Research Article

Isolation and Identification of Acholeplasma sp. from the Mud Crab, Scylla serrata

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For the first time, a mollicute-like organism (MLO) was cultured from moribund mud crabs (Scylla serrata) during an outbreak of clearwater disease in Zhejiang Province, China. The MLO displayed a fried-egg colony morphology in culture, did not possess a cell wall, and was not retained by 0.45 μm and 0.2 μm filters. It was able to ferment glucose, sucrose, lactose, and maltose, but it did not utilize arginine and urea. The MLO grew in the absence of bovine serum and was not susceptible to digitonin. Sequence analysis of the 16S rRNA gene revealed that this MLO had 99% identity with Acholeplasma laidlawii PG-8A, which indicates that the organism isolated from mud crabs is a member of the genus Acholeplasma.

1. Introduction

The class Mollicutes represents a unique category of bacteria, the members of which are characterized by a small cell size, the absence of a cell wall, a reduced genome, and a simplified metabolic pathway [1]. They can be pathogenic or saprophytic and commensal [2]. To date, mollicutes have been observed and identified in many vertebrate, insect, and plant hosts [2]. Mollicutes also have been reported from several aquatic animals, such as fish [3], shrimp [4–8], crab [9], oyster [10], crayfish [11, 12], and bryozoan [13, 14]. However, mollicutes of aquatic animals, especially those of crustaceans, have not been studied extensively. Only a few mollicutes associated with crustaceans have been isolated, purified, and had their taxonomic status confirmed [4, 9].

The mud crab, Scylla serrata (Forska), traditionally called the green crab, is an economically important marine species cultured in the Chinese provinces of Zhejiang, Fujian, Guangdong, Guangxi, and Hainan. Since the 1990s, the S. serrata aquaculture industry has experienced rapid growth. However, the industry also is facing increasing economic losses caused by the outbreak of various diseases, such as sleeping disease (SD) [15] and milk disease [16]. In 2005, an epidemic of clearwater disease (CD) broke out in Zhejiang Province. The symptoms of this disease included debility, weak grip strength of pincers, hydroabdomen, white carapace, drying of gill filaments, and weak blood coagulation capacity. The estimated mortality at the affected farms was ~80%. Mollicute-like organisms (MLOs) together with reo-like viruses (unpublished data) have been implicated as causes of CD. However, the MLO has not been isolated and cultivated, thus the precise taxonomic status and pathogenesis of the MLO in S. serrata have been unclear.

In this study, the MLO from mud crabs showing signs of CD was isolated and cultivated. The taxonomic classification of this organism was determined by morphology, physiological properties, and DNA analysis, and its pathogenesis was investigated.

2. Materials and Methods

2.1. Mud Crab. Two male and three female moribund or dead mud crabs with CD were obtained from a pond of a mud crab farm during the CD outbreak in August 2005 in Sanmen County, Zhejiang Province. Using electron microscopy, two different organisms were detected in the five crabs: reo-like viruses and MLOs (unpublished data).

2.2. Culture. In a previous study, we found that the MLO was present mainly in the epithelium of gill cells (unpublished
was spotted onto MSM plates. The plates were incubated in an atmosphere containing 5% CO₂ at 37°C for 7 days. This process was repeated three times.

2.4. Purification Experiment. Isolated MLOs were purified using the single colony technique [4]. A single colony was removed by cutting out a small block of agar using a sterile scalpel. The colony was transferred into a tube containing 3 mL of MLM and incubated for 48 h. The culture was diluted 1/10 and 1/100 in MLM, and 50 μL of each dilution were spotted onto MS plates and incubated in a humidified atmosphere containing 5% CO₂ at 37°C for 7 days. This purification procedure was repeated three times.

2.5. Ultramicroscopy. For ultrathin sectioning, MLOs on MLM medium were pelleted by centrifugation (12,000 g for 10 min at 4°C), resuspended in 2.5% glutaraldehyde, embedded in 4% Noble agar, placed on Formvar-coated copper grids for solidification, and fixed again in 2.5% glutaraldehyde in phosphate buffered saline (PBS; 0.1 mol L⁻¹, pH 7.2) at 4°C for 2 h. After several rinses with PBS, the samples were post-fixed with 1% OsO₄ for 1 h. Subsequently, the tissues were dehydrated in an ethanol series and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (TEM).

2.6. Biochemical Tests. The mud crab MLO’s metabolism of glucose, sucrose, lactose, and maltose [18, 19] was examined, as was its hydrolysis of arginine and urea [20, 21] and its reduction of tetrazolium chloride and methylene blue [4]. All plates and tests were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 7 days.

2.7. Sterol Requirement. The MLO’s sterol requirement was established by testing the susceptibility of the isolates to digitonin and by placing the isolates in an MLM lacking serum [22].

2.8. Haemolysis and Hemadsorption. The isolated MLO was examined for hemolytic activity and hemadsorption using sheep, chicken, and rabbit erythrocytes using previously described methods [23].

2.9. Filtration Studies. MLO cultures were diluted 1:10 in a liquid medium and filtered through membrane filters (Millipore) with pore diameters of 0.22 μm and 0.45 μm. The numbers of colony-forming unit (CFU) per milliliter in the filtrates were determined by plating the filtrates onto agar and were compared with the numbers of CFU per milliliter in an unfiltered culture dilution [24].

2.10. Reversion Experiment. Isolated MLOs were subcultured eight consecutive times in liquid or solid growth medium lacking ampicillin or thallium acetate to determine whether the organisms reverted to bacterial L forms. Agar plates and fluid cultures of all passages were examined for alterations in the morphology of clones and cells, respectively. In addition, the agar colonies of each clone were stained with Dienes stain and examined with low power light microscopy.

2.11. Analysis of Partial 16S rRNA Gene Sequence. DNA for phylogenetic analysis was extracted from mid-log phase cultures after five passages of a clonal MLO isolate (strain ZJ2005) using the QiAamp DNA Mini kit (Qiagen). The 16S rRNA gene was amplified using M1 and M2 primers [24], cloned into the pMD18-T vector (TaKaRa), and then transformed into E. coli Top 10 competent cells. Plasmid DNA, which was purified using the QiAprep Spin Miniprep kit (Qiagen), was sequenced afterwards. The obtained 16S rRNA gene was compared to archived genetic sequences using BLAST searches within the GenBank database [25]. Highly similar sequences were selected for phylogenetic tree construction. The phylogenetic tree was constructed with the neighbor-joining method using MEGA 4.1 software [26].

2.12. Experimental Infection. The pathogenesis of ZJ2005 was tested in a mud crab bioassay. ZJ2005 cultures were grown in 5 mL of MLM at 37°C for 48 h, after which a
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3. Results

3.1. Cultivation of Clinical Samples. MLOs were removed from all moribund mud crabs \((n = 5)\). Isolated and cultured MLOs decreased the pH of the MLM and formed typical fried-egg shaped colonies (Figure 1). The colonies were readily stained with Dienes reagent, which confirmed that the isolate was a true member of the Mollicutes rather than a bacterial L form [4].

3.2. Morphology. Ultrathin sections showed two morphological types of cells: (i) markedly electron-dense filamentous lobulated cells of various shapes, but often they were curved \((0.5–2 \mu m)\) and (ii) considerably larger cells \((0.1–0.5 \mu m)\) of a more oval shape with a less compact and a less dense cytoplasm (Figure 2). The cells were bounded by a single unit membrane and contained densely packed ribosomes, between which were found fine strands of less dense material that were presumed to be portions of the cell’s nuclear structure.

3.3. Biochemical Tests. The MLO of mud crabs was able to ferment glucose, sucrose, lactose, and maltose without utilizing arginine and urea. The MLO grew in the absence of bovine serum and was not susceptible to digitonin. It was haemolytic for all three types of erythrocytes tested, but it did not haemadsorb these cells. No dye reduction occurred when the MLO was grown in MSM containing tetrazolium chloride or methylene blue. It grew in MLM containing a NaCl concentration from 0.5 to 3%.

3.4. Filtration Studies. Cultures were diluted 1 : 10 in MLM and then sequentially passed through membrane filters with 0.45 \(\mu m\) and 0.22 \(\mu m\) pore diameters. Filtration reduced the colony number from \(2.35 \times 10^7\) CFU mL\(^{-1}\) in the unfiltered culture to \(9.00 \times 10^6\) CFU mL\(^{-1}\) in the 0.45 \(\mu m\) filtrate and to \(6.59 \times 10^4\) CFU mL\(^{-1}\) in the 0.22 \(\mu m\) filtrate.

3.5. Reversion Experiments. The isolate was diluted 1 : 10 in an MLM medium without antibiotics and incubated at 37°C for a total of eight passages. Each passage was subcultured on agar without antibiotics, and the cultures were examined for differences in colony morphology. No reversion was observed.

3.6. 16S rRNA Gene Sequence Analysis. The 16S rRNA gene nucleotide sequence of ZJ2005 is 1425 nt in length (GenBank accession no. GU985440). Overall, the 16S rRNA gene nucleotide sequence similarity data placed strain ZJ2005 in the *Acholeplasma laidlawii* phylogenetic clade (Figure 3), where its closest relative (similarity score: 0.99) was an isolate provisionally named *A. laidlawii* PG-8A (GenBank accession no. FJ226559).

3.7. Experimental Infections. Cumulative mortality by 15 days was 4/10 for Group 1 (1 on day 4, 1 on day 6, and 2 on day 7). For Group 2, mortality by 15 days was 3/10 (1 on day 8 and 2 on day 12). Interestingly, no clinical signs were observed in any of the dead experimental crabs, but MLOs were isolated from the gut and gill of all of the dead crabs.
A. laidlawii
ZJ2005
A. equifetale (AY538165)
A. hippikon (AY538167)
A. polakii (AF031479)
A. laidlawii PG-8A (CP000896)
A. laidlawii REP (EU925161)
A. laidlawii PG8 (U14905)
A. axanthum (AJ311394)
A. parvum (AY538170)
A. oculi 19L (U14904)
A. oculi ISM (U14906)
A. granularum (AY538166)

Figure 3: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of ZJ2005 and some members of the Acholeplasma group. Strain designations have been reported, and GenBank accession numbers are included. Bootstrap confidence level percentage values obtained from 1000 resamplings of the dataset are shown at the nodes. (bar, distance equivalent to 5 substitution per 100 nucleotides).

experimental crabs. No mortality, clinical signs, or MLOs were found in the unaffected experimental crabs and the crabs in control group.

4. Discussion

The properties of the MLO isolated from mud crabs fulfilled the essential criteria for Mollicutes as proposed by the International Committee on Systematic Bacteriology Subcommittee on Taxonomy (1995): it had a typical fried-egg colony form in culture, a polymorphic cell form, absence of a cell wall, passage through 0.45 μm and 0.2 μm filters, lack of reversion to bacteria, and resistance to ampicillin [1]. The results of 16S rRNA gene analysis and the biological, biochemical, and morphological studies indicated that the isolated MLO is a member of the genus Acholeplasma. Taxonomically, Acholeplasma belongs to the kingdom Bacteria, division Firmicutes, class Mollicutes, order Acholeplasmatales, family Acholeplasmataceae, and genus Acholeplasma. There are 15 recognized species in this genus, including saprotrophic and pathogenic species [27–30]. Although Acholeplasma spp. are widely distributed in nature and can be detected and isolated from different plant, avian, and mammalian sources [31–33], they have not been reported previously in aquatic animals. Our detection of Acholeplasma in S. serrata increases our knowledge about the host ranges of these organisms and should lead to further investigation of other possible aquatic hosts and to studies of possible relationships between terrestrial and aquatic hosts.

The MLO in our study had 99% identity with A. laidlawii based on 16S rRNA genes. The three most useful criteria in Acholeplasma taxonomy are the 16S rRNA gene sequence, DNA-DNA hybridization analysis, and serology. The highest resolution is provided by 16S rRNA gene sequence analysis, which is useful for the discrimination of most species [9]. Our study has shown that the organism isolated from mud crabs is indeed a member of the genus Acholeplasma. However, further studies are needed to precisely identify the actual species. It is closely related to A. laidlawii, but it may represent a new species.

The MLO in the experimentally infected crabs did not cause high mortality or result in clinical signs of disease, which is not surprisingly because most Acholeplasma diseases are influenced by a variety of host and environmental factors. Moreover, a virulent strain can occur naturally, and some animals might carry Acholeplasma with no signs of disease until they are stressed [2]. However, the isolation of pure MLO from epithelium of gill and gut tissues of dead crabs suggests that the MLO might be only a cofactor for a reo-like virus, which was thought to be the main pathogen causing CD in mud crabs [34].

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