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*Full Length Research Paper*

# Analysis of bacteria associated with *Acropora solitaryensis* by culture-dependent and -independent methods

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**Diversity of bacteria associated with *Acropora solitaryensis*, the main species in Hermatypic corals of XiSha Island, was investigated using culture-dependent and culture-independent methods (denature gradient gel electrophoresis, DGGE). It shows rich diversity of coral-associated bacteria with abundant novel species or genus. However, the diversity gained by the two methods was different. Among the bacteria identified by DGGE, XSLJ4 (*Psychrobacter sp. KOPRI 25503*), XSLJ6 (*Rhizobium* sp.), XSLJ11, (Uncultured Pseudomonadales), XSLJ12 (*Ochrobactrum* sp. *Yw28*) and XSLJ 13 (*Ochrobactrum* sp. *B10B*）are the predominant species, while the vibrios were the predominant ones identified by bacterial culture method.**

**Key words:** *Acropora solitaryensis*, diversity of bacterium denature gradient gel electrophoresis (DGGE).

## INTRODUCTION

Coral reefs, known as the oasis in desert and tropical rainforest in sea for its biodiversity and high productivity (Bjornsen et al., 1991; Rohwer et al., 2002; Rosenberg et al., 2007). However, due to the global warming and human activity, survival of coral reefs are faced with serious threat (Rosenberg et al., 2002, Luna et al., 2007). Wilkinson (2008) reported that, by 2008, the area of global coral reefs has been reduced by 19%, and nearly 35% of coral reefs were in emergency or dangerous state. It is controversial how this global destruction of coral reefs occurred, but more and more evidence has pointed to the micro-organisms associated with coral reefs (Kushmaro et al., 1998; Ben-Haim et al., 1999, 2003; Barash et al., 2005). As the largest creature of the world, coral reef is a giant symbiont called “holobiont” which contained large, diverse and specific population of microorganisms (Siboni et al., 2008; Thurber et al., 2009). In addition to the algae of zooxanthellae, bacteria, archaea and eukaryote as well as viruses are all involved in construction of the holobiont. Among them, diverse bacteria are extensively distributed in the coral reefs in a species specific, tissue specific and location unspecific manner (Gast et al., 1998). Moreover, over 99% of bacteria in the coral reef are unculturable and many novel species were identified, but the roles of these bacteria in coral health and disease are largely unknown (Rohwer et al., 2001). It has become an intriguing topic for the microbiologists and substantial progress has been made in recent years.

This paper attempts to use culture-dependant and culture-independent (DGGE) techniques to explore and analyse bacteria diversity of *Acropora solitaryensis,* that is, the main species in Hermatypic corals of XiSha, laying the foundation for the future research of coral bacterial diseases.

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## MATERIALS AND METHODS

### Sampling

Staghorn coral (*Acropora solitaryensis*) were collected from 8 colonies in Yongxing isle, Hainan province, China (Figure 1). Ca 0.5 cm fragment was grinded with sterilized mortar and pestle and suspended in 2 ml PBS buffer followed by centrifugation at 3000 rpm for 4 min to remove the big debris. 100 µl of the supernatant was spread on the 2216E plates and incubated at 30°C overnight.

### Identification of the culturable bacterium

The bacteria growing on the 2216E plates were purified and identified by 16S rDNA sequencing. Briefly, the bacteria grow in the 2216E medium and genomic DNA was prepared using a Bacteria DNA Extraction kit (Takara, Dalian, China). The fragment of 16S rDNA for sequencing was amplified with the pair of primers 63F(CAGGCCTAACACATGCAAGTC) and 1389R(ACGGGCGGTGTGTACAAG) in a 25 ul PCR mixture containing 10x PCR Buffer 2.5 ul, dNTP (each, 2.5mM) 2.0 ul, each primer (20 uM) 0.25 ul, rTaq (5 U/ul) 0.25 ul and 1 ul of purified DNA. PCR was performed with an initial denaturation of 95°C for 5 min; followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min;. The PCR products were separated in 1.5% agarose gel and documented with image viewer. (Bio-rad, USA). 16S rDNAs were sequenced by Invitrogen (Guangzhou, China), and the sequences were aligned with Blastn in NCBI website.

### DNA preparation for denature gradient gel electrophoresis (DGGE)

Total DNA was extracted using a modified method referred to by Zhou et al. (1996) following the standard phenol/chloroform extractions. (i) 5 g coral sample was grinded with mortar and pestle in liquid $N_2$. (ii) 13.5 ml DNA lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM $Na_2EDTA$, 100 mM $Na_3PO_4$, 1.5 M NaCl, 1% Cetyltrimethyl ammonium Bromide[CTAB],) and lysozyme(final concentration: 1mg/ml) was added and shaked at 240 rpm for 30 min at 37°C, followed by addition of proteinase K (final concentration: 0.2 mg/ml) and shaked for 20 min. (iii) 100 ul of SDS (20%) was added in the tubes which were incubated in the water bath tank at 65°C for 2 h, mixing the tubes every 15 to 20 min gently. Then centrifuged at 6,000 rpm for 10 min to remove the debris. (iv) The supernatant was extracted with equal volume of phenol-chloroform (24:1) twice and 0.1 volume of NaAC (pH=5.2) and 0.6 volume of isopropanol was used to precipitate the DNA at -20°C for at least 1 hr, followed by centrifugation at 14,000 g for 10 min. The DNA was washed twice with 70% cold ethanol and dissolved in 80 ul sterile water.

### Amplification of 16S rDNA for denature gradient gel electrophoresis (DGGE) analysis

Two rounds of PCR amplification for the variable region of 16S rDNA were performed. The first round was carried out as described above except with 15 cycles, then continued the second round of PCR with the same volume and the primers are GC-341f(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and 534r (ATTACCGCGGCTGCTGG) (Muyzer et al., 1993). Touch-down PCR (Davies et al., 2004) was performed with an initial denaturation step of 95°C for 5 min; followed by 20 cycles at 94°C for 1 min, 65°C (-0.5°C per cycle) for 45 s, and 72°C for 1 min; followed by 10 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. The PCR products were separated in 1.5% agarose gel stained with goldview and visualized using the gel Chem. Doc (Bio-Rad, USA).

### Denature gradient gel electrophoresis (DGGE)

DGGE was done by using the Bio-Rad D-CODE system. 6% acrylamide gel was prepared with the range of gradient from 30 to 50% (100% denaturants gels defined as 7 M urea and 40% deionized formamide) and stayed at room temperature for at least 7 h before use. Twenty micro liter PCR products which have been mixed with 2×loading buffer (Takara, Dalian, China) were loaded in each well. And the tank was filled with 7L 0.5× TAE as running buffer. The electrophoresis was performed at the constant voltage of 80V at 60°C for 18 h. Gels were stained with EB (Sigma) diluted in 0.5× TAE buffer (1:10,000) for 30 min and visualized by using the Gel Doc system (Bio-Rad, USA). The prominent bands on the DGGE gel were excised by using sterile scalpel blades, washed with 200 ul ultra-pure water twice and soaked in 50 ul ultra-pure water at 4°C overnight. The supernatant was harvested with centrifugation for 10 min at 12,000 rpm at 4°C. 0.5 µl of the supernatant was re-amplified with the set of primers 534r and 341f-nc which has the same sequence as 341f but containing no GC clamp as described above. The PCR products was purfied and sequenced. The sequences were aligned with Blastn. Two bands which share the identity by more than 97% were considered to be the same phylotype (Stackebrandt et al., 1994), Mega 4.1 was employed to construct the phylogenetic trees based on neighbor-joining algorithm (Saitou et al., 1987). Bootstrap analysis with 1,000 replicated was applied to assign confidence levels to the nodes in the trees.

## RESULTS

### Bactrial communities based on culturale technique

Less than 20 bacterial colonies from each sample grow on the 2216E plates which are incubated for more than 2 days. 9 isolates were selected according to their colony morphology. Based on their sequences of 16S rDNA, one strain was identified as *Halobacillus* sp. (100% identity with *H.* sp) and one strain was *Alteromonas* sp. (with 99% identity). 7 strains were identifed as *vibrio* sp. It is suprising that only few colonies were isolated from all the samples, indicating that the most of the bacteria associated with coral are unculturable under high concentration of nuitritions, and the predominant heterotrophic bacteria are vibrios.

### Bactrial communities based on denature gradient gel electrophoresis (DGGE)

Before DGGE analysis, PCR products were checked on 1.5% agarose gel. The fragments about 250 bp are expected (data not shown). DGGE analysis with a denaturing gradient from 35 to 50% showed good resolution and separation. More than 30 bands are obtained and the predominant bands are concomitant in all three samples (Figure 2). Fifteen bands that were clear and intensive were excised from acrylamide gel, re-amplified and subjected to sequencing. The sequences from 14 bands were successfully determined. As shown

**Figure 1.** Overview the Yongxing isle. The mark represents the sampling site.

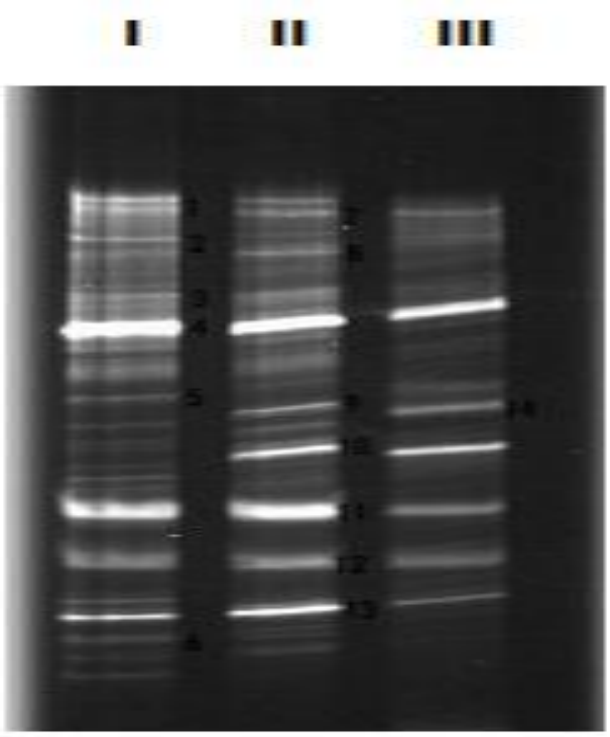


**Figure 2.** The DGGE pattern of 16SrDNA-V3 of three *Acropora solitaryensis*. I,II,III separately represent of three differernt *Acropora solitaryensis,* 1-14 represent the bands that were excised.

in Table 1, all bands are belonged to proteobacteria. Among them, 9 are Gamma-proteobacteria. 2 (XSLJ12 and XSLJ13) are alpha-proteobacteria and 3 (XSLJ6, XSLJ9 and XSLJ14) are unclassified_"Proteobacteria". Most of the sequences show identities with 16S rDNA of uncultured bacterium clone from marine environmental samples. Interestingly, none of the sequences is attributed to a defined species. The most intensive band XSLJ4 is identified as a bacterium that belongs to *Psychrobacter*. Bands XSLJ 2 and XSLJ 3 are also related to this genus. Bands XSLJ 12 and XSLJ 13 which are the second intensive bands are similar to the genus *Ochrobactrum*. Band XSLJ 6 is close to *Rhizobium* sp. *3C6-41.* The phylogenetic relationships of all the 14 sequenced bands are shown in Figure 3. It clearly shows that these sequences are generally aligned into 3 clades. XSLJ2-4 is attributed to one group which is represented by culturable bacteria *Psychrobacter.* XSLJ1, 5, 7-11 and 14 gather into a group which has close relationship with uncultured bacterium clones. XSLJ6, 12, 13 form a clade which is represented by *Rhizobium* sp. and *Ochrobactrum* sp.

## DISCUSSION

As the result shows, the diversity of bacteria associated with *Acropora solitaryensis* determined by the method of culture-dependant or culture-independent exhibited great discrepancy. A very few bacteria colony and species were identified by culture on 2216E marine agar, suggesting that a large portion of bacteria associated with healthy *Acropora solitaryensis* are unable to be cultured under the experimental conditions. Interestingly, each method identified a distinctive group of species, and the most abundant one identified by both approaches are different, in agreement on that in many marine habitats, the most abundant microbial phylotypes have no close relationship with that have been cultured.

With the culture-dependant method, three different bacterium genuses were identified. Among them, vibrios accounted for nearly 75% (7 out of 9 strains). It implied that vibrios are the dominant heterotrophic bacteria species in the niche of coral reef, which is supported by the similar results found in other coral species (Bourne et al., 2005; Ritchie, 2006). However, compared with the coastal area, the concentration of vibrios isolated from the deep sea coral is at a low level (Penn et al., 2006). Interestingly, two of the vibrio species, *Vibrio shiloi* and *Vibrio corallyticus*, have been demonstrated to infect the corals and cause pandemic coral bleaching (Reshef et al., 2006). It might be possible that the vibrios are able to be evolved in a pathogen under the stress conditions.

On the contrary, the diversity of bacteria identified by DGGE is much more complex, and most of the bacteria are close to uncultured species, moreover, the identified species are novel genus or species. As suggested by Rohwer et al. (2002), 97% of the identity of 16S rDNA is used as the objective boundary for species circumscription and the identity between 97 and 93% is considered as the boundary of genus. According to this

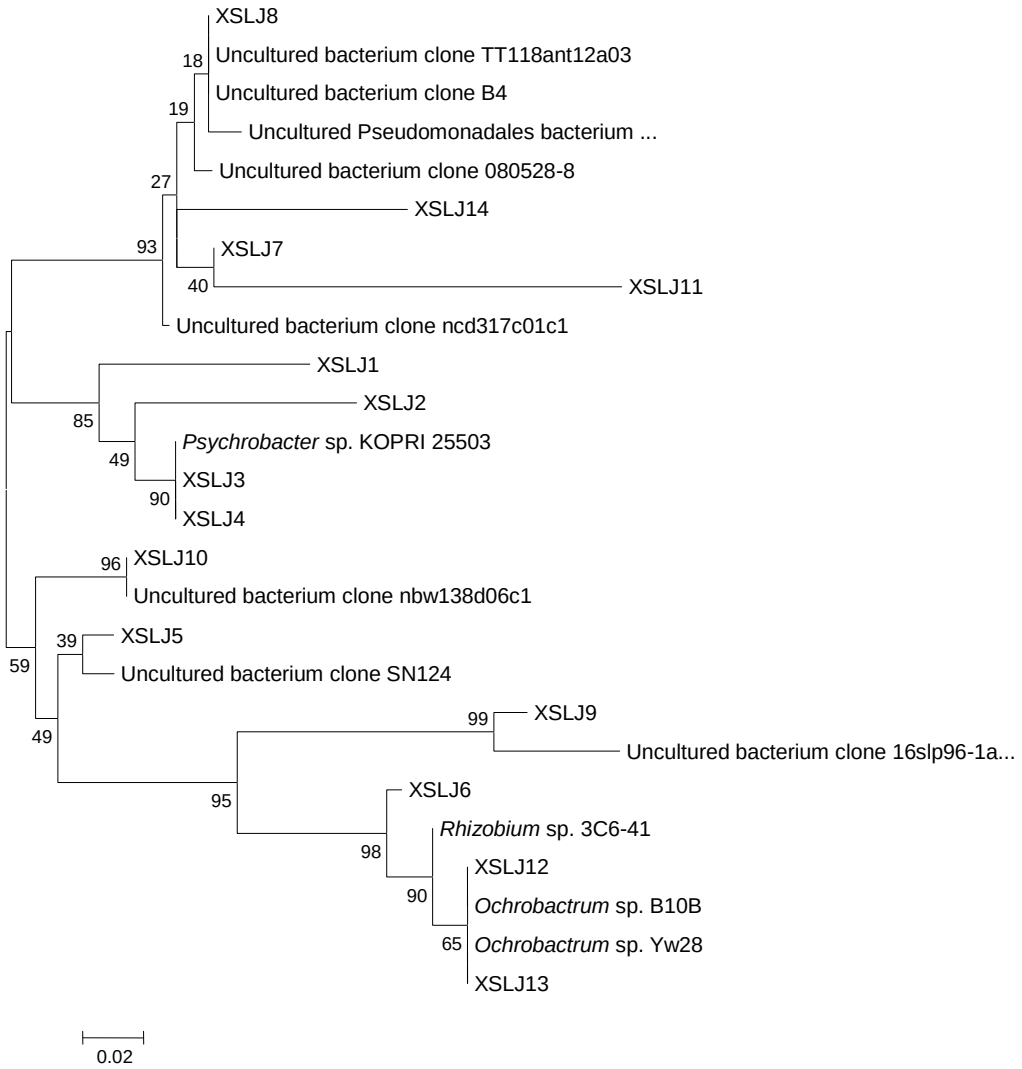


**Figure 3.** Phylogenetic tree constructed based on bacterial 16S rDNA V3 region fragments from bacterium associtated with *Acropora solitaryensis*. The trees were drawn from ClustalW generated multiple sequence alignment of nucleotide sequences using the neighbor-joining method within the MEGA (4.1) package.

**Table 1.** Blast of the V3 region sequence of 16S rDNA of bands excised.

| S/N | Name | Taxon | The closest relatives | Similarity (%) |
|---|---|---|---|---|
| 1 | XSLJ1 | Gammaproteobacteria | Uncultured bacterium clone 080528-8 | 91 |
| 2 | XSLJ2 | Gammaproteobacteria | *Psychrobacter sp. KOPRI 25503* | 92 |
| 3 | XSLJ3 | Gammaproteobacteria | *Psychrobacter sp. KOPRI 25504* | 100 |
| 4 | XSLJ4 | Gammaproteobacteria | *Psychrobacter sp. KOPRI 25503* | 100 |
| 5 | XSLJ5 | Gammaproteobacteria | Uncultured bacterium clone SN124 | 92 |
| 6 | XSLJ6 | unclassified_"Proteobacteria | *Rhizobium sp. 3C6-41* | 98 |
| 7 | XSLJ7 | Gammaproteobacteria | Uncultured bacterium clone ncd317c01c1 | 98 |
| 8 | XSLJ8 | Gammaproteobacteria | Uncultured bacterium clone B4 | 100 |
| 9 | XSLJ9 | unclassified_"Proteobacteria | Uncultured bacterium clone 16slp96-1a04.p1k | 93 |
| 10 | XSLJ10 | Gammaproteobacteria | Uncultured bacterium clone nbw138d06c1 | 100 |
| 11 | XSLJ11 | Gammaproteobacteria | Uncultured Pseudomonadales bacterium clone E203G05 | 89 |
| 12 | XSLJ12 | Alphaproteobacteria | *Ochrobactrum sp. Yw28* | 100 |
| 13 | XSLJ13 | Alphaproteobacteria | *Ochrobactrum sp. B10B* | 100 |
| 14 | XSLJ14 | unclassified_"Proteobacteria | Uncultured bacterium clone TT118ant12a03 | 91 |

standard, XSLJ1, XSLJ2, XSLJ5, XSLJ11 and XSLJ14 are attributed to new genus and XSLJ9 belongs to new species. As identified by DGGE, XSLJ4 which is identical to *Psychrobacter sp. KOPRI 25503* is the dominant species. It was reported that *Psychrobacter sp.* can synthesis an enzyme that can adsorb heavy metal ions such as $Hg^{2+}$ that may assist *Acropora solitaryensis* to resist the effect of external environment toxic substances(Xuejiang et al., 2010). XSLJ6 is identical to *Rhizobia sp*. These genuses of bacteria are chemoautotrophic. It may provide nutrients such as the carbon and nitrogen sources for the coral (Child, 1975). Further more, it was reported to secrete polysaccharides which could enhance the immune system of *Acropora solitaryensis* (Djordjevic et al., 1987; Xiao-bo et al., 2006; Yan-Li et al., 2010). XSLJ12 and XSLJ13 are attributed to *Ochrobactrum sp.* which belongs to pale coli genera. It often lives in the niche where there are rich phosphorus, for an instance, $Ca_3PO_4$ and phosphate minerals (Palaniappan et al., 2010). It can fix nitrogen and degrade toxic organic chemicals such as phenol as well as absorb heavy metal ions (Ozdemir et al., 2003; Ngom et al., 2004; Wei et al., 2008), this function may assist *A. solitaryensis* to adapt to harmful stress environment.

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