Gordonia cholesterolivorans sp. nov., a cholesterol-degrading actinomycete isolated from sewage sludge

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The taxonomic position of the cholesterol-degrading strain Chol-3^T, isolated from a sewage sludge sample, was clarified using a polyphasic taxonomic approach. Phylogenetic analysis of its 16S rRNA gene sequence, whole-cell fatty acid profile and mycolic acid composition revealed that this isolate is a member of the genus *Gordonia* with the species *Gordonia sihwensis*, *G. hydrophobica* and *G. shandongensis* being the nearest phylogenetic neighbours. The results of DNA–DNA hybridization against its phylogenetically closest neighbours as well as the results of physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain Chol-3^T from the other *Gordonia* species with validly published names. Strain Chol-3^T therefore merits recognition as a member of a novel species within the genus *Gordonia*, for which the name *Gordonia cholesterolivorans* sp. nov. is proposed. The type strain is Chol-3^T (=CECT 7408^T =DSM 45229^T).

The genus *Gordonia* (formerly *Gordona*), proposed by Tsukamura (1971), belongs to the mycolic acid-containing group of the actinomycetes and to the suborder *Corynebacterineae*, which forms a distinct phyletic line in the 16S rRNA gene phylogenetic tree (Goodfellow *et al.*, 1998; Stackebrandt *et al.*, 1997). The suborder *Corynebacterineae* currently encompasses the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Millisia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *Skermania*, *Smaragdicoccus*, *Tsukamurella* and *Williamsia*. The members of these genera can be distinguished by using a combination of biochemical, chemical, morphological and genetic markers (Goodfellow *et al.*, 1999; Arenskötter *et al.*, 2004).

Gordoniae appear to be widely distributed in nature and strains have been isolated from environments such as soil, wastewater, estuary sand, mangrove rhizosphere, oil-producing wells and activated sludge foam (Takeuchi & Hatano, 1998; Kummer *et al.*, 1999; Kim *et al.*, 2003; Maldonado *et al.*, 2003; Xue *et al.*, 2003; Shen *et al.*, 2006; Soddell *et al.*, 2006; Luo *et al.*, 2007; Le Roes *et al.*, 2008), as well as from clinical samples (Iida *et al.*, 2005; Kageyama *et al.*, 2006; Blaschke *et al.*, 2007).

The isolation of strains of the genus *Gordonia* with special metabolic abilities has increased the potential of applying this genus to biodegradation and bioremediation projects (Arenskötter *et al.*, 2004). Some isolates are able to degrade xenobiotic contaminants or macromolecules such as rubber, (di)benzothiophene, 3-ethyl- and 3-methylpyridine and alkanes partially or totally (Kummer *et al.*, 1999; Linos *et al.*, 1999; Kim *et al.*, 1999, 2000; Lee *et al.*, 2001). Recently published studies have expanded the metabolic potential of the genus *Gordonia*, as some isolated strains metabolize butyl benzyl phthalates (e.g. *Gordonia* sp. MTCC 4818; Chatterjee & Dutta, 2003) and even hazardous nitro compounds like the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (hexogen), a compound that is difficult for bacteria to degrade (e.g. *Gordonia* sp. KTR9; Thompson *et al.*, 2005; Gorontzy *et al.*, 1994). All these data show the richness of metabolic activities of *gordoniae* and widen our view about the possible industrial and environmental application of these bacteria.

The ability to convert steroid compounds is well documented and has been utilized by the pharmaceutical industry to synthesize novel steroid products. With the objective of finding novel and potent aerobic steroid degraders or transformers, we carried out screening experiments with cholesterol, testosterone and other steroid compounds as the only carbon and energy source for bacterial growth. A few fast-growing strains were isolated from a sewage sludge sample. The aim of this study was to clarify the taxonomic position of one of these newly

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Chol-3^T is EU244645.

Mycolic acid and cellular fatty acid profiles of strain Chol-3^T and related type strains of the genus *Gordonia* and RiboPrint data are available as supplementary materials with the online version of this paper.

isolated steroid-degrading strains, named strain Chol-3^T, by using a polyphasic taxonomic approach.

Strain Chol-3^T was isolated from a sewage sludge sample from a wastewater treatment plant in Ciudad Real, Spain, by enriching a mixed culture in minimal medium (MM; DSMZ medium 457) broth with cholesterol as the only carbon and energy source under aerobic conditions at 30 °C in a rotary shaker (250 r.p.m.) for 4 days. Thereafter, strain Chol-3^T was isolated by repeatedly streaking on Luria–Bertani (LB) agar plates until a pure culture was achieved. Strain Chol-3^T was then again incubated with MM broth and cholesterol to confirm its ability to utilize cholesterol as growth substrate. Cholesterol (>99 % purity; Sigma-Aldrich) was added to the broth directly as pure powder at a theoretical concentration of 2 mM (39 mg in 50 ml). Due to the very low water solubility of cholesterol, this cholesterol suspension served as a reservoir from which the bacteria could grow for a long time. The strain was maintained on LB agar plates at 4 °C for short-term storage and stored as a cell suspension in 50 % (v/v) glycerol–LB broth at –80 °C.

Bacterial biomass for chemotaxonomic and molecular biological studies was routinely obtained by growing strain Chol-3^T in LB broth in shake flasks for 2 days at 30 °C in a rotary shaker at 250 r.p.m. The harvested cells were washed twice in distilled water for all investigations. The type strains of *Gordonia sihwensis* (DSM 44576^T) and *Gordonia hydrophobica* (DSM 44015^T) were obtained from the DSMZ for comparative studies.

Strain Chol-3^T was tested for a broad range of phenotypic characteristics, including substrate assimilation, and the presence of mycolic acids, determined by high-temperature gas chromatography (Linos *et al.*, 1999), and 2,6-diaminopimelic acid, as described by Kim *et al.* (1999). The assimilation of auxonographic substrates was detected photometrically by means of reduction of the redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. For comparative fatty acid methyl ester analysis, biomass was harvested at the late exponential growth phase. The whole-cell fatty acid composition was determined by means of capillary GC analysis and preparation was carried out using a method described by Miller & Berger (1985).

Genomic DNA extraction and PCR-mediated amplification of the 16S rRNA gene were carried out using the procedures described by Rainey *et al.* (1996). To amplify the almost-complete 16S rRNA-encoding sequence (1445 nt) of strain Chol-3^T, PCR primers were used with an annealing temperature of 55 °C for 30 cycles. The PCR product was purified by using the QIAquick Spin PCR purification kit (Qiagen) as described by the manufacturer. The *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) was used to sequence the PCR product directly according to the manufacturer's protocol. Sequence reaction mixtures were separated with an Applied Biosystems 373S DNA sequencer. The resulting 16S rRNA

gene sequence was aligned with the corresponding sequences of type strains of species of the genus *Gordonia* with validly published names (with *Williamsia serinedens* IMMIB SR-4^T as the outgroup) retrieved from the GenBank/EMBL/DDBJ databases using the MEGALIGN expert sequence analysis software (version 6.1 from DNASTAR). Tree topologies were evaluated by performing neighbour-joining, maximum-parsimony and maximum-likelihood analyses with different sets of filters. Only sequences of at least 1400 nt were used for the calculation of different trees. Tree topologies were evaluated by bootstrap analysis based on 1000 resamplings.

Spectroscopic DNA–DNA hybridization experiments were carried out in 2 × SSC with 10 % formamide at 70 °C between DNA extracted from strain Chol-3^T and its phylogenetically closest neighbours *G. sihwensis* DSM 44576^T and *G. hydrophobica* DSM 44015^T according to the protocols of De Ley *et al.* (1970) and Huß *et al.* (1983). Southern hybridization experiments (Bruce, 1996; Clark, 1997) were performed on *Pvu*II-digested chromosomal DNA of strain Chol-3^T and *G. sihwensis* DSM 44576^T with an automated RiboPrinter system (DuPont Qualicon) using a non-radioisotopically labelled 5.5 kb *Escherichia coli* *rrnB* probe specific to the 5S–16S–23S rRNA operon. The RiboPrinter system was also used to display a similarity index of *G. sihwensis* DSM 44576^T to strain Chol-3^T (which was set as 100 %, or 1.0) to assess the relatedness between these two strains. Both DNA–DNA hybridization and riboprinting were done by the Identification Service of the DSMZ.

Most of the morphological, physiological and chemotaxonomic characteristics of strain Chol-3^T were consistent with the diagnostic properties of the type strains of the genus *Gordonia* (Stackebrandt *et al.*, 1988; Goodfellow & Maldonado, 2006). Strain Chol-3^T shows an elementary branching rod–coccus growth cycle that is commonly found among strains of the genus *Gordonia*. Upon cultivation on LB agar, strain Chol-3^T exhibited beige and matt colonies. Cells were aerobic, Gram-positive-staining, non-motile and non-spore-forming.

The results of physiological tests revealed that strain Chol-3^T was able to utilize a set of carbon sources that distinguish this novel isolate from closely related type strains within the genus *Gordonia* (Table 1). When compared to *G. sihwensis* DSM 44576^T, the most closely related strain according to 16S rRNA gene sequence similarity, the substrate utilization pattern of strain Chol-3^T is quite different. The two strains showed many differing characteristics; in addition to the substrates listed in Table 1, strain Chol-3^T also utilizes acetate, *N*-acetyl-D-glucosamine, D-arabitol, cholesterol, 2-hydroxyvalerate, lactate, malate, L-proline, succinate, sucrose and turanose for growth, but not acetamide, L-alanine, L-aspartate, 3-hydroxybenzoate, 4-hydroxybenzoate, *myo*-inositol, protocatechuate, putrescine, pyruvate, quinate, D-ribose or tyramine.

Table 1. Carbon source utilization patterns that distinguish strain Chol-3^T from representatives of closely related *Gordonia* species

Strains: 1, strain Chol-3^T; 2, *G. sihwensis* DSM 44576^T; 3, *G. hydrophobica* DSM 44015^T; 4, *G. namibiensis* DSM 44568^T; 5, *G. hirsuta* DSM 44140^T; 6, *G. amicalis* DSM 44461^T; 7, *G. westfalica* DSM 44215^T; 8, *G. bronchialis* DSM 43247^T; 9, *G. amarae* DSM 43392^T. Data for reference type strains were taken from Kim *et al.* (2003). ND, No data available. +, OD₅₄₀ >0.13; –, OD₅₄₀ ≤0.13.

Carbon source	1	2	3	4	5	6	7	8	9
4-Aminobutyrate	–	+	–	–	ND	–	–	–	ND
Benzoate	+	–	–	–	–	–	+	–	–
Caprate	–	+	–	–	ND	+	+	+	ND
Citrate	–	+	+	–	–	+	+	–	+
D-Galactose	–	+	+	+	+	+	–	+	+
Glucarate	+	–	+	–	–	+	–	–	–
Gluconate	–	+	–	+	–	+	–	+	–
D-Glucosaminic acid	–	+	–	–	–	+	+	–	–
L-Leucine	–	+	+	–	–	+	–	+	–
2-Oxoglutarate	–	+	+	–	+	+	+	–	+
Phenylacetate	+	+	–	–	–	+	–	+	–
Pimelate	–	+	–	–	–	+	–	–	–
L-Rhamnose	+	–	+	–	–	+	–	+	–
L-Serine	–	+	+	+	–	+	–	–	–
L-Valine	–	+	+	–	–	–	–	+	+

The chemotaxonomic characteristics of strain Chol-3^T were also consistent with its classification in the genus *Gordonia* (Goodfellow & Maldonado, 2006). The whole-cell hydrolysate of the isolate contained *meso*-diaminopimelic acid as the only diamino acid of the peptidoglycan and arabinose and galactose as major cell-wall sugars, which places strain Chol-3^T in cell-wall chemotype IV according to Lechevalier & Lechevalier (1970). The main mycolic acid components of strain Chol-3^T and some *Gordonia* type strains are listed in Supplementary Table S1 (available in IJSEM Online). The organism possesses short-chain mycolic acids, ranging in length from C₄₈ to C₅₆, which is comparable with only a few other members of the genus *Gordonia* and sets it close to *G. sihwensis*, *Gordonia amarae*, *Gordonia amicalis* and *Gordonia shandongensis*. Most of the *Gordonia* type strains possess long-chain mycolic acids, ranging in length from C₅₄ to C₆₄.

The fatty acid pattern of strain Chol-3^T contained straight-chain saturated and unsaturated fatty acids plus tuberculostearic acid (10-methyl C_{18:0}) (Supplementary Table S2). The major fatty acids were C_{16:1cis7} (14%), C_{16:0} (37%), C_{18:1} (24%) and 10-methyl C_{18:0} (16%). Although this pattern, with major proportions of palmitic (C_{16:0}), oleic (C_{18:1}) and tuberculostearic acids, is quite similar to those of all *Gordonia* species, there are qualitative and quantitative species-specific differences. As can be seen in Supplementary Table S2, the novel isolate contained a relatively large proportion of C_{16:1cis7}, a fatty acid that

Gordonia westfalica and *Gordonia bronchialis* also contain as a major component. All other type strains of *Gordonia* that have been described so far show minor proportions or the complete absence of this fatty acid.

The assignment within the genus was also confirmed by 16S rRNA gene sequence analysis. The almost-full-length 16S rRNA gene sequence (1445 bp) of strain Chol-3^T was used for BLAST searches against the GenBank/EMBL/DDBJ databases, which showed that this strain is related to members of the genus *Gordonia* with an intrageneric relatedness ranging from 91.8 to 99.8% sequence similarity. Strain Chol-3^T lay in a monophyletic clade together with *G. sihwensis* DSM 44576^T, *G. hydrophobica* DSM 44015^T and *G. shandongensis* SD29^T in the neighbour-joining tree (Fig. 1) and shared the highest gene sequence similarity with these strains (99.8, 95.7 and 95.4%, respectively).

The phenotypic properties of strain Chol-3^T and its closest neighbour in the phylogenetic tree, *G. sihwensis* DSM 44576^T, clearly demonstrate the differences between these two strains. In contrast to *G. sihwensis* DSM 44576^T, it is very interesting that strain Chol-3^T is a foam-producing bacterium when grown in rich media, a characteristic that is well-known for *G. amarae* (Iwahori *et al.*, 2001; Pagilla *et al.*, 2002; Carr *et al.*, 2006) and that has also recently been found for *Gordonia defluvii* and other *Gordonia* species (Soddell *et al.*, 2006; Shen *et al.*, 2007). More differences between strain Chol-3^T and *G. sihwensis* can also be found in colony colour and form. The novel isolate produces beige and matt colonies on agar plates, whereas the colonies of *G. sihwensis* are white and rough (Kim *et al.*, 2003).

Additionally, the completely different RiboPrint patterns of these two strains presented in Supplementary Fig. S1 clearly show that these strains are not identical. The comparison of *Pvu*II-digested and probe-hybridized DNA of the two strains shows bands with different sizes for the two strains and the similarity index was only about 0.54 for *G. sihwensis* DSM 44576^T when compared directly with strain Chol-3^T. Thus, the patterns demonstrated that strain Chol-3^T does not represent a subspecies of *G. sihwensis*. Finally, analysis of DNA–DNA relatedness between strain Chol-3^T and its two phylogenetically closest neighbours in the 16S rRNA gene tree, *G. sihwensis* DSM 44576^T and *G. hydrophobica* DSM 44015^T, revealed values of 8.9 ± 0.7% and 8.8 ± 3.0%, respectively; these values are well below the 70% cut-off point recommended by Wayne *et al.* (1987) for the recognition of genomic species.

It is evident from the above-mentioned data that the tested organism can be distinguished from the type strains of all *Gordonia* species with validly published names. It is, therefore, clear from both genotypic and phenotypic results that strain Chol-3^T should be recognized as a member of a novel species within the genus *Gordonia*, for which the name *Gordonia cholesterolivorans* sp. nov. is proposed. The degradation of cholesterol by a member of

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing the phylogenetic position of strain Chol-3^T within the genus *Gordonia*. The out-group used to root the tree was *Williamsia serinedens* IMMIB SR-4^T. Asterisks indicate branches that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at nodes are percentage bootstrap values; only values above 50% are given. Bar, 0.01 substitutions per nucleotide position.

the genus *Gordonia* is here demonstrated for the first time. This broadens the range of metabolic potential of this genus and therefore makes it more attractive for environmental and industrial applications.

Description of *Gordonia cholesterolivorans* sp. nov.

Gordonia cholesterolivorans (cho.les'te.ro.li.vo'rans. N.L. n. *cholesterol* cholesterol; L. part. adj. *vorans* eating, devouring; N.L. part. adj. *cholesterolivorans* cholesterol-devouring).

Gram-positive-staining, aerobic, non-motile, slightly acid-fast actinomycete that fragments into rod/coccus-like elements. Colonies are beige and matt on LB agar plates. The steroid cholesterol is utilized as a sole carbon and energy source for aerobic growth in MM. Additional properties such as carbon utilization and cell-wall characteristics are mentioned in the text and in Table 1. Hydrolyses the chromogenic substrates *p*-nitrophenyl phosphorylcholine and 2-deoxythymidine-5'-*p*-nitrophenyl phosphate, but not *p*-nitrophenyl β -D-xyloside. The major fatty acids are C_{16:1}cis7, C_{16:0}, C_{18:1} and 10-methyl C_{18:0}. Possesses short-chain mycolic acids ranging in length from C₄₈ to C₅₆, with C₅₀, C₅₂ and C₅₄ being the principal mycolic acids.

The type strain, Chol-3^T (=CECT 7408^T =DSM 45229^T), was isolated from sewage sludge from a sewage treatment plant in Ciudad Real, Spain.

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