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

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A novel keratinolytic actinobacterium, strain WCC-2265^T, was isolated from bovine hoof keratin 'baited' into composting bovine manure from southern Alberta, Canada, and subjected to phenotypic and genotypic characterization. Strain WCC-2265^T 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-2265^T could be differentiated from other species of the genus *Actinomadura* by DNA–DNA hybridization, morphological and physiological characteristics and the predominance of iso-C_{16:0}, iso-C_{17:0} and 10-methyl C_{17:0} fatty acids. The broad range of phenotypic and genetic characters supported the suggestion that this organism represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura keratinilytica* sp. nov. is proposed; the type strain is strain WCC-2265^T (=DSM 45195^T=CCUG 56181^T).

The ability to degrade keratin, the insoluble structural protein of feathers, wool and horn (Bradbury, 1973), is not widely distributed among bacteria. Among the actinobacteria, this activity has been well characterized in many species within the genus *Streptomyces* (Noval & Nickerson, 1959; Young & Smith, 1975; Mukhopadhyay & Chandra, 1990) and keratinolytic activity has been reported from species belonging to the genera *Saccharomonospora*, *Amycolatopsis* and *Terrabacter* (Al-Zarban *et al.*, 2002; Al-Musallam *et al.*, 2003; Montero-Barrientos *et al.*, 2005).

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(H₄), MK-9(H₆) and MK-9(H₈). 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-2265^T and *Actinomadura rubrobrunea* DSM 43750^T are EU637009 and EU637008, respectively.

A table showing the fatty acid profiles of strain WCC-2265^T and related strains and a figure showing the growth of strain WCC-2265^T on keratin agar are available with the online version of this paper.

Fig. 1. Morphology of strain WCC-2265^T viewed by: (a) light microscopy showing substrate hyphae grown in ISP 2 medium for 14 days; (b) SEM of a vapour-fixed sample showing clusters of mature conidia on keratin agar (KA); (c) SEM of a glutaraldehyde-fixed sample showing aerial hyphae and conidia on KA; (d) SEM of a glutaraldehyde-fixed sample showing differentiation of aerial hyphae into conidia on KA. Bars, 1 µm.

relative to other *Actinomadura* species (Kroppenstedt *et al.*, 1990).

Here, the polyphasic characterization and classification of strain WCC-2265^T, isolated in 2007 from composting bovine manure in Lethbridge, Alberta, Canada, are described. Strain WCC-2265^T 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^T was isolated from coarsely ground bovine hoof (25 g) that had been enclosed in 5 × 10 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 KH₂PO₄ (pH 7.5), 8.5 mM NaCl, 2 mM MgSO₄ and 1/1000 (v/v) trace-salt solution (1 g l⁻¹ each of FeSO₄·7H₂O, MnCl₂·4H₂O and ZnSO₄·7H₂O), amended with 10 g l⁻¹ 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^T 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. rubrobrunea* DSM 43750^T and *A. viridilutea* DSM 44433^T 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^T. 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^T were consistent with those of members of the genus *Actinomadura*. Strain WCC-2265^T 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^T produced white floccose aerial

hyphae after 48 h incubation at 45 °C. Aerial hyphae were wider in diameter, on average, than substrate hyphae (1 µm compared with approximately 0.3 µm, respectively), were extensively branched and differentiated into straight or flexuous chains of up to 15 spiny spores with a mean diameter of 1.2 µm (Fig. 1). Mature spores were spherical and, en masse, were blue–green in colour and powdery in appearance. Aerial hyphae did not undergo autolysis upon sporulation, which is a characteristic feature of both *A. rubrobrunea* and *A. viridilutea* (Agre & Guzeva, 1975). Strain WCC-2265^T 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 GPYB broth (Greiner-Mai *et al.*, 1987), pelleted, resuspended in distilled water containing 0.85% NaCl (6 McFarland standard) and incubated in the test strip cupules 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-2265^T 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-xylose and, to a lesser extent, arabinose as sole carbon sources. It could not use mannitol or raffinose. Strain WCC-2265^T could be differentiated from many close phylogenetic neighbours by spore shape, ornamentation and arrangement. It could be differentiated from *A. rubrobrunea* DSM 43750^T and *A. viridilutea* DSM 44433^T 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-2265^T, *A. rubrobrunea* DSM 43750^T and *A. viridilutea* DSM 44433^T 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), α-glucosidase, leucine arylamidase, trypsin and valine arylamidase; negative for α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase and α-mannosidase; and inconclusive for

N-acetyl-β-glucosaminidase, α-chymotrypsin, β-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-2265^T with sequences from the NCBI nucleotide database. Sequence alignments were generated using CLUSTAL W 1.82 (Chenna *et al.*, 2003) and optimized visually using GENEDOC (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. TREEVIEW (Page, 2001) was used to generate rooted phylogenetic trees. DNA G+C content determination and DNA–DNA hybridization were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). DNA for G+C content determination and hybridization studies was purified on hydroxyapatite as described by Cashion *et al.* (1977). DNA G+C content was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

A 1488 bp 16S rRNA gene sequence was obtained for strain WCC-2265^T. BLAST analysis indicated that strain WCC-2265^T was phylogenetically most closely related to members of the genus *Actinomadura*. The neighbour-joining dendrogram confirmed that strain WCC-2265^T was a member of the genus *Actinomadura*, phylogenetically most closely related to *A. rubrobrunea* DSM 43750^T and *A. viridilutea* DSM 44433^T, but it formed a clade (100% bootstrap support) that was distinct from these two species (Fig. 2). A comparison of strain WCC-2265^T 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-2265^T, DNA–DNA hybridization was carried out. Results indicated that strain WCC-2265^T was genetically distinct from its closest phylogenetic neigh-

Table 1. Morphological and physiological characteristics that differentiate strain WCC-2265^T from the type strains of closely related species of the genus *Actinomadura*

Strains: 1, WCC-2265^T; 2, *A. rubrobrunea* DSM 43750^T; 3, *A. viridilutea* DSM 44433^T; 4, *A. oligospora* NRRL 15878^T; 5, *A. namibiensis* DSM 44197^T; 6, *A. hibisca* ATCC 53557^T; 7, *A. kijaniata* ATCC 31588^T; 8, *A. atramentaria* JCM 6250^T. Data from: this study; Agre & Guzeva (1975); Horan & Brodsky (1982); Mertz & Yao (1986); Greiner-Mai *et al.* (1987); Miyadoh *et al.* (1987); Tomita *et al.* (1990); Wink *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.

Characteristic	1	2	3	4	5	6	7	8
Conidia								
Chain arrangement	fl–st	cv	cv–tw	fl–hk	sp	st	fl–sp	st
Number in chains	5–15	6–20	6–20	10–50	20	10–50	≥ 10	2–5
Shape/ornamentation	gl/ac	gl/ac	gl/ac	obl/sm	obl/sm	obl/sm	obl/sm	obl/sm
Colour of colony on:								
ISP 2	ye–or	ye–or	ye–or	br	pi	red	gr/br	br
ISP 3	gy–wh	gy–wh	gy–wh	ye–gy	pi	ND	gr	cl
ISP 4	gy–wh	gy–wh	gy–wh	ye–gy	pi	ND	gr/br	cl
Aerial hyphae:								
ISP 2	TR	–	–	–	–	wh	wh	wh
ISP 3	–	wh/bl–gr	wh/bl–gr	TR	–	ND	wh	wh
ISP 4	–	TR	–	TR	wh	wh	–	wh
Autolysis	–	+	+	ND	ND	ND	ND	ND
Soluble pigment:								
ISP 2	br	–	–	br	–	red	–	br
ISP 3	–	–	–	–	–	–	–	–
ISP 4	–	–	–	–	–	–	gr	–
Growth at/in:								
30 °C	+	–	–	+	+	+	+	+
60 °C	–	+	+	–	ND	–	–	–
>6 % NaCl	+	–	–	–	ND	–	ND	–
Utilization of:								
Arabinose	+ / –	–	+ / –	–	+	+	+	–
D-Fructose	+	+ / –	+	+ / –	–	+	+	–
D-Glucose	+	+	+	+	+	+	+	+
myo-Inositol	+	+ / –	+	–	+ / –	+	+	–
Mannitol	–	+	+	–	+ / –	+	–	–
Raffinose	–	+ / –	–	–	–	+	–	–
Rhamnose	+	+	+	–	+	+	+	+
Sucrose	+	+	+	–	+	+	+	–
Xylose	+	+ / –	+	–	+ / –	+	+	–

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

Biomass for quantitative fatty acid analysis was collected by centrifugation from cultures grown in GPYB broth (Greiner-Mai *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 *et al.* (1990), *A. rubrobrunea* and *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 *Actinomadura*. Strain WCC-2265^T also produced a relatively large proportion of iso-branched fatty acids, predominantly 14-methyl-pentadecanoic acid (iso-C_{16:0}) and 15-methylhexadecanoic acid (iso-C_{17:0}). Strain WCC-2265^T could be differentiated from *Actinomadura* species, including *A. rubrobrunea* and *A. viridilutea*, by the presence of a large proportion of 10-methyl heptadecanoic acid (10-methyl C_{17:0}). The complete cellular fatty acid profiles of strain WCC-2265^T, *A. rubrobrunea* DSM 43750^T and *A. viridilutea* DSM 44433^T determined in this study can be found in Supplementary Table S1 (available in IJSEM Online).

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

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain WCC-2265^T within the radiation of the genus *Actinomadura*. The sequence of *Nonomuraea pusilla* IFO 14684^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.

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 Stanek & Roberts (1974). Consistent with its classification in the genus *Actinomadura* (Zhang *et al.*, 2001), the cell wall of strain WCC-2265^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(H₆) (accounting for 50 % of the total), MK-9(H₈) (15 %), MK-9(H₄) (10 %) and MK-9(H₂) (3 %).

Morphological, physiological and chemotaxonomic analyses indicated that strain WCC-2265^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^T. Therefore, it is concluded that strain WCC-2265^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].

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-xylose 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}, iso-C_{17:0} 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 menaquinones are MK-9(H₆), MK-9(H₈), MK-9(H₄) and MK-9(H₂).

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%.

Acknowledgements

The work of a number of individuals contributed to the completion of this project. We wish to thank the identification services at the DSMZ (Braunschweig, Germany) for DNA–DNA hybridization and DNA base composition analysis, analysis of the respiratory lipoquinones and cell-wall analysis. Thanks to Tara Shelton and Kathaleen House (Agriculture and Agri-Food Canada Research Centre, Lethbridge) for technical support. We are grateful to Byron Lee (AAFC, Lethbridge) for conducting the scanning electron microscopy. Determination of the cellular fatty acids and preparation of lyophilized stocks by Jay Yanke (AAFC, Lethbridge) is also gratefully acknowledged. Financial support was provided by the: (1) Alberta Prion Research Institute; (2) Canadian Centre for Security Science (Chemical, Biological, Radiological, and Nuclear Research and Technology Initiative); (3) Agriculture and Agri-Food Canada SRM targeted funding; and (4) Canada-Alberta Beef Industry Development Fund.

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