Endozoicomonas montiporae sp. nov., isolated from the encrusting pore coral *Montipora aequituberculata*

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A Gram-negative, aerobic, rod-shaped bacterium, designated strain CL-33^T, was isolated from the encrusting pore coral *Montipora aequituberculata* collected from seawater off the coast of southern Taiwan. 16S rRNA gene sequence analysis showed that the strain clustered closely with *Endozoicomonas elysicola* MKT110^T (96.7 % similarity). The novel strain required NaCl for growth and exhibited optimal growth at 25 °C and in the presence of 2–3 % NaCl. Predominant cellular fatty acids were summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 39.6 %), summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c; 32.8 %) and C_{16:0} (12.0 %). The DNA G + C content of strain CL-33^T was 50.0 mol%. The results of physiological and biochemical tests allowed the clear phenotypic differentiation of this isolate from *E. elysicola*. It is evident from the genotypic, phenotypic and chemotaxonomic data presented that strain CL-33^T represents a novel species of the genus *Endozoicomonas*, for which the name *Endozoicomonas montiporae* sp. nov. is proposed. The type strain is CL-33^T (=LMG 24815^T =BCRC 17933^T).

The genus *Endozoicomonas*, proposed by Kurahashi & Yokota (2007), belongs to the order *Oceanospirillales* of the class *Gammaproteobacteria*. At the time of writing, the genus comprises only one recognized species, *Endozoicomonas elysicola*, which was isolated from the sea slug *Elysia ornata* collected from off the coast of Izu-Miyake Island, Japan (Kurahashi & Yokota, 2007). The aim of the present study was to determine the taxonomic position of an *Endozoicomonas*-like bacterium, strain CL-33^T, isolated from the encrusting pore coral *Montipora aequituberculata* collected from off the coast of southern Taiwan.

A coral sample (50 g) was collected in a sterile bag from the Kenting coast (Pingtung County, southern Taiwan) at a depth of 10–15 m, stored at 4 °C and transported to the laboratory within 3–4 h. The coral sample was ground completely and plated on marine agar 2216 (MA; BD Difco) by using the standard dilution plating method. After incubation of the plates at 25 °C for 5 days, strain CL-33^T was purified as a single colony. The strain was preserved at –80 °C as a 20 % (v/v) glycerol suspension in marine broth

2216 (MB; BD Difco) or by lyophilization with 20 % (w/v) skimmed milk. Reference strain *E. elysicola* JCM 21568^T was obtained from the Japan Collection of Microorganisms.

Bacterial cells were observed by phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth to ascertain their morphology. Cell motility was tested by using the hanging drop method. The Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. A Gram-stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction. Accumulation of poly-β-hydroxybutyrate granules was investigated by light microscopy after staining of the cells with Sudan black. Colony morphology was examined by using a stereoscopic microscope (SMZ 800; Nikon). The optimum pH range for growth was examined in MB by using the following biological buffers: pH 3.0–4.0, glycine/HCl; pH 4.0–8.0, citrate/Na₂HPO₄; pH 6.0–8.0, phosphate buffer; 9.0–11.0, glycine/NaOH (at 1.0 pH unit intervals). The pH was adjusted prior to sterilization and post-sterilization controls revealed only minor changes in pH. Tolerance to various NaCl concentrations was tested on nutrient broth prepared according to the formula of the BD Difco

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain CL-33^T is FJ347758.

medium, except that the NaCl concentration was altered as required (0, 0.5 and 1.0–5.0 %, w/v, at increments of 1.0 %). Growth at various temperatures (4–45 °C) was measured in MB. Cellular growth was determined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH values, NaCl concentrations and temperatures. Anaerobic cultivation was performed on MA by using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). The 16S rRNA gene sequence of strain CL-33^T was obtained by using a DNA sequencer (ABI Prism 310; Applied Biosystems) and was then assembled by using the Fragment Assembly System program from the Wisconsin package (GCG, 1995). An almost-complete 16S rRNA gene sequence (1464 nt) of the strain was compared against 16S rRNA gene sequences available from the EzTaxon (Chun *et al.*, 2007), Ribosomal Database Project (Maidak *et al.*, 2001) and GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) databases. Multiple sequence alignment with strain CL-33^T and its closest relatives was performed by using BioEdit software (Hall, 1999) and MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm by using the Jukes & Cantor (1969) distance model; bootstrap analysis for the neighbour-joining tree was performed on the basis of 1000 resamplings. A comparison of the 16S rRNA gene sequence of strain CL-33^T with those of members of genera of the order *Oceanospirillales* in the *Gammaproteobacteria* showed that the novel strain fell within the evolutionary radiation occupied by the genus *Endozoicomonas* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm,

strain CL-33^T formed a coherent clade with *E. elysicola* MKT110^T. A similar topology was obtained in phylogenetic trees generated by the maximum-parsimony and maximum-likelihood algorithms (Fig. 1). According to pairwise sequence comparisons, strain CL-33^T was related most closely to *E. elysicola* MKT110^T (96.7 % 16S rRNA gene sequence similarity). Levels of 16S rRNA gene sequence similarity between strain CL-33^T and the type strains of other recognized species within the order *Oceanospirillales* of the *Gammaproteobacteria* were below 92 %.

The DNA G + C content of strain CL-33^T was estimated as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by means of HPLC. The DNA G + C content of strain CL-33^T was 50.0 ± 1.0 mol% (mean ± SD of three determinations).

Biomass of strain CL-33^T and *E. elysicola* JCM 21568^T was obtained after growing the strains on MA at 25 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI Inc.) (Sasser, 1990). The predominant fatty acid constituents of strain CL-33^T were summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c), summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c) and C_{16:0} (Table 1). Isoprenoid quinones were purified by the methods outlined in Minnikin *et al.* (1984) and were analysed by HPLC as described by Collins (1985). The isoprenoid quinones of strain CL-33^T were ubiquinone Q-9 (91 %) and Q-8 (5 %).

DNA–DNA hybridization experiments were performed with *E. elysicola* JCM 21568^T by using the methods described by Ezaki *et al.* (1989). The results indicated low levels of DNA–DNA relatedness between strain CL-33^T and *E. elysicola* JCM 21568^T (12.7 ± 5.7 %; reciprocal, 14.5 ± 4.8 %) (means ± SD of three determinations). These values are clearly below the 70 % cut-off point recommended

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences and constructed by using the neighbour-joining algorithm with the distance model of Jukes & Cantor (1969), showing the phylogenetic position of strain CL-33^T within the genus *Endozoicomonas*. GenBank accession numbers are shown in parentheses. Numbers at nodes are bootstrap percentages (of 1000 resampled datasets) based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms; only values ≥ 50 % are shown. Filled circles indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony algorithms; open circles indicate nodes that were also recovered in the tree generated with the maximum-parsimony algorithm only. Bar, 0.01 substitutions per nucleotide position.

Table 1. Major fatty acids of strain CL-33^T and *E. elysicola* JCM 21568^T

Data are from this study; the two strains were grown on MA at 25 °C for 3 days. Values are percentages of the total fatty acids; —, <0.5 % or not detected.

Fatty acid	CL-33 ^T	<i>E. elysicola</i> JCM 21568 ^T
C _{10:0} 3-OH	2.9	3.3
C _{12:0} 3-OH	—	2.8
C _{14:0}	8.5	6.1
C _{16:0}	12.0	19.7
C _{18:0}	—	2.1
Summed feature 2*	1.5	4.5
Summed feature 3*	39.6	50.0
Summed feature 8*	32.8	9.9

*Summed feature 2 comprised C_{14:0} 3-OH and/or iso-C_{16:1} I; summed feature 3 comprised C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 8 comprised C_{18:1}ω7c and/or C_{18:1}ω6c.

for the assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Strain CL-33^T was examined for a broad range of phenotypic properties. Catalase, oxidase, DNase and lipase activity and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined according to standard methods (Gerhardt *et al.*, 1994; MacFaddin, 2000). The commercially available API 20NE (bioMérieux), API ZYM (bioMérieux) and MicroPlate GN2 (Biolog) microtest systems were used to determine the biochemical properties, enzyme activities and carbohydrate utilization pattern of strain CL-33^T. API ZYM strips were read after 4 h incubation at 37 °C, whereas API 20NE and MicroPlate GN2 results were read after 72 h at 37 °C. Bacterial inocula were suspended in saline solution (3 % NaCl, w/v) containing 10 % (w/v) MB for Biolog tests, whereas cell suspensions (2 % NaCl, w/v) were used for API 20NE tests (Thompson *et al.*, 2006). API ZYM tests were performed according to the methods outlined by the manufacturer. Sensitivity to antibiotics was tested after spreading cells (0.5 McFarland standard) on MA and by using discs containing the following antibiotics: ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 µg), rifampicin (5 µg), streptomycin (10 µg), sulfamethoxazole plus trimethoprim (23.75/1.25 µg) and tetracycline (30 µg). The effects of antibiotics on cell growth were assessed after 3 days incubation, and susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. Cells were scored as susceptible if the distance was greater than 3 mm, as moderately susceptible if the distance was 1–3 mm and as resistant if the distance was less than 1 mm. Detailed results from the phenotypic and biochemical analyses of strain CL-33^T are provided in Table 2 and in the species description. Distinctive

characteristics of the genus *Endozoicomonas* are colony colour, maximum growth temperature, DNA G+C content and hydroxy fatty acid profile (Kurahashi & Yokota, 2007); data for these characteristics for strain CL-33^T were consistent with its assignment to the genus *Endozoicomonas*. It is therefore clear that strain CL-33^T belongs to the genus *Endozoicomonas*. Phenotypic characteristics that differentiate strain CL-33^T from *E. elysicola* MKT110^T are presented in Table 2.

On the basis of 16S rRNA gene sequence comparisons, strain CL-33^T occupies a distinct position within the genus *Endozoicomonas*. This genotypic distinctiveness was supported by the unique combination of chemotaxonomic characteristics (Tables 1 and 2) and biochemical traits (Table 2) for the strain. It is clear from the genotypic and phenotypic data presented that strain CL-33^T represents a

Table 2. Differential genotypic and phenotypic characteristics between strain CL-33^T and *E. elysicola* MKT110^T

Data for the isolation source and the DNA G+C content of *E. elysicola* MKT110^T were taken from Kurahashi & Yokota (2007); all other data were obtained in the present study. +, Positive reaction or growth; —, negative reaction or no growth; w, weakly positive reaction.

Characteristic	CL-33 ^T	<i>E. elysicola</i> MKT110 ^T
Isolation source	Coral	Sea slug
Growth at 4 °C	—	+
Oxidase activity	+	w
Alkaline phosphatase	—	+
C8 esterase	+	—
N-Acetyl-β-glucosaminidase	—	+
Oxidation (Biolog) of:		
Dextrin	+	—
Tween 40	+	w
Tween 80	+	w
Cellobiose	w	—
L-Fucose	—	+
Melibiose	w	—
Pyruvic acid methyl ester	w	—
Succinic acid monomethyl ester	w	—
Acetic acid	w	—
Citric acid	—	+
Propionic acid	w	—
Succinic acid	+	—
Bromosuccinic acid	+	—
L-Alaninamide	+	—
L-Alanine	+	—
L-Serine	+	—
Inosine	w	—
Uridine	+	—
Thymidine	+	—
Glycerol	+	—
DNA G+C content (mol%)	50.0	50.4

novel species of the genus *Endozoicomonas*, for which the name *Endozoicomonas montiporae* sp. nov. is proposed.

Description of *Endozoicomonas montiporae* sp. nov.

Endozoicomonas montiporae (N.L. gen. fem. n. *montiporae* of *Montipora*, referring to the isolation of the type strain from a coral belonging to the genus *Montipora*).

Cells are Gram-negative, motile rods (0.5–0.7 µm in diameter and 1.0–3.0 µm long). Colonies on MA are beige, circular and convex with entire edges. Colonies are approximately 1.0–2.0 mm in diameter on MA after 72 h incubation at 25 °C. Growth occurs at 15–35 °C, at pH 6–10 and in the presence of 1–3 % NaCl. Optimum growth occurs at 25 °C, at pH 8.0 and in the presence of 2–3 % NaCl. Cells are aerobic. No accumulation of poly-β-hydroxybutyrate granules. Positive for oxidase, catalase, DNase and hydrolysis of Tween 20. Negative for lipase (corn oil) and hydrolysis of casein, starch and Tweens 40, 60 and 80. Positive for the following: nitrate reduction, aesculin hydrolysis, alkaline phosphatase, C4 esterase, C8 esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Negative for the following: indole production, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The following carbon substrates (Biolog GN2) are oxidized: dextrin, Tweens 40 and 80, N-acetyl-D-glucosamine, cellobiose, D-galactose, α-D-glucose, maltose, melibiose, methyl pyruvate, monomethyl succinate, acetic acid, α-ketobutyric acid, α-ketoglutaric acid, propionic acid, succinic acid, bromosuccinic acid, L-alaninamide, L-alanine, inosine, uridine, thymidine and glycerol. Does not oxidize α-cyclodextrin, glycogen, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, D-fructose, L-fucose, gentiobiose, myo-inositol, lactulose, D-mannitol, D-mannose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, DL-lactic acid, malonic acid, quinic acid, D-saccharic acid, sebamic acid, succinamic acid, glucuronamide, D-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate or D-glucose 6-phosphate. Sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin, sulfamethoxazole

plus trimethoprim and tetracycline. The predominant fatty acids are summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c), summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c), C_{16:0} and C_{14:0}. The major respiratory quinone is ubiquinone 9 and the DNA G+C content of the type strain is 50.0 mol%.

The type strain, CL-33^T (=LMG 24815^T =BCRC 17933^T), was isolated from the encrusting pore coral *Montipora aequituberculata* collected from seawater off southern Taiwan.

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