Chitinilyticum litopenaei sp. nov., isolated from a freshwater shrimp pond, and emended description of the genus *Chitinilyticum*

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Strain c1^T, originally isolated from surface water of a freshwater pond located in Pingtung (southern Taiwan) used for culture of Pacific white shrimp (*Litopenaeus vannamei*), was subjected to a polyphasic taxonomic analysis. The strain exhibited strong chitinolytic activity and was able to grow under aerobic and anaerobic conditions by utilizing chitin exclusively as the carbon, nitrogen and energy source. Phylogenetic analysis of the 16S rRNA gene sequence revealed a clear affiliation to the *Betaproteobacteria*, with the closest relatives being *Chitinilyticum aquatile* C14^T and *Chitinibacter tainanensis* S1^T, respectively showing 96.7 and 93.6% 16S rRNA gene sequence similarity. The predominant fatty acids detected in cells of strain c1^T were C_{16:0}, C_{18:1ω7c} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The G + C content of the genomic DNA was 62.2 ± 1.0 mol%. On the basis of phylogenetic analysis, DNA–DNA hybridization data, physiological and biochemical characteristics and fatty acid compositions, the organism was shown to belong to the genus *Chitinilyticum* whilst representing a novel species within this genus, for which we propose the name *Chitinilyticum litopenaei* sp. nov. (type strain c1^T = DSM 21440^T = BCRC 17609^T).

Chitin is a complex molecule of natural polymers that is a common constituent of the 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, it is an insoluble homopolymer 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 (Shigemasa & Minami, 1996).

Enormous amounts of chitin are synthesized in the biosphere annually, as much as 10¹⁰–10¹¹ metric tons (Gooday, 1990), but only traces remain in the environ-

ment. Turnover of the polysaccharide is attributed primarily to micro-organisms that degrade chitin, allowing carbon and nitrogen to return to the ecosystem (Gooday, 1990). Therefore, it is anticipated that shrimp-culturing ponds would be a rich source of chitin-degrading bacteria. Shrimp (prawn) farming has contributed significantly to the economy of southern Taiwan. There are many different types of shrimp pond in the Pingtung area of southern Taiwan. Some are close to the coast, but others are deep inland. The pond water ranges from fresh through brackish to seawater. These diverse culture types provide dramatically different ecosystems for micro-organisms to develop.

In our previous studies, two strains of chitin-degrading bacteria, *Chitinimonas taiwanensis* cf^T and *Chitinilyticum aquatile* C14^T, were isolated from two distinct freshwater ponds of shrimp culture (Chang *et al.*, 2004, 2007). In this study, a water sample was collected from a freshwater pond for Pacific white shrimp (*Litopenaeus vannamei*) culture located in the countryside of Pingtung. The pond water was approximately 25 °C and pH 7.0. Chitin-degrading bacteria were enriched from 100 ml sample water amended with 0.5% (w/v) autoclaved colloidal chitin. The original

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Abbreviation: GlcNAc, *N*-acetyl-β-D-glucosamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain c1^T is EU682455.

An HPLC profile of products of chitin degradation by strain c1^T is available as supplementary material with the online version of this paper.

culture was incubated at 25 °C with shaking at 125 r.p.m. (orbital shaker, 25 mm radius; Firstek Company). Colloidal chitin was prepared from commercial chitin (Ohka Chemical) as described by Chang *et al.* (2004). After 5 days of incubation, the enrichment broth was diluted with sterile distilled water and spread onto chitin basal (CB) medium (Chang *et al.*, 2004). A bacterial strain, showing large clear zones around the colonies due to chitin degradation, was isolated and designated c1^T. The colony morphology of strain c1^T was clearly different from that of *Chitinimonas taiwanensis* cf^T but similar to that of *Chitinilyticum aquatile* C14^T (Chang *et al.*, 2004, 2007).

Bacterial cells of strain c1^T were observed by phase-contrast microscopy (Leica DM 2000) in the lag, exponential and stationary phases of growth to ascertain their morphology. The motility of cells was examined by the hanging drop method. Flagellum staining was performed using the Spot Test flagella stain (BD Difco). Gram staining was performed using the Gram stain set S (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Poly- β -hydroxybutyrate granule accumulation was observed by light microscopy after Sudan black staining.

The pH range for growth was determined by measuring the OD₅₉₅ of the culture grown in tryptic soy broth (TSB; BD Difco). The medium was adjusted to pH 3–11 at intervals of 1.0 pH unit by using appropriate biological buffers; glycine/HCl, sodium citrate/Na₂HPO₄, phosphate buffer and glycine/NaOH were respectively used below pH 4 and at pH 4.0–8.0, 6.0–8.0 and 9.0–11.0. For determination of the temperature range for growth, cells were incubated in CB broth at 4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C using an orbital water-bath shaker (125 r.p.m.). Tolerance of NaCl was determined by adjusting the salinity of CB medium to 0, 0.25, 0.3, 0.5, 0.75, 1, 2 and 3 % (w/v) NaCl. Anaerobic cultivation was performed on CB and R2A medium (BD Difco) under 85 % N₂, 5 % CO₂ and 10 % H₂ in an anaerobic chamber (Forma Scientific) and in the AnaeroGen system (Oxoid). Culture supernatant of strain c1^T was used to detect chitinolytic production by HPLC with a standard of *N*-acetylchitooligosaccharides from the monosaccharide to hexasaccharide [(GlcNAc)_{*n*}, *n*=1–6; Sigma] as described previously (Chang *et al.*, 2004, 2007).

Strain c1^T formed visible colonies (approx. 1.0–1.5 mm in diameter) on CB agar after 2 days of incubation at 35 °C. The colonies were cream-white in colour, circular and convex with entire edges. Surrounding the colony, a colourless, clear zone was observed with a diameter extended to more than double the colony size, indicating that chitinolysis was performed by strain c1^T during growth. With a medium containing only minerals and chitin, strain c1^T still exhibited a reasonable growth rate under both aerobic and anaerobic conditions, indicating that the organism could use chitin as the sole carbon, nitrogen and energy source for growth. Strain c1^T could also be grown on nutrient and tryptic soy medium. Strain c1^T grew at 15–40 °C, pH 7.0–11.0 and 0–0.75 % (w/v)

NaCl. Optimal growth conditions were approx. 30–35 °C, pH 8 and 0.25 % (w/v) NaCl. When the chitinolytic products were examined by HPLC and hydrolysis of fluorogenic substrates, the main products that remained in the culture medium were monosaccharides and disaccharides [GlcNAc and (GlcNAc)₂] (Supplementary Fig. S1, available in IJSEM Online). In this respect, strain c1^T could be distinguished from *Chitinimonas taiwanensis* cf^T, which produced chitotriose [(GlcNAc)₃] (Chang *et al.*, 2004), *Chitinibacter tainanensis* S1^T, which produced GlcNAc (Chern *et al.*, 2004), and *Chitinilyticum aquatile* C14^T, which produced disaccharides [(GlcNAc)₂] (Chang *et al.*, 2007), as the major products of chitin cleavage.

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene was carried out as described previously (Chen *et al.*, 2001). Sequence reaction fragments were separated using a DNA sequencer (ABI PRISM 310; Applied Biosystems) and sequence assembly was achieved using the Fragment Assembly System program from the Wisconsin Package 9.1 (GCG, 1995). The nearly complete 16S rRNA gene sequence of strain c1^T was compared with corresponding sequences taken from the GenBank, Ribosomal Database Project II and EzTaxon (Chun *et al.*, 2007) databases. Multiple sequence alignment including strain c1^T and its closest relatives was carried out by using the BioEdit program (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic reconstruction was inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the distance model of Jukes & Cantor (1969) and bootstrap analysis (1000 resamplings).

The 16S rRNA gene sequence of strain c1^T revealed that the strain belonged to the *Betaproteobacteria*. The highest similarity was obtained from the comparison with *Chitinilyticum aquatile* C14^T (96.7 % similarity; Chang *et al.*, 2007). Strain c1^T and *Chitinilyticum aquatile* C14^T were found to form a well-separated, monophyletic group (Fig. 1). The 16S rRNA gene sequence similarity of strain c1^T to members of all other genera within the *Betaproteobacteria* was less than 94 %, such as *Chitinibacter tainanensis* S1^T (93.6 %; Chern *et al.*, 2004), *Deefgea rivuli* WB 3.4-79^T (92.1 %; Stackebrandt *et al.*, 2007), *Iodobacter fluviatilis* ATCC 33051^T (92.1 %; Logan, 1989), *Silvimonas terrae* KM-45^T (91.5 %; Yang *et al.*, 2005), *Chitinimonas taiwanensis* cf^T (91.3 %; Chang *et al.*, 2004) and *Formivibrio citricus* DSM 6150^T (91.1 %; Hippe *et al.*, 1999). The overall topologies of the phylogenetic trees obtained with the neighbour-joining, maximum-likelihood and maximum-parsimony methods were similar.

Genomic DNA of strain c1^T was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by reversed-phase HPLC equipped with a Cosmosil 5C18 column (Waters). Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

Fig. 1. Neighbour-joining phylogenetic tree of strain c1^T and relatives within the Betaproteobacteria based on 16S rRNA gene sequence comparisons. Bootstrap values (%) are indicated at branches from 1000 resamplings; only values >50% are shown. Asterisks indicate branches of the tree that were also recovered using both the maximum-likelihood and maximum-parsimony tree-making algorithms. GenBank accession numbers are shown in parentheses. Bar, 1% sequence dissimilarity (1 substitution per 100 nucleotide positions).

Each experiment was conducted in triplicate. The G+C content of strain c1^T was 62.2 ± 1.0 mol% (mean $\pm$ SD of triplicate measurements), which is lower than the value reported previously for *Chitinilyticum aquatile* C14^T (69.5 mol%; Chang *et al.*, 2007).

Fluorometric DNA–DNA hybridization experiments were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). Hybridization was conducted in 50% formamide at 50 °C. Each experiment was done in triplicate and the values quoted are means $\pm$ SD of triplicate experiments. Strain c1^T showed relatively low DNA–DNA binding with its closest phylogenetic neighbours *Chitinilyticum aquatile* C14^T (38.6 ± 5.1 %) and *Chitinibacter tainanensis* S1^T (11.0 ± 3.2 %).

Cellular fatty acid analysis was carried out by the Identification Service of the Bioresource Collection and Research Center (BCRC, Taiwan). Cellular fatty acids were saponified and methylated and then extracted according to the standard protocol of the Microbial Identification System (MIDI, 1999). Fatty acids were separated by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package. The major cellular fatty acids of strain c1^T were C_{16:0}, C_{18:1}ω7c and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). Detailed fatty acid compositions of strain c1^T and its close phylogenetic neighbours are shown in Table 1. The fatty acid profile of strain c1^T was similar to those of *Chitinilyticum aquatile* C14^T and *Chitinibacter tainanensis* S1^T, which both contained predominantly C_{16:0}, C_{18:1}ω7c and summed feature 3 (Table 1).

Strain c1^T was characterized biochemically by using the Biolog GN2 and API 20NE and API ZYM (bioMérieux) microtest systems according to the manufacturers' instructions. Catalase activity was determined by bubble production in a 10% (v/v) H₂O₂ solution. Oxidase activity was

Table 1. Comparison of fatty acid profiles of strain c1^T and its phylogenetically closest relatives

Strains: 1, c1^T; 2, *Chitinilyticum aquatile* C14^T (data from Chang *et al.*, 2007); 3, *Chitinibacter tainanensis* S1^T (Chern *et al.*, 2004). Values are percentages of total fatty acids; –, not detected/not reported. The position of the double bond in unsaturated fatty acids is obtained by counting from the methyl (ω) end of the molecule.

Fatty acid	1	2	3
C _{12:0}	0.3	2.8	2.6
C _{12:0} 3-OH	3.5	2.6	3.9
C _{14:1} ω5c	0.3	0.8	–
C _{14:0}	1.9	2.5	0.5
C _{15:1} ω8c	–	–	0.3
C _{15:1} ω6c	–	–	0.3
C _{15:0}	–	–	0.8
C _{16:1} ω5c	0.9	0.7	0.6
C _{16:0}	26.2	23.0	16.7
C _{16:0} 3-OH	0.3	0.3	1.3
C _{17:0} cyclo	–	–	0.3
C _{17:1} ω6c	0.3	0.2	–
C _{17:0}	–	–	0.3
C _{18:1} ω7c	24.2	23.3	19.9
C _{18:1} ω5c	–	–	0.2
C _{18:0}	0.2	0.1	0.8
11-Methyl C _{18:1} ω7c	–	–	0.1
10-Methyl C _{19:0}	–	2.4	2.0
C _{19:1} ω6c	0.3	–	–
Summed feature 3*	41.0	41.3	48.6
Summed feature 4*	0.2	–	–

*Summed feature 3 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 comprises C_{14:0} 3-OH and/or iso-C_{16:1} I.

determined on filter paper moistened with a 1% (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Strain c1^T was also examined for a broad range of phenotypic properties using conventional methods (MacFaddin, 2000). Susceptibility to antimicrobial agents was determined by the disc diffusion assay. A cell suspension was diluted in sterile saline (to 0.5 McFarland) after reaching the exponential growth phase and then spread onto CB medium and incubated at 35 °C. Antimicrobial discs (Difco) used in this experiment separately contained amikacin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), rifampicin (5 µg), penicillin G (10 U), streptomycin (10 µg) and tetracycline (30 µg). The effect of antibiotics on cell growth was assessed after 3 days of incubation at 35 °C and susceptibility was scored based on the distance from the edge of the clear zone to that of the disc. If the distance was greater than 3 mm, 1–3 mm or less than 1 mm, the strain was respectively classified as susceptible, moderately susceptible or resistant.

Differences in phenotypic and biochemical characteristics between strain c1^T and related phylogenetic neighbours are summarized in Table 2. Strain c1^T can be distinguished clearly from *Chitinilyticum aquatile* C14^T by the absence of oxidase, glucose fermentation, gelatin hydrolysis and β -galactosidase. Different phenotypic characteristics between strain c1^T and *Chitinibacter tainanensis* S1^T include oxygen requirement, oxidase, nitrate reduction, red pigment production and assimilation of mannose.

Strain c1^T probably represents a novel species belonging to the genus *Chitinilyticum*, since the 16S rRNA gene sequence similarity to the closest relative with a validly published name, *Chitinilyticum aquatile* C14^T, is 96.7%. Moreover, strain c1^T was readily distinguished from its nearest phylogenetic neighbours, *Chitinilyticum aquatile* C14^T and *Chitinibacter tainanensis* S1^T, by phenotypic and biochemical characteristics (Table 2). Therefore, based on phenotypic and phylogenetic criteria, we suggest that strain c1^T should be assigned to a novel species, for which the name *Chitinilyticum litopenaei* sp. nov. is proposed. We also present an emended description of the genus *Chitinilyticum* to take into account the properties of strain c1^T.

Emended description of the genus *Chitinilyticum* Chang et al. 2007

The description of the genus *Chitinilyticum* (Chang et al., 2007) is emended as follows. Oxidase activity is variable for different species. The DNA G+C content is 62.2–69.5 mol%.

Description of *Chitinilyticum litopenaei* sp. nov.

Chitinilyticum litopenaei (li.to.pen.ae'i. N.L. n. *Litopenaeus* the scientific name of a genus of shrimp; N.L. gen. n. *litopenaei* of *Litopenaeus*, referring to the isolation of the

Table 2. Differentiating characteristics between strain c1^T and its phylogenetically closest relatives

Strains: 1, c1^T; 2, *Chitinilyticum aquatile* C14^T; 3, *Chitinibacter tainanensis* S1^T. Results were acquired in the present study with the exception of DNA G+C contents for *Chitinilyticum aquatile* C14^T and *Chitinibacter tainanensis* S1^T, which were respectively obtained from Chang et al. (2007) and Chern et al. (2004).

Characteristic	1	2	3
Source	Fresh water	Fresh water	Soil
O ₂ requirement*	FAN	FAN	SA
Red pigment production	–	–	+
Oxidase	–	+	+
API 20NE tests			
Nitrate reduction	+	+	–
Glucose fermentation	–	+	–
Gelatin hydrolysis	–	+	–
Assimilation of mannose	+	+	–
Oxidation of (Biolog GN2):			
Acetic acid	–	+	–
DL-Lactic acid	–	+	–
α -Hydroxybutyric acid	–	+	–
D-Gluconic acid	–	+	+
L-Asparagine	–	+	–
L-Aspartic acid	–	+	–
L-Glutamic acid	–	+	–
Maltose	+	–	–
Sucrose	–	+	–
D-Fructose	+	+	–
Trehalose	+	+	–
DNA G+C content (mol%)	62.2	69.5	56

*FAN, Facultatively anaerobic; SA, strictly aerobic.

type strain from a culture pond for Pacific white shrimp, *Litopenaeus vannamei*).

Cells are Gram-negative rods, 3.0–4.5 µm long and 0.3–0.5 µm in diameter. They occur singly and are motile by single polar flagella. Poly- β -hydroxybutyrate granules are present. Endospores are not formed. Colonies on CB medium are surrounded by a large clear zone, derived from degradation of chitin. Grows well by using chitin as the sole carbon, nitrogen and energy source under both aerobic and anaerobic conditions. No diffusible pigments are produced. Growth occurs at 15–40 °C, pH 7.0–11.0 and 0–0.75% NaCl. Positive for catalase but negative for oxidase activity. API 20NE tests show positive reactions for nitrate reduction, assimilation of mannose, *N*-acetyl-D-glucosamine and gluconate and negative reactions for glucose fermentation and gelatin hydrolysis, indole production, arginine dihydrolase, urease, aesculin hydrolysis, β -galactosidase and assimilation of arabinose, mannitol, maltose, caprate, adipate, malate, citrate and phenylacetate. In API ZYM tests, shows positive reactions for alkaline phosphatase, C4 esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase

and negative reactions for α -glucosidase, β -glucuronidase, α -galactosidase, β -galactosidase, β -glucosidase, C14 lipase, valine arylamidase, cystine arylamidase, C8 lipase, α -mannosidase, α -fucosidase, trypsin and α -chymotrypsin. The following carbon sources are oxidized (positive in the Biolog GN2 system): dextrin, glucose, D-fructose, glycogen, maltose, sucrose, cellobiose, mannose, trehalose, turanose, N-acetyl-D-glucosamine, methyl pyruvate and gentiobiose. The following substrates are not oxidized (negative in the Biolog GN2 system): gluconate, D-glucuronic acid, acetic acid, α -ketobutyric acid, α -ketovaleric acid, DL-lactate, α -hydroxybutyric acid, glucose 6-phosphate, α -cyclodextrin, Tweens 40 and 80, arabinose, lactulose, psicose, melibiose, itaconic acid, L-fucose, inosine, α -lactose, D-galactose, raffinose, L-rhamnose, mannitol, xylitol, glycerol, arabitol, i-erythritol, D-galacturonic acid, D-sorbitol, *myo*-inositol, adonitol, 2,3-butanediol, N-acetyl-D-galactosamine, DL- α -glycerol phosphate, glucose 1-phosphate, methyl β -D-glucoside, 2-aminoethanol, monomethyl succinate, citrate, formic acid, D-galactonic acid lactone, malonic acid, α -ketoglutaric acid, propionic acid, urocanic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, *p*-hydroxyphenylacetic acid, *cis*-aconitic acid, quinic acid, sebacic acid, γ -aminobutyric acid, β - and γ -hydroxybutyric acids, D-glucosaminic acid, phenylethylamine, L-glutamic acid, L-histidine, L-aspartic acid, L-leucine, L-threonine, L-phenylalanine, L-asparagine, L- and D-alanine, L- and D-serine, L-proline, hydroxy-L-proline, glycyl L-glutamic acid, L-ornithine, DL-carnitine, L-pyroglutamic acid, glycyl L-aspartic acid, L-alanyl glycine, putrescine, alaninamide, glucuronamide, thymidine and uridine. Sensitive to amikacin (30 μ g), ampicillin (10 μ g), chloramphenicol (30 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), novobiocin (30 μ g), rifampicin (5 μ g) and tetracycline (30 μ g). Resistant to erythromycin (15 μ g), gentamicin (10 μ g), penicillin G (10 U) and streptomycin (10 μ g). The major fatty acid components are C_{16:0}, C_{18:1} ω 7c and summed feature 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH). The DNA G+C content of the type strain is 62.2 mol%.

The type strain, c1^T (=BCRC 17609^T =DSM 21440^T), was isolated from surface water of an aquaculture pond containing Pacific white shrimp (*Litopenaeus vannamei*) at Pingtung in southern Taiwan.

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