vanadium-accumulating bacteria exposed to 500 μM vanadium-containing NaCl medium to increase our understanding of the applications of vanadium-resistant bacterial strains for decontaminating vanadium-containing wastewater at any pH. We also examined the ability of vanadium-resistant bacterial strains in accumulating several heavy metals ions, because in our previous studies the vanadium-binding protein was able to absorb heavy metal ions other than vanadium (Ueki et al. 2003b; Samino et al. 2012), and it should lead to development of a superior metal accumulator that could be widely used to remediate effluents contaminated with metals.

Materials and Methods

Isolation and cultivation of vanadium-resistant bacteria from the intestine of A. sydneiensis samea

Five adult *A. sydneiensis samea* collected from Kojima Port, Okayama Prefecture, Japan, were aseptically dissected and the intestinal contents were removed and diluted with sterile artificial sea water (ASW). Aliquots (10 μL) of 10^{-2} to 10^{-4} dilutions were inoculated/spread on the surfaces of agar culture medium in 20 mL sterile dishes. Four culture media were prepared in artificial seawater: (1) standard medium: yeast extract, 2.5 g/L; peptone, 5 g/L; glucose, 1.0 g/L; agar, 15 g/L (Atlas 2005); (2) 1/2 TZ agar medium: yeast extract, 0.5 g/L; peptone, 2.5 g/L; HEPES, 4.77 g/L; $MnCl_2$-$4H_2O$, 0.2 g/L; agar, 15 g/L

(Maruyama et al. 1993); (3) Posgate medium B (PB): yeast extract, 0.2 g/L; sodium lactate, 2.5 g/L; KH_2PO_4, 0.25 g/L; NH_4Cl, 0.5 g/L; agar, 0.2 g/L (Antipov et al. 1998); and (4) Difco™ Marine Agar 2216 (MA) medium (Hansen and Sørheim 1991). For screening, the strength of artificial seawater was varied by 0.75-times or 1.0-time by dissolving 27 or 36 g salt (Marine Art SF1, Tomita Pharmaceutical, Japan) per 1 L deionized water. The pH of each medium was set to neutral conditions (pH 7.0). Each medium was supplemented with 0, 0.5, 1, 2.5, 5, and 10 mM Na_3VO_4 and incubated at 20°C for 48 h. Bacterial colonies that grew in media containing 10 mM Na_3VO_4 were selected for identification.

Identification of vanadium-resistant bacteria isolated from the intestine of A. sydneiensis samea by 16S rRNA gene sequencing

Colonies of bacteria were selected randomly from each medium and the whole cell of each colony was used as PCR template. The 16S rRNA gene was amplified using specific primers: 306F (5'-CCA GAC TCC TAC GGG AGG CAG C-3') and 935R (5'-CGA ATT AAA CCA CAT GCT CCA C-3') in a PCR reaction that contained an appropriate amount of bacterial cells, 0.2 mM each dNTP, 1 μM each of primers 306F and 935R, 1× reaction buffer, and 2.5 U Taq DNA polymerase (TaKaRa, Inc.) in a 20 μL reaction volume. After denaturation at 94°C for 2 min, 30 cycles of PCR were performed (94°C for 30 s, 50°C for 40 s, and 72°C for 40 s) followed by a final extension at 72°C for 2 min. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide (EtBr). The band of the expected size (~650 bp) was excised, cloned, and sequenced with the 306F primer. DNA sequencing analyses was performed at the Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University, Japan. The sequences of 16S rRNA

genes from each isolate were used as query to determine the closest prokaryotic species available in the GenBank database (http://blast.ddbj.nig.ac.jp/blastn?lang=en). The nucleotide sequences were submitted to DDBJ/EMBL/Genbank under accession number of LC108850 through LC108858. Sequences were aligned using the CLUSTAL X 2.0 software (Larkin et al. 2007), in which several regions with an alignment gap were completely removed from analysis and only 569 nucleotides sites were used to construct a phylogenetic tree using MEGA 6.0 software (Tamura et al. 2013). To provide confidence estimates for branch support, a bootstrap analysis was performed using 1000 trial replications.

Measurement of vanadium and other heavy metals

Medium for the measurement of metal accumulation, 1/2 TZ medium, was supplemented with either 200 and 500 μM of vanadyl sulfate ($VOSO_4$, nH2O, n = 3–4, 99%) or sodium orthovanadate (Na_3VO_4). For other heavy metals accumulation, standard medium was supplemented with 10μM of either copper (II) chloride ($CuCl_2$. $2H_2O$), cobalt (II) sulfate ($CoSO_4$. $7H_2O$), nickel (II) chloride ($NiCl_2$. $6H_2O$), or sodium molybdate (VI) (Na_2MoO_4. $2H_2O$). Original 1/2 TZ medium without supplementation of any metal is denoted as "metal-free" medium.

Bacterial cells in the lag phase (OD_{600} = 0.70 – 1.00) were used as inoculum and cultured in a specific media of 15 mL in 50 mL conical tubes with rotation at 180 rpm at for 24 h. The culture was set as 20°C, which is a habitat temperature for ascidian animal. Bacterial cells were harvested by centrifugation at 8000 rpm for 3 min, washed three times with metal-free medium, and the cell pellet was heated overnight at 65°C. After obtaining the dry weight, 300 μL 1 N HNO_3 was added to each sample and heated overnight at 65°C. Then

each sample was centrifuged at 10000 rpm for 10 min and the supernatant was used to measure vanadium and the contents of other heavy metals by atomic absorption spectrometry (AAS). Vanadium and other heavy metal contents were expressed as the weights per weight of dried cells (ng per mg).

Growth of the strains at 15–35°C was determined in standard medium containing 0, 200, and 500 μM vanadium. The growth of bacterial cells was assessed every 2 h by measuring their optical density (OD) at 600 nm.

To determine the effects of pH on vanadium uptake, we used a protocol developed by López et al. (2000). Aliquots (150 μL) of bacterial cells were grown in standard vanadium-free medium at 25°C and rotated at 180 rpm until the late exponential phase for 24 h. Bacterial cells were harvested by centrifugation and washed three times. A vanadium accumulation experiment was performed by suspending the harvested cells in NaCl medium consisting of 0.5 M NaCl, 500 μM vanadium (IV) or V(V), and 50 mM sodium phosphate buffer at the desired pH in a total volume of 15 mL rotated at 180 rpm at 25°C. Control-lacking cells were included in a similar manner. After 6 or 24 h of incubation, bacterial cells were harvested, washed, dried, and then 300 μL 1 N HNO_3 was added to measure vanadium contents by AAS.

Distribution of vanadium in bacterial cells

The distribution of vanadium in bacterial cells under both acidic and neutral pH was determined following the procedures of Pabst et al. (2010) and Desaunay and Martins (2014). Bacterial cells initially exposed to 0.5 mM V(V) for 6 h of incubation were harvested by centrifugation at 8000 × *g* for 5 min, and bacterial cell pellets were treated with 20 mM EDTA

for 5 min with gentle shaking. These suspensions were centrifuged at 5000 × *g* for 5 min, and then the vanadium contents of the supernatant, which is supposed to be initially associated with membrane compartments, was measured by AAS. The bacterial cell pellets were digested with 1 N HNO_3 at 90°C overnight, and the vanadium contents, assumed to be retained in the cell's cytoplasm after internalization (intracellular compartment), was determined by AAS. The sum of the two measurements of vanadium was considered the amount of vanadium accumulated by the whole cells.

Statistical analysis

All experiments on bioaccumulation of vanadium and other heavy metals by vanadium-resistant bacteria were conducted in triplicate and the averages of each measurement for each treatment are reported with their standard error (SE). A two-way analysis of variance (ANOVA) was performed to evaluate the effects of vanadium concentration, type of bacterial strain, and different pHs on V(V) and V(IV) accumulation. A one-way ANOVA was used to evaluate the effects of different bacterial strains on the accumulation of copper, cobalt, nickel, and molybdate ions. The significance of any differences was tested using Fisher's least significant difference (LSD) test. The statistical significance of differences in the growth of vanadium-accumulating strains under different temperatures and vanadium concentrations was analyzed using a two-tailed Student's t-test.

Results

Identification of vanadium-resistant bacteria from the intestine of the vanadium-rich ascidian Ascidia sydneiensis samea

At the beginning of this study, we determined the vanadium concentration in the intestinal contents of *A. sydneiensis samea*. The vanadium concentration of intestinal contents was 0.67 ± 0.07 mM (mean ± SD, n = 2). This value corresponds to about 20,000 times higher than that of seawater. The pH of the intestinal contents was also directly measured using a portable electric pH meter and was 8.03 ± 0.05 (mean ± SD, n = 3). This value is similar to that of natural seawater. Thus, the intestinal contents of this ascidian species was shown to be a vanadium-rich environment.

Samples of intestinal contents of the vanadium-rich ascidian *A. sydneiensis samea* were then screened for the presence of bacteria resistant to vanadium using agar plates made with four media (standard, 1/2 TZ, PB, and marine agar medium) containing 0 to 10 mM sodium orthovanadate under aerobic conditions. This concentration is reported to be the upper limit, in which microorganisms such as bacteria (Hernández et al. 1998) or fungi (Bisconti et al. 1997) could tolerate the toxicity of vanadate. In each medium, numerous bacteria grew on the plate without supplementation with vanadium, but by increasing the concentration of vanadium, the number of colonies decreased, but some colonies appeared on the plate with 10 mM sodium orthovanadate. No significant difference in the number of bacterial colonies was observed in each medium supplemented with different salt concentrations. Bacterial colonies that were able to grow in the presence of 10 mM sodium orthovanadate with different colorations from the four media were selected at random and used for identification.

Of the 51 bacterial colonies characterized based on their 16S rRNA gene sequences and compared to the GenBank Database, the majority (~80%) were closely related to *Vibrio*. Ten isolates of strain V-RA-1 were closely related to *Vibrio splendidus* strain 630 (99.89 ± 0.13%), 11 isolates (V-RA-2) to *Vibrio splendidus* strain N08 (99.87 ± 0.09%), 8 isolates (V-RA-3 and V-RA-4) to *Vibrio splendidus* partial 16S (99.95 ± 0.13%) and *Vibrio tasmaniensis* (99.93 ± 0.13%), and 2 isolates to *Vibrio tapetis* (99.07 ± 0.81%). The remaining 20% of isolates were closely related to the genus *Shewanella*; 4 isolates (S-RA-6 and S-RA-7) were related to *Shewanella kaireitica* (99.87 ± 0.17%) and *Shewanella pasifica* strain KMM (99.64%), 3 isolates to *Shewanella pasifica* strain UDC (99.88 ± 0.10%), and 1 isolate to *Shewanella olleyana* (98.72 %) (Table 1).

To classify these vanadium-resistant bacterial strains, a phylogenetic tree was constructed using the neighbor-joining method (Tamura et al. 2013). This tree was based on the 569 nucleotides sites as described in the Materials and Methods and rooted using the genus *Marinobacter* as an outgroup. Fig. 1 is a dendrogram that shows the phylogeny of vanadium-resistant bacterial strains, in which five bacterial isolates belonged to the genus *Vibrio* and four to the genus *Shewanella*.

Screening for vanadium-resistant bacteria that can accumulate vanadium (V) and (IV)

To determine whether vanadium-resistant bacterial strains isolated from the intestine of *A. sydneiensis samea* were capable of accumulating vanadium, we determined the ability of each strain to accumulate both V(V) and V(IV) ions. All vanadium-resistant bacterial strains significantly accumulated V(V) and V(IV) ions at every concentration ($P< 0.05$) and straightly differed from control or in the absence of vanadium. Moreover, multiple

comparisons using the LSD test revealed highly significant differences between bacterial strains in accumulating V(V) ($P< 0.05$), of which strains V-RA-4 (300 ± 86 ng mg^{-1} dw) and S-RA-6 (376 ± 68 ng mg^{-1} dw) showed greater accumulation than the other strains (Fig. 2A). In addition, no significant differences between bacterial strains in accumulating V(IV) were observed (Fig. 2B).

Characterization of growth of vanadium-accumulating strains V-RA-4 and S-RA-6 at various temperatures and vanadium concentrations

Due to the highest vanadium (V) accumulation by strains V-RA-4 and S-RA-6, we examined the growth of these two bacterial strains at 15–35°C in the presence of 0, 200, and 500 μM vanadium. The optimal temperature for growth strains V-RA-4 and S-RA-6 ranged from 20°C to 25°C (Fig. 3A and B), and the exponential phase occurred after 8–10 h of incubation. The growth strains V-RA-4 and S-RA-6 was not significantly affected by the vanadium concentration ($P >0.05$) (Fig. 3C and D).

Effect of pH on vanadium uptake by the vanadium-accumulating strains V-RA-4 and S-RA-6

For application purposes, it is important to explore the pH dependency of metal accumulation. To determine the effect of pH on vanadium uptake, the vanadium-accumulating strains V-RA-4 and S-RA-6 were cultured in 0.5 M NaCl medium containing 500 μM V(V) and V(IV) at pH 3, 7, or 9 at 25°C. Cultures were incubated for 6 and 24 h to assess vanadium accumulation during the lag phase or stationary phase. After harvesting, bacterial cells were dried and the vanadium contents was determined by AAS.

Different pHs significantly affected vanadium accumulation by the two bacterial

strains V-RA-4 and S-RA-6 ($P > 0.05$), in which the greatest accumulation of V(IV) and V(V) was detected under acidic conditions at pH 3. Total accumulation of V(IV) ions at pH 3 by strain V-RA-4 was 5,450 ± 1,348 ng mg^{-1} dw or five-fold higher than at pH 7 (1,225 ± 113 ng mg^{-1} dw) and pH 9 (924 ± 29 ng mg^{-1} dw). V(IV) accumulation by strain S-RA-6 was 4,126 ± 530 ng mg^{-1} dw in pH 3 or four-fold higher than at pH 7 (956 ± 67 ng mg^{-1} dw) and pH 9 (964 ± 35 ng mg^{-1} dw) (Fig. 4A).

Significant V(V) accumulation by strain V-RA-4 was observed at pH 3 after 6 h of incubation (19,405 ± 2,096 ng mg^{-1} dw) (Fig. 4B), and the total accumulation doubled after 24 h of incubation (33,471 ± 6,477 ng mg^{-1} dw) (Fig. 4C). High accumulation of V(V) at pH 3 was also detected in strain S-RA-6 after 6 h (19,493 ± 2,278 ng mg^{-1} dw), but decreased after 24 h of incubation (18,509 ± 544 ng mg^{-1} dw). This suggests that the highest accumulation in strain S-RA-6 occurred before 24 h. In contrast, a small amount of vanadium accumulation was detected at pH 7 and 9; under these conditions, total accumulation was lower than 500 ng mg^{-1} dw.

Strains V-RA-4 and S-RA-6 removed 16 and 13% of V(IV) ions, respectively, from aqueous solution at pH 3 (Table 2). In addition, these two vanadium-accumulating strains reduced ± 80% V(V) after 6 h, and accumulation was 1.5-fold higher after 24 h of incubation. In contrast, no significant removal of V(IV) and V(V) was observed at pH 7 or 9. Thus, these strains removed both V(IV) and V(V) effectively at pH 3.

To determine whether the highest uptake of vanadium at pH 3 was actually caused by bacteria and not by precipitation due to a chemical interaction between vanadium and components of the medium, we tested bacteria-free controls incubated with 500 μM vanadium. The total vanadium detected in bacteria-free controls was less than 1% of the total vanadium

accumulated under the same conditions with bacterial cells suggesting that we can neglect the precipitation due to the chemical interaction between vanadium and components of the medium.

Distribution of vanadium in bacterial cells

A set of experiments to evaluate the subcellular distribution of vanadium in two bacterial strains, V-RA-4 and S-RA-6, was performed. To accomplish this, after 6 h of incubation in medium containing 0.5 mM vanadium (V), and harvesting, the bacterial cells were washed with EDTA to indirectly quantify vanadium associated with extracellular and intracellular compartments.

A total of 2,866 ± 655 ng/mg dw and 3,810 ± 2,797 ng/mg dw vanadium appeared to be bound in the extracellular compartment strains V-RA-4 and SRA-6 at pH 3, respectively, and 8 ± 7 ng/mg dw (strain V-RA-4) and 17 ± 2 ng/mg dw (strain S-RA-6) at pH 7. The amount of vanadium retained in the cytoplasm (intracellular compartments) was approximately 80% of the total vanadium accumulated. The cytoplasm contained 10,356 ± 2,745 ng/mg dw (strain V-RA-4) and 14,955 ± 2,509 ng/mg dw (strain S-RA-6) at pH 3 and 108 ± 8 ng/mg dw (strain V-RA-4) and 184 ± 74 ng/mg dw (strain S-RA-6) at pH 7, respectively (Fig. 5). Thus, a small proportion of vanadium, ca. 20%, was released from strains V-RA-4 and S-RA-6 by EDTA extraction under both acidic and neutral pH, suggesting that vanadium was primarily accumulated in the intracellular compartments.

Heavy metal accumulation by vanadium-resistant bacteria

One of the strategies for enhancing the effectivity of bioremediation technology is to

search for novel microorganism having a wide range of uptake capacities of heavy metal ions. In this study we examined the ability of each vanadium-resistant bacterial strain to accumulate other heavy metals: copper (Cu), cobalt (Co), molybdate (Mo), and nickel (Ni) ions. Bacterial cells were cultured overnight in standard medium containing 10 μM of each metal at 25°C with rotation at 180 rpm. After harvesting and drying, the metal contents of bacterial cells were measured by AAS.

Each vanadium-resistant bacterial strain exhibited significant bioaccumulation of copper and cobalt ions (Fig. 6A and B). The strains with the highest accumulation of copper ions were V-RA-3 (349 ± 23 ng mg^{-1} dw), V-RA-4 (282 ± 24 ng mg^{-1} dw), and S-RA-6 (267 ± 9 ng mg^{-1} dw). In addition, vanadium-resistant bacteria removed a significant percentage of copper and cobalt ions from the medium. The removal of copper ions reached 30%, while that of cobalt ions reached 24% (Tables 3 and 4). In contrast, the accumulation of nickel and molybdate by each vanadium-resistant bacterial strain was relatively low; the nickel and molybdate ion contents of bacterial cells ranged from 0.50 to 9.50 ng mg^{-1} dw (Fig. 6C and D).

Discussion

Isolation of microorganisms from metal-rich environments has gained attention due to their resistance to high levels of metals, and because they may be used as a model for bioremediation technology. In this study, we first measured the V concentration in the intestinal contents of the vanadium-rich ascidian *A. sydneiensis samea*, which was 20,000 times higher than in natural seawater. Then we screened the intestinal contents of this ascidian

for the presence of intestinal bacteria able to grow in the presence of 0–10 mM sodium orthovanadate. We found that the number of bacterial colonies decreased with increasing vanadium concentrations in the medium. Each bacterial colony that was able to grow in the presence of 10 mM vanadium showed various colorations from a clear halo to yellow and blue-dark ones. These colorations were indicated as a response to the toxicity of high concentrations of vanadate and a preliminary marker of vanadium accumulation or reduction and possible sequestration by bacterial cells (Hernández et al. 1998; Myers et al. 2004; van Marwijk et al. 2009). Thus, these bacterial colonies were selected at random and identified based on their 16S rRNA sequences.

We successfully isolated nine strains of vanadium-resistant bacteria that are able to grow in the presence of 10 mM vanadium; five strains belong to the genus *Vibrio* and four belong to *Shewanella*. The most abundant strains of genus *Vibrio*, V-RA-2 and V-RA-4, corresponded to *V. splendidus* (99.87 ± 0.09%) and *V. tasmaniensis* (99.93 ± 0.13%), respectively, which have been associated with mortality in crustaceans, mollusks, and fish (Thompson et al. 2003; Faury et al. 2004; Romalde et al. 2014) or in ascidian they have been associated with biofilm communities (Behrendt et al. 2012; Blasiak et al. 2014). Strains S-RA-6 and S-RA-7 were the most abundant strains of the genus *Shewanella*, and corresponded to *S. kaereitica* (99.87 ± 0.17%) and *S. pasifica* (99.67%), which are commonly found in seawater, deep-sea sediment, and as biofilm formations on natural substrates (Ivanova et al. 2003; Jiang et al. 2007; Finnegan et al. 2011). They occur in very low numbers compared to other predominate intestinal bacteria; *Vibrio* and *Shewanella* are known to inhabit the gut of *Ciona intestinalis* with 1.5% and 4% OTU, respectively (Dishaw et al. 2014), although the data for *A. sydneiensis samea* is not available yet. These facts suggested

that the selection by vanadium-rich medium worked well for selecting specific types of bacteria.

Of the nine strains, strain V-RA-4, corresponding to *Vibrio tasmaniensis*, and S-RA-6, corresponding to *Shewanella kaireitica*, accumulated higher levels of vanadium ions than the other strains when they were incubated with vanadate V(V) ions at concentrations of 200 and 500 μM, which are similar to the concentrations in the gut of *A. sydneiensis samea*. These two bacterial strains exhibited good growth in media containing various concentrations of vanadium (V) at an optimal temperature of 25°C (Fig. 3). The ability of these two bacterial strains to accumulate vanadate ions was 20-fold greater than genetically modified *E. coli* cells expressing AgVanabin2 (Samino et al. 2012) (264 ± 165 vs. 10.12 ± 0.64 ng mg^{-1} dw, respectively). In addition, these two strains have advantages in that they are natural bacteria and may not cause any ethical problems for their application.

Use of the genus *Vibrio* in vanadium recovery is less popular than *Saccharomyces* (Willsky et al. 1985; Henderson et al. 1989; Kanik-ennulat et al. 1995; Bisconti et al. 1997), *Enterobacter* (Hernández et al. 1998; van Marwijk et al. 2009), *Pseudomonas* (Antipov et al. 1998, 2000; Shirdam et al. 2006), and *Shewanella* (Carpentier et al. 2003, 2005; Myers et al. 2004). To the best of our knowledge, this is the first report of vanadium accumulation by the genus *Vibrio*, which may increase our understanding of vanadium accumulation by various types of microorganisms. In contrast, *Shewanella*, particularly *S. oneidensis*, is the most versatile bacterium investigated to date and is widely used as a vanadium-reducing bacterium that reduces highly toxic vanadate to the less toxic vanadyl (Carpentier et al. 2003, 2005; Myers et al. 2004).

The efficacy of heavy metal bioaccumulation by bacterial cells is affected by the pH

of the culture medium (López et al. 2000; Esposito et al. 2002). Prior to real-world applications, it is important to characterize the properties of vanadium under different pHs of bacterial culture. In this study, vanadium uptake by the two bacterial strains V-RA-4 and S-RA-6 increased at pH 3 and decreased linearly at pH 7 and 9. Both strains had the same optimal absorption pH for vanadate and vanadyl ions. The optimal pH of 3 for vanadium absorption reported in this study is consistent with that reported previously for vanadium absorption by *Halomonas sp.* GT-83 (Ghazvini and Mashkani 2009). These findings may provide an alternative bioremediation technology for vanadium wastewater recovery under acidic conditions, for which another bacterium such as *Marinobacter sp.* MW1 is unsuitable, which was reported by Kamika and Momba (2014) to be unable to reduce vanadium pollution from acidic mine water.

To provide further evidence of the occurrence of both intracellular and cell surface absorption of vanadium, we examined the distribution of vanadium in bacterial cells exposed to 0.5 mM vanadium at different pHs. Our experimental results showed that EDTA treatment removed only 20% of bound vanadium both at pH 3 and 7. Accordingly, these two bacterial strains exhibited mostly intracellular accumulation of vanadium.

It has been reported that intracellular accumulation is dominantly found in heavy metal decontamination by living cells (Shirdam et al. 2006; Desaunay and Martins 2014; Huang et al. 2014). In this type of accumulation, cell walls only function as a filter for heavy metals ions and control their diffusion towards the cytoplasm. The next steps are complex processes such as localization of the metal within specific organelles, enzymatic detoxification, and efflux pumps inside bacterial cells (Bowman 1983; Kanik-ennulat and Neff 1990; Antipov et al. 2000; Zhang et al. 2014; Huang et al. 2014). The majority of metal

ions in intracellular accumulation are commonly stabilized inside the cell and rarely released toward their environment, except when the cells die (Desaunay and Martins 2014). However, intracellular accumulation of vanadium reported in *Neurospora craspa* is followed by intracellular reduction of V(V) by the high concentration of intracellular reducing agents, and are subsequently removed as vanadyl V(IV) ions (Bowman 1983). From an ecological point of view, if accumulation and reduction are coupled in bacteria in the ascidian intestine, it may have important consequences in terms of metal mobility in a microenvironment such as the ascidian intestine, where uptake and release of vanadium between intestinal bacteria and intestinal cells of ascidians may occur. Therefore, our future studies will focus on vanadium reduction by vanadium-resistant bacterial strains to reveal these relationships.

We also tested the ability of all strains of vanadium-resistant bacteria isolated from *A. sydneiensis samea* to accumulate other heavy metals. All strains exhibited high accumulation of Cu(II) and Co(II) ions, but low accumulation of Mo(IV) and Ni(II) ions. However, Cu(II) ion accumulation capacities of the bacterial strains were 1.5- and 7-fold lower than those reported by Samino et al. (2012) for copper ion accumulation. The bacterial strains identified here have safety advantages because they were obtained from a natural habitat. Several strains of vanadium-resistant bacteria were also able to remove up to 24% of Co(II) ions, which is the first report of cobalt accumulation by intestinal bacteria isolated from an ascidian.

In conclusion, we identified nine strains of vanadium-resistant bacteria from the intestine of the vanadium-rich ascidian *A. sydneiensis samea*. Two of these strains can accumulate high levels of vanadium. Vanadium is primarily accumulated in the intracellular compartment. The growth profile and pH dependency of accumulation were characterized. All nine bacteria can also accumulate copper and cobalt ions. Therefore, these vanadium-resistant

bacteria could be used for the decontamination of metal-containing wastewater.

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Table 1 Vanadium-resistant bacteria isolated from the intestinal content of *Ascidia sydneiensis samea.*

Name of strain	Most closely-related genus and species	Number of colonies	% similarity (s_ab score*)
V-RA-1	*Vibrio splendidus* strain 630	10	99.89 ± 0.13
V-RA-2	*Vibrio splendidus* strain N08	11	99.87 ± 0.09
V-RA-3	*Vibrio splendidus* partial 16S	8	99.95 ± 0.13
V-RA-4	*Vibrio tasmaniensis* strain 007	8	99.93 ± 0.13
V-RA-5	*Vibrio tapetis* P502	2	99.07 ± 0.81
S-RA-6	*Shewanella kaireitica* strain c931	4	99.87 ± 0.17
S-RA-7	*Shewanella pasifica* strain KMM	4	99.64
S-RA-8	*Shewanella olleyana* strain WA6	1	98.72
S-RA-9	*Shewanella pasifica* strain UDC	3	99.88 ± 0.10
	Total	51	

Note: * S_ab score is identity score for individual sequences ± standard deviation

Table 2 Bioaccumulation of V(IV) and V(V) ions by strains V-RA-4 and S-RA-6 in NaCl medium containing 500 μM vanadium with different pH after 6 and 24 hours of culture.

pH	Strain	Chemical species of vanadium	Initial amt of vanadium in medium (ng)	Culture time (h)	Amt of vanadium accumulated by bacteria (ng)[a]	Removal of vanadium from medium (%)
3	V-RA-4	V(IV)	382,065	6	61,452 ± 16,675	16
	S-RA-6				48,888 ± 3,686	13
7	V-RA-4				14,650 ± 1,834	4
	S-RA-6				11,531 ± 125	3
9	V-RA-4				11,355 ± 1,033	3
	S-RA-6				12,439 ± 711	3
3	V-RA-4	V(V)	382,065	6	308,853 ± 26,388	81
	S-RA-6				317,995 ± 30,152	83
7	V-RA-4				1,975 ± 131	1
	S-RA-6				2,160 ± 5	1
9	V-RA-4				3,365 ± 435	1
	S-RA-6				2,419 ± 689	1
3	V-RA-4	V(V)	382,065	24	507,741 ± 151,414	133
	S-RA-6				329,926 ± 15,530	86
7	V-RA-4				23,285 ± 2,730	6
	S-RA-6				33,622 ± 21,803	9
9	V-RA-4				3,299 ± 290	1
	S-RA-6				3,214 ± 684	1

[a] data represent the means ± standard deviations of three independent experiments

Table 3 Bioaccumulation of copper (II) ions by vanadium-resistant bacteria isolated from the intestine of *Ascidia sydneiensis samea.*

Strain	Initial copper conc. in medium (μM)	Initial amount of copper in medium (ng)	Amount of copper accumulated by bacteria (ng) [a]	Removal Cu from medium (%)
V-RA-1	10	9,532	1,772 ± 405	19
V-RA-2			2,048 ± 684	21
V-RA-3			2,818 ± 521	30
V-RA-4			1,865 ± 98	20
V-RA-5			1,675 ± 527	18
S-RA-6			2,303 ± 587	24
S-RA-7			2,550 ± 678	27
S-RA-8			2,832 ± 759	30
S-RA-9			1,138 ± 634	12

[a] data represent the means ± standard deviations of three independent experiments

Table 4 Bioaccumulation of cobalt (II) ion by vanadium-resistant bacteria isolated from the intestine of *Ascidia sydneiensis samea.*

Strain	Initial cobalt conc. in medium (μM)	Initial amount of cobalt in medium (ng)	Amount of cobalt accumulated by bacteria (ng) [a]	Removal cobalt from medium (%)
V-RA-1	10	8,840	960 ± 269	11
V-RA-2			214 ± 8	2
V-RA-3			283 ± 25	3
V-RA-4			2,057 ± 140	23
V-RA-5			1,821 ± 437	21
S-RA-6			2,106 ± 12	24
S-RA-7			958 ± 267	11
S-RA-8			553 ± 262	6
S-RA-9			1,744 ± 144	20

[a] data represent the means ± standard deviations of three independent experiments

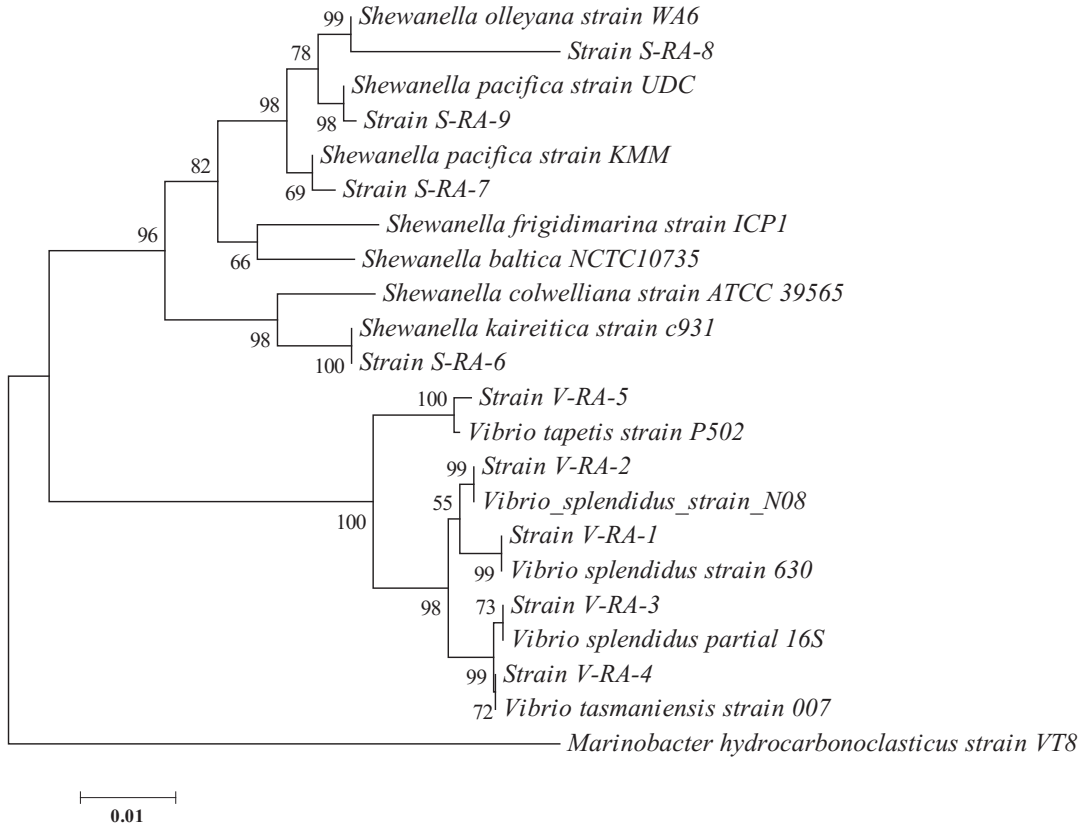

Fig. 1 Phylogenetic tree of vanadium-resistant bacteria isolated from the intestine of *A. sydneiensis samea* constructed using the neighbor joining (NJ) method based on 16S rRNA sequences. The percentage values are bootstrap possibilities determined for 1000 replicates.

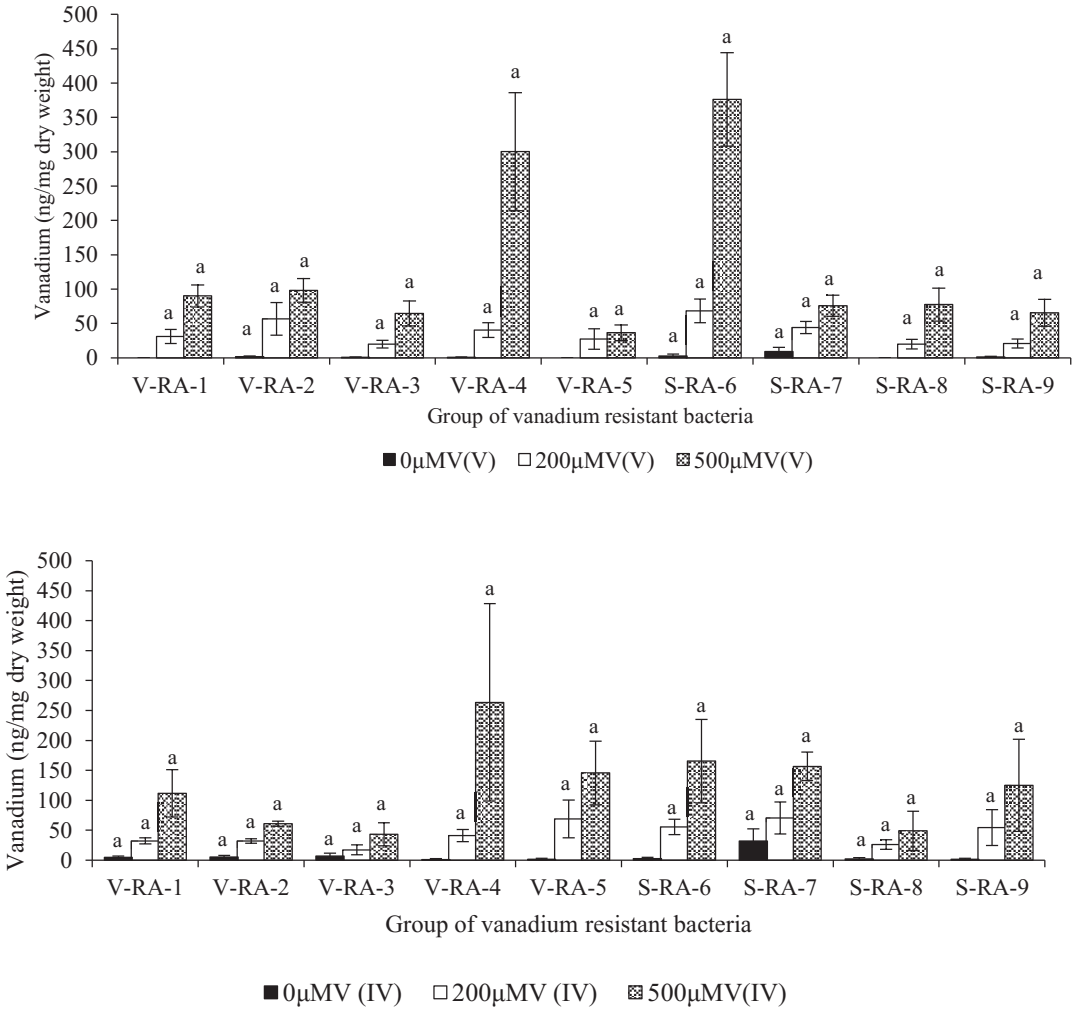

Fig. 2 Bioaccumulation of V(V) (A) and V(IV) (B) by vanadium-resistant bacteria isolated from the intestinal contents of *A. sydneiensis samea* in medium containing 0, 200, and 500 μM vanadium. Error bars show standard errors of averages calculated from three samples. For each bacterial strain, different letters in the each concentration of vanadium (0, 200, and 500 μM) indicate a significant difference in accumulating vanadium between each strain at the level of $P < 0.05$.

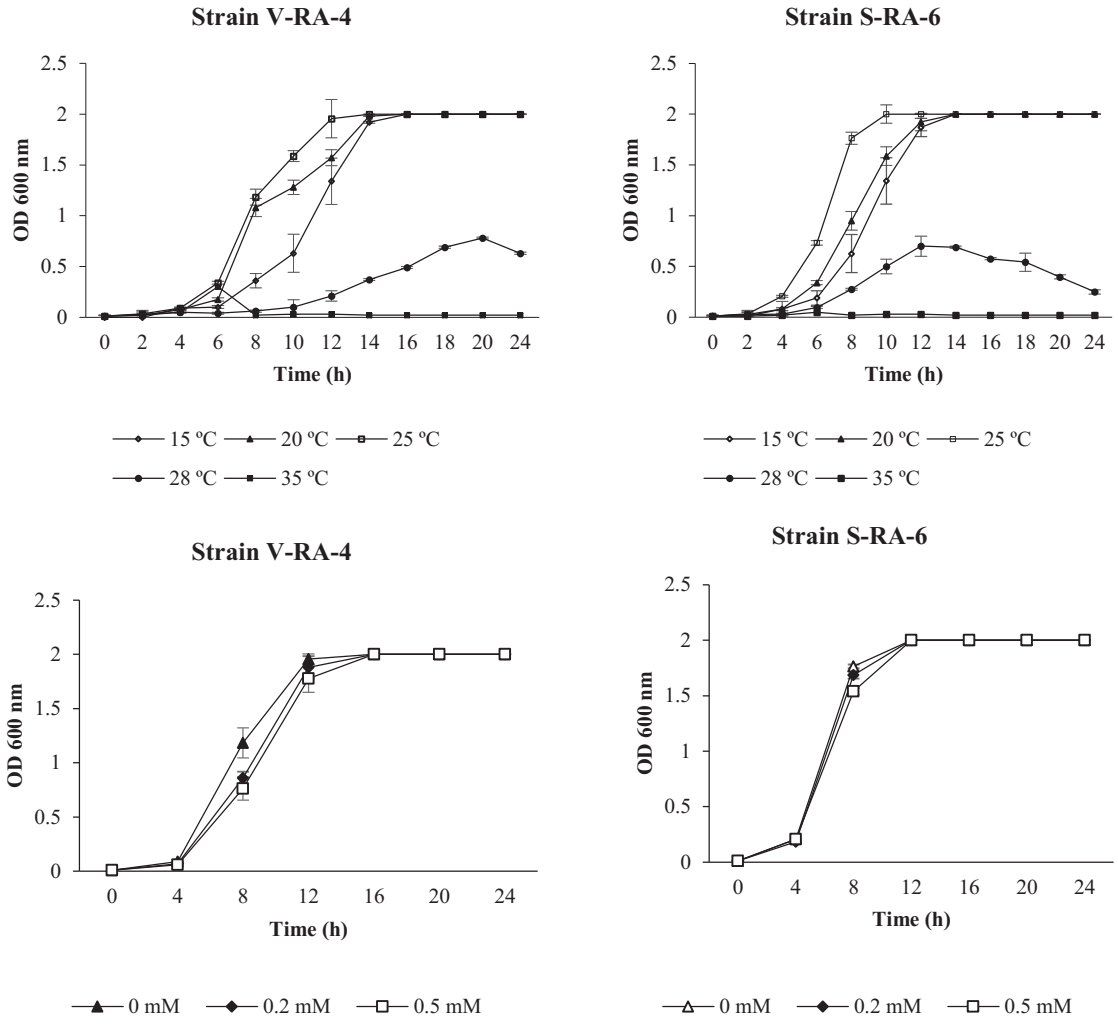

Fig. 3 The effects of temperature (A and B) and vanadium concentrations (C and D) on the growth of the vanadium-accumulating bacterial strains V-RA-4 and S-RA-6. Error bars show standard errors of averages calculated from three samples.

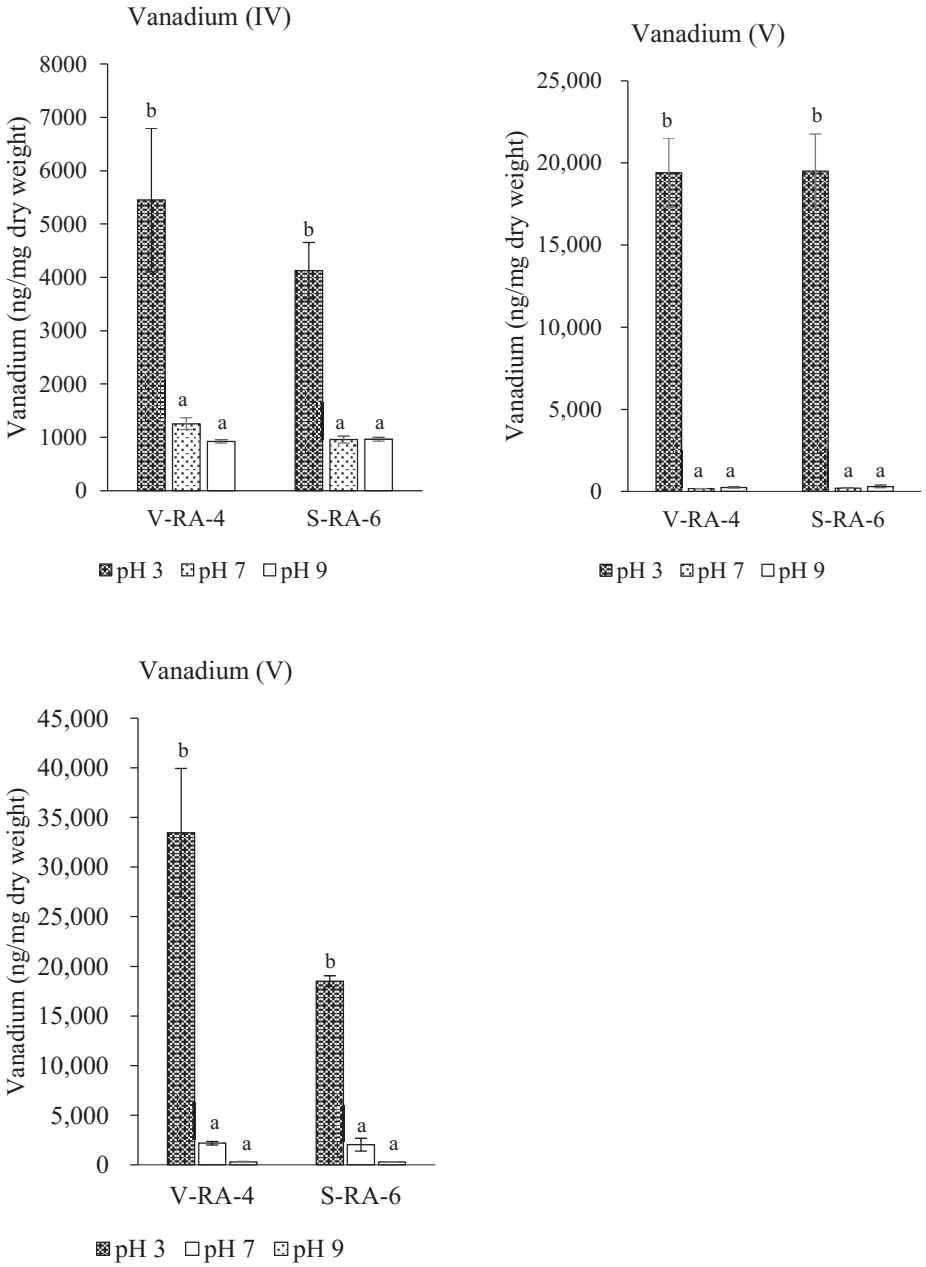

Fig. 4 Effect of pH on vanadium uptake by the vanadium-accumulating bacterial strains V-RA-4 and S-RA-6. Cells were cultured in 500 μM vanadium at pH 3, 7, and 9 with rotation at 180 rpm at 25ºC. V(IV) and V(V) uptake was monitored after 6 (A, B) and 24 h (C) of incubation. For each pH, different letters in the same bacterial strains indicate a significant difference between each pH at the level of $P<0.05$.

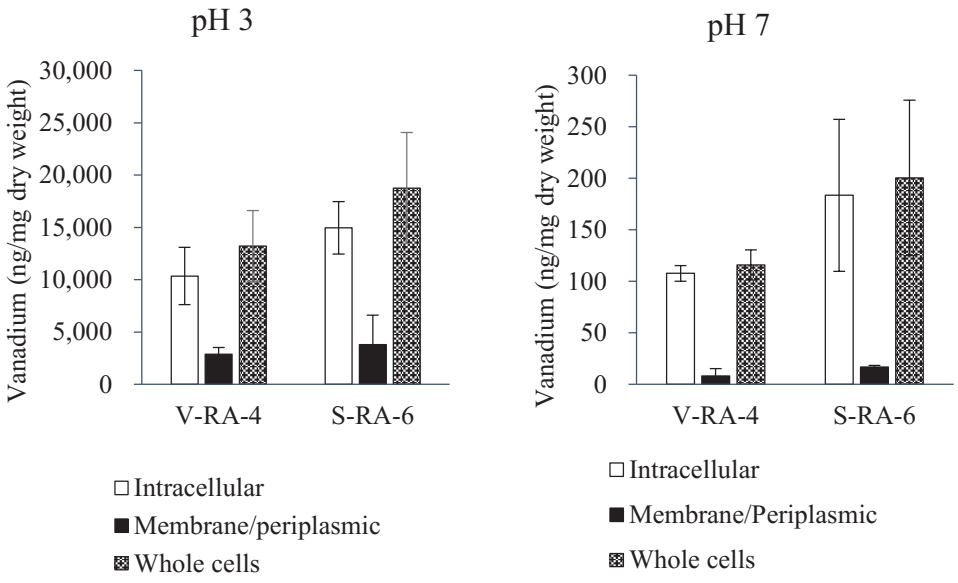

Fig. 5 Vanadium concentration in the subcellular compartments of cells of strains V-RA-4 and S-RA-6 obtained by EDTA chemical washing of cells previously exposed to 0.5 mM vanadium at pH 7 (A) and pH 9 (B). Error bars show standard errors of averages calculated from three samples.

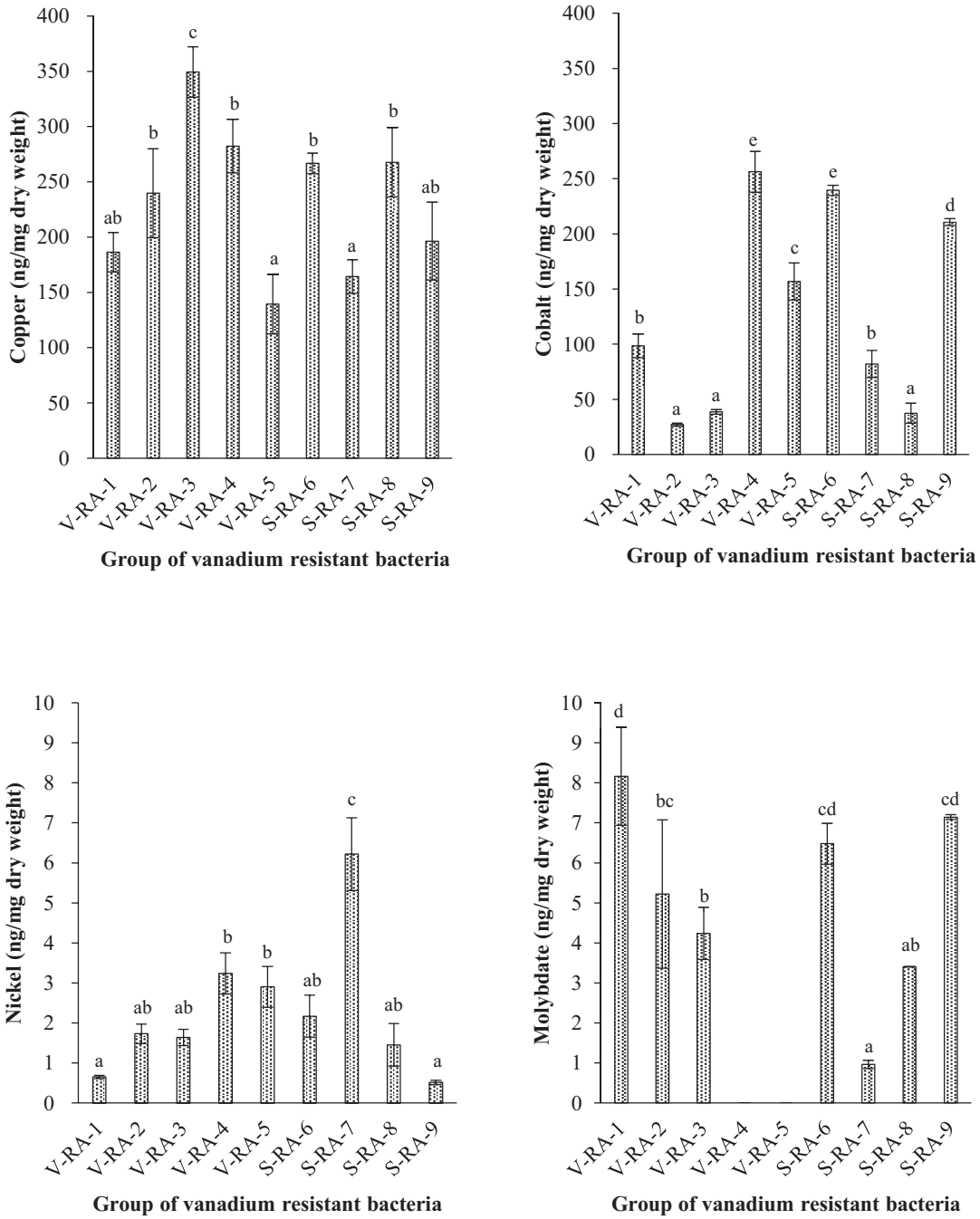

Fig. 6 Accumulation of copper (II) (A), cobalt (II) (B), nickel (II) (C), and molybdate (IV) (D) ions by vanadium-resistant bacteria isolated from the intestine of *A. sydneiensis samea*. Bacterial cells were cultured in standard medium containing 10 µM of each metal in a total volume of 15 mL with rotation at 180 rpm at 25ºC for 24 h in a 50 mL conical tube. Error bars show standard errors of averages calculated from three samples. Bars with different letters are significantly different at $P < 0.05$.

III. General Discussion

In recent years, the disposal of vanadium due to industrial activities has increased the environmental concern, especially to overcome the vanadium pollution. Isolation of microorganisms from vanadium-rich environments has promising an effective bioremediation strategy due to their resistance to high levels of metals. In this study, isolation of intestinal bacteria coping with vanadium ions from a vanadium-rich ascidian animal was motivated by not only the need for seeking microorganisms suitable in decontaminating vanadium and other heavy metals toxicity, but also in the future studies, I would also focus for seeking the evidence on intestinal bacteria contribution to mediate the cycling of vanadium ions in the intestine of ascidian animal as hyperaccumulator of vanadium.

At the beginning of this study, I measured the V concentration in the intestinal contents of the vanadium-rich ascidian *A. sydneiensis samea*, which was 20,000 times higher than in natural seawater. Then I screened the intestinal contents of this ascidian for the presence of intestinal bacteria able to grow in the presence of 0–10 mM sodium orthovanadate. I found that the number of bacterial colonies decreased with increasing vanadium concentrations in the medium. Each bacterial colony that was able to grow in the presence of 10 mM vanadium showed various colorations from a clear halo to yellow and blue-dark ones. These colorations were indicated as a response to the toxicity of high concentrations of vanadate and a preliminary marker of vanadium accumulation or reduction and possible sequestration by bacterial cells (Hernández et al. 1998; Myers et al. 2004; van Marwijk et al. 2009). Thus, these bacterial colonies were selected at random and identified based on their 16S rRNA sequences.

I successfully isolated nine strains of vanadium-resistant bacteria that are able to grow in the presence of 10 mM vanadium; five strains belong to the genus *Vibrio* and four

belong to *Shewanella*. The most abundant strains of genus *Vibrio*, V-RA-2 and V-RA-4, corresponded to *V. splendidus* (99.87 ± 0.09%) and *V. tasmaniensis* (99.93 ± 0.13%), respectively, which have been associated with mortality in crustaceans, mollusks, and fish (Thompson et al. 2003; Faury et al. 2004; Romalde et al. 2014). In oyster, *V. tasmaniensis* expresses coper related gene to fight againts antibactericidal effects released by hemocyte (Vanhove et al. 2016), while in ascidian they have been associated with biofilm communities (Behrendt et al. 2012; Blasiak et al. 2014). Strains S-RA-6 and S-RA-7 were the most abundant strains of the genus *Shewanella*, and corresponded to *S. kaereitica* (99.87 ± 0.17%) and *S. pasifica* (99.67%), which are commonly found in seawater, deep-sea sediment, and as biofilm formations on natural substrates (Ivanova et al. 2003; Jiang et al. 2007; Finnegan et al. 2011). They occur in very low numbers compared to other predominate intestinal bacteria; *Vibrio* and *Shewanella* are known to inhabit the gut of *Ciona intestinalis* with 1.5% and 4% OTU, respectively (Dishaw et al. 2014). By using metagenomic analysis on the intestinal content, Ueki's group also found the similar data for the occurrence of genus *Vibrio* and *Shewanella* in the intestine of *A. sydneiensis samea*. These facts suggested that the selection by vanadium-rich medium worked well for selecting specific types of bacteria.

The data for selecting specific types of bacteria from an ascidian animal by using vanadium-rich medium were also reported by Russian researcher around last two decades (Lyalikova and Yurkova 1992). They successfully isolated genus *Pseudomonas* from the intestine of ascidian that are cultured in 10 mM of vanadate, even though the species of an ascidian animal was not mentioned in their study. This genus was also reported to highly resist to high concentration of vanadium up to 50 mM. Similar high resistance to vanadate was also shown in my recent study on minimum inhibitory concentration of

vanadium-resistant bacterial strains isolated from the intestine of *A. sydneiensis samea* that could tolerate vanadium up to 40 – 50 mM (unpublish data).

Of the nine strains, strain V-RA-4, corresponding to *Vibrio tasmaniensis*, and S-RA-6, corresponding to *Shewanella kaireitica*, accumulated higher levels of vanadium ions than the other strains when they were incubated with vanadate V(V) ions at concentrations of 200 and 500 μM, which are similar to the concentrations in the gut of *A. sydneiensis samea*. These two bacterial strains exhibited good growth in media containing various concentrations of vanadium (V) at an optimal temperature of 25°C. The ability of these two bacterial strains to accumulate vanadate ions was 20-fold greater than genetically modified *E. coli* cells expressing AgVanabin2 (Samino et al. 2012). In addition, these two strains have advantages in that they are natural bacteria and may not cause any ethical problems for their application.

Use of the genus *Vibrio* in vanadium recovery is less popular than *Saccharomyces* (Willsky et al. 1985; Henderson et al. 1989; Kanik-ennulat et al. 1995; Bisconti et al. 1997), *Enterobacter* (Hernández et al. 1998; van Marwijk et al. 2009), *Pseudomonas* (Lyalikova and Yurkova 1992; Antipov et al. 2000; Shirdam et al. 2006), and *Shewanella* (Carpentier et al. 2003, 2005; Myers et al. 2004). To the best of my knowledge, this is the first report of vanadium accumulation by the genus *Vibrio*, which may increase my understanding of vanadium accumulation by various types of microorganisms. In contrast, *Shewanella*, particularly *S. oneidensis*, is the most versatile bacterium investigated to date and is widely used as a vanadium-reducing bacterium that reduces highly toxic vanadate to the less toxic vanadyl (Carpentier et al. 2003, 2005; Myers et al. 2004).

The efficacy of heavy metal bioaccumulation by bacterial cells is affected by the pH of the culture medium (López et al. 2000; Esposito et al. 2002). Prior to real-world

applications, it is important to characterize the properties of vanadium under different pHs of bacterial culture. In this study, vanadium uptake by the two bacterial strains V-RA-4 and S-RA-6 increased at pH 3 and decreased linearly at pH 7 and 9. Both strains had the same optimal absorption pH for vanadate and vanadyl ions. The optimal pH of 3 for vanadium absorption reported in this study is consistent with that reported previously for vanadium absorption by *Halomonas sp.* GT-83 (Ghazvini and Mashkani 2009). These findings may provide an alternative bioremediation technology for vanadium wastewater recovery under acidic conditions, for which another bacterium such as *Marinobacter sp.* MW1 is unsuitable, which was reported by Kamika and Momba (2014) to be unable to reduce vanadium pollution from acidic mine water.

To provide further evidence of the occurrence of both intracellular and cell surface absorption of vanadium, I examined the distribution of vanadium in bacterial cells exposed to 0.5 mM vanadium at different pHs. The experimental results showed that EDTA treatment removed only 20% of bound vanadium both at pH 3 and 7. Accordingly, these two bacterial strains exhibited mostly intracellular accumulation of vanadium.

It has been reported that intracellular accumulation is dominantly found in heavy metal decontamination by living cells (Shirdam et al. 2006; Desaunay and Martins 2014; Huang et al. 2014). In this type of accumulation, cell walls only function as a filter for heavy metals ions and control their diffusion towards the cytoplasm. The next steps are complex processes such as localization of the metal within specific organelles, enzymatic detoxification, and efflux pumps inside bacterial cells (Bowman 1983; Kanik-ennulat and Neff 1990; Antipov et al. 2000; Zhang et al. 2014; Huang et al. 2014). The majority of metal ions in intracellular accumulation are commonly stabilized inside the cell and rarely released

toward their environment, except when the cells die (Desaunay and Martins 2014). However, intracellular accumulation of vanadium reported in *Neurospora craspa* is followed by intracellular reduction of V(V) by the high concentration of intracellular reducing agents, and are subsequently removed as vanadyl V(IV) ions (Bowman 1983).

In my recent study, vanadium-resistant bacteria strains also have ability to reduce V(V) to V(IV) ions and involve enzymatic mechanism. I detected both V(V) and V(IV) ions that exist in intra and extracellular compartment of bacterial cells as well they are also detected in supernatant. From this finding, it indicated that there are several possibilities by which vanadium accumulated in intracellular compartments (cytoplasm) of bacteria cells. The fist possibility is that V(V) ions are firstly reduced to V(IV) in extracellular compartments (membrane) and later transported to cytoplasm. In this case, I detected vanadate reductase activity which is predominantly exists in membrane fraction. The second possibility is that V(V) ions are transported directly from extracellular compartment to cytoplasm by transmembrane transporter or by simple diffusion, and later V(V) ions are reduced to V(IV) by enzymatic reduction as I also detected in cytoplasmic fraction. The final step, V(IV) ions are pumped out of the cell or released to supernatant. In line with these findings, previous result of vanadium reduction study in *S. cerevisiae* revealed that V(IV) and V(V) ions can enter the cell through the phosphate transport system, while V(V) ions are reduced thereafter to V(IV) by thiols and other reducing agents to takes place in the cytoplasm (Willsky et al. 1984; Zoroddu et al. 1991; Zoroddu et al. 1996; Zoroddu and Masia 1997). It also indicated from data in Fig. 1 that vanadium-resistant bacterial strain significantly could accumulate V(V) and V(IV) ions. From an ecological point of view, if accumulation and reduction are coupled in bacteria in the ascidian intestine, it may have important consequences in terms of

metal mobility in a microenvironment such as the ascidian intestine, where uptake and release of vanadium between intestinal bacteria and intestinal cells of ascidians may occur. It might be concluded that vanadium-resistant bacterial strain may also contribute indirectly to vanadium accumulation and reduction in ascidian by preparing the reduced-V(IV) ions before transported to intestinal lumen.

I also tested the ability of all strains of vanadium-resistant bacteria isolated from *A. sydneiensis samea* to accumulate other heavy metals. All strains exhibited high accumulation of Cu(II) and Co(II) ions, but low accumulation of Mo(IV) and Ni(II) ions. However, Cu(II) ion accumulation capacities of the bacterial strains were 1.5- and 7-fold lower than those reported by Samino et al. (2012) for copper ion accumulation. The bacterial strains identified here have safety advantages because they were obtained from a natural habitat. Several strains of vanadium-resistant bacteria were also able to remove up to 24% of Co(II) ions, which is the first report of cobalt accumulation by intestinal bacteria isolated from an ascidian.

In conclusion, I identified nine strains of vanadium-resistant bacteria from the intestine of the vanadium-rich ascidian *A. sydneiensis samea*. Two of these strains can accumulate high levels of vanadium. Vanadium is primarily accumulated in the intracellular compartment. The growth profile and pH dependency of accumulation were characterized. All nine bacteria can also accumulate copper and cobalt ions. Therefore, these vanadium-resistant bacteria could be used not only for the decontamination of metal-containing wastewater, but also to support my hypothesis on their contribution in vanadium accumulation and reduction in ascidian animal.

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IV. General Summary

Isolation of microorganisms from heavy metals or vanadium-rich environment are recommended for the bioremediation purposes in order to get the candidate of bacterial strains having highest resistance to the toxicity of heavy metals ions. In this study, I found that the vanadium concentration in the intestine of the vanadium-rich ascidian *Ascidia sydneiensis samea* could reach 0.67 mM, and thus I isolated vanadium-resistant bacteria from the intestinal contents and determined the ability of each bacterial strain to accumulate vanadium and other heavy metals.

Nine strains of vanadium-resistant bacteria were successfully isolated, in which five strains belong to the genus *Vibrio* and four to genus *Shewanella.* Two bacterial strains, V-RA-4 and S-RA-6, accumulated vanadium at a higher rate than did the other strains. Further characterization of the two bacterial strains revealed that they exhibited good growth in media containing various concentrations (200- and 500-μM) of vanadium (V) at an optimal temperature of 25°C. The maximum vanadium absorption by these bacteria was achieved at pH 3, and intracellular accumulation was the predominant mechanism.

Each vanadium-resistant bacterial strain also strongly accumulated copper and cobalt ions, but accumulation of nickel and molybdate ions was relatively low. These bacterial strains can be applied to protocols for bioremediation of vanadium and heavy metal toxicity.

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Abbreviations

AAS, atomic absorption spectrometry; EDTA, ethylene diamine tetra acid; EtBr, ethidium bromide; OD, optical density; OTU, operational taxonomic unit; PB medium, postgate medium B; PCR, polymerase chain reaction.

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