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# Chitinase-Producing Marine Bacteria Isolated from Jeju Island

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**Abstract:** Chitinase-producing marine bacteria were isolated, and chitinase activity of selected bacteria was measured. Seawater, sediment, and marine animals were collected from the coastal area of Jeju Island, Korea, diluted in sterile seawater, and spread on the selective plate medium containing colloidal chitin. A total 22 strains of clear-hole formation bacteria were isolated as chitinase producers. Chitinase activity of these bacteria was measured by detecting the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide (PNG), and 9 strains showing more than 0.1 unit/h of chitinase activity were finally selected. In particular, *Microbulbifer* sp. CJ11049 and *Microbulbifer* sp. CJ11075 showed high chitinase activity. Taxonomic identification for the 9 strains was performed using phylogenetic analysis of 16S rDNA: *Vibrio* sp. CJ11027, *Bacillus* sp. CJ11043, *Microbulbifer* sp. CJ11049, *Microbulbifer* sp. CJ11052, *Microbulbifer* sp. CJ11064, *Thalassospira* sp. CJ11073, *Microbulbifer* sp. CJ11075, *Bacillus* sp. CJ11076, *Cellulomonas* sp. CJ10597. Among them, 16S rDNA sequences of 4 strains (CJ11049, CJ11052, CJ11064, and CJ11073) showed less than 97% of similarities with previously reported species; therefore, they seem to be new genus or species.

**Key words:** chitinase, marine bacteria, *Vibrio*, *Bacillus*, *Thalassospira*, *Microbulbifer*, *Cellulomonas*.

## INTRODUCTION

Chitinase (EC 3.2.1.14) is the enzyme that catalyzes random hydrolysis of β-1,4-linkages in chitin, one of the most abundant organic compounds in the marine environment. Chitin, a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) residues linked by β-1-4 bonds, is a common constituent of insect exoskeletons, shells of crustaceans, and fungal cell walls (Deshpande, 1986). For the entire aquatic biosphere, it is estimated that more than $10^{11}$ metric tons of chitin are produced annually (Keyhani and Roseman, 999). Production of *N*-acetylglucosamine (NAG) from chitin is important not only for chemical and pharmaceutical purposes but also for use in food products as sweeteners and growth factors of intestinal bacteria.

Chitinases are found in a wide variety of organisms, for instance, bacteria, fungi, insects, plants, and animals, and the corresponding genes have been cloned and characterized. To date, various chitinases have been isolated from bacteria such as *Aeromonas* sp. (Shiro et al., 1996; Ueda

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et al., 1995), *Alteromonas* sp. (Tsujibo et al., 1993), *Bacillus amyloliquefaciens* (Wang et al., 2002), *Bacillus circulans* (Alam et al., 1995; Watanabe et al., 1992), *Pseudomonas aeruginosa* (Folders et al., 2001), *Pseudomonas* sp. PE2 (Kitamura and Kamei, 2003), *Serratia liquefaciens* (Joshi et al., 1988), *Serratia marcescens* (Watanabe et al., 1997), *Streptomyces olivaceoviridis* (Blaak et al., 1993; Radwan et al., 1994), *Streptomyces plicatus* (Robbins et al., 1988), *Vibrio furnissi* (Bassler et al., 1991), and *Vibrio* sp. strain Fi:7 (Bendt et al., 2001).

In this study, we isolated and cultured marine bacteria with chitinase activity and identified them by phylogenetic analysis using 16S rDNA sequences.

## Materials and Methods

### Sample Collection and Screening of Chitin-Degrading Microorganisms

Seawater, sediment, and marine animals were collected from the coastal area of Jeju Island, Korea. The marine animals were ground in sterile seawater. Samples were diluted 10- to 1000-fold in sterile seawater and spread on chitin-containing minimal agar plates [colloidal chitin 10 g, $(NH_4)_2SO_4$ 2 g, $KH_2PO_4$ 0.7 g, $Na_2HPO_4 \cdot 7H_2O$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 1 mg, $MnSO_4 \cdot 5H_2O$ 1 mg, agar 15 g, distilled water 500 ml, and aged sea water 500 ml, pH 7.0]. Colloidal chitin was prepared by the method of Rodriguez–Kabana et al. (1983), by partial hydrolysis of chitin (Sigma) with 10 N HCl for 2 h at room temperature. The colloidal chitin was washed several times with large volumes of distilled water to adjust the pH to 7.0. After incubation for 7 days at room temperature, clear-hole-forming bacteria were selected as the chitinase producer.

### Assay of Chitinase Activity

Chitinase activity was measured by determining the release of *p*-nitrophenol from PNG on the basis of the method of Roberts and Selitrennikoff (1988) with modification. One hundred milliliters of enzyme solution were added to 100 µl of 10 mM PNG (Sigma) and 300 µl of 0.1 M citrate–phosphate buffer (pH 6.0). After incubation at 37°C for 30 min, the reaction was stopped with the addition of 500 µl of 1 M $Na_2CO_3$. The amount of *p*-nitrophenol was measured at 405 nm. One unit of chitinase was defined as the amount of enzyme needed to release 1 mmol of *p*-nitrophenol per hour.

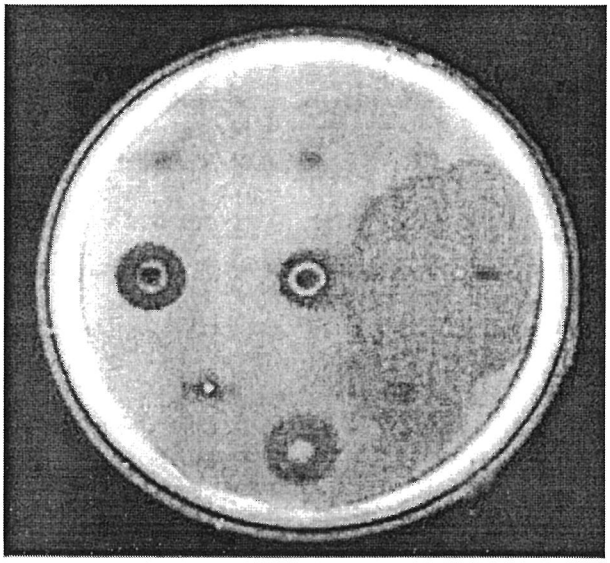

**Figure 1.** Clear zone formation of chitinase-producing bacteria.

* Reduce to 80% this figure in size.

### Culture Conditions

The isolates were precultured in the same medium as described above without agar for 4 days at 30°C with stirring at 150 rpm. The medium (500 ml) was inoculated with 1% of preculture in a 2-L Erlenmeyer flask and incubated for 4 days at 30°C on a reciprocal shaker. The purified isolates were then cultured in ZoBell 2216e media and stored at −70°C in a fresh medium that contained 10% (v/v) sterile glycerol.

### DNA Extraction and PCR Amplification

The genomic DNA was extracted from 1 ml of isolate cultured in the ZoBell 2216e buffer using the AccuPrep genomic DNA Extraction kit (Bioneer). From the genomic DNA, nearly full-length 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ and 1522R (5′-AAGG AGGTTATCCANCCRCA-3′). The PCR mixture consisted of 5 µl of 10× PCR buffer (final concentrations: 100 mM KCl, 20 mM Tris-HCl, pH 8.0), 2.5 mM $MgCl_2$, 2.5 mM each dNTP, 1 µl of each primer, 1 µl of the template DNA, and 5.0 units of *Taq* polymerase (TaKaRa) for a total volume of 50 µl. The thermal cycling program used was as follows: initial denaturation at 95°C for 5 min; 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension step consisting of 72°C for 7 min. Amplified PCR products were analyzed by agarose

**Table 1.** Chitinase Activity Estimation of Nine Chitinase-Producing Bacteria

| Strain | Closest species | No. of 16S rDNA sequences | Chitinase activity (unit/h) |
|---|---|---|---|
| CJ11027 | *Vibrio campbellii* | AF246980 | 0.1429 |
| CJ11043 | *Bacillus subtilis* | AF500205 | 0.1504 |
| CJ11049 | *Microbulbifer salipaludis* | AF500206 | 0.2583 |
| CJ11052 | *Microbulbifer hydrolyticus* | AF500207 | 0.1505 |
| CJ11064 | *Microbulbifer hydrolyticus* | AF500211 | 0.1533 |
| CJ11073 | *Thalassospira lucentensis* | AF500210 | 0.1406 |
| CJ11075 | *Microbulbifer hydrolyticus* | AF500209 | 0.2667 |
| CJ11076 | *Bacillus thuringiensis* | AF500212 | 0.1488 |
| CJ10597 | *Cellulomonas cellulans* | AF500208 | 0.1630 |

* Center this column.

gel electrophoresis, purified with High pure PCR product Purification Kit (Roche), and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

### Sequence Analysis

The sequencing was performed using primer 518R (5′-GTATTACCGCGGCTGCTGG-3′), and sequences of the 16S rDNA between 362 and 484 bp (average-451 bp) were submitted to the Advanced BLAST search program of NCBI to identify whether they aligned with closely related organisms. The related sequences were preliminarily aligned with the default settings of CLUSTAL X (Thompson et al., 1997), and complete sequence alignments were performed using PHYDIT (Chun, 1995) and manual comparison to secondary structures. The phylogenetic analysis was performed with PHYLIP (Felsenstein, 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei, 1987).

## Results and Discussion

We isolated 22 strains, which had formed clear-hole on chitin-containing minimal agar plates (Figure 1). After measuring chitinase activity of the 22 strains, we finally selected 9 strains showing more than 0.1 unit/h of chitinase activity (Table 1). In particular, *Microbulbifer* sp. CJ11049 and *Microbulbifer* sp. CJ11075 showed high chitinase activity.

Taxonomic identification for the 9 strains was performed using phylogenetic analysis of 16S rDNA: *Vibrio* sp. CJ11027, *Bacillus* sp. CJ11043, *Microbulbifer* sp. CJ11049, *Microbulbifer* sp. CJ11052, *Microbulbifer* sp. CJ11064, *Thalassospira* sp. CJ11073, *Microbulbifer* sp. CJ11075, *Bacillus* sp. CJ11076, and *Cellulomonas* sp. CJ10597. Among them, 16S rDNA sequences of 4 strains—CJ11049, CJ11052, CJ11064, and CJ11073—showed less than 97% of similarities with previous sequences; therefore, they seemed to be new genus or species.

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