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Chitinase gene diversity at a deep sea station of the east Pacific nodule province

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Abstract The Pacific nodule province covered about 4.5 million km^2 in the east tropical Pacific with an abundance of polymetallic nodules at the seafloor. In view of the environmental protection and resource preservation, the survey of biodiversity was important during the reconnaissance and exploitation in this area. As one of the important component of the deep sea ecosystem, the microbial community in the Pacific nodule province was still largely unknown. The chitinolytic bacteria diversity in deep-sea sediment of a station within the Pacific nodule province was examined by molecular technology. A total of 18 chitinase genes were detected by a set of degenerate PCR primer specific for *chiA* gene fragment of family 18 chitinase. Most of them belonged to the *Serratia*-like chitinase. Eight genes had different amino acid sequences in the conserved motif, encompassing the catalytic site among the ChiA protein of family 18 glycosyl hydrolases, and clustered in an independent clade on the phylygenetic tree.

Keywords Chitinase · *chiA* · Diversity · Deep sea · Pacific nodule province

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Introduction

Chitin is an abundant and one of the important sources of nutrients and energy in marine environment (Gooday 1990). Microorganisms play a critical role in the degradation of chitin, which is the key step in the cycling of nutrients in the environment (Gooday 1990; Poulicek et al. 1998; Tsujibo et al. 2002). The partial hydrolysis of polymeric insoluble chitin is necessary to be assimilated by microbial cells, which is mainly accomplished by the chitinase (EC 3.2.1.14) (Chrost 1992; Cottrell et al. 1999; Henrissat 1991). Bacterial chitinases are often associated with the outer membrane or are secreted as extracellular enzymes, which suggests that they must be adapted to the function under the physicochemical conditions of the environment (Keyhani and Roseman 1999; Kirchman and White 1999; LeCleir et al. 2004). Consequently, their gene and biochemical properties may be selected by different environmental conditions, especially by the extreme conditions such as low temperature and high press in the deep sea.

Chitinases are classified into family 18 and 19 of the glycosyl hydrolases, based on the similarity of amino acid sequence (Henrissat 1991; Henrissat and Bairoch 1993). The vast majority of bacterial chitinases fall within family 18, which is subdivided into three groups, ChiA, ChiB, and ChiC, based on the differences in the amino acid sequences of their catalytic domains (Suzuki et al. 1999). ChiA and ChiB are processive chitinases that degrade chitin chains in opposite directions, while ChiC is a nonprocessive endochitinase (Horn et al. 2006). Due to their prevalence in nature, ChiA have been used for studying the diversity and distribution of chitinolytic bacteria in terrestrial sys-

tems as well as aquatic environment (Cottrell et al. 1999; Kirchman and White 1999; Krsek and Wellington 2001; Metcalfe et al. 2002; Ramaiah et al. 2000; Tsujibo et al. 2003; Williamson et al. 2000), including the marine environment (Cottrell et al. 1999, 2000; Metcalfe et al. 2002; Ramaiah et al. 2000). However, there have been a few attempts to investigate the diversity of chitinase genes in the deep-sea sediment, which is rich in chitin from the principal exoskeletal material of crustaceans and many mollusks.

The Pacific nodule province stretches from 118° to 157°W, and from 9° to 16°N in the international waters and has an area of about 4.5 million km^2. It is named for the abundant polymetallic nodules at the seafloor, which is mainly composed of manganese, iron, cobalt, copper, and nickel. The activities in the deep sea of the Pacific nodule province is organized and controlled by the International Seabed Authority, an autonomous international organization headquarters in Jamaica. One of the key responsibilities of the authority is to ensure that the deep-sea environment is not disturbed by the reconnaissance and exploitation in this area. In view of the protection and preservation, the survey of the biodiversity in nodule province is important. Microorganisms are believed to play vital roles in the deep sea ecosystem, but the study of them in this area is still largely unknown (Xu et al. 2005), especially the chitinolytic bacteria and their chitinase genes, which play an important role in the cycling of substances in the deep sea environment. Here, we report the first molecular assessment of chitinase diversity in the deep-sea sediment of a station within the east Pacific nodule province by using a pair of primers specifically for group A chitinase belonging to family 18. The results revealed the diversity of chitinase genes and chitinolytic bacteria including some novel sequences in this deep-sea sediment. It may be served as one of the bio-monitors for the environment evaluation in the future.

Materials and methods

Sample collection and bulk DNA extraction

The deep-sea sediment was collected from a Pacific nodule province station (ES0304, 145°23′03″W, 8°19′50″N, 5246 m) in 2003 by the multi-core sampler. It was brown silicon ooze. The sub-sampling was carried out by discarding the surface in a clean bench immediately, transferring it to sterile falcon tubes, and keeping in –20°C. The surface of the sample was discarded again in a clean bench after it was brought back to the lab. Only the central section was subjected to further analysis. The microbial bulk DNA was extracted by combining chemical lysis and enzyme digestion, and then purified by DNA adsorbent resin (Zeng et al. 2005).

Molecular detection of chitinase genes

The primers ChiA_F1/ChiA_R1 or ChiA_F2/ChiA_R2 (Hobel et al. 2005) that were designed by comparing the amino acid sequences of chitinase belonging to family 18 group A from GenBank and Pfam (Sonnhammer et al. 1997), choosing from the highly conserved active site of chitinase gene (Watanabe et al. 1993), were used to amplify the chitinase genes from the extracted bulk DNA. The sequences were as follows: ChiA_F1, 5′-ACG GCG TGG ACA TCG AYT GGG ART-3′; ChiA_R1, 5′-CCC AGG CGC CGT AGA RRT CRT AYS-3′; ChiA_F2, 5′-CGT GGA CAT CGA CTG GGA RTW YCC-3′; ChiA_R2, 5′-CCC AGG CGC CGT AGA RRT CRT ARS WCA-3′ (Hobel et al. 2005). The predicted size of the amplicons was ~270–300 bp. The mixture of DNA, dNTP, primers and reaction buffer was applied to a hot start of 95°C for 6 min. Then *Ex-Taq* DNA polymerase (Takara, Dalian, China) was added. The reaction cycles included 25 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min, and a final step of 72°C for 5 min. Five replicate PCRs were mixed together to reduce random biases within individual PCR. The PCR products were purified from agarose gel and cloned into pTA2 vector (Toyobo, Osaka, Japan). Recombinant transformants were selected by blue-white screening and were checked for correct insert size by colony PCR using the forward and reverse M13 primers. The positive clones were subjected to second PCR using primer pair ChiA_F1/ChiA_R1 or ChiA_F2/ChiA_R2. The restriction fragments length polymorphism (RFLP) was performed, by digesting the PCR products with *Msp*I. The identification of transformants was performed, by comparing the size and the number of the digested fragments after electrophoreses through a 3% agarose gel. The clones with different digestion profile were picked and sequenced subsequently.

Phylogenetic analysis

The chitinase gene sequences retrieved from the constructed library were analyzed by BlastX program in the NCBI databank (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of deduced amino acid sequences and the construction of phylogenetic trees

were carried out by DNAMAN (Version 5.2.2) program. All the 18 partial chitinase gene sequences, obtained in this study, have been deposited in the EMBL database under the accession numbers of AM268400–AM268417.

Results

A total of 18 different RFLP types were identified from 100 clones, which were picked randomly from the constructed chitinase gene clone library. The representative clones for each RFLP types were sequenced and compared with the known chitinase genes at the amino acid level by BlastX. The results revealed that they all belonged to the chitinase gene, showing 70–95% similarity to those sequences retrieved from the GenBank database at the amino acids level. These putative chitinase genes showed the best match with five chitinase sequences in the database (Table 1). Most of them were closely related to chitinase of the *Serratia* group isolated both from soil (Chernin et al. 1997; Metcalfe et al. 2002) and aquatic environment (LeCleir et al. 2004). The types CHIE2 and CHIE10 that showed high homology to a chitinase from an upland pasture (Metcalfe et al. 2002) were most prevalent in the library, occupying, respectively, 19 and 17% of the total clones (Table 1).

The phylogenetic tree based on deduced amino acid sequences showed that all these clones fell within four major clades (Fig. 1), including three independent clades within *chiA* groups and one *chiB* group. Despite the high similarity to the referenced matches, our sequences were clustered in independent clades, indicating their different phylogenesis, which might relate to the deep-sea environment. It was interesting that clones CHIE14 and CHIE3 were closely related to a chitinase gene of an uncultured organism from the lake sediment of Antarctic Ardley Island, indicating their similar adaptation to cold environment.

A motif encompassing the catalytic site, [DG]-G-[LIV]-[DG]-[IV]-[DH]-W-[EG], was found to be conserved among the ChiA protein of family 18 glycosyl hydrolases (LeCleir et al. 2004; Papanikolau et al. 2001). Most of our clones had the same motif, but eight clones had different sequences in this domain (Fig. 2). Accordingly, these clones clustered in independent clade on the phylogenetic tree (Fig. 1), suggesting that they were different chitinase sequences of ChiA group.

Discussion

From an ecological point of view, there is great interest evinced in the enumeration of chitinolytic microorganisms by rapid, reliable detection techniques. In this study, we quote a set of degenerate primers for *chiA* (Hobel et al. 2005) to detect the diversity of chitinase genes in a deep sea sediment sample of the nodule province in the east Pacific. Although our primers were identical to Hobel's (Hobel et al. 2005), none of our sequences showed close identity to their sequences from the seashore sediment. On the other hand, three clones (CHIE9, CHIE14, and CHIE3) in our study were found clustered within the *chiB* group (Fig. 1). The results indicated that the primers were able to

Table 1 Identification of cloned chitinase gene from deep-sea sediment of nodule province

RFLP types	No. of clones	Similarity (%)[a]	Closest database match[b]
CHIE1	3	71	*Serratia plymuthica chiA* (AAB49933.1)
CHIE2	13	83	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)
CHIE3	1	93	Uncultured organism clone L59–28 chitinase (AAS19506.1)
CHIE4	2	70	*Serratia plymuthica chiA* (AAB49933.1)
CHIE5	3	95	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)
CHIE6	7	72	*Serratia liquefaciens chiA* (AAK07482.1)
CHIE7	1	82	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)
CHIE8	3	72	*Serratia liquefaciens chiA* (AAK07482.1)
CHIE9	2	98	*Serratia liquefaciens chiB* (AAN03597.1)
CHIE10	12	84	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)
CHIE11	4	71	*Serratia plymuthica chiA* (AAB49933.1)
CHIE12	2	97	*Serratia liquefaciens chiA* (AAK07482.1)
CHIE13	5	–	–
CHIE14	1	90	Uncultured organism clone L59–28 chitinase (AAS19506.1)
CHIE15	5	78	*Serratia liquefaciens chiA* (AAK07482.1)
CHIE16	2	94	*Serratia plymuthica chiA* (AAB49933.1)
CHIE17	2	83	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)
CHIE18	1	95	Uncultured bacterium clone controlC8S chitinase (AAL89744.1)

[a] The scores were determined based on amino acid identity

[b] The number in the bracket was the accession number of each sequence in the GenBank

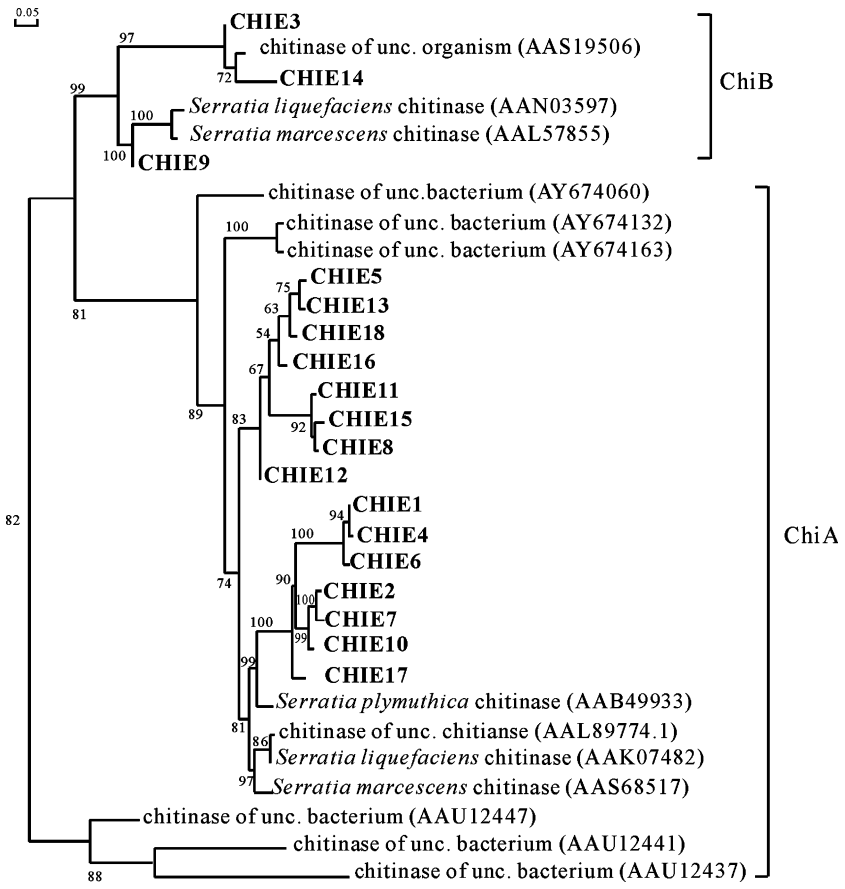

Fig. 1 Phylogenetic tree based on ChiA amino acid sequences. Clones in this study were showed in *bold face*. The numbers in brackets were the GenBank accession numbers of the ChiA amino acid sequences. The tree was constructed by maximum-likelihood method. The *scale bar* represents 0.05 amino acid substitution per position. *Numbers* refer to bootstrap values for each node out of a total of 100 replicate resamplings. "*unc.*" was the abbreviation of "uncultured"

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                    310       320       330       340       350       360
Serratia plymuthica 1   LQTWKFFDGVDIDWEFPGGGGANPKLGNAQ.DGATYVQLMKDLRAMLDQLSAETGR...KY
Serratia plymuthica 2   LQTWKFFGGADIDWEFPGGQGANPKLGSAQ.DGATYVQLMKDLRAMLDQLSAETGR...KY
Serratia liquefaciens   LQTWKFFDGVDIDWEFPGGQGANPKLGSTQ.DGATYVQLMKELRAMLDQLSAETGR...KY
Serratia marcescens     LQTWKFFDGVDIDWEFPGGKGANPNLGSPQ.DGETYVLLMKELRAMLDQLSAETGR...KY
uncultured bacterium 1  .......VDIDWEYPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDQLSAETGR...KY
uncultured bacterium 2  LRTWKFFDGIDIDWEYPGGGGANPDMGDPATGGATYLSLMTELRVMLDRLSAEKGR...DY
uncultured bacterium 3  LQTWKFFDGVDIDWEFPGGKGVNANLGEAAKDGLTYQLLMQELRAMLDELSAETGR...SY
uncultured bacterium 4  LQTWKFFDGVDIHWEFPGGKGVNANLGEAAKDGLTYQLLMQELRAMLDELSAETGR...SY
CHI12   .......DGVDIDWEFPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI13   .......DGVDIDWEFPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI15   .......DGVDIDWEFPGGGGENPALGSTA.DGDTYVQLMKELRTMLNELSAQTGE...TY
CHI16   .......DGVDIDWEFPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI18   .......DGVDIDWEFPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI3    .......GVDIDWEYPN.......TEAE..KNQFTKLIQSLRTKLDAQGLKDDK...YY
CHI5    .......GVDIDWEFPGGQGANPKLGSAQ.DGATYVQLMKELRAMLDHLSAQTGR...KY
CHI8    .......GVDIDWEFPGGGGENPALGSTA.DGDTYVQLMKELRTMLNELSAQTGK...TY
CHI9    .......GVDIDWEYPQ.......SSE..VDGFVAALQEIRTLLNQQTQADGRQALPY
CHI11   .......GVDIDWEFPGGGGENPALGSAA.DGDTYVQLMKELRTMLNELSAQTGK...TY
CHI10   .......RGHRLGVSG.RAGRPETGQAQ.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI17   .......RGHRLGVSG.RAGRPETGQCS.GGATYVQLMKELRAMLDQLSAQTGR...KY
CHI6    .......RGHRLGVSG.RWRKSCTGQTA.DGDTYVQLMKELRTMLNELSAQTGK...TY
CHI4    ......RRGHRLGVSG.RWRKSCTGQHG.DGDTYVQLMKELRTMLNELSAQTGK...TY
CHI2    ......RRGHRLGVPG.RAGRPETGQCS.DGATYVQLMKELRAMLDQLSAQTGR...KY
CHI1    ......RRGHRLGVSG.RWRKSCTGQHG.DGDTYVQLMKELRTMLNELSAQTGK...TY
CHI14   ......RRGHRLGYPN.......TEAE..KNQFTKLIQSLRTKLDAQGLKDDK...YY
CHI17   .......RGHRLGIPR.RAGRPETGQCS.DGATYVQLMKELRAMLDQLSAQTGR...KY
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Fig. 2 Amino acid sequence alignment of the catalytic site of deduced *chiA* gene. Conserved residues were shown in the frame. The accession number of sequences obtained from GenBank database were *Serratia plymuthica* 1 (AAB49933), *Serratia plymuthica* 2 (CAD32933), *Serratia liquefaciens* (AAK07482), *Serratia marcescens* (AAS68517), uncultured bacterium 1–4 (AAL89774, AY674060, AY674132, and AY674163, respectively)

detect *chiA* gene of family 18 chitinase and *chiB* gene synchronously.

The *Serratia*-like chitinase gene sequences were found to dominate in our deep-sea sediment sample. Their close matches came from all kinds of environment (Table 1) (Chernin et al. 1997; Metcalfe et al. 2002; Woytowich et al. 2000), indicating that the *Serratia*-like chitinase genes were prevalently distributed in the natural environment. It is noticeable that the chitinase clones in this study tended to locate in independent clades on the phylogenetic tree, although they showed close similarity to the referenced sequences.

The chitinase gene in ES0304 deep sea sediment was not of high diversity (Table 1). Considering that the

Pacific nodule province is a specific area with polymetallic nodules abundant in the seabed, and the content of the total organic carbon in ES0304 sediment was estimated to be only 0.71% by the burning oxidation-nondispersive infrared method, the low diversity of chitinase genes might be due to the relatively poor quality and quantity of substances in this deep sea sediment. The previous researches had shown that the amount and diversity of chitinolytic bacteria and chitinase gene was closely related to the concentration of chitin in various environments (Metcalfe et al. 2002; Hobel et al. 2005; LeCleir et al. 2004; Xiao et al. 2005). As an abundant and important nutrient in deep- sea sediment, the changes in chitin, which mainly come from sedimentation and benthic crustaceans, would result in the changes of the microbial chitinase gene. Therefore, the study on the changes of chitinase gene might be used as a biomarker to evaluate the environmental disturbance in the deep sea of east Pacific nodule province during the reconnaissance and mining in the future.

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