Amycolatopsis keratiniphila sp. nov., a novel keratinolytic soil actinomycete from Kuwait

Azza A. Al-Musallam,¹ Sheikha S. Al-Zarban,¹ Yunis A. Fasasi,¹ Reiner M. Kroppenstedt² and Erko Stackebrandt²

Correspondence
Reiner M. Kroppenstedt
kdt@dsmz.de

¹Kuwait University, Department of Biological Sciences, Microbiology Division, P.O. Box 5969, 13060 Safat, Kuwait

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, 38124 Braunschweig, Germany

A keratinolytic, nocardioform actinomycete, strain D2^T, was isolated from Kuwait marsh soil by the bait technique using animal wool. Strain D2^T was an aerobic, Gram-positive organism that produced light-grey aerial mycelium but no specific spore chains. 16S rDNA sequence analyses and chemotaxonomic markers were consistent with the classification of strain D2^T in the genus *Amycolatopsis*, i.e. it had *meso*-diaminopimelic acid in its peptidoglycan, arabinose and galactose as its diagnostic sugars, the polar lipids phosphatidylinositol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and diphosphatidylglycerol, menaquinone MK-9(H₄) and an *iso*-/anteiso-branched fatty acid pattern combined with 10-methyl-branched and 2-hydroxy-branched fatty acids. *Amycolatopsis japonica* was the closest phylogenetic neighbour of strain D2^T, showing 99.4% 16S rDNA sequence similarity with the novel strain. *A. japonica* and strain D2^T could be clearly separated from each other on the basis of their low DNA–DNA reassociation value (55.9%). These data, together with its distinct physiological traits, led to the conclusion that strain D2^T represented a novel species within the genus *Amycolatopsis*, for which the name *Amycolatopsis keratiniphila* (type strain D2^T = DSM 44409^T = NRRL B24117^T) is proposed.

The ability to degrade hair or other keratin-based substances, such as horn or feathers, is not widely distributed among bacteria. The degradation of natural keratin by *Lysobacter* spp. has been reported previously (Reichenbach, 1992). Martin & So (1969) isolated a strain of myxobacteria that could use autoclaved feathers and wool as sole C and N sources, but it was unable to attack untreated material. Among the actinomycetes, members of the genera *Cellulomonas* (Goodfellow, 1971), *Streptomyces* (Jensen, 1930) and *Pilimelia* (Kane, 1966) show keratinolytic activities. Species of the genus *Pilimelia* are well known for their ability to degrade keratin, as they are frequently isolated from soil using the hair bait technique. Species of this genus are easily differentiated from all other actinomycetes by their typical sporangia, which release zoospores when the mycelium is flooded with water (Vobis *et al.*, 1986). Until now, there have been no reports concerning keratin-degrading *Amycolatopsis* spp. The aim of this study was to clarify the taxonomic position of a novel keratin-degrading *Amycolatopsis* isolate, strain D2^T, by morphological, physiological, chemotaxonomical and molecular biological methods.

Strain D2^T was isolated from marsh soil in Kuwait that had been baited with sterilized and defatted wool; the strain was deposited with the DSMZ as *Amycolatopsis keratiniphila* DSM 44409^T. Determinations of morphological traits, colour of the aerial and substrate mycelia, and of soluble pigments were performed as described previously (Shirling & Gottlieb, 1966). Biochemical tests were done as described by Lechevalier *et al.* (1986). Strain D2^T showed the typical macroscopic and microscopic appearance of a member of the genus *Amycolatopsis* (Henssen *et al.*, 1987; Kothe *et al.*, 1989; Embley, 1992; Holt *et al.*, 1994), having dirty-white aerial mycelium which became light-grey in ageing cultures grown on GYM medium (4 g glucose l⁻¹, 4 g yeast extract l⁻¹, 10 g malt extract l⁻¹). No specific pigments were produced by the strain. In older cultures, aerial hyphae disintegrated into spore-like structures.

Cell material used for chemotaxonomic analyses was obtained in trypticase soy broth (BBL) after 4 days incubation of strain D2^T at 28 °C on a rotary shaker. The cell material was harvested by centrifugation and washed twice with distilled water. Analyses of amino acids and sugars were carried out using the methods of Stanek & Roberts (1974). Menaquinones and polar lipids were extracted following the procedure of Minnikin *et al.* (1984). Polar lipids and menaquinones were analysed by

The GenBank/EMBL accession number for the 16S rDNA sequence of *Amycolatopsis keratiniphila* D2^T is AJ278496.

TLC (Minnikin *et al.*, 1977) and HPLC (Kroppenstedt, 1982, 1985), respectively. Analyses of fatty acids were performed following the methods of Miller (1982) and Sasser (1990). The presence of mycolic acids was checked by the method of Minnikin *et al.* (1975). Whole-cell hydrolysates of strain D2^T contained *meso*-diaminopimelic acid as the diamino acid of the peptidoglycan and galactose and arabinose as the major cell-wall sugars. Menaquinone with a tetrahydrogenated isoprenoid chain of nine isoprene units [MK-9(H₄)] was found as the dominant isoprenoid quinone. The polar lipid pattern was composed of phosphatidylinositol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and diphosphatidylglycerol. This pattern matched quite well with the patterns for other *Amycolatopsis* spp. reported by Lechevalier *et al.* (1986). The fatty acid pattern was composed mainly of iso-/anteiso-branched fatty acids. Of diagnostic value is the combination of 10-methyl-branched iso-branched fatty acids together with 2-hydroxy-branched fatty acids (fatty acid pattern 3f *sensu* Kroppenstedt & Evtushenko, 2002), i.e. iso-C_{14:0} (13.9%), iso-C_{15:0} (8.0%), iso-C_{16:0} (33.5%), iso-C_{17:0} (0.8%), anteiso-C_{15:0} (1.9%), anteiso-C_{17:0} (1.3%), 10-methyl-iso-C_{17:0} (1.0%), 2-hydroxy-iso-C_{16:0} (7.4%), 2-hydroxy-anteiso-C_{15:0} (0.9%), C_{15:0} (7.1%), C_{16:0} (2.0%), C_{16:1} (4.9%), C_{17:0} (7.6%) and C_{17:1} (9.9%). Mycolic acids were not detected. All chemotaxonomic properties of strain D2^T were consistent with its classification in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Henssen *et al.*, 1987; Yassin *et al.*, 1993).

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). The AE2 editor (Maidak *et al.*, 1999) was used to align the almost-complete 16S rDNA sequence of strain D2^T (1439 nt) against the 16S rDNA gene sequences of representatives of the main actinobacterial lineages and then against members of the genus *Amycolatopsis*. Phylogenetic analyses (De Soete, 1983; Felsenstein, 1993) followed described methods. The almost-complete 16S rDNA sequence of strain D2^T, consisting of 1439 nt, was compared to 16S rDNA sequences of members of the class *Actinobacteria*. With intrageneric relationships ranging between 99.4 and 94.3% sequence similarity, strain D2^T was most closely related to members of the genus *Amycolatopsis*, especially to *Amycolatopsis japonica* (99.4% sequence similarity). A phylogenetic dendrogram (Fig. 1) was constructed from a distance matrix using the treeing algorithm of De Soete (1983). Distance-matrix and maximum-likelihood analyses gave consistent results, in that strain D2^T clustered with the type strains of *A. japonica*, *Amycolatopsis azurea* and *Amycolatopsis orientalis* (Fig. 1).

Strain D2^T was able to utilize all of the tested carbon sources (Table 1) and could grow in the presence of 5% NaCl. The strain grew at 10 °C but not at 45 °C; the optimal growth temperature was between 25 and 28 °C. In addition, strain D2^T could grow on feathers as sole C

Fig. 1. Dendrogram (De Soete, 1983), based on 16S rDNA sequences, displaying the phylogenetic positions of strain D2^T and related members of the genus *Amycolatopsis*. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 resamplings. Bar, sequence divergence. More distantly related members of the genus *Amycolatopsis* served as a root for the dendrogram (Kim *et al.*, 2002). Strain numbers and 16S rDNA accession numbers (in parentheses) of reference organisms are given.

and N sources, and tolerated 25% NaCl for growth. Based on these properties, strain D2^T could be distinguished from its closest phylogenetic relatives (Table 1).

DNA was isolated by chromatography on hydroxyapatite by the method of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). The DNA–DNA relatedness of strain D2^T to *A. japonica* was 59.5%, indicating that they were distinct species based on the criteria set forth by Wayne *et al.* (1987) for strains of the same species.

Based on phenotypic and genotypic data, we conclude that strain D2^T merits separate species status within the genus *Amycolatopsis*. We, therefore, propose the name *Amycolatopsis keratiniphila* for strain D2^T, the sole strain of this species.

Description of *Amycolatopsis keratiniphila* sp. nov.

Amycolatopsis keratiniphila (ke.rat.i.ni'phi.la. N.L. n. *keratinum* keratin; Gr. adj. *philos* loving; N.L. adj. *keratiniphila* keratin-loving, referring to the ability of the species to degrade keratin).

Aerobic, Gram-positive, non-motile actinomycete, which forms light-grey aerial mycelium. No specific endo- or exopigments are produced. In ageing cultures, aerial and

Table 1. Characteristics useful for distinguishing between species of the *Amycolatopsis orientalis*–*Amycolatopsis mediterranei* group

Strains: 1, *Amycolatopsis keratiniphila* D2^T; 2, *Amycolatopsis alba* 44262^T; 3, *A. azurea* DSM 43854^T; 4, *Amycolatopsis coloradensis* DSM 44425^T; 5, *A. japonica* DSM 44123^T; 6, *A. mediterranei* DSM 43304^T; 7, *A. orientalis* subsp. *orientalis* DSM 40040^T; 8, *A. orientalis* subsp. *lurida* DSM 43134^T. Data from Lechevalier *et al.* (1986), Henssen *et al.* (1987), de Boer *et al.* (1990), Labeda (1995) and Chun *et al.* (1999). –, Negative or absent; +, positive or present; ND, no data; w, weak positive. None of the strains produced a yellowish-green or dirty-white to grey aerial mycelium. All strains were able to utilize D(+)–cellobiose, D-fructose, mannitol and D-xylose.

Characteristic	1	2	3	4	5	6	7	8
Colour of aerial mycelium:								
Blue	–	–	+	–	–	–	–	–
Purple	–	–	+	–	–	–	–	–
White	–	+	–	–	+	+	+	+
White to olive-buff	–	–	–	+	–	–	–	–
Production of soluble pigment	–	–	+	+	–	–	–	–
Growth on sole carbon source (1 %, w/v):								
Adonitol	+	+	+	–	+	–	+	+
L(+)–Arabinose	+	+	+	–	+	+	+	+
D-Galactose	+	+	–	+	+	–	+	ND
Glycerol	+	+	+	ND	ND	+	+	+
Inositol	+	+	+	+	+	+	+	w
Lactose	+	+	+	–	+	+	+	+
Melibiose	+	+	+	–	+	+	–	–
Raffinose	+	+	+	–	+	+	+	–
L(+)–Rhamnose	+	–	–	–	–	–	+	–
Sucrose	+	+	+	+	+	+	+	–
Growth at:								
10 °C	+	–	+	+	+	+	+	–
45 °C	–	–	–	–	–	–	+	–
Growth in the presence of 5 % (w/v) NaCl	+	–	+	+	w	w	w	+

substrate mycelia disintegrate into spore-like structures. Optimal growth is obtained on GYM medium (4 g glucose l^{–1}, 4 g yeast extract l^{–1}, 10 g malt extract l^{–1}) at 28 °C. Whole-cell hydrolysates contain *meso*-diaminopimelic acid, arabinose and galactose. The predominant menaquinone is MK-9(H₄). The polar lipids are phosphatidylinositol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and diphosphatidylglycerol. Major fatty acids are iso-C_{16:0} (33.5 %), iso-C_{14:0} (13.9 %), C_{17:1} (9.9 %), iso-C_{15:0} (8.0 %), C_{17:0} (7.6 %), 2-hydroxy-iso-C_{16:0} (7.4 %) and C_{15:0} (7.1 %); minor fatty acids are C_{16:1} (4.9 %), C_{16:0} (2.0 %), anteiso-C_{15:0} (1.9 %), anteiso-C_{17:0} (1.3 %), 10-methyl-iso-C_{17:0} (1.0 %), 2-hydroxy-anteiso-C_{15:0} (0.9 %) and iso-C_{17:0} (0.8 %). Mycolic acids are not synthesized. Other characteristics of the species can be found in Table 1.

The type strain of *Amycolatopsis keratiniphila* is D2^T

(=DSM 44409^T=NRRL B24117^T). Isolated from agricultural soil in Kuwait using animal wool as bait.

Acknowledgements

We thank Ina Kramer, Gabriele Pötter, Michaela Schmidt, Bettina Sträubler and Jolantha Swiderski for their technical assistance.

References

- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- Chun, J., Kim, S. B., Oh, Y. K. & 7 other authors (1999). *Amycolatopsis thermoflava* sp. nov., a novel soil actinomycete from Hainan Island, China. *Int J Syst Bacteriol* **49**, 1369–1373.
- de Boer, L., Dijkhuizen, L., Grobben, G., Goodfellow, M., Stackebrandt, E., Parlett, J. H., Whitehead, D. & Witt, D. (1990). *Amycolatopsis methanolica* sp. nov., a facultatively methylotrophic actinomycete. *Int J Syst Bacteriol* **40**, 194–204.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Embley, T. M. (1992). The family *Pseudonocardiaceae*. In *The Prokaryotes*, 2nd edn, pp. 1188–1213. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, USA.
- Goodfellow, M. (1971). Numerical taxonomy of some nocardioform bacteria. *J Gen Microbiol* **69**, 33–80.
- Henssen, A., Kothe, H. W. & Kroppenstedt, R. M. (1987). Transfer of *Pseudonocardia azurea* and “*Pseudonocardia fastidiosa*” to the genus *Amycolatopsis*, with emended species description. *Int J Syst Bacteriol* **37**, 292–295.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stayley, J. T. & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore: Williams & Wilkins.
- Huss, V. A. R., Festl, H. & Schleifer, K.-H. (1983). Studies on the spectrometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K.-D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- Jensen, H. J. (1930). Decomposition of keratin by soil microorganisms. *J Agric Sci* **20**, 390–398.
- Kane, W. D. (1966). A new genus of *Actinoplanaceae*, *Pilimelia*, with a description of two species, *Pilimelia terevasa* and *Pilimelia anulata*. *J Elisha Mitchell Sci Soc* **82**, 220–230.
- Kim, B., Sahin, N., Tan, G. Y. A., Zakrzewska-Czerwinska, J. & Goodfellow, M. (2002). *Amycolatopsis eurytherma* sp. nov., a thermophilic actinomycete isolated from soil. *Int J Syst Evol Microbiol* **52**, 889–894.

- Kothe, H. W., Vobis, G., Kroppenstedt, R. M. & Henssen, A. (1989). A taxonomic study of mycolateless, wall chemotype IV actinomycetes. *Syst Appl Microbiol* **12**, 61–69.
- Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP-18) and a silver-loaded ion exchanger. *J Liq Chromatogr* **5**, 2359–2367.
- Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics*, no. 20 SAB Technical Series, pp. 173–199. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Kroppenstedt, R. M. & Evtushenko, L. I. (2002). The family *Nocardiopsaceae*. In *The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*, 3rd edn (release 3.11). Edited by M. Dworkin. New York: Springer (<http://et.springer-ny.com:8080/prokPUB/index.htm>).
- Labeda, D. P. (1995). *Amycolatopsis coloradensis* sp. nov., the avoparcin (LL-AV290)-producing strain. *Int J Syst Bacteriol* **45**, 124–127.
- Lechevalier, M. P., Prauser, H., Labeda, D. P. & Ruan, J.-S. (1986). Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. *Int J Syst Bacteriol* **36**, 29–37.
- Maidak, B., L., Cole, J. R., Parker, C. T., Jr & 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acid Res* **27**, 171–173.
- Martin, S. M. & So, V. (1969). Solubilization of autoclaved feathers and wool by myxobacteria. *Can J Microbiol* **15**, 1393–1397.
- Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanols. *J Gen Microbiol* **88**, 200–204.
- Minnikin, D. E., Patel, P. V., Alshamaony, L. & Goodfellow, M. (1977). Polar lipid composition in the classification of *Nocardia* and related bacteria. *Int J Syst Bacteriol* **27**, 104–117.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athaly, M., Schaal, K. P. & Parlett, J. H. (1984). An integrated procedure for the extraction of isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete. lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.
- Reichenbach, H. (1992). The genus *Lysobacter*. In *The Prokaryotes*, 2nd edn, pp. 3256–3275. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *USFCC Newsl* **20**, 1–6.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Vobis, G., Schäfer, D., Kothe, H.-W. & Renner, B. (1986). Description of *Pilimelia columellifera* (ex Schäfer 1973) nom. rev. and *Pilimelia columellifera* subsp. *pallida* (ex Schäfer 1973) nom. rev. *Syst Appl Microbiol* **8**, 67–74.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Yassin, A. F., Haggenei, B., Budzikiewicz, H. & Schaal, K. P. (1993). Fatty acid and polar lipid composition of the genus *Amycolatopsis*: application of fast atom bombardment-mass spectrometry to structure analysis of underivatized phospholipids. *Int J Syst Bacteriol* **43**, 414–420.