Actinomadura keratinilytica sp. nov., a keratin-degrading actinobacterium isolated from bovine manure compost

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A novel keratinolytic actinobacterium, strain WCC-2265T, was isolated from bovine hoof keratin ‘baited’ into composting bovine manure from southern Alberta, Canada, and subjected to phenotypic and genotypic characterization. Strain WCC-2265T produced well-developed, non-fragmenting and extensively branched hyphae within substrates and aerial hyphae, from which spherical spores possessing spiny cell sheaths were produced in primarily flexuous or straight chains. The cell wall contained meso-diaminopimelic acid, whole-cell sugars were galactose, glucose, madurose and ribose, and the major menaquinones were MK-9(H6), MK-9(H8), MK-9(H4) and MK-9(H2). These characteristics suggested that the organism belonged to the genus Actinomadura and a comparative analysis of 16S rRNA gene sequences indicated that it formed a distinct clade within the genus. Strain WCC-2265T could be differentiated from other species of the genus Actinomadura by DNA–DNA hybridization, morphological and physiological characteristics and the predominance of iso-C16 : 0, iso-C17 : 0 and 10-methyl C17 : 0 fatty acids.

The description of the family Thermomonosporaceae was recently amended and the family currently contains four genera: Actinomadura, Actinocorallia, Spirillospora and Thermomonospora (Zhang et al., 2001). The revised description of the genus Actinomadura by Zhang et al. (2001) accommodates aerobic, Gram-positive, non-acid-fast, non-motile actinobacteria that typically produce well-developed, extensively branched, non-fragmenting vegetative hyphae and aerial hyphae that differentiate into chains of spores of various shapes and ornamentation. All members of the genus Actinomadura possess type III cell walls (containing meso-diaminopimelic acid) and the acyl group of the muramic acid is of the acetyl type. Madurose can be found in the whole-cell sugars of most species. The predominant menaquinone types are MK-9(H6), MK-9(H8) and MK-9(H2). The total cellular fatty acid pattern is type 3a (saturated, unsaturated and branched fatty acids plus tuberculostearic acid) (Kroppenstedt & Goodfellow, 1991), except for Actinomadura rubrobrunea and Actinomadura viridilutea, which can be distinguished from other species of the genus Actinomadura by the relatively high proportions of iso-branched and low proportions of 10-methyl-branched fatty acids they produce. These differences have been attributed to the thermophilic nature of A. rubrobrunea and A. viridilutea.

Abbreviation: SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain WCC-2265T and Actinomadura rubrobrunea DSM 43750T are EU637009 and EU637008, respectively.

A table showing the fatty acid profiles of strain WCC-2265T and related strains and a figure showing the growth of strain WCC-2265T on keratin agar are available with the online version of this paper.
relative to other *Actinomadura* species (Kroppenstedt et al., 1990).

Here, the polyphasic characterization and classification of strain WCC-2265<sup>T</sup>, isolated in 2007 from composting bovine manure in Lethbridge, Alberta, Canada, are described. Strain WCC-2265<sup>T</sup> had genotypic and phenotypic properties that are typical of species of the genus *Actinomadura* and displayed the ability to degrade keratin and utilize it as a sole carbon and nitrogen source.

Strain WCC-2265<sup>T</sup> was isolated from coarsely ground bovine hoof (25 g) that had been enclosed in 5610 cm polyester bags (pore size, 50 μm; ANKOM Technology) and buried within a model compost system that consisted of a matrix primarily composed of bovine manure (Xu et al., 2009). Baited hoof samples were removed periodically over a 10 month period and 1 g material was homogenized in 10 ml PBS (100 mM, pH 7.2) amended with 0.01 % (v/v) Tween 80 with a Stomacher 80 Biomaster (Brinkmann). This suspension was serially diluted with 100 mM PBS, spread in duplicate onto keratin agar (KA), and incubated at 45 °C for 2, 4, 7 and 14 days. KA was a basal medium containing 5 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.5), 8.5 mM NaCl, 2 mM MgSO<sub>4</sub> and 1/1000 (v/v) trace-salt solution (1 g l<sup>−1</sup> each of FeSO<sub>4</sub>·7H<sub>2</sub>O, MnCl<sub>2</sub>·4H<sub>2</sub>O and ZnSO<sub>4</sub>·7H<sub>2</sub>O), amended with 10 g l<sup>−1</sup> bovine hoof or poultry feathers that had been milled to a fine powder with an MM-200 ball grinder (Retsch). Following isolation, strain WCC-2265<sup>T</sup> was maintained on tryptic soy agar and KA; it was also kept as a hyphal fragment and spore suspension in 20 % (v/v) glycerol at −80 °C.

For comparative purposes, *A. rubrobrunnea* DSM 43750<sup>T</sup> and *A. viridilutea* DSM 44433<sup>T</sup> were included in the study because phylogenetic analysis with all recognized species of the genus *Actinomadura* indicated that they were the most closely related to strain WCC-2265<sup>T</sup>. The strains that were tested were grown on KA, yeast/malt extract agar [International Streptomyces Project medium 2 (ISP 2)], oatmeal agar (ISP 3), and inorganic salts-starch agar (ISP 4) (Shirling & Gottlieb, 1966) at 45 °C following inoculation with a fresh spore suspension. Growth and morphology were examined at 2, 7 and 14 days. The colour of the substrate hyphae was determined from the reverse side as described by Shirling & Gottlieb (1966). Cell morphology was observed and measurements were made under a light microscope with an ocular micrometer and by scanning electron microscopy (SEM). For SEM, the agar blocks containing the organism (cultivated on KA and ISP 2 for 7 and 14 days at 45 °C) were fixed with either 2 % glutaraldehyde and critical-point-dried or with the vapour of 1 % osmium tetroxide and air-dried. Samples were sputter-coated with gold and viewed with a Hitachi S-570 microscope operated at 20 kV.

The morphological characteristics of strain WCC-2265<sup>T</sup> were consistent with those of members of the genus *Actinomadura*. Strain WCC-2265<sup>T</sup> grew well on all of the media tested and colonies could be observed after 24 h. The bacterium produced well-developed, non-fragmenting and extensively branched substrate hyphae that often formed long open coils (Fig. 1a). Colonies were opaque, raised and rubbery in consistency, with a wrinkled surface on ISP 2. On ISP 3 and ISP 4, colonies were raised and smooth. The substrate hyphae of the colonies were yellow-orange on ISP 2 and grey-white on ISP 3 and ISP 4. On KA, strain WCC-2265<sup>T</sup> produced white flocose aerial growth.
Sporulation, which is a characteristic feature of both A. rubrobrunea and A. viridilutea (Agre & Guzeva, 1975). Strain WCC-2265T could break down insoluble hoof and feather keratin within KA, producing an obvious zone of clearing (see Supplementary Fig. S1, available in IJSEM Online).

Growth was tested over a range of temperatures (25–65 °C), NaCl concentrations (0–9 % w/v) and pH values (pH 4.0–10.0) on ISP 2 and ISP 3. For pH adjustments, media were amended with 75 mM sodium citrate (pH 4.0–7.0) or BIS/TRIS/propane (pH 7.0–10.0) at the respective pH. Carbohydrate utilization was tested using ISP 9 medium as described by Shirling & Gottlieb (1966). Constitutive enzymic activities were tested using an API ZYM test kit according to the manufacturer’s instructions (bioMérieux; Humble et al., 1977). Cells were grown for 48 h in GYPB broth (Greiner-Mai et al., 1987), pelleted, resuspended in distilled water containing 0.85 % NaCl (6 McFarland standard) and incubated in the test strip cups for 4 h at 45 °C. Colour intensity scores of 4 or 5 were taken as positive for enzyme activity, 0 or 1 were negative and 2 or 3 were considered inconclusive. All physiological tests were performed in triplicate.

The physiological characteristics of strain WCC-2265T are consistent with those of members of the genus Actinomadura. The temperature range for growth on ISP 3 was 30–55 °C, with optimal growth at 45 °C. The strain grew at all tested pH values, but optimal growth was displayed at pH 6.0–9.0. The strain could tolerate up to 7 % NaCl in the medium and utilized d-fructose, d-glucose, myo-inositol, rhamnose, sucrose, d-xyllose and, to a lesser extent, arabinose as sole carbon sources. It could not use mannitol or raffinose. Strain WCC-2265T could be differentiated from many close phylogenetic neighbours by spore shape, ornamentation and arrangement. It could be differentiated from A. rubrobrunea DSM 43750T and A. viridilutea DSM 44433T by its production of soluble pigment, lack of aerial hyphae on ISP 3, tolerance for NaCl, lower growth temperature and inability to use mannitol (Table 1).

Strain WCC-2265T, A. rubrobrunea DSM 43750T and A. viridilutea DSM 44433T could not be differentiated based on the results of the API ZYM test kit. All strains were: positive for acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), z-glucosidase, leucine arylamidase, trypsin and valine arylamidase; negative for z-fucosidase, z-galactosidase, z-galactosidase, z-glucuronidase and z-mannosidase; and inconclusive for N-acetyl-z-glucosaminidase, z-chymotrypsin, z-glucosidase, lipase (C14) and naphthol-AS-BI-phosphohydrolase.

Genomic DNA was extracted and purified from cell cultures using the Qiagen DNeasy blood and tissue kit as described in the supplied protocol for DNA extraction from Gram-positive bacteria (Qiagen). The 16S rRNA gene was amplified with HotStar Taq (Qiagen) using the eubacterial primers UNI27F and UNI1492R (Lane, 1991). The almost complete 16S rRNA gene sequence data were obtained by direct sequencing with an ABI 3130 Automated Genetic Analyzer (Applied Biosystems) and the primers UNI27F, UNI338F, UNI1100R and UNI1492R (Lane, 1991). BLAST analysis (Altschul et al., 1990) was used to compare the 16S rRNA gene sequence of strain WCC-2265T with sequences from the NCBI nucleotide database. Sequence alignments were generated using CLUSTAL W 1.82 (Chenna et al., 2003) and optimized visually using GENEODOC (Nicholas et al., 1997). Phylogenetic trees were constructed using the neighbour-joining method. The reliability of the phylogenetic estimates was evaluated with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package (Felsenstein, 2005) and based on 1000 replications.

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The 16S rRNA gene sequence of strain WCC-2265T was phylogenetically most closely related to members of the genus Actinomadura. The neighbour-joining dendrogram confirmed that strain WCC-2265T was a member of the genus Actinomadura, phylogenetically most closely related to A. rubrobrunea DSM 43750T and A. viridilutea DSM 44433T, but it formed a clade (100 % bootstrap support) that was distinct from these two species (Fig. 2). A comparison of strain WCC-2265T with the type strains of other Actinomadura species revealed a sequence similarity of 97 % with A. rubrobrunea and A. viridilutea and sequence similarities of 92–96 % with all other species. The sequence dissimilarity was thus equal to or greater than the 3 % divergence typically used to define different species based on 16S rRNA gene sequences (Stackebrandt & Ludwig, 1988). To assess the validity of the novel species status of strain WCC-2265T, DNA–DNA hybridization was carried out. Results indicated that strain WCC-2265T was genetically distinct from its closest phylogenetic neigh-

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bours. The mean DNA–DNA relatedness values (duplicate measurements) of strain WCC-2265T with \textit{A. rubrobrunea} DSM 43750\textsuperscript{T} and \textit{A. viridilutea} DSM 44433\textsuperscript{T} were 43.1 ± 1.2\% and 38.3 ± 3.1\%, respectively, clearly indicating that strain WCC-2265T represents a novel species according to the 70\% DNA–DNA relatedness threshold recommended by Wayne \textit{et al.} (1987) for the recognition of genomic species. The DNA G+C content of strain WCC-2265T was 73.7 mol\%.

Biomass for quantitative fatty acid analysis was collected by centrifugation from cultures grown in GPYB broth (Greiner-Mai \textit{et al.}, 1987) for 48 h at 45°C. The cellular fatty acid profiles were determined by GLC (MIDI) using the standard protocol (Sasser, 1990). Consistent with the observations of Kroppenstedt \textit{et al.} (1990), \textit{A. rubrobrunea} and \textit{A. viridilutea} contained high amounts of iso-branched fatty acids and a low proportion of 10-methyl-branched fatty acids relative to other species of the genus \textit{Actinomadura}. Strain WCC-2265T also produced a relatively large proportion of iso-branched fatty acids, predominantly 14-methyl-pentadecanoic acid (iso-C16:0) and 15-methyl-hexadecanoic acid (iso-C17:0). Strain WCC-2265T could be differentiated from \textit{Actinomadura} species, including \textit{A. rubrobrunea} and \textit{A. viridilutea}, by the presence of a large proportion of 10-methyl-heptadecanoic acid (10-methyl C17:0). The complete cellular fatty acid profiles of strain WCC-2265T, \textit{A. rubrobrunea} DSM 43750\textsuperscript{T} and \textit{A. viridilutea} DSM 44433\textsuperscript{T} determined in this study can be found in Supplementary Table S1 (available in IJSEM Online).

Table 1. Morphological and physiological characteristics that differentiate strain WCC-2265\textsuperscript{T} from the type strains of closely related species of the genus \textit{Actinomadura}

| Strains: 1, WCC-2265\textsuperscript{T}; 2, \textit{A. rubrobrunea} DSM 43750\textsuperscript{T}; 3, \textit{A. viridilutea} DSM 44433\textsuperscript{T}; 4, \textit{A. oligospora} NRRL 15878\textsuperscript{T}; 5, \textit{A. namibiensis} DSM 44197\textsuperscript{T}; 6, \textit{A. hibiscus} ATCC 53557\textsuperscript{T}; 7, \textit{A. kijaniata} ATCC 31588\textsuperscript{T}; 8, \textit{A. atramentaria} ICMP 6250\textsuperscript{T}. Data from: this study; Agre & Guzeva (1975); Horan & Brodsky (1982); Mertz & Yao (1986); Greiner-Mai \textit{et al.} (1987); Miyadoh \textit{et al.} (1987); Tomita \textit{et al.} (1990); Wink \textit{et al.} (2003). cv, Curved; fl, flexuous; hk, hooked; sp, spiralled; st, straight; tw, twisted; gl, globose; obl, oblong; ac, aculeate; sm, smooth; bl, blue; br, brown; cl, colourless; gr, green; gy, grey; or, orange; pi, pink; wh, white; ye, yellow. +, Positive or present; −, negative or absent; +/−, inconclusive; ND, no data; TR, trace.

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Menaquinone analyses were carried out by the DSMZ Identification Service and B. J. Tindall (DSMZ). Quinones were extracted from 100 mg freeze-dried cell material using...
methanol/hexane based on the two-stage method described by Tindall (1990a, b). Quinones were separated by TLC on silica gel, removed from the plate and further analysed by HPLC. Cell wall analysis was also performed by the DSMZ Identification Service. Preparation of cell walls and determination of peptidoglycan structure were carried out using the methods described by Rhuland et al. (1955), Schleifer & Kandler (1972), Schleifer (1985) and MacKenzie (1987). Cell-wall sugars were analysed by a modification of the method of Staneck & Roberts (1974). Consistent with its classification in the genus Actinomadura (Zhang et al., 2001), the cell wall of strain WCC-2265\textsuperscript{T} contained meso-diaminopimelic acid, the cell-wall sugars were of type B (madurose, galactose, glucose and ribose were present) and the major menaquinones were MK-9(H\textsubscript{6}) (accounting for 50 % of the total), MK-9(H\textsubscript{8}) (15 %), MK-9(H\textsubscript{4}) (10 %) and MK-9(H\textsubscript{2}) (3 %).

Morphological, physiological and chemotaxonomic analyses indicated that strain WCC-2265\textsuperscript{T} belongs to the genus Actinomadura; 16S rRNA gene sequence and DNA–DNA hybridization analyses indicated that it represents a novel species. Phenotypic characters and fatty acid analysis are also consistent with novel species status for strain WCC-2265\textsuperscript{T}. Therefore, it is concluded that strain WCC-2265\textsuperscript{T} represents a novel species within the genus Actinomadura, for which the name Actinomadura keratinilytica sp. nov. is proposed. The epithet pertains to the recovery of the strain from composting keratin, as well as its ability to degrade and utilize keratin as its sole carbon and nitrogen source. This ability is due in part to the action of at least one highly active, keratin-degrading serine protease that has been cloned from A. keratinilytica (A. A. Puhl, L. B. Selinger, T. A. McAllister and G. D. Inglis, unpublished data).

**Description of Actinomadura keratinilytica sp. nov.**

Actinomadura keratinilytica [ke.rat.in.i.ly'ti.ca. N.L. n. keratinum keratin; N.L. adj. lyticus -a -um (from Gr. adj. lutikos) able to dissolve; N.L. fem. adj. keratinilytica keratin-dissolving.]

*Fig. 2.* Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain WCC-2265\textsuperscript{T} within the radiation of the genus Actinomadura. The sequence of *Nonomuraea pusillia* IFO 14684\textsuperscript{T} was used as an outgroup. Bar, 0.01 nt substitutions per base. Numbers at nodes (≥50 %) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps). NCBI accession numbers are presented in parentheses.
Aerobic, Gram-positive. Cells grow well on ISP 2, ISP 3, ISP 4 and KA, forming well-developed, extensively branched substrate hyphae. A brown soluble pigment is produced on ISP 2. White aerial hyphae are formed on KA, which differentiate into flexuous or straight chains of 5–15 spherical and spiny spores. Spore maturation is marked by a distinct change in the colour of the aerial hyphae to blue-green; no autolysis of the aerial hyphae occurs. Optimal growth is at 45°C and pH 6–9. D-Fructose, D-glucose, myo-inositol, rhamnose, sucrose, D-xylene and, to a lesser extent, arabinose are used as carbon sources, but not mannitol or raffinose. Tolerates up to 7 % NaCl. Keratin can be used as the sole carbon and nitrogen source. The predominant cellular fatty acids are iso-C_{16:0}, C_{17:0} \text{iso}, C_{17:0} \text{10-methyl} and 10-methyl C_{17:0}. The diagnostic amino acid of the cell-wall peptidoglycan is meso-diaminopimelic acid. The cell-wall sugars are madurose, galactose, glucose and ribose. The major menaquiones are MK-9(H_6), MK-9(H_8), MK-9(H_4) and MK-9(H_2).

The type strain, WCC-2265^T ( =DSM 45195^T=CCUG 56181^T), was isolated from composting bovine manure in southern Alberta, Canada. The DNA G+C content of the type strain is 73.7 mol%.

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