Phylogenetically Diverse ureC Genes and Their Expression Suggest the Urea Utilization by Bacterial Symbionts in Marine Sponge Xestospongia testudinaria

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Abstract

Urea is one of the dominant organic nitrogenous compounds in the oligotrophic oceans. Compared to the knowledge of nitrogen transformation of nitrogen fixation, ammonia oxidation, nitrate and nitrite reduction mediated by sponge-associated microbes, our knowledge of urea utilization in sponges and the phylogenetic diversity of sponge-associated microbes with urea utilization potential is very limited. In this study, Marinobacter littoralis isolated from the marine sponge Xestospongia testudinaria and the slurry of X. testudinaria were found to have urease activity. Subsequently, phylogenetically diverse bacterial ureC genes were detected in the total genomic DNA and RNA of sponge X. testudinaria, i.e., 19 operative taxonomic units (OTUs) in genomic DNA library and 8 OTUs in cDNA library at 90% stringency. Particularly, 6 OTUs were common to both the genomic DNA library and the cDNA library, which suggested that some ureC genes were expressed in this sponge. BLAST and phylogenetic analysis showed that most of the ureC sequences were similar with the urease alpha subunit of members from Proteobacteria, which were the predominant component in sponge X. testudinaria, and the remaining ureC sequences were related to those from Magnetococcus, Cyanobacteria, and Actinobacteria. This study is the first assessment of the role of sponge bacterial symbionts in the regenerated utilization of urea by the detection of transcriptional activity of ureC gene, as well as the phylogenetic diversity of ureC gene of sponge bacterial symbionts. The results suggested the urea utilization by bacterial symbionts in marine sponge X. testudinaria, extending our understanding of nitrogen cycling mediated by sponge-associated microbiota.

Introduction

Nitrogen can be a limiting nutrient and nitrogen availability in the marine environment may therefore be a major factor in controlling biomass production. Most nitrogen cycling-related studies focus on nitrate and ammonium as the primary sources of nitrogen available to coral reefs [1], because they are generally the preferred forms for assimilation [2,3]. But tropical marine ecosystems rely heavily on regenerated nitrogen sources [4]. In low nitrate systems, regenerated nitrogen such as urea or ammonium can provide up to 75% of the requirements of phytoplankton [5,6]. Urea represents the single dominant component of the diverse group of organic nitrogenous compounds in the oligotrophic oceans [7]. Urea in the oceans originates from a variety of sources [8–12]. For example toadfish (Opsanus beta) can excrete over 90% of their waste nitrogen as urea [13]. On the other hand, urea can be used directly by many organisms such as hard corals [3], phytoplankton [5], benthic macroalgae [14], protozoans and bacteria [15,16]. However, in total, the role of regenerated utilization of urea in the nitrogen cycle of marine ecosystem is poorly understood, especially for marine microbial symbionts.

Marine sponges (phylum Porifera), the oldest multicellular animals (metazoans), are one of the main components in coral reefs ecosystem. Sponges are filter feeders, pumping large amounts of seawater every day. Their ecological significance has attracted much research interest [17,18,19]. It is known that sponges host abundant and diverse symbiotic microorganisms including bacteria, archaea, unicellular algae and fungi [20–32]. In oligotrophic seawater where the nitrogen level is very low, symbiotic microorganisms may contribute to the nitrogen budget of sponges via fixation of atmospheric nitrogen [18]. The release of nitrate from incubated sponges provided the first indication of nitrification within these organisms, with estimated rates often far exceeding those for other benthic substrata [19]. To date, the potential of sponge microbial symbionts in nitrogen fixation, ammonia oxidation and nitrite reduction have been suggested by the analysis of nitrogen cycle-related functional genes such as nifH, amoA, nirK or nirS [21,33–39]. But, till now, the regenerated utilization of urea by sponge bacterial symbionts remains nearly unknown [38].

Urease is one of the important enzymes in nitrogen cycle [40]. Most organisms that use urea as a source of nitrogen rely on a urease such as urea amidohydrolase (EC 3.5.1.5), that can catalyze...
the hydrolysis of urea to yield ammonia and carbamate [41–44]. Bacterial urease is a trimer of three subunits (encoded by ureA, ureB, and ureC) and requires up to four accessory proteins for activation and Ni²⁺ incorporation (most commonly encoded by ureD, ureE, ureF, and ureG) [45]. The urease peptides have highly conserved active sites and Ni²⁺ binding residues [44]. According to the single-cell genomics analysis of Poribacteria in sponge *Aplysina aerophoba*, a 10 ORF containing urease gene cluster including three urease subunits ureA, ureB, ureC was identified, suggesting that the *Poribacteria* are capable of using urea to cover their nitrogen needs [38]. However, we don’t know whether urease genes are active in sponges and what is the phylogenetic diversity of urease genes of microbial community in sponges.

The ureC gene was chosen as the target gene for urease analysis because it is the largest of the genes encoding urease functional subunits and contains several highly conserved regions that are suitable as PCR priming sites [46]. In this study, using ureC gene as marker, the phylogenetically diverse ureC genes and their expression in the marine sponge *X. testudinaria* were investigated for the first time, suggesting an important role of phylogenetically diverse sponge bacterial symbionts in the regenerated utilization of urea for sponge host.

**Methods**

**Ethics Statement: N/A**

This study and the collection of sponges were approved by the ethics committee for scientific study at Shanghai Jiao Tong University.

No legislation was required for the sampling of sponges around Yongxing island (112°20’E, 16°50’N). The government of China permits the sampling of sponge samples around the Yongxing Island in the South China Sea for scientific research, and no specific permissions were required for these locations/activities, the location is not privately-owned or protected in any way, the field studies did not involve endangered or protected species. The sponges were collected by us.

**Sample Collection**

Specimens of the marine sponge *X. testudinaria* were collected by scuba diving at a depth of ca. 20 m from Yongxing Island (latitude 112°20’E, longitude 16°50’N) in the South China Sea, in 2010. Sponge samples were transferred directly to plastic bags to avoid the contact of sponge tissue with air and immediately stored at −80°C. Sponge samples for RNA extraction were immediately stored in RNA later (Qiagen, Valencia, CA, USA).

**Urease Activity Assay**

Sponge samples were washed with sterile artificial seawater (1.1 g CaCl₂, 10.2 g MgCl₂·6H₂O, 31.6 g NaCl, 0.75 g KCl, 1.0 g Na₂SO₄, 2.4 g Tris·HCl, 0.02 g NaHCO₃, 1 L distilled water, pH 7.6) to remove the contaminants from sea water or sediment. Three sponge cubes (1 cm²) from different parts of the sponge were grounded into slurry using a mortar and pestle. Sponge slurry was centrifuged at 100 g for 2 min. The supernatant was then reacted in urease activity using a colorimetric urea agar method at 28°C [47]. The medium contains 1 g peptone, 1 g glucose, 2 g KH₂PO₄, 0.012 g phenolsulfophthalein, 20 g agar, 1 L artificial seawater (ASW). The medium was supplemented with 40% (w/v) urea solution [47]. Cultures were scored positive for urease activity if the agar color changed from pale orange (pH 6.9) to pink or fuchsia (pH 7.6 or higher). The color change is because of the dead cells. The uninoculated control should be still blank.

**Total Genomic DNA and RNA Extraction**

Three cubes (~1cm³) from different parts of the sponge were dissociated in sterilized CMFSW (31.6 g NaCl, 0.75 g KCl, 1.0 g Na₂SO₄, 2.4 g Tris·HCl, 0.02 g NaHCO₃, 7.2 g EDTA, 1 L distilled water, pH 7.6) and grinded into slurry using a mortar and pestle. Sponge slurry was centrifuged at 100 × g for 15 min to separate and discard the supernatant. The supernatants were freeze-dried and grinded into powder, which was washed twice with 1 mL TE and centrifuged at 12,000 × g for 1 min. Total DNA and RNA were extracted by AllPrep DNA/RNA Mini Kit (Qiagen, Germany) simultaneously; RNA extraction were treated with amplification grade DNase I (Invitrogen) according to the manufacturer’s protocol to remove any residual DNA prior to reverse transcription for cDNA synthesis. The amount and purity of DNA or RNA were determined by NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, USA), based on the 260 nm absorption and the 260/280 ratio, respectively.

**Reverse Transcription and cDNA Synthesis**

RNA was reverse-transcribed into cDNA using the SuperScript III First Strand Synthesis System (Invitrogen). Reverse transcription was carried out with random hexamer primers in 25 μl reaction system containing 100 ng template RNA, 500 nmol of reverse primer, 1.5 μl dNTPs, H₂O was added to reach 12.5 μl. After incubation at 65°C for 5 min, the 25 μl reaction mixture was placed on ice for 1 min, added 12.5 μl cDNA Synthesis Mix (10×RT Buffer 2.5 μl, 25 mM MgCl₂ 5 μl, 0.1 M DTT 2.5 μl, RNaseOUT 1.25 μl, SuperScript III RT 1.25 μl), incubated at 50°C for 50 min and the reaction was terminated at 85°C for 5 min [40]. The cDNA obtained was stored at −20°C until further use.

**PCR Amplification of Urease (ureC) Genes from Genomic DNA and cDNA**

Bacterial urease (ureC) genes were amplified with the primer set L₅F (5’-ATCGGYAAARGCNGGN AAYCC-3') and L₅R (5’-GTBHNCACCARTYCTCRTG-3’) [46] from genomic DNA and cDNA. Reactions were performed in Mastercycler personal (Eppendorf). The PCR mixture (20 μl) contained 100 ng template DNA, 500 nmol of each primer, 10 μl ×2PCR Mix, 0.5 μl DNA polymerase and H₂O was added to reach 20 μl. PCR amplification began with a 5 min denaturing step at 94°C, followed by 30 cycles at 94°C for 1 min, 57°C for 1.5 min, and 72°C for 2 min. Extension was achieved at 72°C for 10 min. PCR products (390 bp) were purified by electrophoresis on a 2% (wt/vol) agarose gel and recovered using a gel purification kit (Takara, Dalian, China).

**PCR Amplification of 16S rRNA Gene from Genomic DNA**

Bacterial 16S rRNA genes were amplified with the domain bacteria-specific primer 27F (5’-GAGTTTGATCCTGGCT-AGAAG-3’) and universal primer 1500R (5’-AGAAAGGAGGT-GATCCAGCC-3’) [49]. PCR Kit (Takara, Dalian, China) was used in the PCR amplification. The PCR mixture (20 μl) contained 100 ng template DNA, 500 nmol of each primer, 10 μl ×2PCR Mix, 0.5 μl DNA polymerase and H₂O was added to reach 20 μl. Cycling conditions were as follows: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, and a final extension of 10 min at 72°C [50]. PCR products of 1500 bp were purified by electrophoresis on a 1% (wt/vol) agarose gel and recovered using a gel purification kit (Takara, Dalian, China).
Clone Library Construction and Sequencing

The libraries of ureC (from genomic DNA and cDNA) and 16S rRNA genes were separately constructed using the pMD18-T Cloning kit (Takara, Dalian, China) following the manufacturer’s instructions. Transformants were inoculated into LB broth (with 100 µg/ml ampicillin) and incubated overnight at 37°C. The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indolyl-b-D-Galactopyranoside) - IPTG (isopropyl- b-D-thiogalactopyranoside) - ampicillin-tetracycline indicator plates by color-based recombinant selection. Positive clones were identified by PCR amplification with pMD18-T vector primer pairs: M13F (5’-TGTAAACGACGAGCCGCTAG-3’) and M13R (5’-CAGGAAACGCTATGACC-3’), using the same program as the PCR amplification of ureC gene or 16S rRNA gene.

Species Richness Estimation and Phylogenetic Analysis

Operational taxonomic unit (OTU) was defined at the 97% similarity (3% difference) for 16S rRNA gene and 90% similarity (10% difference) for ureC gene, respectively. Chimera checking was carried out using the program Bellerophon v3.0 from the Greengenes website to check the bacterial 16S rRNA genes [51]. Meanwhile Abundance-based coverage estimator (ACE), Shannon, Simpson and rarefaction analysis were performed using DOTUR [52]. One representative clone was selected from each OTU for further phylogenetic analysis. The nearest relatives of each sequence were obtained from the GenBank database using the blastn tool (http://blast.ncbi.nlm.nih.gov/). The 16S rRNA gene sequence was used for the BLAST analysis of bacterial diversity and the deduced amino acid sequence was used for the BLAST analysis of ureC gene. The unrooted phylogenetic tree was constructed using Clustal X 2.0 and Mega 5.0. At the same time, all 16S rRNA gene sequences were classified using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/index.jsp) classifier with a confidence threshold of 70%.

Nucleotide Sequence Accession Numbers

The gene sequences were deposited in the GenBank database under the accession numbers: JQ359624-JQ359642 (ureC gene in genomic DNA library), KC152840-KC152847 (ureC gene in cDNA library), JQ359612-JQ359623 (16S rRNA gene library), KC763777 (16S rRNA gene of Marinobacter litoralis).

Results

The Urea Utilization by Sponge X. testudinaria

The urea utilization test of sponge X. testudinaria, the color of the agar plate which was coated with sponge slurry changed from yellowish to pink within several hours (Figure 1A), the color of the negative control didn’t change (Figure 1B). Apparently, the homogenates of the sponge contained urease which might be produced by sponge itself or its associated microorganisms. In the former study, a bacterium Marinobacter litoralis, which was isolated from the sponge X. testudinaria, showed similar color change in the agar plate (Figure 1C). M. litoralis was also tested to be urease-positive by Yoon et al. [53]. Thus, it is suggested that sponge-associated bacteria may participate in the urea hydrolysis. In order to reveal the diversity of ureC gene of the bacterial symbionts in X. testudinaria, the bacterial ureC gene was analyzed.

The Detection of ureC Gene and its Expression in Sponge X. testudinaria

After the ureC gene was successfully amplified from the total genomic DNA and cDNA, two clone libraries of ureC genes were constructed, respectively named ureC-D library (genomic DNA library) and ureC-R library (cDNA library). The coverage ratios of ureC-D and ureC-R libraries were 84.61% and 88.16%, respectively (Figure 2), suggesting that the sequencing of clones was nearly saturated.

A total of sixty five positive clones were randomly selected from the ureC-D library for sequencing. Finally, 19 OTUs were obtained at the 10% dissimilarity level. BLAST analysis using the deduced amino acid sequence and phylogenetic analysis showed that the most similar reference sequences were from Proteobacteria, Cyanobacteria, and Actinobacteria (Figure 3).

In the ureC-R library, a total of forty five positive clones were randomly selected for sequencing, resulting in 8 OTUs. In particular, six OTUs were common to both the genomic DNA library and the cDNA library: ureC-D6(3) and ureC-R6(3), ureC-D7(13) and ureC-R3(2), ureC-D10(2) and ureC-R4(1), ureC-D17(7) and ureC-R5(1), ureC-D10(2) and ureC-R7(2), ureC-D19(2) and ureC-R2(1)). Therefore, RT-PCR results showed that some of the sponge-associated ureC genes were metabolically active. BLAST analysis showed that these active ureC genes were of diverse microbial origin, suggesting various groups of sponge-associated microbes might participate in the urea transformation for sponge host. The finding of phylogenetically diverse bacterial urease genes and their expression strongly suggested the bacterial role in the urea utilization of sponge X. testudinaria.

Phylogenetic Diversity of Bacteria in Sponges

Fifty nine clones from the 16S rRNA gene library were sequenced successfully. At the 3% dissimilarity level, these sequences could be divided into 12 OTUs. Rarefaction analysis indicated that the bacterial library well represented the microbial communities because the rarefaction curve was approaching plateau. The Shannon and ACE richness estimators predicted 16.03 and 12.58 OTUs at the species level for sponge X. testudinaria. The Shannon and ACE richness estimators predicted nearly one half sequences had a very low similarity (≤94%) with related sequences in GenBank indicating the novelty of bacteria in the sponge X. testudinaria from the South China Sea. All bacterial sequences were classified using the RDP classifier with a confidence threshold of 70%.

The predominant bacterial phylum observed in sponge X. testudinaria was Proteobacteria, including Alphaproteobacteria (28%), Deltaproteobacteria (68%), and Gammaproteobacteria (4%). The second abundant phylum was Gemmatimonadetes (27%). The remaining belonged to Acidobacteria (14%), Actinobacteria (3%), Chloroflexi (10%), Nitrospira (2%) and Lentisphaerae (2%) (Figure 5).

Discussion

Marine sponge X. testudinaria and M. litoralis which was isolated from the sponge can transfer urea into ammonia, which suggests the potential for urea utilization by sponge symbionts. This suggestion was supported by the detection of bacterial ureC gene
and its expression in this sponge. Among the observed 19 OTUs of ureC genes observed in the ureC-D library, only 6 OTUs were detected in the ureC-R library, suggesting that not all the ureC genes were active. In contrast, two OTUs, ureC-R1 which was closely related to Staphylococcus epidermidis VCU071, and ureC-R8 which was closely related to Mycobacterium tusciae JS617, were unique in cDNA library. It was probably due to the incomplete sequencing of clones in the two libraries. Unexpectedly, in ureC-D library, we also acquired one ureC gene which was most similar to Cenarchaeum symbiosum A. Previous study showed that Thaumarchaeota from arctic deep waters had high abundance of urease genes [55]. This finding indicated that archaea might also play an important part in urea utilization.

Montalvo et al. [54] analyzed the bacterial diversity of X. testudinaria from the Manado Bay using gene library-based method, and found that Chloroflexi, Acidobacteria, Actinobacteria and Deltaproteobacteria were dominant in the bacterial community. Using 454 pyrosequencing, Lee et al. [30] revealed that Proteobacteria, Firmicutes and Chloroflexi were predominant bacterial phyla in X. testudinaria from the Red Sea. In this study, the bacterial community in X. testudinaria from the South China Sea was found to be composed of Proteobacteria (42%), Gemmatimonadetes (27%), Acidobacteria (14%), Chloroflexi (10%), Actinobacteria (3%), Nitrospira (2%) and Lentisphaerae (2%). As Montalvo et al. [54], dominant Deltaproteobacteria (68%), Chloroflexi, Acidobacteria and Actinobacteria were observed in X. testudinaria, and meanwhile as Lee et al. [30], Proteobacteria and Chloroflexi were detected. Previous studies concluded that uniform microbial community occurred in sponges from different oceans [56–59]. According to the comparison above, though the sponge X. testudinaria species were collected from

Figure 1. Urease activity of marine sponge X. testudinaria. A: negative control; B: activity of sponge slurry; C: Marinobacter litoralis (bacterium isolated from the sponge).
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Figure 2. Rarefaction curves of ureC and 16S rRNA gene sequences. (Clusterization stringency at 90% and 97% for ureC and 16S rRNA gene, respectively.).
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Figure 3. Unrooted phylogenetic tree based on urease alpha subunit (130aa) of sponge X. testudinaria using Neighbour-joining method. The scale bar represents 0.05 substitutions per amino acids position. Bootstrap values (1,000 replicates) higher than 50% are shown. ○ mark and ureC-D mean the OTU in genomic DNA library, and ●mark and ureC-R mean the OTU in cDNA library. The number inside the parenthesis means the number of sequences within each OTU.

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Figure 4. Unrooted phylogenetic tree based on bacterial 16S rRNA gene sequences (ca.1,400bp) of sponge *X. testudinaria* using Neighbour-joining method. The scale bar represents 0.05 substitutions per nucleotide position. Bootstrap values (1,000 replicates) higher than 50% are shown. The number in brackets shows the number of sequences in each OTU. ● mark means sequences from sponge *X. testudinaria*. 

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different ocean areas, it had similar predominant bacterial groups, i.e. Proteobacteria, Chloroflexi, Acidobacteria and Actinobacteria might represent its core bacterial community. This is supported by Figure 4, where X. testudinaria-derived 16S rRNA gene sequences were clustered together suggesting some bacteria are X. testudinaria specific. Besides, this study, together with that of Lee et al. [30], Montalvo et al. [54], and Schmitt et al. [31], suggested the sponge species-specific bacterial components which might come from the different marine environment, since sponge microbial symbionts might be acquired by both horizontal transfer and vertical transmission approaches [26,28].

The phylogenetic tree based on ureC genes indicated that Proteobacteria, Cyanobacteria, Actinobacteria and Crenarchaeota might produce urease in the sponge X. testudinaria. However, according to the phylogenetic analysis of 16S rRNA genes, the dominant bacterial phyla existing in the sponge X. testudinaria were Proteobacteria, Gemmatimonadetes, Acidobacteria and Chloroflexi. Apparently, 16S rRNA-based phylogeny showed partial congruence to the ureC-based phylogeny. Probably, the discrepancy may be due to events of horizontal transfer of ureC among ureolytic bacterial species. Investigating the 16S rRNA gene can reveal comprehensive community structure of sponge-associated bacteria, but is not a good approach for ecological investigation of ureolytic bacterial species, while the ureC gene can provide a better estimation of bacteria with urea utilization potential. So, integrated approach combining the 16S rRNA gene (phylogenetic marker) and ureC gene (functional marker), should be more accurate in the analysis of ureolytic bacteria.

Most of the marine sponges live in coral reefs ecological system, where it is generally limited by nitrogen. The nickel metalloenzyme urease catalyses the hydrolysis of urea to ammonia and carbamate, and thus generates the preferred nitrogen source of many organisms. Urease can be produced by diverse bacterial species [60]. Urea in sponges may come from the sponge host excretion, seawater, bacterial degradation of nucleic and amino acids, and is therefore a possible product to be encountered in the sponge mesohyl. Only the potential of sponge-associated bacteria in urea utilization was suggested by genome analysis before this study [38]. In this study, the plate assay and phylogenetic analysis suggested the function of sponge bacterial symbionts in urea utilization. This study provided the first insight into the bacterial potential in urea utilization by detecting the transcriptional activity of ureC gene as well as the phylogenetic diversity of bacteria with ureC gene.

Author Contributions

Conceived and designed the experiments: JS LJ QJ ZL. Performed the experiments: JS LJ. Analyzed the data: JS LJ ZL. Contributed reagents/materials/analysis tools: WS FZ. Wrote the paper: JS ZL.

Figure 5. Pie charts illustrating the bacterial community based on 16S rRNA gene libraries of sponge X. testudinaria. Sequences in libraries were classified using the Classifier Tool provided by the Ribosomal Database Project. Hierarchical taxa assignment was based on RDP naive Bayesian rRNA Classifier. Percentage represented specific value between the number of clones in each taxon and the number of all clones in 16S rRNA gene library.

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