Chitinimonas taiwanensis gen. nov., sp. nov., a Novel Chitinolytic Bacterium Isolated from a Freshwater Pond for Shrimp Culture

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Summary

A bacterial strain, designated cfT was isolated from surface water of a freshwater pond for shrimp (Macrobrachium rosenbergii) culture at Ping-Tung (Southern Taiwan). Cells of this organism were Gram-negative, slightly curved rods which were motile by means of a single polar flagellum. Strain cfT utilized chitin as the exclusive carbon, nitrogen, and energy source for growth, both under aerobic and anaerobic conditions. Optimum conditions for growth were between 25 and 37 °C, 0 and 1% NaCl and pH 6 to 8. Strain cfT secreted two chitinolytic enzymes with approximate molecular weight 52 and 64 kDa, which hydrolyzed chitin to produce chitotriose as major product. Sequence comparison of an almost complete 16S rDNA gene showed less than 92% sequence similarity with known bacterial species. Phylogenetic analysis based on the neighbour-joining and other methods indicated that the organism formed a distinct lineage within the β-subclass of Proteobacteria. The predominant cellular fatty acids of strain cfT were hexadecanoic acid (about 29%), octadecenoic acid (about 12%) and summed feature 3 (16:1 ω7c or 15 iso 2-OH or both [about 49%]). Its DNA base ratio was 62.8 mol% G+C. We propose to classify strain cfT (= CCRC 17210T = LMG 22011T) as Chitinimonas taiwanensis gen. nov., sp. nov.

Key words: Chitinimonas taiwanensis – chitinolytic enzymes – chitotriose – shrimp culture – taxonomy

Introduction

Chitin, a complex molecule of natural polymers, is a common constituent of shells of crustaceans, cell walls of fungi and certain green algae, and exoskeletons of insects and is the second most abundant polysaccharide in nature (after cellulose). Chemically, chitin is an insoluble homopolymer and composed of linear chains of β-1,4-linked N-acetyl-β-D-glucosamine (GlcNAc) residues which are highly cross-linked by hydrogen bonds. Chitin and its partially deacetylated derivatives exhibit interesting properties and constitute a valuable raw material for biomedical, agricultural, and cosmetic applications [18, 21].

Enormous amounts of chitin are synthesized in the biosphere to the level about 10^10 to 10^11 metric tons annually [6], but only traces of chitin remain in the environment. The turnover rate of the polysaccharide is attributed primarily to microorganisms that degrade chitin, allowing carbon and nitrogen to return to the ecosystem [11]. Bacteria are the principal mediators of chitin degradation and their abilities to promote hydrolysis of chitin play an important role in the recycling of chitin in nature [7, 9]. Chitinolytic bacteria produce chitinase to hydrolyze the glycosidic bonds between GlcNAc residues for utilization of chitin as a carbon and energy source [17]. The capability to degrade chitin is widespread among various groups of bacteria and archaea [4, 10].

Shrimp farming has significantly contributed to the economics of Southern Taiwan. Shrimps have a hard shell made of chitin and will moult periodically. Therefore, pond water for shrimp culture contains enormous

Accession: The DDBJ/EMBL/GenBank accession number for the 16S rDNA sequence of the strain cfT is AY323827
amounts of suspended chitin and bacteria which rely on chitin for growth substrate. Some chitinolytic bacteria are also been known as pathogens implicated in shell disease of crabs and shrimps [25]. In the present study, we describe a novel chitinolytic organism isolated from a freshwater pond for shrimp culture, and the enzymes secreted from the organism were also partially characterized.

Materials and Methods

Isolation and growth condition
In August 2000, a sample of surface water was taken from a freshwater pond for shrimp (Macrobrachium rosenbergii) culture at Ping-Tung County, Southern Taiwan. The chitin enrichment culture containing 200 ml of sample water and 0.5% (w/v) autoclaved colloidal chitin was incubated at 25 °C with shaking at 125 r.p.m. The colloidal chitin was prepared from commercial chitin (Ohka chemical, Taiwan) using the method of Rodriguez-Kabana et al. [20] with minor modifications. Chitin powder was partially hydrolysed with 12 N HCl, followed by repeated washings with tap water and then dialysis against distilled water until the colloidal chitin gave a final pH of 6.0 to 6.5. After 5 days of culture, the enrichment broth was diluted with sterile distilled water and spread onto chitin basal (CB) medium incubated at 35 °C. CB medium (l−1) contained 0.5% (w/v) colloidal chitin, 0.1 g of MgSO4 · 7H2O, 0.1 g of CaCl2 · 2H2O, 0.1 g of Na2MoO4 · 2H2O, 0.08 g of CuSO4 · 5H2O, 0.1 g of FeCl3 and 0.14 g of Na2HPO4. The agar plates were prepared by adding 1.0 ml trace element solution. The pH was adjusted to 6.5. The trace element solution (l−1) contained 2.86 g of H3BO3, 2.03 g of MnSO4 · 4H2O, 0.22 g of ZnSO4 · 7H2O, 0.08 g of CuSO4 · 5H2O, 0.1 g of FeCl3, and 0.14 g of Na2HPO4 · 2H2O. The agar plates were prepared by adding 1.5% (w/v) Bacto agar (Difco) to the CB media. A bacterial strain, designated cfT was isolated and shown to produce chitinolytic activity.

Morphological and physiological tests
Strain cfT was grown on CB medium for 3 days at 35 °C. Morphology was observed by a light microscope (Zeiss Axioskop) and transmission electron microscope (Hitachi model H600). Negative staining was performed with an aqueous solution (1%, w/v) of phosphotungstic acid adjusted to pH 7, as described by Robinson et al. [19]. The motility of exponential-phase cells was examined by the hanging drop method. Polyβ-hydroxybutyrate granule accumulation was observed by light microscopy after being stained with Sudan black [5].

The pH range for growth was determined by adjusting the pH of CB medium to values between 4.0 and 11.0 with 1N NaOH or 1N HCl. For the determination of the temperature range for growth, cells were incubated in CB medium at temperatures between 4 and 45 °C. Tolerance to NaCl was determined by adjusting the salinity of CB medium to values between 0 and 3.0% NaCl. Anaerobic cultivation was performed on CB medium under 85% N2, 5% CO2, and 10% H2, in an anaerobic chamber (Forma Scientific).

Biochemical analysis
Characteristics presented in the API 20NE, API ZYM (BioMérieux) and BBL Crystal Enteric/Nonfermenter ID (Becton Dickinson) microtest systems were determined according to the recommendations of the manufacturers. For carbon substrate metabolism tests, Biolog GN II microtitre test plates (Biolog) were used. Early log phase cultures were used as inoculum for the test plate (150 µl well−1). Plates were incubated at 28 °C and examined after 24 h and 48 h to allow for the development of a purple color indicative of substrate oxidation.

Antimicrobial susceptibility testing
The susceptibility to antimicrobial agents was determined by disk diffusion. The density of the bacterial suspension from exponential-phase culture was adjusted with sterile saline to the turbidity corresponding to 0.5 of the McFarland standard and spread onto CB medium for further incubation incubated at 35 °C. The following antimicrobial disks (Dispens-O-Susceptibility Test Disks, Difco) were used including ampicillin (10 µg discs), penicillin G (10 µ), cefotaxime (30 µg), cefotaxime (30 µg), amikacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), netilmicin (30 µg), streptomycin (10 µg), erythromycin (10 µg), tetracycline (30 µg), nalidixic acid (30 µg), chloramphenicol (30 µg), rifampin (5 µg), imipenem (10 µg) and trimethoprim (1.25 µg)/sulfamethoxazole (23.75 µg). Resistance was defined as confluent growth up to the edge of a disk.

16S rDNA sequencing and phylogenetic analysis
Amplification and sequence analysis of the 16S rRNA gene was performed as described previously [1]. The sequence was compared with others available in GenBank and Ribosomal Database Project II. The multiple-sequence alignment including strain cfT and its closest relatives were using the BioEdit program [8]. The phylogenetic reconstruction was inferred by using the neighbour-joining, UPGMA, maximum-likelihood and Fitch-Margoliash methods using the BioEdit software [8]. A bootstrap analysis (confidence values estimated from 1000 replications of each sequence) was performed for the neighbour-joining analysis using the CLUSTAL w 1.7 program [22]. A phylogenetic tree was drawn using the TREEVIEW program [14]. Sequence identities were calculated using the BioEdit program [8].

Determination of the DNA base composition
DNA was prepared as described by Pitcher et al. [16] and degraded enzymatically into nucleosides as described by Mesbah et al. [13]. The obtained nucleoside mixture was then separated by high-performance liquid chromatography using a Waters Symmetry Shield C8 column thermostated at 37 °C. The solvent was 0.02 M NH4H2PO4 (pH 4.0) with 1.5% acetonitrile. Nonmethylated lambda phage DNA (Sigma) was used as the calibration reference.

Fatty acid methyl ester analysis
Cells were grown for 24 h at 28 °C on Trypticase Soy Agar (BBL). Cellular fatty acids were analyzed as methyl esters by GC according to the instructions of Mico (Identification System (M IDI); M ( icrobial ID) described elsewhere [23].

Characterization of chitinolytic activity
In order to characterize chitinolytic enzymes produced by this organism, one ml of late-exponential-phase culture was inoculated to 100 ml CB medium and incubated with shaking (125 r.p.m) at 25 °C. The culture broth was collected periodically for cell counting and the enzyme activity in supernatant of broth was determined after centrifugation with 10,000 × g for 20 min at 4 °C. Chitinase activity was measured by the extent of hydrolysis of fluorogenic substrate analogues 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUF-GlcNAc; Sigma), 4-methylumbelliferyl-β-D-N-acetylchitobioside (MUF-diGlcNAc; Sigma) and 4-methylumbelliferyl-β-D-N,N′-diacetylchitotrioside (MUF-triGlcNAc; Sigma). Fluorogenic substrates were made up with 1 mM stock solutions in dimethyl sulfoxide (DM SO). Enzyme activity was determined by adding 60 µl culture supernatant to the substrate solution which contained 50 µM fluorogenic substrate in a sodium phosphate buffer (75 mM, pH 7.0; the final reaction volume was 700 µl). The mixture was incubated at 37 °C for 30 min, and terminated by
addition of 300 µl of 3 M sodium carbonate. The fluorescence derived from 4-methylumbelliferone was determined with an excitation wavelength of 390 nm and emission wavelength of 485 nm using a fluorescence spectrophotometer (Hitachi Spectrophotometer F2500). 4-M ethylumbelliferone (M UF; Sigma) was used as a reference compound.

N-acetylchitoooligosaccharides in the culture supernatant were analyzed by high performance liquid chromatography. One ml of culture supernatant was filtrated through 0.45 µm Millipore membrane to remove fine particle, and 20 µl filtrate was injected into APS-2 Hypersil column (Thermo Hypersil-Keystone, 250 × 4.5 mm, 5 µm in particle size). The samples were eluted with a concentration gradient beginning with 20/80 (H2O/acetonitrile, v/v) and ending with 50/50 at 40th min. The gradient was performed with a Hitachi L-7100 pump and eluted N-acetylchitoooligosaccharides were detected at 205 nm with a Hitachi Diode Array detector. A mixture of authentic GlcNACn, n = 1–6, sigma) was made up with 1 mg each component in 1 ml H2O, as external standards.

A separate sample of culture supernatant collected from 7 days old culture broth was also used to determine the molecular weight of enzyme and hydrolyzed chitin. Estimation of molecular weight of the enzymes was performed with discontinuous SDS-PAGE according to Laemmli [12]. The culture supernatant was concentrated by lyophilization, and about 20 µg of total protein was dissolved in sample buffer without 2-mercaptoethanol. Electrophoresis of proteins was conducted in a 10% (w/v) polyacrylamide gel containing 0.5% (w/v) colloidal chitin and 0.1% (w/v) SDS using a Bio-Rad Mini Protein III apparatus at a constant voltage of 140 V for 1.5 h at room temperature. After electrophoresis, SDS was removed from the gel by soaking in 25% (v/v) isopropanol in 10 mM acetate buffer pH 5.0 for 10 min, and then equilibrated in 10 mM acetate buffer pH 5.0 for 15 min. The gel was incubated at 37 °C for 4 h in a plastic container under moist conditions. Proteins with enzymatic activity were detected on gels by staining with 0.01% fluorescent brightener 28 (Sigma) in 50 mM Tris pH 8.3 for 5 min, followed by destaining in distilled water. Clear zones indicating chitinolytic activity were visualized by placing the gel on a UV transilluminator. Proteins on gel were also stained with Coomassie brilliant blue G-250. The molecular weights of the chitinases were estimated by comparing with ProSieve protein standard markers (FM C BioProducts).

**Results and Discussion**

**Identification of strain cf**

Strain cf was isolated from surface water of a freshwater pond for shrimp (Macrobrachium rosenbergii) culture using the chitin enrichment culture. Strain cf formed colonies on CB agar after 3 days of incubation at 35 °C, making large clear zones caused by degradation of chitin around the colonies. No diffusible pigments were produced on CB medium. With a limited medium containing only minerals and chitin, strain cf still exhibited a reasonable growth rate under aerobic and anaerobic conditions, indicating that the organism could use chitin as the exclusive carbon, nitrogen, and energy source for growth. Vitamins and yeast extract were not needed as supplements in mineral medium for growth. Strain cf could be grown on nutrient or trypticase soy agar when the colonies were transferred from CB medium. However, the subcultures died very quickly and colonies grown on nutrient or trypticase soy agar could not be easily transferred again.

Strain cf grew at temperatures ranging from 4 to 39 °C, pH values between 4 and 10, and NaCl concentrations between 0 and 1%. Optimal growth conditions (as determined by measuring optical densities) were around 25–37 °C, 0–1% NaCl and pH 6–8. Light microscopic examination revealed that cells of strain cf were Gram-negative, slightly curved rods, 0.3–0.4 µm wide and 1.2–1.8 µm long. Poly-β-hydroxybutyrate granules were present. Cells were motile by means of single polar flagella. A transmission electron micrograph of strain cf is shown in Fig. 1.

Strain cf exhibited the following biochemical characteristics as determined using the results of API 20NE, API ZYM, BBL Crystal Enteric/Nonfermenter ID and Biolog GNII microtest galleries: nitrate reduction; esculin hydrolysis; oxidase, catalase, urease, β-galactosidase, N-acetylglucosaminidase, C4 esterase, C8 lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, β-glucosidase, and naphthol-AS-BI-phosphohydrolase activity; assimilation of dextrin, glycogen, tween 80, cellobiose, fructose, glucose, mannitol, N-acetylglucosamine, maltose, methyl pyruvate, β-hydroxybutyric acid, D-arabitol, D,L-lactic acid, L-asparagine, L-aspatic acid, L-glutamic acid, L-leucine, p-nitrophenyl α-β-glucoside, proline nitroanilide and p-nitrophenyl-N-acetyl glucosamine.

The following biochemical characteristics were absent: indole production; glucose acidification; arginine dihydrolase, tetrazolium reduction; alkaline phosphatase, α-galactosidase, α-mannosidase and α-fucosidase activity; assimilation of caprate, adipate, malate, mannose, glucose, phenyl-acetate, α-cyclodextrin, N-acetyl-D-galactosamine, adonitol, arabinose, i-erythritol, L-fucose, D-galactose, gentiobiode, m-inositol, lactulose, D-melibiose.

**Fig. 1.** Electron micrograph of a negatively stained cell of Chitinimonas taiwanensis strain cf. Bar, 1 µm.
Fig. 2. Neighbour-joining phylogenetic tree of Chitinimonas taiwanensis strain cfT and related bacteria of β-Proteobacteria based on 16S rRNA sequence comparisons. Scale bar indicates 1% sequence dissimilarity (one substitution per 100 nt). Bootstrap values (%) are indicated at the branches from 1000 replications. Only bootstrap values >50% are shown. Representative sequences in the dendrogram obtained from GenBank which were almost complete sequences were used in the phylogenetic analysis (GenBank accession numbers are shown in parentheses). *, No strain designation provided. The sequence of E. coli K-12 (L10328) was used as an outgroup.
β-methyl D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, mono-methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, ρ-hydroxyphenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromo succinic acid, succinamic acid, glucic acid, quinic acid, D-saccharic acid, sebacic acid, phosphate.

**Taxonomic relationships of strain cfT**

Comparison of the 16S rRNA gene sequence of strain cfT (GenBank nucleotide sequence accession number is AY323827) with available 16S rRNA gene sequences in public databases revealed that it belonged to the proteobacteria (Fig. 2). The highest similarity values were obtained towards the name Pandoraea pnomenusa LMG 9035^T, and that it should be classified in a new genus, Pandoraea, with the type species Pandoraea pnomenusa LMG 9035^T. The similarity levels towards other bacterial species belonging to the β-Proteobacteria were below 91%. The overall topologies of the phylogenetic trees obtained by the neighbour-joining, UPGMA, and Fitch-Margoliash methods were similar (data not shown).

The guanine-plus-cytosine (G+C) content of strain cfT DNA was 62.8 mol%. Its major cellular fatty acid components were 16:0 (29.2%), 18:1 ω7c (11.6%) and a large amount (49.4%) of a mixed unresolved peak corresponding to 16:1 ω7c or 15 iso 2-OH or both (summed feature 3). Other cellular fatty acid components present in minor amounts were 12:0 (3.7%), 12:0 2-OH (1.8%), 14:0 (1.3%), 16:1 ω5c (0.6%), and 18:0 (0.6%). The presence of a large amount of summed feature 3, and trace amounts of 17:0 cyclo, 16:0 3-OH, 19:0 cyclo ω8c seem to differentiate strain cfT from members of the genera Burkholderia and Pandoraea [2, 3, 24], although this observation needs to be confirmed by the analysis of these bacteria under identical growth conditions.

These results indicate that strain cfT represents a presently rather distinct phylogenetic branch within the β-Proteobacteria, and that it should be classified in a species belonging to a novel genus for which we propose the name Chitinimonas taiwanensis gen. nov., sp. nov.

**Chitinolytic activity of strain cfT**

In this study, the change of chitinase activity during the cell growth was also examined. The chitinolytic activity of extracellular proteins in culture supernatant was measured by assessing the release of the fluorescent MUF in reaction mixtures with fluorogenic analogues of disaccharide (MUF-GlcNAc), trisaccharide (MUF-diGlcNAc) and tetrasaccharide (MUF-triGlcNAc) of chitin derivatives. The growth curve, pH value and production of chitinase during cultivation in CB medium are shown in Fig. 3. There was almost no detectable activity against MUF-GlcNAc and minor activity against MUF-diGlcNAc during cell growth, indicating low exochitinases (β-D-glucosaminidase and chitobiosidases activity in the culture supernatant. The highest activity was against MUF-triGlcNAc suggesting that the primary function of the enzyme in culture supernatant is the removal of chitotriose from chitin chains. These results suggest that the chitinolytic activity exhibited by strain cfT was caused by chitotriosidase activity.

The main products of chitin cleavage after cultivation in CB medium were examined by HPLC. The major product was a chitotriose (GlcNAc)3 in the culture supernatant as shown in Fig. 4. This indicated that the exclusive end product hydrolyzed by chitinase of strain cfT was chitotriose. Although there was still some minor activity on the fluorogenic analogue MUF-diglucosamine in the culture supernatant (Fig. 3), no corresponding product, (GlcNAc)2, was revealed by HPLC analysis (Fig. 4). Patil et al. [15] have reported that the substrates with substituted aglyconic moieties are more prone to be hydrolyzed by chitinase than substrates with real sugar residue.

Strain cfT was susceptible to all antibiotics tested.
SDS-PAGE analysis revealed the presence of two chitinases with approximate molecular weights of 52 and 64 kDa as major constituents of the extracellular proteins in 7 days old culture supernatants (Fig. 5a). Chitinase activities were restored after SDS-PAGE analysis and detected by the degradation of colloidal chitin in situ in the gel (Fig. 5b).

**Description of Chitinimonas gen. nov.**

Chitinimonas (Chi.ti.ni.mo’nas N.L. n. chitinum chitin; Gr. n. monas unit, monad; N.L. fem. n. Chitinimonas a chitin-utilizing monad) cells are Gram-negative rods motile by means of single polar flagella. Poly-β-hydroxybutyrate granules are stored as reserve material. Endospores are not formed. Grows well by using chitin as the exclusive carbon, nitrogen and energy source both under aerobic and anaerobic conditions. Growth occurs at 4 to 39 °C, pH 4 to 10 and 0 to 1% NaCl salinity. Catalase and oxidase activity is present. Nitrate is reduced and esculin is hydrolysed. The major fatty acid components are 16:0, 18:1 ω7c and summed feature 3 (16:1 ω7c or 15 iso 2-OH or both). The type species is Chitinimonas taiwanensis.

**Description of Chitinimonas taiwanensis sp. nov.**

Chitinimonas taiwanensis (tai.wan.en’sis N.L. fem. adj. Taiwanensis, of Taiwan, where the type strain was isolated) cells are Gram-negative rods with slightly curved shape, 1.2–1.8 µm long and a diameter of 0.3–0.4 µm. They occur singly and are motile by single polar flagella. Colonies on CB medium display a large clear zone around it, which is derived from degradation of chitin. No diffusible pigments are produced.

Growth characteristics, biochemical and chemotaxonomic details are listed above. The type strain, cfT, was isolated from surface water of an aquaculture shrimp (Macrobrachium rosenbergii) pond at Ping-Tung Country in Southern Taiwan. The G+C content of its DNA is 62.8 mol%.

The type strain has been deposited in the CCRC Bacteria Collection (Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan) as CCRC 17210T and in BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium) as LMG 22011T.

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**Fig. 4.** HPLC profile of chitin digested products of Chitinimonas taiwanensis strain cfT after cultivation in CB medium. (a) Standard of N-acetylchitooligosaccharides from the monosaccharide to hexasaccharide (GlcNAc<sub>n</sub>, n = 1–6). (b) Chitin digested products of Chitinimonas taiwanensis strain cfT after cultivation in CB medium.

**Fig. 5.** Detection of chitinolytic enzymes in extracellular proteins from Chitinimonas taiwanensis strain cfT. (a) SDS-PAGE electropherogram; (b) Chitinolytic activity after separation by SDS-PAGE was detected in situ with the substrate of colloidal chitin. Lane 1, ProSieve SDS-PAGE standards (FMC BioProducts) were used as size markers. Lane 2 and 3, extracellular proteins from strain cfT grown in CB medium.
References


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