Corynebacterium atrinae sp. nov., isolated from the gastrointestinal tract of a pen shell, Atrina pectinata

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A novel Gram-stain-positive, non-motile, facultatively anaerobic and rod-shaped bacterium, strain PSPT56T, was isolated from the gastrointestinal tract of a pen shell (Atrina pectinata). Optimal growth of strain PSPT56T was ascertained to occur at 30 °C, pH 8.0 and in the presence of 1–2 % (w/v) NaCl. The strain was catalase-positive and oxidase-negative. The major cellular fatty acids were C18:1ω9c, C16:0, C17:1ω8c and C17:0. Tuberculostearic acid was not present. The major cell-wall sugars were ribose, galactose, glucose and arabinose. Peptidoglycan amino acids were meso-diaminopimelic acid, alanine and glutamic acid. The predominant isoprenoid quinone was MK-8(H2). Strain PSPT56T contained phosphatidylglycerol, diphosphatidylglycerol, an unidentified phospholipid, two unidentified lipids and two unidentified amino-lipids. Mycolic acids were detected as constitutive components of the cell wall. A phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strain PSPT56T was most closely related to Corynebacterium testudinoris M935/96/4T and Corynebacterium felinum M714/95/5T with 98.69 % and 97.01 % similarity, respectively. DNA–DNA hybridization experiments indicated less than 29.9 % relatedness to the phylogenetically closest species. The G+C content of genomic DNA was 67.6 mol%. The phenotypic, phylogenetic and genotypic analyses indicated that strain PSPT56T represents a novel species within the genus Corynebacterium, for which the name Corynebacterium atrinae is proposed. The type strain is PSPT56T (=KACC 17525T=JCM 19266T).

The genus Corynebacterium, which was created by Lehmann & Neumann (1896), contains more than 100 species of widely known Gram-positive, asporogenous bacteria in the phylum Actinobacteria. The members of the genus Corynebacterium are characterized by chemotaxonomic features such as tuberculostearic acid (TBSA) among cellular fatty acids, menaquinone MK-8(H2) and/or MK-9(H2) as isoprenoid quinones (Collins et al., 1985; Collins et al., 1982), meso-diaminopimelic acid (DAP)-containing A1γ-type cell-wall peptidoglycan (Bernard & Funke, 2012) and arabinose and galactose as major cell-wall sugar components. Many of the constituents of the genus Corynebacterium were isolated from human clinical specimens: Corynebacterium diphtheriae (Lehmann & Neumann, 1896), C. falsenii (Sjödén et al., 1998), C. lipophiloflavum (Funke et al., 1997) and C. tuberculostearicum (Feurer et al., 2004). Additionally, various animals and food materials have been sources of species of the genus Corynebacterium: Corynebacterium nuruki (Shin et al., 2011), C. casei, C. mooreparkense (Brennan et al., 2001), C. epidermidicaris (Frischmann et al., 2012), C. spheniscorum (Goyache et al., 2003a), C. sphenisci (Goyache et al., 2003b), C. testudinoris and C. felinum (Collins et al., 2001). Here, we described a novel species of the genus Corynebacterium, designated strain PSPT56T, based on physiological, biochemical and genotypic investigations.

Strain PSPT56T was isolated from the gastrointestinal tract (GIT) of a pen shell (Atrina pectinata) collected from offshore of Yeosu, Jeollanam-do, Korea. Homogenized GIT tissue was serially diluted with filtered PBS buffer and spread on trypticase soy agar (TSA; Bacto) plates. These plates were cultured at 25 °C for 1 week. Cultivation on fresh media was performed repeatedly to obtain a pure culture. All tests were conducted in triplicate. For phylogenetic analysis, amplification of the 16S rRNA gene of the isolate was carried out through colony-PCR, as described by Kim et al. (2013), with PCR pre-mix (iNtRon Biotechnology) and two universal bacterial-specific primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGAATTTCCGTAGACCTT-3′).
and 9) or 10 mM Na₂HPO₄ (for pH 10). The NaCl 10 mM MES (for pH 4, 5 and 6), 10 mM TAPS (for pH 7, 8 Aldrich) and 1 M NaOH solution (Sigma Aldrich) with medium was adjusted using 6 M HCl solution (Sigma 532 International Journal of Systematic and Evolutionary Microbiology 65 components of TSB except NaCl. The turbidity of each culture and 15 %, w/v), which was prepared by including all com-

The assembled near-full-length 16S rRNA gene sequence was compared with other sequences of type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The 16S rRNA gene comparison revealed that the isolate shared high similarity with C. testudinoris M935/ 96/4T (98.69 %) and C. felinum M714/95/5T (97.01 %). The 16S rRNA gene sequences of strain PSPT56T and representa-
tives of species of the genus Corynebacterium were aligned using the multiple alignment program CLUSTAL W (Thompson et al., 1994). BioEdit software was used for checking the aligned sequences manually (Hall, 1999). Phylogenetic trees based on the 16S rRNA gene sequences of the isolate and the closely related members of the genus Corynebacterium were reconstructed using the MEAG5 software (Tamura et al., 2011). Neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (MP; Kluge & Farris, 1969) and maximum-likelihood (ML; Felsenstein, 1981) methods with 1000 bootstrap replicates were used to confirm phylogenetic correlations (Felsenstein, 1985). The conjoined phylogenetic consensus tree (branch reconstructed by neighbour-joining algorithm with bootstrap value of corresponding branches from MP/ML) based on the 16S rRNA genes of strain PSPT56T and the type strains of species of the genus Corynebacterium is shown in Fig. 1. We obtained C. testudinoris CCUG 41823T (=M935/ 96/4T) and C. felinum CCUG 39943T (=M714/95/5T) from the Culture Collection, University of Göteborg, Sweden (CCUG) to conduct a more comprehensive characterization of strain PSPT56T.

Temperature and pH ranges for growth and optimal growth conditions of the isolate were examined by cultivation at various temperatures (4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C) and at pH 4.0–10.0 (at intervals of 1.0 pH unit) in tryp
ticasoy broth (TSB; Bacto) medium. The pH of the medium was adjusted using 6 M HCl solution (Sigma Aldrich) and 1 M NaOH solution (Sigma Aldrich) with 10 mM MES (for pH 4, 5 and 6), 10 mM TAPS (for pH 7, 8 and 9) or 10 mM Na₂HPO₄ (for pH 10). The NaCl requirement and tolerance were verified in TSB medium with different NaCl concentrations (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v), which was prepared by including all components of TSB except NaCl. The turbidity of each culture was measured at OD₅₀₀ using a spectrophotometer (Synergy MX, BioTek) after 24 h, 48 h, 72 h and 7 days of incubation. Strain PT56T showed growth at 10–37 °C, pH 6.0–8.0 and in the presence of 0–7 % (w/v) NaCl, and showed optimal growth at 30 °C, pH 8.0 and in the presence of 1–2 % (w/v) NaCl. Unless stated otherwise, all experiments were carried out under optimal growth conditions for 24–48 h.

The Gram staining test was performed using a Gram staining kit (bioMérieux) according to the manufacturer’s instructions. Gram staining and cell morphology were observed with a light microscope (ECLIPSE 50i, Nikon). Semi-solid agar media were used for checking cellular motility (Tittsler & Sandholzer, 1936). Growth under anaerobic conditions was identified in an anaerobic chamber (Sheldon Manufacturing), filled with an atmosphere composed of N₂/CO₂/H₂ (90:5:5). Cell morphology tests showed that cells were Gram-stain positive and rod-shaped, and formed orange–cream-coloured, circular (0.5–1.0 mm in diameter), smooth and slightly viscous colonies after 48 h on TSA medium at 30 °C.

Catalase and oxidase activities were confirmed by bubble production in 3 % (v/v) hydrogen peroxide and change in colour of a colony by 1 % (w/v) tetramethyl-p-phenylenediamine (bioMérieux), respectively. The isolate was catalase-positive and oxidase-negative. API ZYM test strips (bioMérieux) and API Coryne test strips (bioMérieux) with API suspension medium (bioMérieux) were used to confirm the enzyme activities of the isolate and the type strains of reference species, according to the manufac-
turer’s instructions. Acid production from various carbohydrates and metabolism of sole carbon sources were ascertained using API 50 CH test strips (bioMérieux) with 50 CHB/E medium (bioMérieux) and GP2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog), respect-
ively. The isolate and C. testudinoris CCUG 41823T differed in their ability to metabolize 3-methyl glucose, succinamic acid and L-asparagine (GN2 MicroPlate), produce acid from amygdalin, cellobiose and gentiobiose (API 50 CH) and their level of enzyme activity for α-gluco
cidase, pyrazinamidase and β-glucosidase (hydrolysis of aesculin) (API Coryne). The complete results of biochemical tests and the differential characteristics of strain PSPT56T are presented in Table 1 and the species description.

Chemotaxonomic analyses were performed with the cell biomass of strain PSPT56T and the type strains of reference species cultured on TSA plates for 24 h. To extract cellular fatty acids, the protocol of the Sherlock Microbial Identification System was applied (MIDI, 1999). The cellular fatty acids were analysed by GC (Agilent 6890 GC, Agilent Technologies). The peaks of the gas chromato
gram were identified using the MIDI software package (Sherlock version 6.2B) (Sasser, 1990) with the enclosed TSBA6 library. The dominant cellular fatty acids (>(10 %) of strain PSPT56T were C₁₈:1ω9c (38.0 %), C₁₆:0 (26.3 %), C₁₇:1ω8c (21.4 %) and C₁₇:0 (11.2 %). Neither the novel isolate nor the type strains of reference species contained tuberculostearic acid (10-methyl C₁₈:0). The complete cellular fatty acid compositions of strain PSPT56T and the type strains of reference species are given in Table 2. Isoprenoid quinone extraction was carried out by steeping in chloroform/methanol (2:1, v/v) overnight, as described by Collins & Jones (1981). Reverse-phase HPLC with a Thermo ODS HYPERSIL (250 x 4.6 mm) column was employed for analysis of isoprenoid quinone composition. Liquid chromatography (LC) (Ultimate 3000, Dionex) analysis using an ion trap-mass spectrometer equipped
with an electrospray ionization probe (HCT, Bruker Daltonics) was performed for intensive discrimination of the quinone constituent, according to the protocol of Taguchi et al. (2005). The predominant isoprenoid quinones of strain PSPT56^T were menaquinones MK-8(H2) (69.6 %), MK-9(H2) (16.2 %) and MK-7(H2) (14.2 %), which were similar to those of *C. testudinoris* (analysis performed in this study) and other species in the genus *Corynebacterium* (Collins et al., 1977). The polar lipid composition was identified by extracting polar lipids according to the protocol described by Xin et al. (2000) and separating the extracted polar lipids using two-dimensional TLC on a silica gel 60 F254 plate (Merck). The reagents used for detecting and identifying the polar lipids were as follows: 10 % ethanolic molybdophosphoric acid reagent for total lipids, ninhydrin reagent for amino-containing lipids and Zinzadze reagent for phospholipids (Tindall, 1990). The phospholipids were identified using one-dimensional TLC with standard compounds (Sigma Aldrich). The polar lipids of strain PSPT56^T included two unidentified lipids (L1–2), two unidentified aminolipids (AL1–2), an unidentified phospholipid (PL1), phosphatidylglycerol (PG) and diphasphatidylglycerol (DPG) (Fig. S1a, available in the online Supplementary Material). The isolate shared three phospholipids (PL1, PG, and DPG) with *C. testudinoris*; however, unidentified lipid components such as AL1, AL2, L1 and L2 were different from those of *C. testudinoris* (Fig. S1b). Cell-wall hydrolysates were extracted as described by Schleifer & Kandler (1972). Cell-wall sugars and standard sugars (ribose, galactose, glucose, mannose, arabinose, xylose and rhamnose) (Schön & Groth, 2006) were spotted on a cellulose TLC plate (Merck) and developed twice in ethyl acetate/pyridine/water (100 : 35 : 25, by vol.). To detect sugars on the TLC plate, aniline-phthalate spray reagent was sprayed. Strain PSPT56^T contained ribose, galactose and arabinose as cell-wall sugars. Cell-wall peptidoglycan amino acids were purified and then hydrolysed according to the method of Bousfield et al. (1985). L-L-DAP and meso-DAP, 2,4-diaminobutyric acid, glutamic acid, glycine, alanine, lysine, threonine and ornithine (Sigma Aldrich) were used as

**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequence comparisons, mainly reconstructed using the neighbour-joining algorithm (NJ). Maximum-parsimony (MP) and maximum-likelihood (ML) algorithms were applied for additional comparison. Filled diamonds indicate branches present in the phylogenetic trees generated using the three different methods. Numbers on the nodes (>70 %) represent bootstrap values as percentages of 1000 replicates (NJ/MP/ML). *Turicella otitidis* 234/92^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.
Table 1. Differential characteristics of strain PSPT56<sup>T</sup> and closely related species of the genus Corynebacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Metabolism of:</td>
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<tr>
<td>3-Methyl glucose, succinamic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Asparagine</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Arbutin, salicin, maltose, D-psisose, N-acetyl-D-glucosamine, trehalose, sucrose, N-acetyl-β-D-mannosamine, methyl β-D-glucoside, D-ribose, α-ketoglutaric acid, β-hydroxybutyric acid, D-lactic acid methyl ester, L-malic acid, pyruvatic acid methyl ester, succinic acid monomethyl ester, pyruvic acid, succinic acid, L-glutamic acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdalin, cellobiose, gentiobiose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin, aesculin, salicin, sucrose, turanose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Enzyme activity and reaction</td>
<td></td>
<td></td>
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<tr>
<td>Reduction of nitrate to nitrite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Esterase (C4), esterase lipase (C8)</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>+</td>
<td>–*</td>
<td>+*</td>
</tr>
<tr>
<td>Pyrroolidonyl arylamidase</td>
<td>–</td>
<td>–*</td>
<td>+*</td>
</tr>
<tr>
<td>β-Glucosidase (hydrolysis of aesculin)</td>
<td>v</td>
<td>–*</td>
<td>–*</td>
</tr>
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</table>

*Data from Collins et al. (2001).

The genomic DNA of strain PSPT56<sup>T</sup>, C. testudinoris and C. felinum was extracted as described by Rochelle et al. (1992) for genotypic analyses. A fluorimetric method with SYBR Gold I (Invitrogen) and a real-time PCR thermocycler (BioRad) was used to estimate the G + C content of the genomic DNA (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA of Escherichia coli K-12 (50.8 mol%), Bacteroides fragilis JCM 11019<sup>T</sup> (43.1 mol%), Bacteroides thetaiotaomicron JCM 5827<sup>T</sup> (42.9 mol%) and Ruegeria pomeroyi DSS-3<sup>T</sup> (64.1 mol%) was used for calibration. The DNA G + C content of the isolate was 67.6 mol%. This value fell within the DNA G + C content range of the genus Corynebacterium (51–74 mol%) (Bernard & Funke, 2012). To elucidate the genetic relatedness between strain PSPT56<sup>T</sup> and the most closely related species, DNA–DNA hybridization (DDH) was performed using the genome-spotted microarray method (Bae et al., 2005; Chang et al., 2008). The DDH values were calculated from the signal-to-noise ratio of the genomic probes (Loy et al., 2005). The values of DDH between the isolate and the type strains of reference species were as follows: 29.9±3.4 % (49.4±17.6 % reciprocal) to C. testudinoris CCG 41823<sup>T</sup> and 6.9±4.2 % (26.4±15.2 % reciprocal) to C. felinum CCG 39943<sup>T</sup>. The DDH values were below the novel genotypic species threshold of 70 % (Wayne et al., 1987).

The results of phylogenetic, phenotypic and genotypic analyses indicate that strain PSPT56<sup>T</sup> represents a novel species of the genus Corynebacterium, for which the name Corynebacterium atrinae sp. nov. is proposed.

**Description of Corynebacterium atrinae sp. nov.**

*Corynebacterium atrinae* (a.tri’nae. N.L. gen. n. *atrinae* of *Atrina*, isolated from the gastrointestinal tract of a pen shell, *Atrina pectinata*). Cells are Gram-stain positive, rod-shaped, non-motile, catalase-positive and oxidase-negative. Colonies are circular, orange-cream-coloured, smooth and slightly viscous on TSA medium after 48 h at 30 °C. Growth occurs at temperatures between 10 and 37 °C, at pH 6.0–8.0 and the presence of 0–7 % (w/v) NaCl. Optimal growth conditions...
are 30 °C, pH 8.0 and in the presence of 1–2 % (w/v) NaCl. Acid is produced from D-ribose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, arbutin, aesculin, salicin, maltose, sucrose, trehalose and turanose (API 50 CH). Metabolizes dextrin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, arbutin, D-fructose, α-D-glucose, maltose, maltotriose, D-mannose, methyl β-D-glucoside, D-psicose, D-ribose, salicin, sucrose, trehalose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketoglutaric acid, D-lactic acid methyl ester, L-lactic acid, L-malic acid, pyruvatic acid methyl ester, succinic acid monomethyl ester, pyruvic acid, succinic acid, L-glutamic acid, adenosine, 2′-deoxyadenosine, inosine, thymidine, uridine and L-asparagine (Biolog GP2 Microplates). The following enzyme activities and reactions are positive based on the API ZYM and API Coryne test strip: esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, α-glucosidase, β-glucosidase (hydrolysis of aesculin) (API ZYM), reduction of nitrate to nitrite, pyrazinamidase and α-glucosidase (API Coryne). The following enzyme activities and reactions are negative based on the API ZYM and API Coryne test strip: alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase (API ZYM), pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, N-acetyl-β-glucosaminidase, β-glucosidase (hydrolysis of aesculin), urease and gelatin hydrolysis (API Coryne). The major cell-wall sugars are ribose, glucose, arabinose and galactose. The predominant quinones are menaquinones MK-8(H2), MK-7(H2) and MK-9(H2). The major cellular fatty acids (>10 %) are C18:1ω9c, C16:0, C17:0ω8c and C17:0. Tuberculostearic acid is absent. Mycolic acids are contained. Polar lipids comprise two unidentified lipids, an unidentified phospholipid, two unidentified aminolipids, phosphatidylglycerol and diphosphatidylglycerol. Alanine, glutamic acid and meso-DAP are contained in the peptidoglycan. Mycolic acids are present.

The type strain is PSPT56T (=KACC 17525T=JCM 19266T), isolated from the gastrointestinal tract of a pen shell (Atrina pectinata) at Yeosu, Jeollanam-do, Korea. The DNA G+C content of the type strain is 67.6 mol%.

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References


