Agromyces atrinae sp. nov., isolated from fermented seafood

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A Gram-staining-positive, aerobic, non-motile and rod-shaped bacterium, designated P27^T, was isolated from a traditional fermented seafood. The isolate grew optimally with 0–2.0 % (w/v) NaCl and at pH 6–7 and 30 °C. The predominant menaquinones were MK-12 and MK-11. The major cellular fatty acids were anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The major cell-wall sugars were galactose, mannose and rhamnose. The peptidoglycan amino acids of strain P27^T were 2, 4-diaminobutyric acid, alanine, glutamic acid and glycine. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. The genomic DNA G+C content of strain P27^T was 69.0 mol%. Based on its 16S rRNA gene sequence, strain P27^T showed highest pairwise similarity with *Agromyces cerinus* subsp. *cerinus* JCM 9083^T (97.0 % similarity). Based on phenotypic, genotypic and phylogenetic studies, strain P27^T represents a novel species in the genus *Agromyces*, for which the name *Agromyces atrinae* sp. nov. is proposed. The type strain is P27^T (=KCTC 19593^T =JCM 15913^T).

The genus *Agromyces* was introduced by Gledhill & Casida (1969). *Agromyces* species are typical Gram-positive, aerobic to microaerophilic, mesophilic actinobacteria. At the time of writing, the genus *Agromyces* contains 19 recognized species (Zgurskaya *et al.*, 1992; Suzuki *et al.*, 1996; Takeuchi & Hatano, 2001; Dorofeeva *et al.*, 2003; Li *et al.*, 2003; Rivas *et al.*, 2004; Jurado *et al.*, 2005a, b, c; Jung *et al.*, 2007; Yoon *et al.*, 2008). They are found mainly in the soil and rhizosphere; however, two species, *Agromyces albus* and *Agromyces ulmi*, have been isolated from plants (Dorofeeva *et al.*, 2003; Rivas *et al.*, 2004). Strain P27^T was isolated from a traditional fermented seafood that is generally made with plenty of salt.

Strain P27^T was isolated using the standard dilution-plating method at 30 °C on marine agar (MA; BBL). Optimum culture conditions for strain P27^T were determined at 4, 10, 15, 20, 25, 30, 37 and 40 °C and pH 3–10 (at intervals of 1 pH unit) on MA and in marine broth (MB; BBL), respectively. NaCl tolerance and requirement were determined in MB containing various NaCl concentrations (0, 1, 2, 3 and 5–30 % at intervals of 5 %, w/v). For the investigation of its morphological and physiological characteristics, strain P27^T was routinely cultivated in MB or on MA under optimum culture conditions. Cell morphology was observed with a light microscope (E600; Nikon). Cultured bacterial cells at the exponential growth phase in liquid medium were stained using a Gram-staining reagent (Difco), according to the manufacturer's instructions. Motility and flagella were determined using semisolid agar (Tittsler & Sandholzer, 1936) and transmission electron microscopy, respectively. Growth under anaerobic conditions was determined after incubation for 7 days at 30 °C on MA in an oxygen-free anaerobic chamber with a $N_2/CO_2/H_2$ (8 : 1 : 1) atmosphere. Catalase and oxidase activities were determined by bubble production in a 3 % (v/v) H_2O_2 solution and with an oxidase reagent (bioMérieux), respectively. API 20NE and API ZYM kits (bioMérieux) with 0.85 % NaCl inoculating fluid were used to investigate enzyme activities. Biolog GP2 metabolic fingerprinting plates with GN/GP inoculating fluid were used to examine substrate utilization from various sole carbon sources, according to the manufacturer's instructions. Hydrolysis of casein, xanthine and hypoxanthine was determined as described by Cowan & Steel (1965).

Strain P27^T was Gram-staining-positive, aerobic, non-motile and rod-shaped (1.0–2.0 × 0.5–1.0 μm) and grew at 15–37 °C, at pH 6–10 and in the presence of 0–4 % NaCl. No growth was detected with NaCl concentrations higher than 5 %. The isolate did not grow under anaerobic conditions. Strain P27^T was catalase-positive and oxidase-negative. Catalase and oxidase activities vary among *Agromyces* species (Zgurskaya *et al.*, 1992). The morpho-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P27^T is FJ607310.

logical, cultural, physiological and biochemical characteristics of strain P27^T and closely related *Agromyces* type strains are summarized in Table 1.

For cellular fatty acid methyl ester analysis, strain P27^T was grown on MA at 30 °C for 2 days and harvested. Fatty acid methyl esters were extracted and analysed using standard protocols provided by the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Whole-cell sugars were determined as described by Komagata & Suzuki (1987). The menaquinone composition was analysed by TLC as described by Hiraishi *et al.* (1996). Polar lipids were extracted according to the procedures described by Xin *et al.* (2000) and were identified using two-dimensional TLC followed by spraying with appropriate detection reagents. The whole cell-wall peptidoglycan was extracted and purified as described by Schleifer & Kandler (1972). The amino-acid composition and sugars of the peptidoglycan were analysed by TLC by using the methods of Schleifer & Kandler (1972).

Table 1. Comparison of characteristics of strain P27^T with its phylogenetically closest relatives in the genus *Agromyces*

Strains: 1, *Agromyces atrinae* sp. nov. P27^T; 2, *A. cerinus* subsp. *cerinus* JCM 9083^T; 3, *A. fucosus* ATCC 51764^T; 4, *A. cerinus* subsp. *nitratus* IMET 11532^T. Data were obtained in this study. +, Positive; −, negative.

Characteristic	1	2	3	4
Enzyme activity				
Esterase (C4)	+	−	−	−
Cystine arylamidase	−	−	+	+
Acid phosphatase	−	+	+	+
β-Galactosidase	+	−	−	−
Utilization of:				
Glycogen	+	−	+	−
Cellobiose	+	−	+	+
D-Fructose	+	−	−	+
L-Fucose	−	+	+	+
D-Gluconic acid	+	−	−	+
α-D-Glucose	+	−	−	−
Maltose	+	−	+	+
D-Mannitol	+	−	−	−
D-Mannose	+	−	+	+
D-Psicose	+	−	+	−
L-Rhamnose	−	+	+	+
D-Ribose	+	+	+	−
Sucrose	+	−	−	−
Trehalose	+	−	−	−
Turanose	−	−	+	+
D-Xylose	+	−	−	−
Acetic acid	+	−	+	−
α-Hydroxybutyric acid	−	+	+	−
γ-Hydroxybutyric acid	−	+	−	−
p-Hydroxyphenylacetic acid	−	+	+	−
L-Lactic acid	−	+	+	−
Pyruvic acid methyl ester	+	+	+	−
Propionic acid	−	−	+	−
L-Alaninamide	−	+	+	−
L-Alanyl glycine	−	+	+	+
Putrescine	−	+	+	−
Inosine	+	+	−	−
Uridine	+	+	−	−
Thymidine 5′-monophosphate	+	−	−	−
Uridine 5′-monophosphate	+	−	−	−

Analyses of fatty acids and their relative amounts and comparison with reference strains are necessary for the identification and classification of novel bacteria (Busse *et al.*, 1996). The major cellular fatty acid methyl esters (contributing more than 5% of the total) in strain P27^T were anteiso-$C_{17:0}$ (40.1%), anteiso-$C_{15:0}$ (33.4%) and iso-$C_{16:0}$ (13.3%). Anteiso-$C_{15:1}$ (4.4%), iso-$C_{15:0}$ (2.3%), iso-$C_{17:0}$ (2.17%) and $C_{16:0}$ (1.5%) were minor components. Other fatty acids ($C_{18:0}$, iso-$C_{18:0}$, $C_{18:1}$, iso-$C_{15:1}$, iso-$C_{14:0}$, $C_{14:0}$ and anteiso-$C_{19:0}$) contributed less than 1% of the total fatty acids. The major cellular fatty acids of the genus *Agromyces* are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$ (Zgurskaya *et al.*, 1992; Jung *et al.*, 2007; Yoon *et al.*, 2008). Thus, the fatty acid profile of strain P27^T was similar to that of other *Agromyces* species. The major cell-wall sugars of strain P27^T were galactose, mannose and rhamnose; glucose and ribose were also detected. The following sugars have been detected in purified cell walls of *Agromyces* species: galactose, glucose, rhamnose, tyvelose, fucose, mannose and ribose (Zgurskaya *et al.*, 1992). The major polar lipids in strain P27^T were diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. The peptidoglycan amino acids were 2,4-diaminobutyric acid, alanine, glutamic acid and glycine. MK-12 (56%) and MK-11 (35%) were the predominant menaquinones; MK-10 (7.1%) and MK-13 (1.9%) were minor components. The predominant menaquinones of strain P27^T were similar to those of closely related strains; in particular, MK-12 is a predominant menaquinone in all recognized *Agromyces* species (Gledhill & Casida, 1969; Zgurskaya *et al.*, 1992; Dorofeeva *et al.*, 2003; Yoon *et al.*, 2008). Thus, chemotaxonomic comparisons with *Agromyces* species showed that strain P27^T belonged to the genus *Agromyces*.

Extraction and purification of chromosomal DNA was carried out as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR using two universal primers as described by Stackebrandt *et al.* (1993). Sequencing and phylogenetic analysis were performed using a previously described procedure (Roh *et al.*, 2008). Phylogenetic analysis was conducted using MEGA 4 version 2.1 and relationships between strain P27^T and representatives of the genus *Agromyces* were determined. The 16S rRNA gene sequence of strain P27^T was compared with known 16S rRNA gene sequences of other strains of the genus *Agromyces* in the GenBank database. Distance matrices were computed as described by Kimura (1980) and used to construct dendrograms using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony

(Kluge & Farris, 1969) methods. Bootstrap analysis was performed with 1000 replications to obtain a consensus tree. The genomic DNA G+C content was determined according to Gonzalez & Saiz-Jimenez (2002).

On the basis of the 16S rRNA gene sequence, strain P27^T showed highest pairwise similarity to *Agromyces cerinus* subsp. *cerinus* JCM 9083^T, *A. ramosus* DSM 43045^T, *A. cerinus* subsp. *nitratus* IMET 11532^T and *A. terreus* DS-10^T (97.0, 96.9, 96.5 and 96.4 %, respectively). The similarity between strain P27^T and other *Agromyces* strains was less than 96.4 %. It has been reported that 16S rRNA gene sequence similarity of less than 98.7 % indicates DNA–DNA reassociation values of less than 70 % (Stackebrandt & Ebers, 2006). Moreover, phylogenetic analysis showed that strain P27^T formed a clade that was separate from strains of other *Agromyces* species (Fig. 1). Thus, strain P27^T can be considered as representing a distinct novel *Agromyces* species.

The genomic DNA G+C content of strain P27^T was 69.0 mol%. G+C contents of *Agromyces* species are in the range 65.3–73.3 mol% (Zgurskaya *et al.*, 1992; Suzuki *et al.*, 1996; Takeuchi & Hatano, 2001; Dorofeeva *et al.*, 2003; Li *et al.*, 2003; Rivas *et al.*, 2004; Jurado *et al.*, 2005a, b, c; Jung *et al.*, 2007; Yoon *et al.*, 2008). Thus, the G+C content of the genomic DNA of strain P27^T was in the range of values for a species of the genus *Agromyces*.

Therefore, on the basis of its phenotypic, genotypic and phylogenetic characteristics, strain P27^T is considered to represent a novel species of the genus *Agromyces*, for which the name *Agromyces atrinae* sp. nov. is proposed.

Description of *Agromyces atrinae* sp. nov.

Agromyces atrinae [a.tri′na.e. N.L. n. *Atrina* zoological name for a genus of bivalve mollusc; N.L. gen. n. *atrinae* of *Atrina*, referring to the isolation of the type strain from a fermented food prepared from *Atrina pectinata* (comb pen shell)].

Cells are Gram-staining-positive, aerobic, non-motile and straight or curved rods (1.0–2.0 × 0.5–1.0 μm) after incubation for 2 days on MA medium at 30 °C. Colonies on MA are circular, smooth, convex and yellow. Growth occurs in the presence of 0–4 % NaCl, at 10–37 °C and at pH 6–10. No growth is detected in an anaerobic chamber on MA. Optimal conditions are 30 °C, pH 6–7 and 2 % NaCl on MA or in MB. Catalase-positive and oxidase-negative. Reduces nitrate to nitrite. The following substrates are utilized as sole carbon and energy sources: dextrin, glycogen, Tweens 40 and 80, arbutin, cellobiose, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, α-lactose, maltose, maltotriose, D-mannitol, D-mannose, D-psicose, D-ribose, salicin, sucrose, trehalose, D-xylose, acetic acid, pyruvic acid methyl ester, pyruvic acid, glycerol, adenosine, inosine, thymidine, uridine, thymidine 5′-monophosphate and uridine 5′-monophosphate. The following substrates are not utilized: α- and β-cyclodextrin, inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, D- and L-arabinose, L-fucose, D-galacturonic acid, gentiobiose, *myo*-inositol, lactulose, melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl D-glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, raffinose, L-rhamnose, sedoheptulosan, D-sorbitol, stachyose, D-tagatose, turanose, xylitol, α-, β-and γ-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, 2′-deoxyadenosine, adenosine 5′-monophosphate,

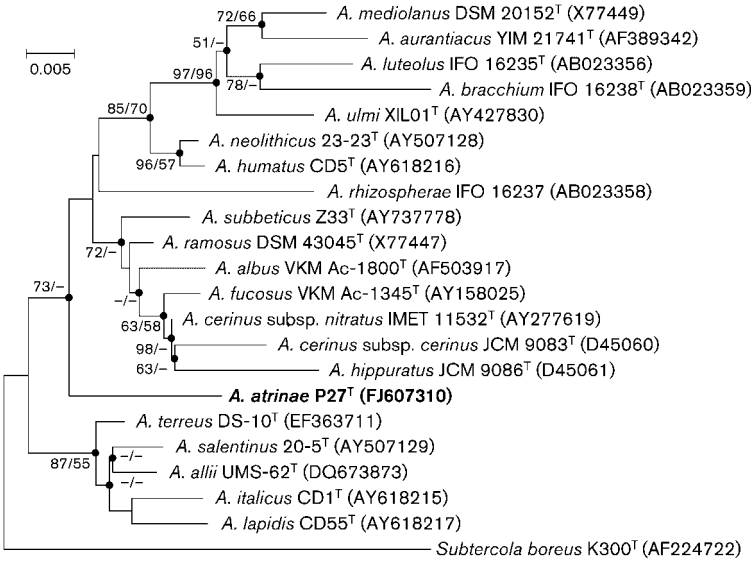

Fig. 1. Phylogenetic tree showing the positions of strain P27^T and *Agromyces* strains based on 16S rRNA gene sequences and generated by the neighbour-joining method. Bootstrap values (>50 %; dashes indicate values below 50 %) based on 1000 replications and calculated by neighbour-joining/maximum-parsimony methods are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered with the maximum-parsimony algorithm. *Subtercola boreus* K300^T served as an outgroup. Bar, 0.005 accumulated changes per nucleotide position.

D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. With the API ZYM system, positive for leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases and α- and β-glucosidases; weakly positive for esterase (C4), esterase lipase (C8) and acid phosphatase; negative for alkaline phosphatase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The major cell-wall sugars are galactose, mannose and rhamnose. The peptidoglycan amino acids are 2,4-diaminobutyric acid, alanine, glutamic acid and glycine. The predominant menaquinones are MK-12 and MK-11; minor components are MK-10 and MK-13. Major cellular fatty acids are anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The DNA G+C content of the type strain is 69.0 mol%.

The type strain, P27^T (=KCTC 19593^T =JCM 15913^T), was isolated from a traditional fermented seafood in Korea.

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