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Micromonospora equina sp. nov., isolated from soil from a racecourse

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Two actinomycete strains were isolated from within the fynbos-rich area surrounded by the horseracing track at Kenilworth Racecourse in Cape Town, South Africa. Rapid molecular identification indicated that the isolates belonged to the family *Micromonosporaceae*. Based on 16S rRNA gene sequence BLAST analysis, the isolates were identified as members of the genus *Micromonospora*. Phylogenetic analysis showed that the isolates clustered with each other and were most closely related to *Micromonospora viridifaciens* DSM 43909^T. Further 16S rRNA gene sequence analysis using EzTaxon revealed that the isolates are closely related to *Micromonospora auratinigra* TT1-11^T, *Micromonospora chaiyaphumensis* MC5-1^T, *Micromonospora eburnea* LK2-10^T, *Micromonospora nigra* DSM 43818^T and *Micromonospora olivasterospora* DSM 43868^T. DNA–DNA hybridization and physiological tests allowed genotypic and phenotypic differentiation of both isolates from related species; however, their high DNA–DNA relatedness showed that they belong to the same genomic species. Strain Y22^T (=DSM 45644^T=NRRL B-24859^T) was selected as the type strain to represent this novel species, for which the name *Micromonospora equina* sp. nov. is proposed.

The genus *Micromonospora* was proposed in 1923 by Ørskov (1923) and belongs to the family *Micromonosporaceae* along with 27 other genera, the majority of which only contain one or two species (Euzéby, 2012; Zhi *et al.*, 2009). The genus can be readily distinguished from the other members of its family by a combination of morphological and chemotaxonomic characters (Cross, 1981; Koch *et al.*, 1996). *Micromonospora* is the type genus of the *Micromonosporaceae* and is the most speciated genus in the family, currently containing 48 members with validly published names. *Micromonospora chalcea* is the type species (Euzéby, 2012).

Members of the genus *Micromonospora* have been isolated from a wide range of sources and are thought to occur in low numbers in soils, but are more abundant in aquatic habitats, including fresh and salt water, for example lakes, river and deep-sea sediments, water samples, beach sand and rice paddy soils (Cross, 1981; Kawamoto, 1989). Micromonosporae have also been isolated from root nodules (Garcia *et al.*, 2010; Trujillo *et al.*, 2006) and leaves (Kirby & Meyers, 2010). Many strains of the genus *Micromonospora* have been isolated as producers of aminoglycoside-type antibiotics (Kasai *et al.*, 2000), with gentamicin, produced by *Micromonospora echinospora*, being the most well-known example (Kasai *et al.*, 2000). The discovery of antibiotic-producing micromonosporae sparked the widespread isolation and screening of members of this genus, resulting in the discovery of producers of almost all of the major classes of antibiotics (Wagman & Weinstein, 1980). As well as containing strains that have the ability to produce antibiotics, this genus contains other interesting members, some of which are capable of degrading natural rubber (Kasai *et al.*, 2000) or surviving in water contaminated with radon, a radioactive by-product of uranium mining (Trujillo *et al.*, 2005). Here we describe a novel species isolated from soil collected from a racecourse in the Western Cape, South Africa.

Strains Y21 and Y22^T were isolated from a soil sample collected from the fynbos-rich area surrounded by the horse racing track at Kenilworth Racecourse, Cape Town, South Africa. The soil was ground with a sterile pestle and mortar before being heated at 60 °C for 1 h. Sterile distilled water (1 ml) was added to 0.1 g ground soil, vortexed vigorously for 1 min and allowed to stand for 5 min. The suspension was then serially diluted in sterile distilled water and 0.1 ml aliquots were plated on International *Streptomyces* Project (ISP) medium 2 (Shirling & Gottlieb, 1966) containing (ml^{-1}) 100 μg cycloheximide and 10 μg nalidixic acid. The plates were incubated at 30 °C for 18 days.

Genomic DNA was isolated as previously described (Everest & Meyers, 2008). Rapid identification of the

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Y21 and Y22^T are JF912510 and JF912511, respectively.

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

isolates to the genus level was achieved by 16S rRNA gene amplification and restriction endonuclease digestion (Cook & Meyers, 2003), using single digestions with *Mbo*I (*Sau*3AI isoschizomer), *Vsp*I (*Asn*I isoschizomer), *Sph*I, *Sna*BI and *Sal*I. Amplification of *gyrB* was attempted as per the methods of Everest & Meyers (2009) using all possible combinations of the primers 7G-gyrB-F (le Roes *et al.*, 2008), 7G-gyrB-R (Everest & Meyers, 2009), GgyrB-F1, GgyrB-F2, GgyrB-R1 (le Roes *et al.*, 2008), GYR-UniF1, KgyrB-F, KgyrB-R and KgyrB-R1892 (Kirby *et al.*, 2010). The PCR products were purified using an MSB Spin PCRapace kit (Invitek) and sequenced. Sequence chromatograms were edited using Chromas version 2.01 (Technelysium) and analysis of all sequences was performed using DNAMAN version 5.2.9 (Lynnon BioSoft). MEGA version 5.05 (Tamura *et al.*, 2011) was used to conduct the phylogenetic analyses. Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Takahashi & Nei, 2000) methods. BLAST (Altschul *et al.*, 1997) and EzTaxon (Chun *et al.*, 2007) analyses were performed to identify the closest relatives.

Physiological tests were performed as described by Williams *et al.* (1989). All plates were incubated at 30 °C for the recommended periods unless otherwise stated. All ISP media were prepared according to Shirling & Gottlieb (1966). Morphological properties were determined on inorganic salts-starch agar (ISP 4). The production of diffusible pigments was determined on glycerol-asparagine agar (ISP 5) and the production of melanin was determined on peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7). Growth in the presence of antibiotics was determined on Bennett's medium (Atlas, 2004) containing the indicated concentration of compounds and incubated for 7 days. NaCl tolerance was tested on ISP 2 incubated for 14 days. Growth at different pHs and temperatures was determined on Bennett's medium incubated for 14 days. Carbon source utilization was tested as per the methods of Shirling & Gottlieb (1966), but with the basal medium modified to include yeast extract to a final concentration of 0.05 % (w/v) to allow growth of the isolates, which failed to grow on the standard medium. All carbon sources were filter sterilized and tested at a concentration of 1 % (w/v), with the exception of the sodium salts of carboxylic acids, which were tested at 0.1 % (w/v). Nitrogen source utilization was performed as per Williams *et al.* (1989), with the basal medium modified to include 0.05 % (w/v) yeast extract. All nitrogen sources were filter sterilized and tested at a concentration of 0.1 % (w/v). The concentration of yeast extract added to both the carbon and nitrogen source utilization tests was the lowest concentration that allowed for growth on the positive control but not the negative control.

Antibiotic activity was determined by agar overlay tests. The isolates were stab-inoculated into ISP 2, Difco Middlebrook 7H9 Agar (Becton Dickinson) supplemented with 10 mM glucose (albumin-glucose-catalase supplement omitted), Modified Czapek solution (MC) agar (Nonomura & Ohara, 1971) and MC agar containing glycerol instead of glucose as the carbon source. The cultures were incubated at 30 °C for 9 days before being tested for activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Mycobacterium aurum* A+. Luria–Bertani sloppy agar (Sambrook *et al.*, 1989) was used to perform the overlays containing the test bacteria.

Analysis of the isomer of diaminopimelic acid present in the cell-wall peptidoglycan and the whole-cell sugar pattern was performed as per the methods of Hasegawa *et al.* (1983) using freeze-dried cells instead of colonies. The solvent system used in the whole-cell sugar analysis was ethyl acetate/pyridine/distilled water (100 : 35 : 25, by vol.). The polar lipid patterns were determined as described by Komagata & Suzuki (1987) and Minnikin *et al.* (1984) using *para*-anisaldehyde, ninhydrin and molybdenum blue reagents, while the presence of mycolic acids was determined as per Minnikin *et al.* (1975). Analysis of respiratory quinones was carried out by the identification service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. These analyses were all performed on freeze-dried cells of a culture of strain Y22^T grown in ISP 2 broth with moderate shaking at 30 °C for 3 days. Fatty acid analysis was performed by the BCCM/LMG Culture Collection as per the recommendations of the commercial identification system MIDI (Microbial Identification System, Inc.; MIDI Sherlock version 3.10; database TSBA 50 rev 5.0) on cells grown at 28 °C for 3 days on tryptic soy agar (11768; BBL). DNA–DNA hybridization analysis was performed by the BCCM/LMG Culture Collection. DNA was isolated using a modification of the method of Gevers *et al.* (2001) using a combination of the enzymes lysozyme, mutanolysin and lysostaphin for the enzymic cell lysis. Hybridization was performed in the presence of 50 % formamide at 54 °C, according to a modification of the method described by Ezaki *et al.* (1989).

Strains Y21 and Y22^T were isolated on ISP 2 agar after 16 and 18 days, respectively. The rapid genus identification method (Cook & Meyers, 2003) showed that the isolates belonged to one of six genera within the family *Micromonosporaceae*. BLASTN analysis of their 16S rRNA gene sequences (1388 nt for strain Y21 and 1375 nt for strain Y22^T) showed that the isolates belonged to the genus *Micromonospora*. The BLAST analysis revealed *Micromonospora viridifaciens* DSM 43909^T as having the highest sequence match with both isolates, and *Micromonospora auratinigra* TT1-11^T and *Micromonospora olivasterospora* MK70^T were also identified as close relatives of the isolates. EzTaxon searches showed that *Micromonospora chaiyaphumensis* MC5-1^T, *Micromonospora eburnea* LK2-10^T and *Micromonospora nigra* DSM 43818^T were also closely related to strains Y21 and Y22^T (⩾98.6 % 16S rRNA gene sequence similarity). The 16S rRNA gene sequences of strains Y21 and Y22^T are identical over 1375 nt. Strain Y21 had 98.9, 98.6, 98.2, 98.4, 99.0 and 99.2 % sequence similarity with *M. auratinigra* TT1-11^T, *M. chaiyaphumensis* MC5-1^T, *M. eburnea* LK2-10^T, *M. nigra*

DSM 43818^T, *M. olivasterospora* DSM 43868^T and *M. viridifaciens* DSM 43909^T, respectively, over 1388 nt. Strain Y22^T had 98.8, 98.6, 98.1, 98.5, 99.0 and 99.2 % sequence similarity with these type strains, respectively, over 1375 nt. The position of strains Y21 and Y22^T within the genus *Micromonospora* was determined by constructing a neighbour-joining phylogenetic tree with the type strains of all species with validly published names from the genus (Fig. S1, available in IJSEM Online, and Fig. 1). Strains Y21 and Y22^T were most closely related to each other and *M. viridifaciens* DSM 43909^T was their closest relative.

Given the high 16S rRNA gene sequence similarity between the isolates and their closest relatives, DNA–DNA hybridization analysis was conducted. This showed that strain Y22^T shared 26.0 ± 3.0 % DNA–DNA relatedness with *M. auratinigra* TT1-11^T, 35.4 ± 0.5 % with *M. chaiyaphumensis* NBRC 106529^T, 38.3 ± 12.3 % with *M. eburnea* LK2-10^T,

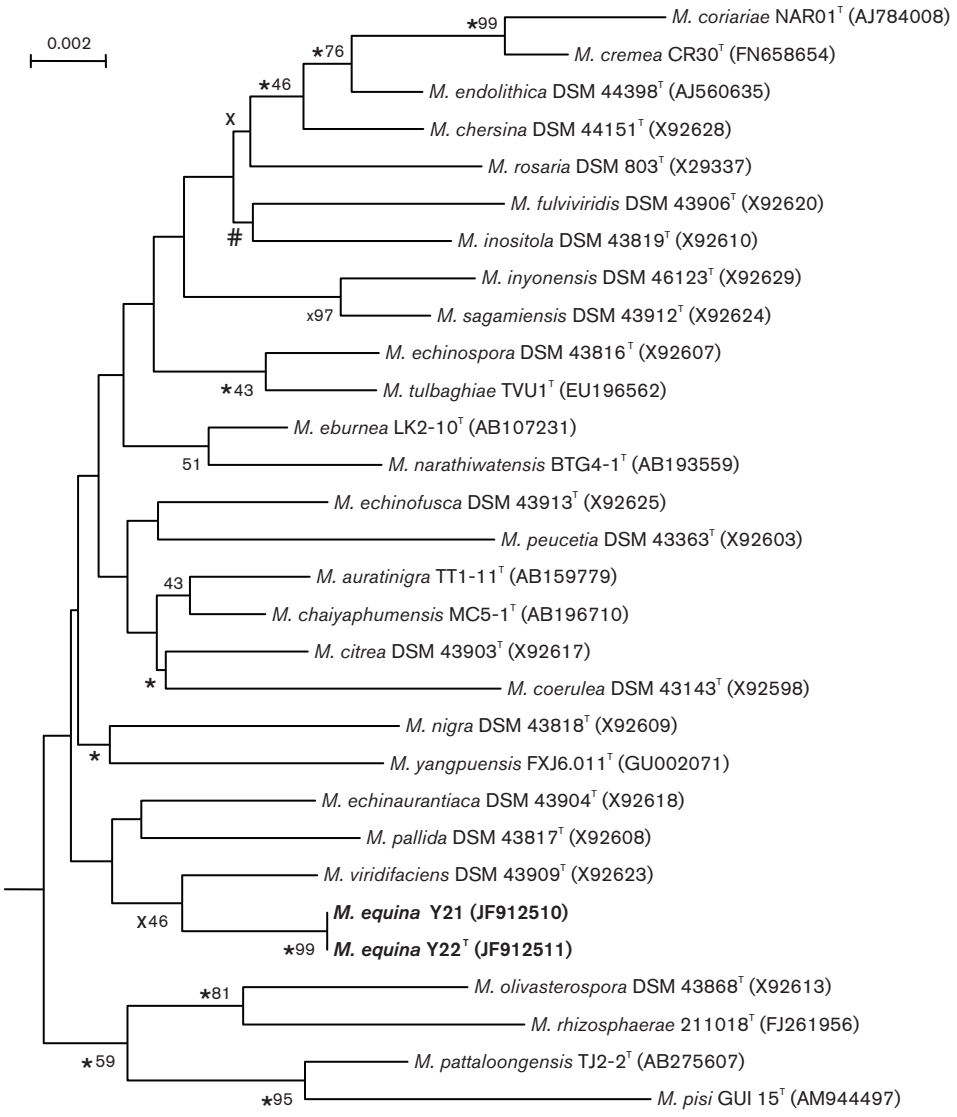

Fig. 1. Subtree of the 16S rRNA gene phylogenetic tree showing the position of strains Y21 and Y22^T within the genus *Micromonospora*. The tree forms part of the full neighbour-joining tree that was constructed with all the type strains belonging to the genus *Micromonospora*, based on 1365 nt of common sequence (Fig. S1). Values at each node are the percentage bootstrap values of 1000 replications (only values ⩾40 % are shown), with asterisks (*) indicating the clades that were conserved in the neighbour-joining, maximum-likelihood and maximum-parsimony trees; the hash (#) indicates the clade that was conserved in the neighbour-joining and maximum-likelihood trees and the crosses (x) indicate the clades that were conserved in the neighbour-joining and maximum-parsimony trees. Accession numbers are given in parentheses after the strain numbers. Bar, 2 nt substitutions per 1000 nt. *Catellatospora citrea* DSM 44097^T was used as an outgroup.

23.8±4.3 % with *M. nigra* NRRL 3097^T, 33.8±7 % with *M. olivasterospora* NRRL 8178^T and 45.4±7.5 % with *M. viridifaciens* NBRC 101887^T. These values are all below the 70 % threshold recommended by Wayne *et al.* (1987) for the recognition of genomospecies. Strains Y22^T and Y21 shared 77.7±6.0 % DNA–DNA relatedness, indicating that they belong to the same genomospecies.

Amplification of *gyrB* was attempted on several occasions, but no amplification was obtained with any of the

Table 1. Physiological and biochemical characteristics allowing differentiation of strains Y21 and Y22^T from their closest relatives in the genus *Micromonospora*

Strains: 1, *Micromonospora equina* sp. nov. Y21; 2, *M. equina* sp. nov. Y22^T; 3, *M. auratinigra* TT1-11^T; 4, *M. chaiyaphumensis* NBRC 106529^T; 5, *M. eburnea* LK2-10^T; 6, *M. nigra* NRRL 3097^T; 7, *M. olivasterospora* NRRL 8178^T; 8, *M. viridifaciens* NBRC 101887^T. Data were from this study unless otherwise stated. ++, Strongly positive; +, positive; w, weakly positive; −, negative.

Characteristic	1	2	3	4	5	6	7	8
Growth with/at:								
3 % (w/v) NaCl	w	−	−	− (+*)	+	−	−	w
4 % (w/v) NaCl	−	−	−	−	w (+†)	−	−	−
pH 5	w	w	−	+	+	−	−	−
45 °C	−	−	− (+*)	w (+*)	+	−	−	+
H_2S production	+	+	+ (−‡)	w	w (− †)	+	w	−
Nitrate reduction	w	w	w (−‡)	−	+	−	− (+§)	−
Degradation of:								
Gelatin	+	+	+	+	+	+ (−†)	+ (w§)	+
L-Tyrosine	+	+	+ (−‡*)	+	+ (−†)	+ (−†)	+ (−‡)	+
Xylan	w	w	w	w	+	+	−	−
Starch hydrolysis	+	+	+ (−†)	+	w	+	+	−
Sole carbon sources								
(+)-L-Arabinose	w	w	+	++ (+*)	+ (−†)	+ (w†)	−	− (wll)
(−)-D-Fructose	+	w	++ (+‡)	+	++ (−†)	w	+	w
Glycerol	−	−	w (−‡*)	+	− (+†)	−	−	−
myo-Inositol	+	−	w	w	w	w	−	− (w)
α-Lactose	w	w	+ (−†)	+	+	+	− (w ‡)	w
Mannitol	−	−	w (−‡)	−	− (w†)	w (−†)	−	− (wll)
(+)-D-Melibiose	w	w	++ (+‡)	++ (+*)	+	++	− (w‡)	−
Raffinose	−	−	+ (−†)	w (+*)	w (+†)	+	w (−‡§)	− (wll)
(+)-L-Rhamnose	−	w	w (−‡)	−	w (+†)	−	− (+‡)	− (wll)
Ribose	+	w	+	w (−*)	+ (−†)	w	w (+‡§)	+
Salicin	−	w	w (+‡/−†)	w (+*)	+	w (+†)	w (−‡§)	w
Sodium acetate	−	w	w	w	++	w	−	w
Sodium citrate	w	−	−	−	−	w	w	−
Sodium succinate	w	−	w	w	w	−	w	w
(−)-D-Sorbitol	+	w	w	−	w	w	−	−
Sole nitrogen sources								
L-Cysteine	−	−	w	+	−	−	w	−
L-Histidine	−	+	−	−	+	++	+	+
L-Methionine	+	+	w	w	+	w	−	+
L-Threonine	−	−	+	++	−	−	+	w
L-Valine	w	−	w	+	−	−	−	w
Resistance to (μg ml^{-1}):								
Lincomycin hydrochloride (100)	−	−	−	+	+	−	w	+
Tobramycin sulfate (50)	−	−	−	−	−	−	− (+§)	−

*Data taken from Jongrungruangchok *et al.* (2008).
†Data taken from Thawai *et al.* (2005).
‡Data taken from Thawai *et al.* (2004).
§Data taken from Kawamoto *et al.* (1983).
||Data taken from Kroppenstedt *et al.* (2005).

combinations of the primers that were tested. Several different genomic DNA samples were prepared and used without success. Efforts to obtain *gyrB* sequences for strains Y21 and Y22^T were therefore abandoned.

The physiological and biochemical differences between strains Y21 and Y22^T and related type strains are presented in Table 1. A full comparison of the fatty acid compositions of the isolates and related type strains is shown in Table S1. Strain Y22^T contained DL-diaminopimelic acid (*meso*-DAP) and glycine in the cell wall and arabinose, glucose, ribose and xylose in the whole-cell hydrolysate (chemotype II cell wall and type D whole-cell sugar pattern; Lechevalier & Lechevalier, 1970). The polar lipid profile comprised phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, unidentified aminolipids, an unidentified glycolipid and unidentified aminophospholipids (Fig. S2). The major menaquinones of the type strain were MK-10(H_4) (30 %), MK-9(H_4) (22 %) and MK-10(H_6) (16 %), with minor amounts of MK-9(H_6) (7 %), MK-10 (3 %), MK-10(H_2) (2 %) and MK-10(H_8) (2 %); remaining HPLC peaks were unidentified. The major fatty acids were iso-$C_{15:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$ and iso-$C_{16:0}$. These chemotaxonomic characteristics are consistent with those of the genus *Micromonospora* (Cross, 1981; Kawamoto, 1989; Koch *et al.*, 1996; Thawai *et al.*, 2004; Kroppenstedt *et al.*, 2005; Thawai *et al.*, 2005; Jongrungruangchok *et al.*, 2008).

Based on the polyphasic taxonomic characterization presented, strains Y21 and Y22^T can be distinguished from each other and from their closest phylogenetic relatives in phenotypic characteristics and DNA relatedness and are thus proposed as representatives of a novel species in the genus *Micromonospora*.

Description of *Micromonospora equina* sp. nov.

Micromonospora equina (e.qui′na. L. fem. adj. *equina* relating to horses, isolated from a soil sample collected near a horse racing track, Kenilworth Racecourse, Cape Town, South Africa).

Cells are Gram-positive. The substrate mycelium appears wrinkled and light orange (strain Y21) to deep orange (strain Y22^T) in colour, with the edges of the colonies darkening and becoming slightly black upon ageing. Melanin is not produced on peptone-yeast extract-iron agar (ISP 6) or tyrosine agar (ISP 7) and diffusible pigments are not formed on glycerol-asparagine agar (ISP5). Nitrate is weakly reduced to nitrite. H_2S is produced. Aesculin, arbutin and starch are hydrolysed. Casein, gelatin, Tween 80 and L-tyrosine are degraded and xylan is weakly degraded, but adenine, allantoin, cellulose, guanine, hypoxanthine, urea and xanthine are not degraded. As sole carbon sources, utilizes (+)-D-glucose, maltose and trehalose, weakly utilizes (+)-L-arabinose, α-lactose and melibiose. The extent of utilization of (+)-D-cellobiose, (−)-D-fructose, (+)-D-mannose, ribose, sorbitol and (+)-D-xylose varies between strains. Unable to utilize adonitol, glycerol, inulin, (−)-D-mannitol, (+)-D-melezitose or raffinose. *myo*-Inositol, (+)-L-rhamnose, salicin, sodium acetate, sodium citrate and sodium succinate utilization is variable depending on strain. As sole nitrogen sources, utilizes L-asparagine, L-methionine, potassium nitrate and L-serine and variably utilizes L-histidine, L-4-hydroxyproline and L-valine, but does not utilize DL-α-amino-*n*-butyric acid, L-cysteine, L-phenylalanine or L-threonine. Growth occurs at pH 5–10 and at 20–37 °C, but not at 45 °C. Grows in the presence of up to 2 % (w/v) NaCl; one strain grows weakly in the presence of 3 % (w/v) NaCl. The cell-wall peptidoglycan contains DL-diaminopimelic acid (*meso*-DAP) and glycine. The whole-cell hydrolysates contain arabinose, glucose, ribose and xylose. Mycolic acids are not present. The polar lipid profile contains phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, unidentified aminolipids, an unidentified glycolipid and unidentified aminophospholipids. The major menaquinones are MK-10(H_4), MK-9(H_4) and MK-10(H_6), with minor amounts of MK-9(H_6), MK-10, MK-10(H_2) and MK-10(H_8). The predominant fatty acids are iso-$C_{15:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$ and iso-$C_{16:0}$. No antibacterial activity is detected against *E. coli* ATCC 25922, *S. aureus* ATCC 25923 or *M. aurum* A+.

The type strain is Y22^T (=DSM 45644^T =NRRL B-24859^T), isolated from a horse racecourse, Cape Town, South Africa. The type strain is resistant to (ml^{-1}) penicillin G (10 U) and weakly resistant to oleandomycin phosphate (100 μg), but sensitive to cephaloridine (100 μg), lincomycin hydrochloride (100 μg), neomycin sulfate (50 μg), rifampicin (50 μg), streptomycin sulfate (100 μg), tobramycin sulfate (50 μg) and vancomycin hydrochloride (50 μg).

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