Endozoicomonas atrinae sp. nov., isolated from the intestine of a comb pen shell *Atrina pectinata*

Dong-Wook Hyun, Na-Ri Shin, Min-Soo Kim, Sei Joon Oh, Pil Soo Kim, Tae Woong Whon and Jin-Woo Bae

Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

Correspondence
Jin-Woo Bae
baejw@khu.ac.kr

A novel bacterium, designated strain WP70^T, was isolated from the gut of a comb pen shell (*Atrina pectinata*) collected from the southern sea of Yeosu in Korea. The isolate was Gram-stain-negative, aerobic, non-motile and rod-shaped. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain WP70^T belonged to the genus *Endozoicomonas*. The highest level of sequence similarity (98.4 %) was shared with *Endozoicomonas elysicola* MKT110^T. Optimal growth occurred in 2 % (w/v) NaCl at 30 °C and at pH 7. The major cellular fatty acids were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and $C_{16:0}$. The main respiratory quinone was Q-9. The polar lipids comprised phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, three unidentified phospholipids, an unidentified aminolipid, an unidentified aminophospholipid and an unidentified lipid. The genomic DNA G+C content was 50.5 mol% and DNA–DNA hybridization values indicated <11 % genomic relatedness to the closest species. Physiological, biochemical, chemotaxonomic and genotypic analyses indicated that strain WP70^T represents a novel species of the genus *Endozoicomonas*, for which the name *Endozoicomonas atrinae* sp. nov. is proposed. The type strain is WP70^T (=KACC 17474^T=JCM 19190^T).

The genus *Endozoicomonas* belongs to the order *Oceanospirillales* within the class *Gammaproteobacteria*, and was first proposed by Kurahashi & Yokota (2007). Members of the genus *Endozoicomonas* are Gram-stain-negative and strictly aerobic or facultatively anaerobic rod-shaped bacteria, and at the time of writing there are five species of the genus with validly published names: *Endozoicomonas elysicola* (Kurahashi & Yokota, 2007), *Endozoicomonas montiporae* (Yang *et al.*, 2010), *Endozoicomonas numazuensis* (Nishijima *et al.*, 2013), *Endozoicomonas euniceicola* and *Endozoicomonas gorgoniicola* (Pike *et al.*, 2013). All species of the genus *Endozoicomonas* were isolated from marine environments; sources include sponges (Nishijima *et al.*, 2013), corals (Pike *et al.*, 2013, Yang *et al.*, 2010) and sea slugs (Kurahashi & Yokota, 2007).

The comb pen shell (*Atrina pectinata*) belongs to the *Pinnidae* family, and is a large wedge- or fan-shaped suspension-feeding bivalve (Okutani, 1997), which is a valuable seafood resource in Korea, China and Japan. It is distributed throughout the coastal waters of East Asia, and its habitat ranges from muddy-to-sandy sediments to tidal flats or shallow subtidal environments up to 20 m deep (Yurimoto *et al.*, 2003). Although three bacterial species have been isolated from *Atrina pectinata*-associated sources, *Agromyces atrinae* and *Kocuria atrinae* from fermented *Atrina pectinata* seafood (Park *et al.*, 2010a, b) and *Polaribacter atrinae* from the gut of *Atrina pectinata* (Hyun *et al.*, 2014), information about the microbiota within live comb pen shells is limited. Here, a novel *Endozoicomonas*-like bacterium, strain WP70^T, was isolated from the intestine of *Atrina pectinata* during a study of bacterial diversity. The isolate was then taxonomically characterized.

Comb pen shells were sampled from the southern sea of Yeosu in Korea. To isolate bacteria, the intestinal tract was detached, homogenized and serially diluted (10-fold) with 0.22 μm-filtered PBS. Samples (diluted 10^{-1}, 10^{-2} and 10^{-3}) were spread-plated onto marine agar 2216 (MA, Difco) and incubated at 20, 25 and 30 °C. Strain WP70^T was isolated from a 10^{-2}-diluted sample after incubation at 30 °C for 72 h and then purified by repeated subculture. The purified isolate was stored at −80 °C as a suspension in marine broth 2216 (MB, Difco) containing 40 % (v/v) glycerol. All physiological, biochemical, chemotaxonomic and genotypic analyses were repeated at least three times.

To identify the taxonomic position of the strain WP70^T, the 16S rRNA gene sequence of the isolate was obtained by

Abbreviations: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WP70^T is KC878324.

A supplementary figure is available with the online version of this paper.

PCR amplification. Cells from a single colony were added to a PCR Premix (*i*NtRon Biotechnology) with two universal bacterial primers: forward 8F (5′-AGAGTTTGATCCTGG-CTCAG-3′) and reverse 1492R (5′-GGYTACCTTGTTACG-ACTT-3′) (Lane, 1991). The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. The reaction mixtures were then analysed in an automated 3730xl DNA Analyser (Applied Biosystems). The 16S rRNA gene sequences were assembled using SeqMan (DNASTAR) and compared with those of other type strains in the EzTaxon-e database (Kim *et al.*, 2012). The results revealed that strain WP70^T is closely related to the genus *Endozoicomonas*. On the basis of 16S rRNA gene sequences, the isolate shared 98.39 % similarity with *Endozoicomonas elysicola* MKT110^T and less than 96 % similarity with other species of the genus *Endozoicomonas*: *Endozoicomonas numazuensis* HC50^T (95.97 %), *Endozoicomonas montiporae* CL-33^T (95.76 %), *Endozoicomonas gorgoniicola* PS125^T (95.70 %) and *Endozoicomonas euniceicola* EF212^T (94.81 %). Phylogenetic consensus trees were reconstructed based on the aligned sequences using the MEGA 5 software package (Tamura *et al.*, 2011) with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. This indicated that strain WP70^T was a member of a monophyletic branch containing recognized species of the genus *Endozoicomonas* (Fig. 1). Strain WP70^T was further characterized by comparison with *Endozoicomonas elysicola*

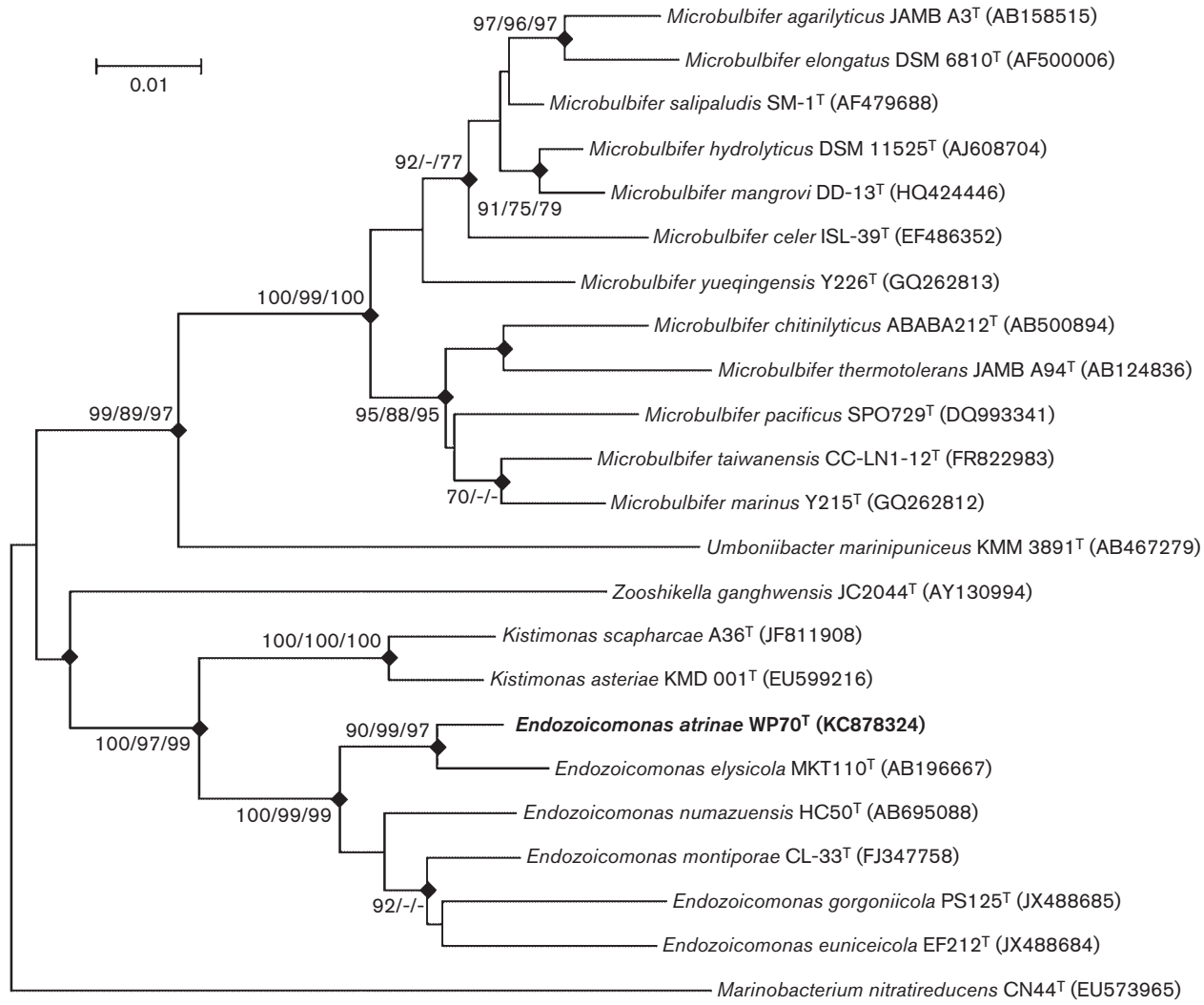

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain WP70^T in relation to species of the genus *Endozoicomonas*. Filled diamonds indicate identical branches generated by all three methods: neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML). Numbers at nodes represent the bootstrap values (NJ/MP/ML) as percentages of 1000 replicates. *Marinobacterium nitratireducens* CN44^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.

Table 1. Differences in the phenotypic characteristics of strain WP70^T and the type strains of closely related species of the genus *Endozoicomonas*

Strains: 1, WP70^T; 2, *Endozoicomonas elysicola* KCTC 23743^T; 3, *Endozoicomonas numazuensis* NBRC 108893^T; 4, *Endozoicomonas montiporae* LMG 24815^T; 5, *Endozoicomonas gorgoniicola* NCCB 100438^T; 6, *Endozoicomonas euniceicola* NCCB 100458^T. All data are from this study, except where otherwise indicated. +, Positive or weakly positive; –, negative. All strains were positive for enzyme activity of alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM); acid production from D-galactose, D-glucose, *N*-Acetylglucosamine and maltose (API 50 CH); utilization of dextrin, Tweens 40 and 80, *N*-acetyl-D-glucosamine, cellobiose, α-D-glucose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, α-ketoglutaric acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanine, L-serine, inosine, uridine and thymidine (Biolog GN2); D-glucose fermentation and Tween 20 hydrolysis. All strains were negative for enzyme activity of lipase (C14), cystine arylamidase, α-chymotrypsin, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase (API ZYM); acid production from erythritol, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, arbutin, salicin, cellobiose, sucrose, trehalose, inulin, melezitose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-arabitol, L-arabitol and 2-ketogluconate (API 50 CH); reduction of nitrates to nitrogen, indole production, L-arginine dihydrolase, gelatin hydrolysis (API 20NE); utilization of α-cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, α-lactose, lactulose, D-mannitol, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, D-alanine, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol and glycerol (Biolog GN2); citrate utilization; H_2S production; hydrolysis of casein, chitin, citrate, H_2S, starch and CM-cellulose.

Characteristic	1	2	3	4	5	6
Growth temperature (°C)						
Range	15–37	4–37*[a]	15–37[b]	15–35[c]	15–30[d]	15–30[d]
Optimum	30	25–30[a]	25[b]	25[c]	22–30[d]	22–30[d]
Aerobic/anaerobic growth	Aerobic	Aerobic[a]	Both[b]	Aerobic[c]	Both[d]	Both[d]
Motility	Non-motile	Motile[a]	Non-motile[b]	Motile[c]	Motile[d]	Motile[d]
Enzyme activities (API ZYM/20NE)						
Esterase (C4),	+	–	–	+	+	+
Esterase lipase (C8)	–	–	+	+	+	+
Trypsin	+	+	+	–	–	–
α-Galactosidase,	+	+	–	+(–[c])	+(–[d])	–
p-Naphthyl-β-D-galactopyranosidase	–	–	–	+(–[c])	+(–[d])	+
β-Glucuronidase, *N*-acetyl-β-glucosaminidase, urease	+	+	–	–	–	–
β-Glucosidase	+	+	–	+	–	–
4-Nitrophenyl-β-D-galactopyranosidase	+	+	–	+	+	+
Utilization of (Biolog GN2 MicroPlate):						
L-Glutamic acid	+	+	+	–	+	+(–[d])
L-Asparagine, L-aspartic acid	+	+	+	–	+	+
L-Threonine	–	+	+	+(–[c])	+(–[d])	+
Maltose	+	+	–	+	–	+
D-Mannose	+	–	+	+(–[c])	+	–
Glycyl L-aspartic acid	–	+	+	–	+	+(–[d])
Glycyl L-glutamic acid, L-proline	–	+	+	–	+	+
α-Ketobutyric acid	–	+	–	+	+(–[d])	+(–[d])
Glycogen, α-D-glucose 1-phosphate	+	+	–	–	+	–
D-Gluconic acid	–	+	+	–	–	+(–[d])
L-Alanylglycine	–	+	–	–	+	+
D-Glucose 6-phosphate	+	+	–	–	–	–
Melibiose	+	–	–	+	–	–
Urocanic Acid, citric acid	+	–	–	–	–	–
DL-α-glycerol phosphate	+	–	–	–	+	–
Acid production from (API 50 CHB):						
Lactose	+	–	–	+	+	+
Gluconate	+	+	+	–	–	–

Table 1. cont.

Characteristic	1	2	3	4	5	6
Glycogen	+	+	−	−	−	+
Aesculin	+	+	−	+	−	−
Starch	+	+	−	−	+	−
Amygdalin	+	+	−	−	−	−
Melibiose	+	−	−	+	−	−
D-Mannose	−	−	+	−	−	+
D-Fructose	−	−	+	−	−	−
D-Arabinose, L-fucose	−	−	−	+	−	−
Raffinose, 5-ketogluconate	+	−	−	−	−	−
Glycerol, inositol	−	+	−	−	−	−
Reduction of nitrates to nitrites	+	+	+	+	−	−
Lipase activity (Spirit blue agar)	+	+	+	−	+	+
DNase activity (DNase test agar)	−	−	− (+[b])	−	+	−
Hydrolysis of:						
Tween 40, Tween 80	+	+	+	−	+	+
Tween 60	+	+	−	−	+	+
Tyrosine	+	+	+	−	−	−
DNA G+C content (mol%)	50.5	50.4[a]	48.7[b]	50.0[c]	47.5[d]	48.6[d]
Isolation source	Comb pen shell	Sea slug[a]	Marine sponge[b]	Pore coral[c]	Octo coral[d]	Octo coral[d]

*Data from: *a*, Kurahashi & Yokota (2007); *b*, Nishijima *et al.* (2013); *c*, Yang *et al.* (2010); *d*, Pike *et al.* (2013).

MKT110^T (=KCTC 23743^T), which was obtained from the Korean Collection for Type Cultures (KCTC) and used as a reference strain. In addition, *Endozoicomonas numazuensis* HC50^T (=NBRC 108893^T) and *Endozoicomonas montiporae* CL-33^T (=LMG 24815^T) were obtained from NITE Biological Resource Center (NBRC) and Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie, Universiteit Gent (BCCM/LMG), respectively, and *Endozoicomonas gorgoniicola* PS125^T (=NCCB 100438^T) and *Endozoicomonas euniceicola* EF212^T (=NCCB 100458^T) were obtained from Netherlands Culture Collection of Bacteria (NCCB) to conduct more comprehensive biochemical features and fatty acid profile comparison with the isolate.

The following characteristics were determined using cells of strain WP70^T grown on MA at 30 °C for 48 h. Gram-staining was performed using a Gram-staining kit (bioMérieux) according to the manufacturer's instructions. Gram-staining and cell morphology were observed under a light microscope (Eclipse 50*i*; Nikon). Cell motility was assessed in semi-solid MA containing 0.4 % agar (Tittsler & Sandholzer, 1936). Gliding motility was also tested using MB according to the method of Bernardet *et al.* (2002). Cells of strain WP70^T were Gram-stain-negative, non-motile and rod-shaped (0.7–1.0 μm wide and 1.2–3.6 μm long). The isolate also formed beige circular colonies that were convex, 0.6–1.1 mm in diameter and had an intact margin. Experiments were conducted to determine the minimal, maximal and optimal growth conditions for strain WP70^T. Cells were incubated in MB at 4, 10, 15, 20, 25, 30, 37, 40, 45, 55 and 65 °C. Growth was also measured in MB at pH 4–11 (at intervals of 1.0 pH unit) to determine the range of tolerance. Broth pH was adjusted using 10 mM MES (for pH 4–6), 10 mM TAPS (pH 7–8) or 10 mM Na_2HPO_4 (pH 9–11). Growth was also measured in the presence of NaCl (0, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 %, w/v) in a medium that contained all of the ingredients of MB except NaCl. The amount of growth after 24 h, 48 h, and 7 days of incubation under each of the culture conditions was determined by measuring the turbidity at 600 nm (OD_{600}) using a spectrophotometer (SYNERGY MX; BioTek). The ability to grow anaerobically was determined by culture in MA at 30 °C for 7 days in an anaerobic chamber filled with $N_2/CO_2/H_2$ (90 : 5 : 5, by vol.). Strain WP70^T grew at 15–37 °C, pH 6–9 and in the presence of 1–4 % NaCl. Optimum growth conditions were 30 °C, pH 7 and 2 % NaCl. Anaerobic growth was not observed. Unless stated otherwise, all tests to characterize the isolate were conducted under optimal growth conditions.

Enzyme activities, hydrolysis, utilization of sole carbon sources and acid production from different carbohydrates were determined for strain WP70^T and the type strains of five species of the genus *Endozoicomonas*. Biochemical tests were conducted using cultures grown on MA under optimal conditions for 48 h. Catalase activity was assessed according to bubble production in the presence of 3 % (v/v) hydrogen peroxide solution, and the presence of oxidase was determined by indophenol blue production using 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). Tests for the hydrolysis of various substrates used MA medium supplemented with each test substrate: 0.5 % (w/v) soluble starch (Junsei); 5 % (w/v) skimmed milk (Difco) for casein (Benson, 1994); 1 % (w/v) Tweens 20, 40, 60 and 80

(Goszczynska & Serfontein, 1998); 0.5 g l^{-1} tyrosine; and 1 % (w/v) CM-cellulose (Sigma) (Teather & Wood, 1982). Hydrolytic activities were determined by the formation of clearance zones around colonies (starch, casein, tyrosine and CM-cellulose) or turbid zones around colonies (Tweens 20, 40, 60 and 80). Citrate hydrolysis, DNase and lipase activity were tested by clearance zone formation. Simmons citrate agar (Difco) for citrate hydrolysis and DNase test agar (Difco) for DNase activity were supplemented with 1.5 % (w/v) NaCl to set the optimal NaCl concentration for growth (2 %, w/v). Lipase reagent and 2 % (w/v) NaCl were added to Spirit blue agar (Difco) for lipase activity tests. Chitin hydrolysis was assessed using the methods of Leisner *et al.* (2008). Additional enzyme activities were tested using API ZYM and API 20NE test strips (bioMérieux), according to the manufacturer's instructions with the minor modification that demineralized water containing 2 % (w/v) NaCl was used as the inoculation fluid. Acid production from a range of different carbohydrates was examined using API 50 test strips (bioMérieux) upon culture in 50 CHB/E medium supplemented with 2 % (w/v) NaCl. Utilization of sole carbon sources was determined using GN2 MicroPlates with GN/GP inoculating fluid (Biolog) supplemented with 2 % (w/v) NaCl, according to the manufacturer's instructions. Strain WP70^T was catalase- and oxidase-positive, and differed from recognized species of the genus *Endozoicomonas* by its utilization of various carbon sources, enzyme activities and acid production from several carbohydrates. The differential biochemical features between strain WP70^T and type strains of recognized species of the genus *Endozoicomonas* are listed in Table 1 and the complete biological test results of the novel isolate are detailed in the species description.

Chemotaxonomic characteristics of strain WP70^T were demonstrated using cell biomass cultured on MA at 30 °C for 48 h. Cellular fatty acids were extracted according to the protocol for the Sherlock Microbial Identification System (MIDI, 1999). The cellular fatty acid composition of the isolate and the five reference strains were identified using gas chromatography (Agilent 6890 gas chromatograph; Agilent Technologies) and the Microbial Identification software package (Sherlock, version 6.2) based on the TSBA6 database (Sasser, 1990). Of the major cellular fatty acids (>5 % of the total) in the novel isolate, 33.9 % were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), 28.0 % were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and 24.4 % were $C_{16:0}$. The fatty acids of summed feature 3, summed feature 8 and $C_{16:0}$ were common to strain WP70^T and the five reference strains; however, there were quantitative differences between the isolate and the type strains of species of the genus *Endozoicomonas*. The complete fatty acid profiles of all the tested strains are presented in Table 2. Isoprenoid quinones were extracted from strain WP70^T using the method of Collins & Jones (1981a). The isoprenoid quinone extracts were purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and identified by HPLC (Collins & Jones, 1981b) using a reverse-phase Hydrosphere C18 (150 × 2.0 mm) column. A more comprehensive analysis of the isoprenoid quinones was performed by liquid chromatography (Ultimate 3000; Dionex) using an ion trap mass spectrometer equipped with an electrospray ionization probe (HCT; Bruker) according to the method of Kaiser *et al.* (2012). The respiratory quinones in strain WP70^T were ubiquinone-9 (Q-9) and ubiquinone-8 (Q-8), which are found in other species of the genus *Endozoicomonas* (Kurahashi & Yokota, 2007, Nishijima *et al.*, 2013, Pike *et al.*, 2013, Yang *et al.*, 2010). In the isolate, Q-9 and Q-8 were constituted in the ratio of 9 : 1. Polar lipids were extracted from the isolate and the reference strains according to the method of Xin *et al.* (2000), and separated by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). Two solvents were used for separation: chloroform/methanol/water (65 : 25 : 4, by vol.) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) for the second dimension. Four spray reagents were used to detect the polar lipids (Tindall, 1990): 5 % ethanolic molybdatophosphoric acid for total lipids, ninhydrin for amino group-containing lipids, Zinzadze's reagent for

Table 2. Cellular fatty acid contents (%) of strain WP70^T and the type strains of closely related species of the genus *Endozoicomonas*

Strains: 1, WP70^T; 2, *Endozoicomonas elysicola* KCTC 23743^T; 3, *Endozoicomonas numazuensis* NBRC 108893^T; 4, *Endozoicomonas montiporae* LMG 24815^T; 5, *Endozoicomonas gorgoniicola* NCCB 100438^T; 6, *Endozoicomonas euniceicola* NCCB 100458^T. All data are from this study. TR, Trace level (<0.5 %); −, not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{12:0}$	0.5	TR	TR	TR	TR	TR
$C_{14:0}$	2.1	9.2	1.4	7.2	9.9	10.2
$C_{16:0}$	24.4	21.7	23.6	17.6	16.8	19.2
$C_{18:0}$	0.5	TR	3.09	1.2	1.7	TR
Unsaturated						
$C_{17:1}\omega8c$	−	TR	−	−	−	0.6
$C_{18:1}\omega9c$	TR	−	1.1	TR	0.8	−
Branched						
$C_{10:0}$ 3-OH	2.7	2.7	2.1	2.8	3.0	2.9
$C_{12:0}$ 3-OH	1.7	2.3	1.2	0.6	0.9	0.8
iso-$C_{15:0}$	−	0.8	−	TR	TR	−
$C_{16:0}$ 3-OH	TR	TR	TR	TR	0.5	TR
Cyclo $C_{17:0}$	1.0	TR	1.6	−	−	−
Summed features*						
2	3.8	3.8	1.7	1.8	2.1	1.5
3	33.9	51.1	31.7	31.2	40.5	45.5
8	28.0	7.1	30.9	35.27	21.3	17.3

*Summed features refer to groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 comprises $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8 comprises $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

phospholipids and α-naphthol for glycolipids. The phospholipids were identified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) with chloroform/methanol/acetic acid/water (50 : 6 : 6 : 1, by vol.) using four standard compounds (SIGMA): phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG). The polar lipids of strain WP70^T comprised DPG, PG, PE, an unidentified aminophospholipid (APL1), three unidentified phospholipids (PL1–3), an unidentified aminolipid (AL1) and an unidentified lipid (L1), which was similar to the type strain of *Endozoicomonas elysicola* (Fig. S1, available in the online Supplementary Material).

Genomic DNA was isolated from both strain WP70^T and *Endozoicomonas elysicola* KCTC 23743^T as described by Rochelle *et al.* (1992). The DNA G+C content of the isolate was estimated fluorometrically with SYBR Gold I (Invitrogen) using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from *Bacteroides thetaiotaomicron* VPI 5482^T, *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T, *Bacteroides fragilis* NCTC 9343^T and *Streptococcus parasanguinis* ATCC 15912^T were used as calibration references. The DNA G+C content of strain WP70^T was 50.5 mol%. The genetic relatedness between the isolate and the type strain of *Endozoicomonas elysicola* was determined by DNA–DNA hybridization using a genome-probing microarray (Bae *et al.*, 2005, Chang *et al.*, 2008). Relatedness values were calculated from the signal-to-noise ratio of the genomic probes (Loy *et al.*, 2005). The DNA–DNA relatedness value for strain WP70^T and type strain of *Endozoicomonas elysicola* was 6.4±0.4 % (10.5±0.4 % in reciprocal). Because the relatedness value was below the threshold of 70 % (Wayne *et al.*, 1987), the isolate was considered to be a distinct genomic species.

On the basis of the physiological, biochemical, chemotaxonomic and genotypic analyses used in this study, it is suggested that strain WP70^T represents a novel species within the genus *Endozoicomonas*, for which the name *Endozoicomonas atrinae* sp. nov. is proposed.

Description of *Endozoicomonas atrinae* sp. nov.

Endozoicomonas atrinae (a.tri′nae. N.L. gen. n. *atrinae* of Atrina, the systematic name of a genus of pen shell, isolated from *Atrina pectinata*).

Cells are Gram-stain-negative, aerobic, rod-shaped (0.7–1.0 μm × 1.2–3.6 μm), non-motile and positive for catalase and oxidase activities. Colonies on MA are circular, convex with an entire margin, beige and 0.6–1.1 mm in diameter after incubation for 48 h at 30 °C. Growth occurs at 15–37 °C (optimum 30 °C), at pH 6–9 (optimum pH 7) and with 1–4 % (w/v) NaCl (optimum 2 %). Sodium ions are an absolute requirement for growth. Positive for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase (API ZYM), urease, D-glucose fermentation, reduction of nitrates to nitrites (API 20NE) and lipase (Spirit blue agar). Aesculin, PNPG (API 20NE), Tweens 20, 40, 60 and 80 and tyrosine are hydrolysed. Acid is produced from D-galactose, D-glucose, *N*-acetylglucosamine, amygdalin, aesculin, maltose, starch, glycogen, gluconate, lactose, melibiose, raffinose and 5-ketogluconate (API 50 CHB). Utilizes dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, cellobiose, α-D-glucose, maltose, D-mannose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, α-ketoglutaric acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-serine, inosine, uridine, thymidine, α-D-glucose 1-phosphate, D-glucose 6-phosphate, melibiose, citric acid, urocanic acid and DL-α-glycerol phosphate (Biolog GN2). The major fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and $C_{16:0}$. The predominant ubiquinone is Q-9 and the minor ubiquinone is Q-8. The polar lipids comprise PG, PE, DPG, an unidentified aminophospholipid, three unidentified phospholipids, an unidentified aminolipid and an unidentified lipid.

The type strain WP70^T (=KACC 17474^T=JCM 19190^T) was isolated from the intestinal tract of a comb pen shell, *Atrina pectinata*, collected from the southern sea of Yeosu in Korea. The DNA G+C content of the type strain is 50.5 mol%.

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References

Bae, J. W., Rhee, S. K., Park, J. R., Chung, W. H., Nam, Y. D., Lee, I., Kim, H. & Park, Y. H. (2005). Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. *Appl Environ Microbiol* **71**, 8825–8835.

Benson, H. J. (1994). *Microbiological Applications: a Laboratory Manual in General Microbiology*, 6th edn. Dubuque, Iowa: W.C. Brown.

Bernardet, J. F., Nakagawa, Y., Holmes, B. & Subcommittee on the taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.

Chang, H. W., Nam, Y. D., Jung, M. Y., Kim, K. H., Roh, S. W., Kim, M. S., Jeon, C. O., Yoon, J. H. & Bae, J. W. (2008). Statistical superiority of genome-probing microarrays as genomic DNA-DNA hybridization in revealing the bacterial phylogenetic relationship compared to conventional methods. *J Microbiol Methods* **75**, 523–530.

Collins, M. D. & Jones, D. (1981a). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbiol Rev* **45**, 316–354.

Collins, M. D. & Jones, D. (1981b). A note on the separation of natural mixtures of bacterial ubiquinones using reverse-phase partition thin-layer chromatography and high performance liquid chromatography. *J Appl Bacteriol* **51**, 129–134.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.

Goszczynska, T. & Serfontein, J. J. (1998). Milk–Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *J Microbiol Methods* **32**, 65–72.

Hyun, D. W., Shin, N. R., Kim, M. S., Kim, P. S., Jung, M. J., Kim, J. Y., Whon, T. W. & Bae, J. W. (2014). *Polaribacter atrinae* sp. nov., a bacterium isolated from the intestine of a comb pen shell *Atrina pectinata*. *Int J Syst Evol Microbiol* **64**, 1654–1661.

Kaiser, P., Geyer, R., Surmann, P. & Fuhrmann, H. (2012). LC-MS method for screening unknown microbial carotenoids and isoprenoid quinones. *J Microbiol Methods* **88**, 28–34.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kurahashi, M. & Yokota, A. (2007). *Endozoicomonas elysicola* gen. nov., sp. nov., a gamma-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst Appl Microbiol* **30**, 202–206.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.

Leisner, J. J., Larsen, M. H., Jørgensen, R. L., Brøndsted, L., Thomsen, L. E. & Ingmer, H. (2008). Chitin hydrolysis by *Listeria* spp., including *L. monocytogenes*. *Appl Environ Microbiol* **74**, 3823–3830.

Loy, A., Schulz, C., Lücker, S., Schöpfer-Wendels, A., Stoecker, K., Baranyi, C., Lehner, A. & Wagner, M. (2005). 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "*Rhodocyclales*". *Appl Environ Microbiol* **71**, 1373–1386.

MIDI (1999). *Sherlock Microbial Identification System Operating Manual, version 3.0*. Newark, DE: MIDI Inc.

Nishijima, M., Adachi, K., Katsuta, A., Shizuri, Y. & Yamasato, K. (2013). *Endozoicomonas numazuensis* sp. nov., a gammaproteobacterium isolated from marine sponges, and emended description of the genus *Endozoicomonas* Kurahashi and Yokota 2007. *Int J Syst Evol Microbiol* **63**, 709–714.

Okutani, T. (1997). Basic data on scarcely wild species in Japan–IV. *Jpn Fish Resour Conserv Assoc Tokyo*, 43–47.

Park, E. J., Kim, M. S., Jung, M. J., Roh, S. W., Chang, H. W., Shin, K. S. & Bae, J. W. (2010a). *Agromyces atrinae* sp. nov., isolated from fermented seafood. *Int J Syst Evol Microbiol* **60**, 1056–1059.

Park, E. J., Kim, M. S., Roh, S. W., Jung, M. J. & Bae, J. W. (2010b). *Kocuria atrinae* sp. nov., isolated from traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **60**, 914–918.

Pike, R. E., Haltli, B. & Kerr, R. G. (2013). Description of *Endozoicomonas euniceicola* sp. nov. and *Endozoicomonas gorgoniicola* sp. nov., bacteria isolated from the octocorals *Eunicea fusca* and *Plexaura* sp., and an emended description of the genus *Endozoicomonas*. *Int J Syst Evol Microbiol* **63**, 4294–4302.

Rochelle, P. A., Fry, J. C., Parkes, R. J. & Weightman, A. J. (1992). DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiol Lett* **100**, 59–65.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Teather, R. M. & Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* **43**, 777–780.

Tindall, B. J. (1990). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Tittsler, R. P. & Sandholzer, L. A. (1936). The use of semi-solid agar for the detection of bacterial motility. *J Bacteriol* **31**, 575–580.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Xin, H., Itoh, T., Zhou, P., Suzuki, K., Kamekura, M. & Nakase, T. (2000). *Natrinema versiforme* sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China. *Int J Syst Evol Microbiol* **50**, 1297–1303.

Yang, C. S., Chen, M. H., Arun, A. B., Chen, C. A., Wang, J. T. & Chen, W. M. (2010). *Endozoicomonas montiporae* sp. nov., isolated from the encrusting pore coral *Montipora aequituberculata*. *Int J Syst Evol Microbiol* **60**, 1158–1162.

Yurimoto, Y., Watanabe, Y., Nasu, H., Tobase, N., Matsui, S. & Yoshioka, N. (2003). Relationship between environmental food and glycogen contents in pen shells. In *Proceedings of 32nd US Japan Symposium on Aquaculture, California, USA*, pp. 16–23.