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# Isolation and Identification of Cultivable Microorganisms Isolated from Sea Squirt (*Ciona savignyi*) Collected from the Jiaozhou Bay, China

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**Abstract:** Sea squirt is an important source for isolation of new bacteria strains that are able to accumulate bio-active compounds. In this study, we isolated and identified of cultivable bacteria isolated from sea squirt *Ciona savignyi*, collected from the Tsingtao Port, Jiaozhou Bay, China. We investigated the phenotypic characteristics and neighbour-joining phylogenetic analysis of this isolated cultivable strains, which suggested the isolated seventeen strains mainly belong to the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* four phyla, of which twelve strains belong to *Proteobacteria*, accounted for 70.6% of total isolated stains, and respectively belonging to *Alphaproteobacteria* and *Gammaproteobacteria*. This identification and analysis of this these marine bacteria will enrich sea squirt-associated bacteria resources, from which more bio-active substances would be discovered.

**Keywords:** Cultivable Microorganisms, Phylogenetic Analysis, Marine Bacteria, Sea Squirt, Isolation And Identification

## Introduction

Marine microorganism is widely distributed, there are some strains living in the sea, some existing on the surface of the sea and some living in sediment or on the surface of mud, while some have the codependent, symbiotic and parasitic or associated relationship with marine animals or plants. Associated bacteria may play an significant role in clearing metabolic waste of the host (Wikjinson C R , 1978) and providing the bio-active substances for the host (Unson M. D. *et al*.; Schmidt E. W. *et al*.; Hentschel U. *et al*.). Because of co-evolution of the associated bacteria with the host for a long time, the associated bacteria often have special metabolic pathway, it is likely to produce new physiological active substances. Therefore many researchers have the considerable interests in the using of the associated marine microorganisms as a source of the natural biological products (Oern P.). Microorganisms have the advantages of the short growth cycle, easy controlling metabolism and easy breeding of strains, therefore, there is wide availability of using and development of the associated microorganism resources. The isolation of bio-active substances from


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associated microorganisms not only protects the environment but also reduces the cost of the commercial production.

There are lots of marine microorganisms living on the surface and in the coelom of the marine invertebrates, and they have highly specific associated relations with the marine invertebrates. At present, the researches of marine invertebrates are mainly concentrated on the sponge, sea anemones, corals and some coelenterate. In recent years, many researchers also found a large amount of novel structure and unique active compounds in the sea squirt (Ryuichi S *et a*l.; Mcdonald L. A. *et a*l.; Williams A. B *et a*l.). These novel active compounds have attracted many researchers' attention, and have gradually become hot research on marine natural products chemistry, sea squirts became the important marine biological resources to obtain significant physiological active substances for human. Because of many marine biological active compounds is produced by the associated marine microorganisms, thus isolated associated-sea squirts microbial resources and studied its phylogenetic diversity, will be able to provide new data for marine bio-active compounds.

This study we isolated and identified of cultivable bacteria isolated from sea squirt *Ciona savignyi*, we investigated the phenotypic and phylogenetic characteristics and neighbour-joining phylogenetic analysis of the isolated strains. Therefore, the identification of marine bacteria will provide the basis for further exploring more bio-active substances and marine biological community distribution.

## Materials and Methods

### Experimental sample

A sea squirt (*C. savignyi*) was collected by scuba diver in the Tsingtao Port (36°04′00″N, 120°19′05″E), Jiaozhou Bay, China, in May 2015, and was used as the source for isolation of squirt-associated bacteria.

### The separation and purification of strains

The inner content of the sea squirt was serially diluted using 0.9% (w/v) NaCl, and then were spread on marine 2216E agar (per litre seawater: 5 g peptone, 1 g, yeast extract, 0.1 g ferric phosphate, 15 g agar; pH 7.6-7.8). The cultivable strains were isolated after 10 days of incubation and was cultivated routinely on MA at 28 °C. Culture purity was confirmed by homogeneity of the cell and colony morphologies and then observed and recorded the colony characteristics of the marine bacteria isolated from *Ciona savignyi*. Stored the isolated strains at -80 °C supplemented with 50% (v/v) glycerol.

### The 16S rRNA gene sequences alignment

For DNA extraction, cell biomass of strains was obtained from cultures grown in marine broth 2216 (per litre seawater: 5 g peptone, 1 g,yeast extract, 0.1 g ferric phosphate; pH 7.6-7.8) at 28 °C for 24h. Chromosomal DNA was extracted and purified according to standard methods (Ausubel *et al*., 1995). The 16S rRNA gene was amplified by PCR with two universal primers (27F,5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTTACGACTT-3'). The components and condition of PCR reaction see Table 1, 2. After purification using an E.Z.N.A gel extraction kit (OMEGA Biotech), the amplified 16S rRNA gene was ligated into the pMD19-T (TaKaRa Clontech), which was further sequenced at TsingKe Biotech (Qingdao, China). Sequence homology values between the 16S rRNA gene from isolated strains and closely related type strains were analysed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al*., 2012).

**Table 1 Components of PCR reaction**

| Components | Volume（μl） |
|---|---|
| 2 × taq PCR MasterMix（TIANGEN Biotech Beijing） | 50 |
| Primer 27F | 5 |
| Primer1492R | 5 |
| DNA | 5 |
| $ddH_2O$ | 35 |
| total | 100 |

**Table 2 Condition of PCR reaction**

| Reaction | Condition | |
|---|---|---|
| Pre-Denaturation | 95 °C 5 min | |
| Denaturation | 95 °C 30 s | 25 cycles |
| Annealing | 56 °C 30 s | |
| Extension | 72 °C 90 s | |
| Post-Extension | 72 °C 5 min | |

**Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequence**

Multiple alignments of the 16S rRNA gene sequence of isolated strains with those of its related strains were performed using the CLUSTAL_X (Thompson *et al*., 1997). Phylogenetic trees were constructed by the neighbour-joining (N-J) (Saitou & Nei, 1987), algorithms using MEGA 6.06 (Tamura *et al*., 2013), and were analyzed using bootstrapping (Felsenstein, 1985) based on 1000 re-samplings.

**Results and Discussion**

**Morphology characteristics of the isolated strains**

We obtained cultivable bacteria isolated from sea squirt (*Ciona savignyi*). They have various morphology characteristics (Table 3) and was distinguished by color shape, height, uniformity, surface, humidity, transparency. Most of the stains were roundness, dimpling, orderliness, smooth, humid and opacity.

**Table 3 The colony characteristics of the marine bacteria isolated from *Ciona savignyi***

| NO. | Color | Shape | Height | Uniformity | Surface | Humidity | Transparency |
|---|---|---|---|---|---|---|---|
| H-1 | Cream white | Roundness | Embossment | Orderliness | Smooth | Humid | Opacity |
| H-3 | Cream white | Roundness | Embossment | Orderliness | Smooth | Wet | Opacity |
| H-4 | Orange | Roundness | Dimpling | Orderliness | Smooth | Humid | Opacity |
| H-6 | Yellow | Roundness | Dimpling | Orderliness | Level | Humid | Opacity |
| H-7 | Orange | Random | Embossment | Irregular | Smooth | Humid | Opacity |
| H-8 | White | Roundness | Flat | Irregular | Unfairness | Middle | Translucent |
| H-9 | White | Random | Flat | Irregular | Level | Middle | Opacity |
| H-10 | brownish | Oval | Blowup | Orderliness | Smooth | Wet | Opacity |
| H-11 | Saffron yellow | Roundness | Embossment | Orderliness | Smooth | Humid | Opacity |
| H-12 | Yellowish white | Roundness | Embossment | Orderliness | Smooth | Humid | Opacity |
| H-15 | Cream white | Roundness | Dimpling | Orderliness | Smooth | Wet | Opacity |
| H-16 | Gray | Roundness | Flat | Irregular | Unfairness | Dry | Opacity |
| H-17 | Yellowish white | Roundness | Dimpling | Orderliness | Level | Middle | Opacity |
| H-18 | Roundness | Roundness | Blowup | Orderliness | Smooth | Humid | Opacity |
| H-19 | White | Oval | Embossment | Orderliness | Smooth | Humid | Opacity |
| H-20 | Saffron | Roundness | Embossment | Orderliness | Smooth | Humid | Opacity |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| H-22 | yellow Saffron yellow | Roundness | Embossment | Orderliness | Smooth | Wet | Opacity |

**The 16S rRNA gene sequences alignment**

We obtained seventeen cultivable bacteria isolated from *Ciona savignyi*. The agarose gel electrophoresis results of PCR products of 16S rRNA gene as shown in Figure 1, gene fragment size is about 1500 b.The results of 16S rRNA gene sequences alignment of the isolated cultivable stains see the Table 4.

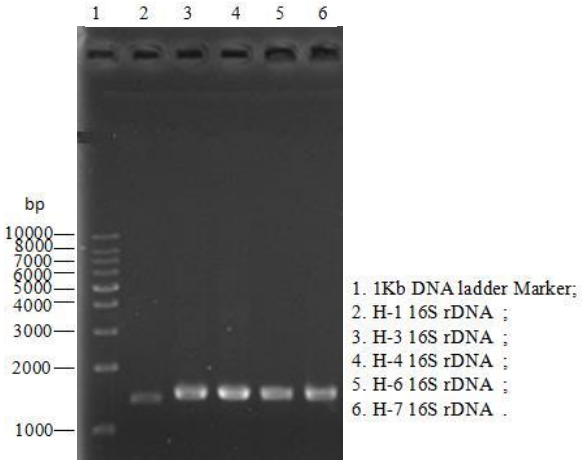


**Figure 1** The agarose gel electrophoresis results of PCR products of 16S rRNA gene

**Table 4** The 16S rRNA gene sequences alignment results of the cultivable bacteria isolated from *Ciona savignyi*

| Phylogenetic groups | NO. | Closest type strain | Similarity(%) |
|---|---|---|---|
| *Proteobacteria*; | H-9 | *Sulfitobacter pontiacus* DSM 10014$^T$ | 99.78 |
| *Alphaproteobacteria* | H-10 | *Phaeobacter inhibens* DSM 16374$^T$ | 99.93 |
| | H-12 | *Amylibacter marinus* 2-3$^T$ | 95.33 |
| | H-25 | *Pseudophaeobacter arcticus* DSM 23566$^T$ | 99.21 |
| | H-18 | *Celeribacter halophilus* ZXM137$^T$ | 98.82 |
| *Proteobacteria*; | H-1 | *Pseudoalteromonas marina* Mano4$^T$ | 99.71 |
| *Gammaproteobacteria* | H-3 | *Vibrio atlanticus* Vb 11.11$^T$ | 99.93 |
| | H-8 | *Vibrio lentus* 4OM4$^T$ | 99.39 |
| | H-11 | *Pseudoalteromonas tetraodonis* IAM 14160$^T$ | 99.38 |
| | H-19 | *Marinomonas aquimarina* CECT 5080$^T$ | 98.22 |
| | H-20 | *Psychrosphaera saromensis* SA4-48$^T$ | 98.48 |
| | H-22 | *Shewanella piezotolerans* WP3$^T$ | 99.67 |
| *Bacteroidetes*; | H-4 | *Polaribacter reichenbachii* 6Alg 8$^T$ | 98.06 |
| *Flavobacteria* | H-6 | *Tenacibaculum soleae* LL04 12.1.7$^T$ | 99.05 |
| | H-7 | *Winogradskyella crassostreae* TYO-19$^T$ | 100.00 |
| *Actinobacteria*; | H-16 | *Streptomyces harbinensis* NEAU-Da3$^T$ | 99.72 |
| *Actinobacteria_c*; | H-17 | *Kocuria palustris* DSM 11925$^T$ | 100.00 |

**Neighbor-joining phylogenetic analysis based on 16S rRNA gene sequences**

The isolated seventeen strains mainly belong to the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* four phyla, of which twelve strains belong to *Proteobacteria*, accounted for 70.6% of total isolated

stains, and respectively belonging to *Alphaproteobacteria* and *Gammaproteobacteria* (Figure 2). *Proteobacteria* is one of the largest groups of bacteria (Bergey, 2003), and this is according to our study. In previous study found that *Alphaproteobacteria* have the advantage on the amount and type, while there are some important species have been found in *Gammaproteobacteria* such as genus *Pseudomonas* and *Vibrios*. In addition, we also isolated strains within the class *Flavobacteria* and *Actinobacteria_c*.

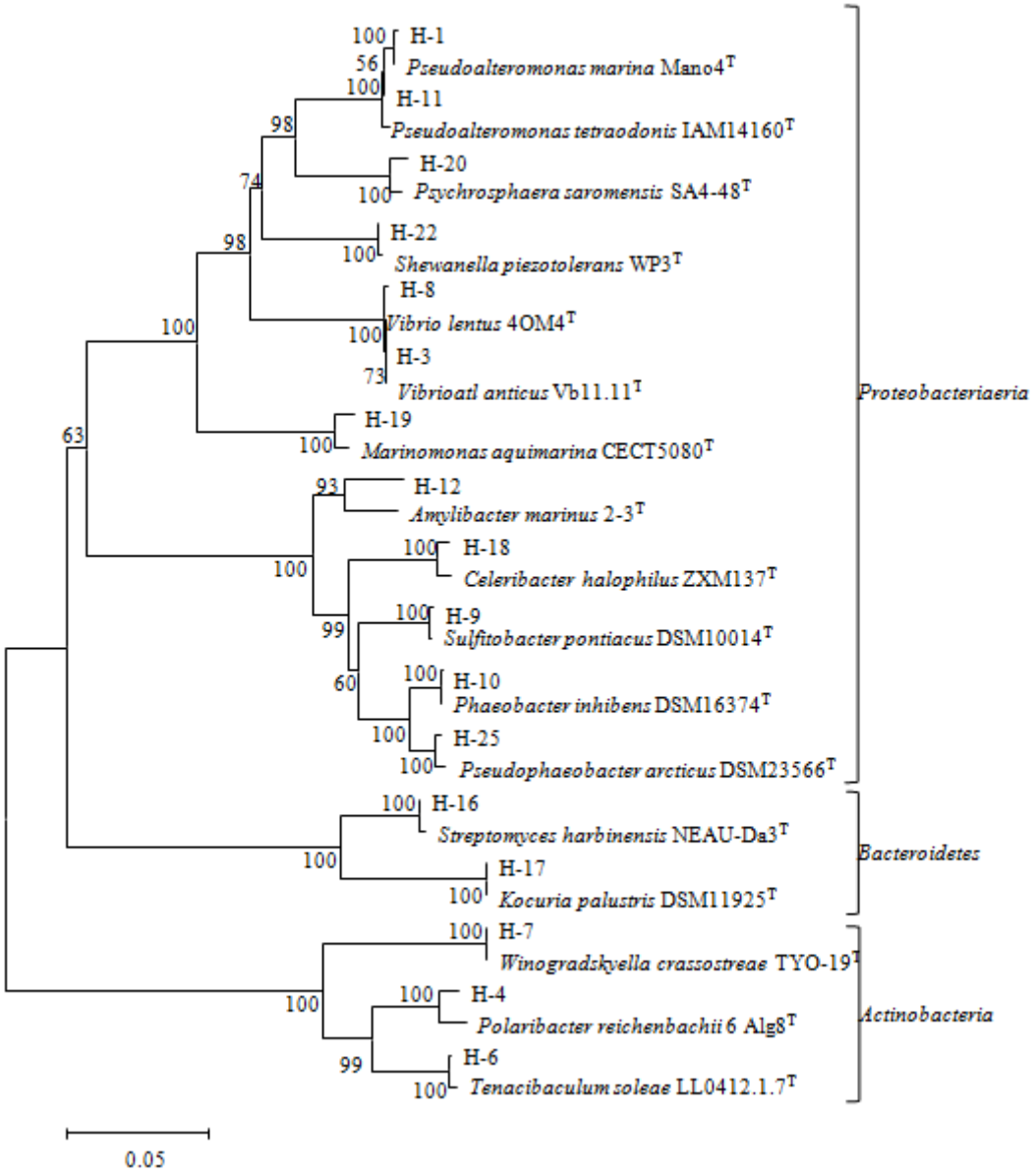


**Figure 2** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the cultivable bacteria isolated from *Ciona savignyi*

**Conclusion**

This study we isolated and identified of cultivable bacteria isolated from sea squirt *Ciona savignyi*, collected from the Tsingtao Port, Jiaozhou Bay, China. We investigated the phenotypic and phylogenetic characteristics and neighbour-joining phylogenetic analysis of this isolated seventeen cultivable strains, which suggested the isolated seventeen strains mainly

belong to the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* four phyla, of which twelve strains belong to *Proteobacteria*, accounted for 70.6% of total isolated stains, and respectively belonging to *Alphaproteobacteria* and *Gammaproteobacteria*. The identification of marine bacteria will provide the basis for further studying the marine biological active compounds and exploring marine biological community distribution.

**Acknowledgment**

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