Gordonia neofelifaecis sp. nov., a cholesterol side-chain-cleaving actinomycete isolated from the faeces of *Neofelis nebulosa*

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A cholesterol side-chain-cleaving bacterial strain, AD-6^T, was isolated from fresh faeces of a clouded leopard (*Neofelis nebulosa*) and was studied using a polyphasic taxonomic approach. 16S rRNA gene sequence analysis showed that the novel strain formed a distinct subline within the genus *Gordonia*, its closest neighbours being the type strains of *Gordonia cholesterolivorans*, *Gordonia sihwensis* and *Gordonia hydrophobica*, with sequence similarity values of 98.2, 97.8 and 97.6 %, respectively. The *gyrB* gene sequence of strain AD-6^T exhibited similarities of 77–91 % with those of the type strains of recognized species of the genus *Gordonia*, being most similar to the type strains of *G. sihwensis*, *G. hydrophobica* and *Gordonia hirsuta* (91, 87 and 84 % similarity, respectively). The results of whole-cell fatty acid analyses and DNA–DNA relatedness data readily distinguished the new isolate from its nearest neighbours. Strain AD-6^T is therefore considered to represent a novel species of the genus *Gordonia*, for which the name *Gordonia neofelifaecis* sp. nov. is proposed. The type strain is AD-6^T (=NRRL B-59395^T =CCTCC AB-209144^T).

The genus *Gordonia*, first described by Tsukamura (1971), belongs to the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997). At the time of writing, the genus comprises 31 recognized species. In recent years, members of the genus *Gordonia* have attracted much interest for their potential use in industrial and environmental biotechnology. Many species of the genus *Gordonia* are able to degrade toxic environmental pollutants, such as rubber by *Gordonia westfalica* (Linos *et al.*, 2002), benzothiophene and dibenzothiophene by *Gordonia desulfuricans* and *Gordonia amicalis* (Kim *et al.*, 1999, 2000), hydrocarbons by *Gordonia paraffinivorans* and *Gordonia alkanivorans* (Kummer *et al.*, 1999; Xue *et al.*, 2003), phenol by *Gordonia kroppenstedtii* (Kim *et al.*, 2009) and cholesterol by *Gordonia cholesterolivorans* (Drzyzga *et al.*, 2009). Moreover, members of the genus *Gordonia* play an important role in bioremediation (Franzetti *et al.*, 2009). Species of the genus *Gordonia* are widely distributed in various environments such as soil, activated sludge, biofilms, industrial wastewater, oil-producing wells, estuaries (le Roes *et al.*, 2008) and the mangrove rhizosphere

(Takeuchi & Hatano, 1998), and some are human pathogens (Kageyama *et al.*, 2006). There are no reports of the isolation of species of the genus *Gordonia* from the faeces of healthy animals.

The aim of the present study was to clarify the taxonomic position of an actinomycete that transformed cholesterol to androsta-1,4-diene-3,17-dione (ADD) by side-chain cleavage. The novel strain was isolated from fresh faeces of a clouded leopard, *Neofelis nebulosa*, a medium-sized feline which is mainly restricted to southern China and mainland south-east Asia. Fresh faecal samples were collected directly from the anus of a healthy specimen of *N. nebulosa* maintained at Chengdu Zoo, China, and were suspended in 10 ml minimal medium (MM, containing 0.1 % NaNO₃, 0.025 % KH₂PO₄, 0.025 % MgSO₄·7H₂O, 0.0001 % FeSO₄ and 0.1 % cholesterol, pH 7.0). The suspension was shaken (200 r.p.m.) at 30 °C for about 3 days. Fresh isolation medium was inoculated with 200 µl of the culture and incubated under the same conditions. As previously described, four subcultures were prepared to dilute the faecal material (Ye *et al.*, 2008). The bacterial suspension was then spread onto agar plates of the minimal medium in 200 µl volumes and incubated at 30 °C for 2–3 days. For screening bacteria capable of transforming cholesterol into ADD and AD (androsta-4-en-3,17-dione), well-developed colonies on agar plates were cultured in 10 ml liquid MM at 30 °C for 48 h with shaking. The bacterial culture liquid was then extracted by adding an equal volume of ethyl

Abbreviations: AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain AD-6^T are FJ938167 and GQ140632, respectively.

A supplementary figure and table are available with the online version of this paper.

acetate and was analysed by TLC on silica gel 60F-254 plates with benzene/ethyl acetate (10:1, v/v) as the solvent system, at room temperature. The ADD and AD yielded by cholesterol side-chain cleavage were detected as dark, UV-absorbing spots under UV illumination and were further identified by spectroscopic techniques, including ^1H NMR, ^{13}C NMR, IR and time-of-flight MS (this work will be reported elsewhere). One colony that was able to degrade the side chain of cholesterol was isolated and subcultured on a Luria–Bertani (LB) agar plate for 2 days at 30 °C; this strain was designated AD-6^T. On this medium the strain formed beige colonies. The organism was subjected to a study utilizing a polyphasic taxonomic approach, which showed that it represented a novel species of the genus *Gordonia*.

Extraction of chromosomal DNA, amplification of the 16S rRNA gene by PCR (employing a pair of universal 16S rRNA gene primers) (Hiraishi, 1992; Hiraishi *et al.*, 1994) and purification of the PCR products were carried out according to the procedures described by Rainey *et al.* (1996). The purified PCR products were sequenced with an ABI PRISM 3730 DNA sequencer. A 16S rRNA gene sequence (1417 nt; 92 % of the 16S rRNA gene sequence of *Escherichia coli*) was obtained from strain AD-6^T. A BLAST search showed that the levels of 16S rRNA gene sequence similarity between strain AD-6^T and the type strains of members of the genus *Gordonia* ranged from 95.4 to 98.2 %. For phylogenetic analysis, reference strains were selected based on these BLAST (Altschul *et al.*, 1997) results. The 16S rRNA gene sequence of strain AD-6^T was aligned with those of recognized species of the genus *Gordonia*. The MEGA version 4.0 software package (Tamura *et al.*, 2007) was used for constructing phylogenetic trees.

Micromorphological properties were observed by light microscopy and transmission electron microscopy as described by Bozzola & Russell (1991). The Ziehl–Neelsen method was used to determine acid-fastness. Carbon source utilization was determined in microtitre plates (Linosa *et al.*, 1999). For mycolic acid and quinone analyses, cells were cultivated in trypticase soy broth at 30 °C for 3 days on a rotary shaker at 200 r.p.m., harvested by centrifugation, washed twice with distilled water and freeze-dried. Isoprenoid quinones were extracted and purified by using the procedure of Collins (1985) and were analysed by HPLC as described by Collins *et al.* (1977). The profile of mycolic acid methyl esters was determined by exposing TLC plates to saturated iodine vapour for 3–5 min. Species of the genus *Gordonia* were cultivated at 30 °C for 3 days on trypticase soy agar (TSA) for fatty acid methyl ester analysis. Fatty acid methyl esters were prepared from fresh cells (Miller, 1982) and were identified by GC-MS (Takeuchi & Hatano, 1998). *Gordonia sihwensis* DSM 44576^T, *G. cholesterolivorans* DSM 45229^T and *Gordonia hydrophobica* DSM 44015^T were used as reference strains.

The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (Fig. 1; Supplementary Fig. S1

available in IJSEM Online) showed that strain AD-6^T formed a distinct subline within the genus *Gordonia*, branching within a subcluster containing *G. sihwensis*, *G. cholesterolivorans* and *G. hydrophobica*. Strain AD-6^T was most closely related to *G. cholesterolivorans* Chol-3^T, sharing 98.2 % 16S rRNA gene sequence similarity, a value that corresponded to 25 nt differences out of 1417 nt. Levels of 16S rRNA gene sequence similarity between strain AD-6^T and *G. sihwensis* DSM 44576^T and *G. hydrophobica* DSM 44015^T were 97.8 and 97.6 %, respectively.

To obtain the *gyrB* gene sequence of strain AD-6^T, degenerate PCR primers GYRBN1 (GGNRTSTCSGTSGK-NAMMGCSCTRTC) and GYRBN2 (CAGCACGATCTTG-TGRTAGCGNAG) were designed based on *gyrB* sequences of members of the genus *Gordonia* in the GenBank database, and were used to amplify a *gyrB* fragment of approximately 1.2 kb from the genomic DNA extracted from strain AD-6^T. To determine the *gyrB* gene sequence of strain AD-6^T, the internal sequencing primer GYRBNF1 (ACTACATCAAGCACCTCAAC) was designed and used with the two forward primers GYRBF2 and GYRBR1 (Shen *et al.*, 2006). The resultant sequence was compared with all available *gyrB* gene sequences for members of the genus *Gordonia*.

Analysis of the *gyrB* gene sequence of strain AD-6^T (1219 bp) indicated that it belonged to the genus *Gordonia*, with sequence similarities ranging from 77 to 91 % with those of recognized species of the genus. Strain AD-6^T formed a distinct phyletic line in the neighbour-joining tree based on *gyrB* gene sequences (Fig. 2). The strain was most closely related to the type strains of *G. sihwensis* (91 % *gyrB* gene sequence similarity), *G. hydrophobica* (87 %) and *Gordonia hirsuta* (84 %), which corresponded to 110, 164 and 190 nt differences out of 1219 nt, respectively. The result supported the phylogenetic analysis based on 16S rRNA gene sequences.

DNA–DNA hybridization analysis was performed between strain AD-6^T, *G. sihwensis* DSM 44576^T and *G. cholesterolivorans* DSM 45229^T. Genomic DNA extraction was carried out by using the procedures described by Rainey *et al.* (1996). The renaturation rates of genomic fragments from pairs of strains were determined spectrophotometrically with a model 1601 UV spectrophotometer equipped with a thermoelectric cell temperature controller (Shimadzu) according to previously described methods (De Ley *et al.*, 1970; Huß *et al.*, 1983).

Strain AD-6^T had morphological properties that were consistent with its classification in the genus *Gordonia* (Stackebrandt *et al.*, 1988). Cells were Gram-positive, non-motile, non-spore-forming, aerobic rods (0.3–0.6 × 0.5–2.1 µm). Colonies on LB agar were beige and rough with irregular margins.

The results of physiological tests revealed that strain AD-6^T was able to utilize a set of carbon sources that distinguished it from closely related type strains within the genus

Fig. 1. Unrooted neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rRNA gene sequences of strain AD-6^T (1417 nt) and its closest relatives. Numbers at nodes are bootstrap percentages (from 1000 replications). Only values above 50% are given. Asterisks indicate branches of the tree that were also recovered using minimum-evolution and maximum-parsimony methods. Bar, 0.005 substitutions per nucleotide position. An expanded version of this tree including the type strains of all recognized species of the genus *Gordonia* is available in Supplementary Fig. S1.

Gordonia (Table 1). The chemotaxonomic properties of strain AD-6^T were also consistent with its classification in the genus *Gordonia* (Stackebrandt *et al.*, 1988). TLC and HPLC patterns of mycolic acids from strain AD-6^T were similar to those of *Gordonia amarae* DSM 43392^T (data not shown). MK-9(H₂) was the predominant menaquinone.

The major fatty acids of strain AD-6^T were C_{16:1} (18%), C_{16:0} (43%), C_{18:1ω9c} (10%) and 10-methyl C_{18:0} (13%) (see Supplementary Table S1 in IJSEM Online). Although this pattern, containing major amounts of C_{16:0} and tuberculostearic acids, was similar to those of other species of the genus *Gordonia*, there were some obvious differences between strain AD-6^T and closely related species. Strain AD-6^T contained a relatively large amount of fatty acid C_{16:1}, whereas the type strains of *G. sihwensis*, *G. cholesterolivorans* and *G. hydrophobica* showed a complete

absence of this fatty acid (Supplementary Table S1). Strain AD-6^T also contained C_{18:1ω9c}, a fatty acid present as a major component only in *G. rhizosphaera* IFO 16068^T and *G. alkanivorans* KCTC 0605BP^T (Takeuchi & Hatano, 1998; Drzyzga *et al.*, 2009). Although fatty acid C_{18:1ω9c} has been detected by others in *G. sihwensis* DSM 44576^T, it was not detected under the cultivation conditions used in this study. The type strains of most other recognized species of the genus *Gordonia* lack this fatty acid (Stackebrandt *et al.*, 1988; Kim *et al.*, 1999, 2000; Drzyzga *et al.*, 2009).

DNA–DNA relatedness studies performed between strain AD-6^T and *G. sihwensis* DSM 44576^T and between strain AD-6^T and *G. cholesterolivorans* DSM 45229^T resulted in reassociation values of 45.5 ± 4.0 and 55.7 ± 4.0%, respectively (values are means ± SD for four replications). From

Fig. 2. Unrooted neighbour-joining tree based on *gyrB* gene sequences of strain AD-6^T (1219 bp) and its closest phylogenetic neighbours. Numbers at nodes are bootstrap percentages (from 1000 replications). Only values above 50% are given. Asterisks indicate clades that were conserved in the minimum-evolution and maximum-parsimony trees. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential carbon source utilization patterns between strain AD-6^T and the type strains of closely related species of the genus *Gordonia*

Strains: 1, AD-6^T; 2, *G. cholesterolivorans* DSM 45229^T; 3, *G. sihwensis* DSM 44576^T; 4, *G. hydrophobica* DSM 44015^T. All data were determined in this study.

Carbon source	1	2	3	4
4-Aminobutyrate	+	–	+	+
Benzoate	–	+	–	–
Caprate	–	–	+	–
Citrate	–	–	+	+
D-Galactose	+	–	+	+
Glucarate	–	+	–	–
Gluconate	–	–	+	–
D-Glucosaminic acid	–	–	+	–
L-Leucine	+	–	+	–
Phenylacetate	–	+	+	–
L-Rhamnose	+	+	–	–
L-Serine	+	–	+	–
L-Valine	–	–	+	+

these data it was evident that strain AD-6^T represented a species distinct from *G. sihwensis* and *G. cholesterolivorans*.

Based on the genotypic and phenotypic data presented, it was concluded that strain AD-6^T represents a novel species of the genus *Gordonia*, for which the name *Gordonia neofelifaecis* sp. nov. is proposed.

Description of *Gordonia neofelifaecis* sp. nov.

Gordonia neofelifaecis (ne.o.fel.i.fae.cis. N.L. fem. n. *Neofelis* a zoological genus name which encompasses the clouded leopard *Neofelis nebulosa*; L. n. *faex faecis* faeces; N.L. gen. n. *neofelifaecis* of the faeces of a clouded leopard).

Gram-positive, aerobic, slightly acid-fast, non-motile rods (0.3–0.6 × 0.5–2.1 µm). Colonies are beige and rough with irregular margins. Colonies are 3.0–5.0 mm in diameter after 3 days at 30 °C on LB and TSA plates. No soluble pigment is produced. When grown in liquid MM medium containing 1 g cholesterol l^{–1}, 87 % of the cholesterol is transformed to ADD and 3 % to AD. The optimum temperature for growth is 30 °C. Utilizes 4-aminobutyrate, D-galactose, L-leucine, L-serine and L-rhamnose as carbon sources, but not benzoate, caprate, citrate, glucarate, gluconate, glucosaminic acid, phenylacetate or L-valine. MK-9(H₂) is the predominant menaquinone. Major fatty acids are C_{16:1}, C_{16:0}, C_{18:1ω9c} and 10-methyl C_{18:0}.

The type strain, AD-6^T (=NRRL B-59395^T=CCTCC AB-209144^T), was isolated from fresh faeces of a specimen of *Neofelis nebulosa* maintained at Chengdu Zoo, China.

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