Amycolatopsis marina sp. nov., an actinomycete isolated from an ocean sediment

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A Gram-positive, aerobic, non-motile actinobacterium, designated strain Ms392A^T, was isolated from an ocean-sediment sample collected from the South China Sea. The isolate contained chemical markers that supported chemotaxonomic assignment to the genus *Amycolatopsis*. On the basis of an analysis of 16S rRNA gene sequence similarities, strain Ms392A^T represents a novel subclade within the genus *Amycolatopsis*, with *Amycolatopsis palatopharyngis* 1BDZ^T as its closest phylogenetic neighbour (99.4 % similarity). However, DNA–DNA hybridization demonstrated that strain Ms392A^T was distinct from *A. palatopharyngis* AS 4.1729^T (48.6 % relatedness). The polyphasic analysis demonstrated that the ocean isolate can be clearly distinguished from recognized species of the genus *Amycolatopsis*. Therefore, strain Ms392A^T represents a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis marina* sp. nov. is proposed. The type strain is Ms392A^T (=CGMCC 4.3568^T =NBRC 104263^T).

The genus *Amycolatopsis*, classified as belonging to the family *Pseudonocardiaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994), was proposed by Lechevalier *et al.* (1986) for aerobic, amycolate, nocardioform actinomycetes and is well defined as a result of chemotaxonomic characterization (Lechevalier *et al.*, 1986; Henssen *et al.*, 1987; Mertz & Yao, 1993; Yassin *et al.*, 1993) and phylogenetic analyses based on the comparison of 16S rRNA gene sequences (Embley *et al.*, 1988; Warwick *et al.*, 1994). The members of the genus *Amycolatopsis* are Gram-positive, non-acid-fast, non-motile actinomycetes that form branched vegetative hyphae that undergo fragmentation into rod-like and squarish elements. In addition, they are represented chemotaxonomically by the following features: wall chemotype IV (*meso*-diaminopimelic acid, arabinose and galactose in cell-wall hydrolysates), a tetrahydrogenated menaquinone with nine isoprene units [MK-9(H_4)] as the major menaquinone, a phospholipid pattern of type II *sensu* Lechevalier *et al.* (1977) (phosphatidylethanolamine as a diagnostic phospholipid), fatty acid profiles that include complex mixtures of saturated and branched-chain acids and the absence of mycolic acids. The members of this genus have DNA G+C contents in the range 66–73 mol%.

At the time of writing, the genus *Amycolatopsis* comprises 38 recognized species, most of which were isolated from various terrestrial environments (Goodfellow *et al.*, 2001; Kim *et al.*, 2002; Saintpierre-Bonaccio *et al.*, 2005; Lee *et al.*, 2006; Tan *et al.*, 2006a; Groth *et al.*, 2007; Carlsohn *et al.*, 2007) or clinical material (Labeda *et al.*, 2003; Huang *et al.*, 2004). On the basis of chemotaxonomic and morphological markers (Kim & Goodfellow, 1999) and analyses using genus-specific oligonucleotide primers (Tan *et al.*, 2006b), *Amycolatopsis* strains were separated from members of the other genera classified within the family *Pseudonocardiaceae*. Recently, there has been an explosion of information about novel bioactive compounds isolated from members of the genus *Amycolatopsis* (Demain & Zhang, 2005; Zhang *et al.*, 2005). In an effort to explore the

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relatively untapped potential of members of this genus and investigate potential applications for their secondary metabolites (Zhang *et al.*, 2007), we attempted to isolate and identify strains from the South China Sea.

Strain Ms392A^T was isolated using the following procedure. Fresh deep-ocean sediment samples were collected in the South China Sea and kept at 4 °C for isolation as soon as possible. Serial dilutions of sample suspensions were transferred onto the selective isolation medium (SM1; Tan *et al.*, 2006b) for the genus *Amycolatopsis* and incubated at 28 °C for 4 weeks.

Strain Ms392A^T was cultivated on ISP 2, ISP 3, ISP 4 and ISP 5 media (Shirling & Gottlieb, 1966) at 28 °C. Spore chains were observed for colonies grown using the coverslip technique of Kawato & Shinobu (1959). Morphological characteristics were examined by using light microscopy (CX41; Olympus) and scanning electron microscopy (S-570; Hitachi). Morphological features were observed on ISP 2 and ISP 4 media at 28 °C. The phenotypic properties of the isolate were consistent with its classification within the genus *Amycolatopsis*. The whitish aerial mycelium, which was produced only on ISP 2 and ISP 4, formed rod-like mycelial fragments. No diffusion pigments were produced on any of the media tested.

Physiological and biochemical characteristics of strain Ms398A^T are given in Table 1 and in the species description. Carbohydrate utilization was tested using ISP 9 (Shirling & Gottlieb, 1966) as the basal medium with filter-sterilized compounds at a final concentration of 1 % (w/v). Urease activity was determined by checking for a colour change in Bacto urea broth (Difco). The production of H_2S was tested on peptone iron agar (Difco). Nitrate reduction, gelatin liquefaction and degradation of elastin and starch were examined by using previously described methods (MacFaddin, 1980). Decomposition of adenine, hypoxanthine, casein, DL-tyrosine and xanthine was examined by using the methods of Gordon *et al.* (1974). Antibiotic susceptibility was investigated as described by Groth *et al.* (2004), using antibiotic discs (Himedia). Growth over a range of temperatures (4–60 °C), pH values and NaCl concentrations was determined on ISP 2 medium. The pH range and optimum for growth and the tolerance of NaCl were examined as described by Tang *et al.* (2003). Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2, and oxidase activity was determined using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovács, 1956).

The procedures used for the identification of cell-wall amino acids and sugars in whole-cell hydrolysates were those described by Staneck & Roberts (1974). Menaquinones were extracted by using the method of Collins *et al.* (1977) and were analysed by means of HPLC, as described by Tamaoka *et al.* (1983). Polar lipids were extracted as described by Minnikin *et al.* (1979) and identified by using two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Biomass for quantitative fatty acid analysis of strain Ms398A^T was prepared by scraping growth from TSB agar plates that had been incubated for 7 days at 28 °C. Fatty acids were extracted, methylated and analysed using the MIDI (Microbial Identification) system. The cell-wall diamino acid in the peptidoglycan layer of strain Ms392A^T was *meso*-diaminopimelic acid, the major sugars in the cell wall were arabinose and galactose (cell-wall chemotype IV according to Lechevalier & Lechevalier, 1980) and the predominant isoprenoid quinones were tetrahydrogenated menaquinones with eight and nine isoprene units. The phospholipids included diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannoside. The major fatty acids were iso-$C_{16:0}$ (40.35 %) and iso-$C_{16:0}$ 2-OH (11.42 %). Others fatty acids that occurred in smaller amounts were $C_{17:1}$ *cis*9 (8.61 %), $C_{16:1}$ *cis*9 (7.79 %), $C_{16:0}$ (7.08 %), $C_{17:0}$ (4.89 %), iso-$C_{17:0}$ (3.46 %), $C_{15:0}$ (2.91 %), iso-$C_{16:1}$ H (2.66 %), anteiso-$C_{17:0}$ (2.28 %), $C_{15:1}$ B (1.63 %), iso-$C_{15:0}$ (1.50 %), 10-methyl $C_{16:0}$ (1.39 %), $C_{18:0}$

Table 1. Differential phenotypic characteristics of strain Ms392A^T and its closest phylogenetic neighbour, *A. palatopharyngis* AS 4.1729^T

Data were taken from this study or from Huang *et al.* (2004). +, Positive; W, weakly positive; −, negative; R, resistant; S, sensitive.

Characteristic	*A. marina* sp. nov. Ms392A^T	*A. palatopharyngis* AS 4.1729^T
Utilization as sole carbon source of:		
(+)-L-Arabinose	−	W
(+)-L-Rhamnose	+	−
(+)-Maltose	+	−
(+)-Raffinose	−	W
Decomposition of:		
L-Lysine	−	+
Starch	W	−
Production of:		
Catalase	+	−
Urease	−	W
Nitrate reductase	−	+
Drug susceptibility/resistance		
Rifampicin	R	S
Erythromycin	S	R
Sulfamethoxazole	R	S
Kanamycin	R	S
Carbenicillin	S	R
Clarithromycin	R	S
Cephalothin	S	R
Growth at/with:		
45 °C	W	−
12 % NaCl	W	−
pH 10.0	−	W

(1.35 %), $C_{18:1}$ *cis*9 (0.97 %), iso-$C_{14:0}$ (0.90 %) and iso-$C_{18:0}$ (0.81).

Extraction of genomic DNA and PCR amplification and 16S rRNA gene sequencing were carried out as described previously (Li *et al.*, 2007). An almost-complete 16S rRNA gene sequence (1485 bp) was generated for the novel isolate. Preliminary comparison of this sequence against those in the GenBank database indicated that the novel isolate was closely related to the members of the family *Pseudonocardiaceae*. A phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA, version 3.1 (Kumar *et al.*, 2004), after multiple alignment of the data using CLUSTAL_X (Thompson *et al.*, 1997). Distances (using distance options according to Kimura's two-parameter model; Kimura, 1980, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (based on 1000 resamplings) was used to evaluate the tree topology of the neighbour-joining data (Felsenstein, 1985).

The phylogenetic analysis (Fig. 1) indicated that isolate Ms392A^T formed a distinct clade within the radiation encompassing the members of the genus *Amycolatopsis*, and revealed that the isolate was most closely associated with *Amycolatopsis palatopharyngis* 1BDZ^T within a separate cluster. The 16S rRNA gene sequence similarities between strain Ms392A^T and *Amycolatopsis* species with validly published names were below 97.0 %, except for *A. palatopharyngis* 1BDZ^T, which showed 99.4 % similarity (corresponding to 9 differences over 1427 locations).

To determine whether strain Ms392A^T represents a distinct species of the genus *Amycolatopsis*, DNA–DNA hybridizations were performed by applying the method of He *et al.* (2005) with five replications for each sample. Strain Ms392A^T displayed low DNA–DNA reassociation with *A. palatopharyngis* AS 4.1729^T (mean value 48.6 %). The result is far below the cut-off point recommended for the circumscription of bacterial genomic species by Wayne *et al.* (1987). The G+C content of the DNA was determined by using the HPLC method (Mesbah *et al.*, 1989) and a mean value of 70.1 mol% was obtained.

Features that serve to differentiate strain Ms392A^T from its closest phylogenetic neighbour, *A. palatopharyngis* AS 4.1729^T, are shown in Table 1. Thus, in conclusion, genotypic, chemotaxonomic and phenotypic data demonstrate that strain Ms392A^T represents a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis marina* sp. nov. is proposed.

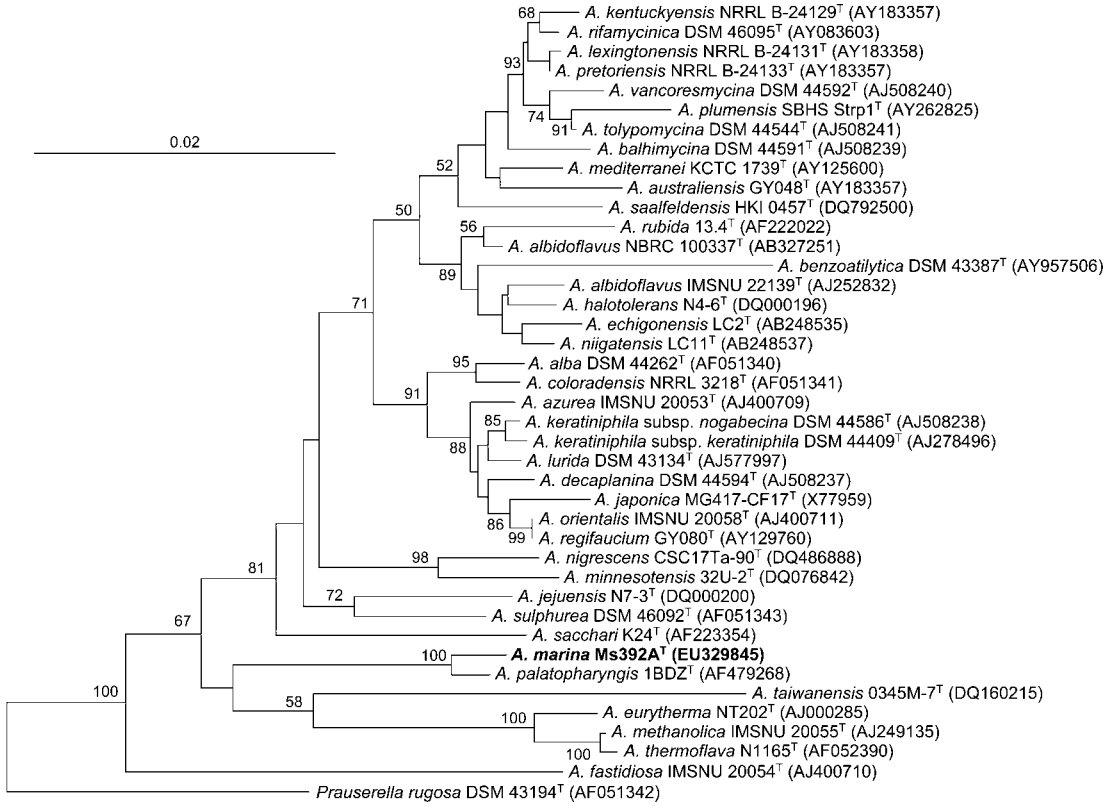

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing the position of strain Ms392A^T within the radiation of the genus *Amycolatopsis*. Bootstrap percentages (based on 1000 resamplings) are shown at nodes. Bar, 0.02 substitutions per nucleotide position.

Description of *Amycolatopsis marina* sp. nov.

Amycolatopsis marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine).

Cells are Gram-positive, aerobic and non-motile and produce white aerial mycelium sparsely on ISP 2 agar medium. The branched yellow to yellow–brown substrate mycelium fragments into rod-like elements. No diffusion pigments are produced on any of the media tested. Catalase-positive and oxidase-negative. Negative for urease and negative for reduction of nitrate to nitrite. H_2S is not produced. Growth occurs between pH 6.0 and 9.0, between 10 and 45 °C and between 0.5 and 12 % NaCl, but not above 45 °C or above 12 % NaCl. The optimum growth pH, temperature and NaCl are 7.0–8.0, 28 °C and 5 % NaCl. Resistant to rifampicin, amikacin, carbenicillin, tobramycin, kanamycin, clarithromycin and penicillin G (each at 30 μg), but sensitive to gentamicin, novobiocin, streptomycin, doxycycline, acetylspiramycin, carbenicillin, midecamycin, minocycline, cephalothin and chloramphenicol. (+)-D-Fructose, (+)-D-galactose, (+)-cellobiose, *myo*-inositol, (+)-L-rhamnose salicin, (+)-maltose, (+)-D-mannitol and (+)-trehalose are utilized as carbon sources, but sorbitol, (+)-D-lactose, dextrin, arabinose, (+)-raffinose and (−)-sucrose are not. Decomposes gelatin, hypoxanthine, xanthine, allantoin and starch, but not L-tyrosine or casein. Cell-wall hydrolysates contain *meso*-diaminopimelic acid, arabinose and galactose. MK-9(H_4) (79 %) is the predominant menaquinone; MK-8(H_4) (13 %) is also present. The phospholipids comprise diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannoside. The major cellular fatty acids are iso-$C_{16:0}$ and iso-$C_{16:0}$ 2-OH. The DNA G+C content of the type strain is 70.1 mol%.

The type strain, Ms392A^T (=CGMCC 4.3568^T =NBRC 104263^T), was isolated from an ocean-sediment sample collected in the South China Sea.

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