Novel members of the family *Micromonosporaceae*, *Rugosimonospora acidiphila* gen. nov., sp. nov. and *Rugosimonospora africana* sp. nov.

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Two novel Gram-positive-staining, acidophilic strains were isolated from soil samples. Both show typical features of filamentous actinomycetes. On the basis of 16S rRNA gene sequence analysis, the strains are members of the family *Micromonosporaceae*. The two strains contain hydroxydiaminopimelic acid, glycine, alanine and glutamic acid in the peptidoglycan. Fatty acid profiles clearly differentiate the two strains: cyclohexyl C_{17:0}, i-C_{16:0} and ai-C_{17:0} are predominant in Delta1^T, while the major components for Delta3^T are ai-C_{17:0} and i-C_{16:0}. The two strains also differ in their major menaquinones, MK-9(H₈, H₄, H₆) for Delta1^T and MK-9(H₈, H₆) for Delta3^T, and in phospholipid patterns; Delta1^T displays phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, methyl phosphatidylethanolamine and an unknown aminophospholipid, while Delta3^T also contains minor amounts of several unknown phospholipids in addition to these phospholipids. The whole-cell sugars of both strains are galactose, arabinose and xylose. The G + C content of the DNA is 72.7 mol% for Delta1^T and 71.9 mol% for Delta3^T. On the basis of chemotaxonomic, physiological and phylogenetic data, we propose *Rugosimonospora* gen. nov. to accommodate the two strains, with the description of *Rugosimonospora acidiphila* gen. nov., sp. nov. (the type species; type strain Delta1^T = DSM 45227^T = NBRC 104874^T) and *Rugosimonospora africana* sp. nov. (type strain Delta3^T = DSM 45228^T = NBRC 104875^T).

The family *Micromonosporaceae* (Krasil'nikov, 1938; Koch *et al.*, 1996; Stackebrandt *et al.*, 1997) currently comprises 21 genera, several of which have been described only recently (Tamura *et al.*, 2006; Thawai *et al.*, 2006; Ara & Kudo, 2007a, b; Ara *et al.*, 2008a, b; Wiese *et al.*, 2008). During a programme aimed at identifying novel filament-

ous actinomycetes (Donadio *et al.*, 2005), we isolated two moderately acidophilic actinomycete strains, Delta1^T and Delta3^T, from two soil samples collected in northern Italy and in Cameroon. Phylogenetic data indicated that the isolates belong to the family *Micromonosporaceae* but form a lineage clearly separated from described genera. The data we report show that the two isolates represent two novel species within a new genus, *Rugosimonospora* gen. nov.

For isolation of strains Delta1^T and Delta3^T, 500 mg dried soil was resuspended in 15 ml water and incubated overnight at 28 °C. The soil suspension was then incubated at 50 °C for 1 h and aliquots were plated on HSA5 medium (Busti *et al.*, 2006). Strains Delta1^T and Delta3^T appeared on isolation plates after incubation at 28 °C for 5–6 weeks. The strains formed small, brown colonies (<3 mm), lacking aerial mycelium. Direct observation of these

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Delta1^T and Delta3^T are FM208261 and FM208262, respectively.

Signature nucleotides within the 16S rRNA gene sequence are detailed in a table available as supplementary material with the online version of this paper.

colonies under a light microscope equipped with a $\times 40$ long-working-distance objective (ULWD-CDPlan; Olympus) and fitted with a 3CCD camera (Sony) revealed small vesicles distributed along the vegetative mycelium, probably corresponding to spores. Inocula for cultural and physiological characterization were obtained by growing strains in baffled flasks of liquid BTT medium (l^{-1} : glucose, 10 g; yeast extract, 1 g; meat extract, 1 g; casitone, 2 g; pH 5.5) for 1 week at 28 °C on rotary shaker at 200 r.p.m.; mycelium was then washed twice with sterile water and suitably diluted according to the test to be performed. The two strains were cultured and characterized as described previously (Busti *et al.*, 2006). Both strains stain Gram-positive and are non-acid-fast. Cultural characteristics of the strains on different media, observed after incubation at 28 °C for 3 weeks, are reported in Table 1. Field emission scanning electronic microscopy, performed as described previously (Busti *et al.*, 2006) after growth on ISP3 medium (pH 5.5) at 28 °C for 5 weeks, confirmed the presence of single spores along vegetative hyphae. Spores are spherical, 0.7–0.8 μm in diameter, and have a rugose surface (Fig. 1). No spore motility was observed. Upon sporulation, colonies develop a green–dark green pigmentation. The pH range for growth was determined on BTT medium solidified with 20 g agar l^{-1} . Both strains grew at pH 4.5–7.2, with an optimum between pH 5 and 6. On BTT medium (pH 5.5), the optimal growth temperature for both strains was between 22 and 28 °C. Strain Delta1^T grew at 17–32 °C, while strain Delta3^T had a broader growth range, 15–37 °C. Carbon source utilization was determined on Gauze1 medium (Gauze *et al.*, 1957) without starch. Both strains grew well

on xylose, mannose and glycerol, while scant growth was observed with glucose, sucrose, L-arabinose, inositol, fructose, raffinose and rhamnose.

All other physiological tests were performed as described previously (Busti *et al.*, 2006). Both strains hydrolyse starch, casein and gelatin, are positive for tyrosinase and produce H₂S, and neither strain reduces nitrate. Both strains are sensitive to lysozyme ($<10 \mu g ml^{-1}$). Nalidixic acid tolerance is $10 \mu g ml^{-1}$ for Delta1^T and $15 \mu g ml^{-1}$ for Delta3^T. Delta1^T cannot grow in the presence of 1% NaCl, while Delta3^T grows in the presence of 1% but not 2% NaCl.

The 16S rRNA gene sequences of the two strains were determined and analysed as described previously (Monciardini *et al.*, 2003). The two strains share 99.6% sequence identity. Comparison with GenBank showed identity values of 97.0–97.7% for both strains with members of different genera of the family *Micromonosporaceae*, among which *Polymorphospora rubra* TT9742^T (97.7 and 97.6% for Delta1^T and Delta3^T, respectively) and *Micromonospora auratinigra* DSM 44815^T (97.5 and 97.4%) share the highest identities. These values are comparable to those found for intergenus comparisons within the family.

The sequences shared all the signature nucleotides described for the family *Micromonosporaceae* (Stackebrandt *et al.*, 1997). However, they did not share any of the signature nucleotide patterns described for known genera of the family (Koch *et al.*, 1996; Maldonado *et al.*, 2005; Ara & Kudo, 2007a, b). A list of signatures that allow differentiation of the two strains from the other genera of

Table 1. Growth characteristics of the novel strains after 3 weeks of incubation at 28 °C

Media were prepared as described by Shirling & Gottlieb (1966) and Suter (1978) but acidified to pH 5.5. Growth is scored as: + + + +, abundant; + + +, good; + +, sufficient; +, scant. Colour codes are given according to Maerz & Paul (1950).

Medium	Vegetative mycelium		Soluble pigment
	Growth	Colour	
Strain Delta1 ^T			
ISP2	+ + +	Yellow (11I15). Spots of green (16L7)	None
ISP3	+ + +	Colourless. Spots of green (16L7)	None
ISP4	+ +	Colourless	None
ISP5	+ +	Colourless	None
ISP7	+ +	Colourless; darkening along the edges	Red–brown 5E12
Suter	+ +	Colourless	None
Suter + Tyr	+ +	Colourless with dark spots	Red–brown 12G12
Strain Delta3 ^T			
ISP2	+ + + +	Colourless to green to orange (10J7) from centre outwards	None
ISP3	+ + +	Colourless with yellowish edges (10E6)	None
ISP4	+ +	Colourless to yellowish	None
ISP5	+ + +	Colourless	None
ISP7	+ + +	Colourless. Spots of dark green	Pink 11C2
Suter	+ + +	Colourless	None
Suter + Tyr	+ + +	Colourless	Pink 11C5

Fig. 1. Scanning electron microscopic images of strains Delta1^T (a) and Delta3^T (b, c). Spore germination is visible in (c). Bars, 2 µm (a, b) and 1 µm (c).

the family is shown in Supplementary Table S1 (available in IJSEM Online). As only a few sequences were available, we consider this list only tentative, and refrain from inserting it in the formal genus description. The sequences of the two strains were aligned with those of representatives of all described genera of the family and the phylogenetic tree in Fig. 2 was calculated as described previously (Monciardini *et al.*, 2003). Strains Delta1^T and Delta3^T formed a cluster sustained by a bootstrap value of 100 %, clearly separated from other lineages within the family. The

topology of the tree was confirmed by maximum-parsimony analysis with the program DNAPARS from the PHYLIP package version 3.6 (Felsenstein, 2005).

Cell-wall amino acids, whole-cell sugars, isoprenoid quinones, cellular fatty acids, polar lipids and DNA G+C content were determined as described previously (Busti *et al.*, 2006). The structures of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1984) and fatty acid methyl esters were confirmed by GC/MS (Schumann *et al.*, 1997) by using a 320 Single Quad instrument (Varian).

The diagnostic diamino acid of the peptidoglycan was hydroxydiaminopimelic acid. In addition, the amino acids alanine, glycine and glutamic acid occurred in a molar ratio of approximately 0.6:1.1:1.0. Within the family *Micromonosporaceae*, *meso*-diaminopimelic acid is present in all other genera of the family, with the exception of *Catenuloplanes* and *Couchioplanes*, which contain lysine. Members of *Hamadaea*, *Catellatospora* and *Pseudosporangium*, as well as some strains of the genera *Micromonospora* and *Actinoplanes*, also present minor amounts of hydroxydiaminopimelic acid (and sometimes also *LL*-diaminopimelic acid) in addition to *meso*-diaminopimelic acid (Komagata & Suzuki, 1987; Ara *et al.*, 2008b). In contrast with those strains, no *meso*- or *LL*-diaminopimelic acid was detected in strains Delta1^T and Delta3^T. Unfortunately, it was not possible to determine the configuration of hydroxydiaminopimelic acid. The acyl type of murein was *N*-glycolyl, as determined by the method of Uchida *et al.* (1999).

The major menaquinones found in Delta1^T were MK-9(H₈), MK-9(H₄) and MK-9(H₆) (11:9:6), while Delta3^T only had MK-9(H₈) and MK-9(H₆) (55:13). Among described genera of the *Micromonosporaceae*, MK-9(H₈) can be found as the major menaquinone only in *Catenuloplanes*, but in this case it is the only component. Only the sporangiate genus *Dactylosporangium* and the pseudosporangiate genus *Krasilnikovia* have MK-9(H₄, H₆, H₈) as major menaquinones, but neither of them has MK-9(H₈) as the predominant component (Ara & Kudo, 2007b).

The whole-cell sugars of both strains are galactose, arabinose and xylose. These sugars are found (sometimes with others) in several genera of the family. Polar lipids detected for Delta1^T were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, methyl phosphatidylethanolamine and an unknown aminophospholipid, while strain Delta3^T contained minor amounts of several unknown phospholipids in addition to these phospholipids. With the exception of *Catenuloplanes*, all described genera of the family *Micromonosporaceae* have similar phospholipid patterns.

For determination of the cellular fatty acids, the two strains were cultured in liquid BTT medium, supplemented with 25 mM MES, pH 6.0, at 30 °C and 200 r.p.m. for 10 days.

Fig. 2. Neighbour-joining (Saitou & Nei, 1987) tree showing the position of strains Delta1^T and Delta3^T within the family *Micromonosporaceae*. The tree is based on 1429 aligned positions of the 16S rRNA gene sequence and was rooted using the sequence of *Streptomyces albus* DSM 40313^T as an outgroup (GenBank accession no. AJ621602; not shown). Numbers at nodes are bootstrap values based on 1000 resampled datasets; only values >500 are shown. Asterisks indicate nodes supported by parsimony analysis with bootstrap values >850. Bar, 1 inferred substitution per 100 nucleotides.

Fatty acid profiles, analysed as described by Miller (1982), are reported in Table 2. The major component found in Delta1^T, cyclohexyl C_{17:0}, is totally absent from Delta3^T, while the amount of ai-C_{17:0} in Delta3^T is more than double that found in Delta1^T. The identity of cyclohexyl C_{17:0} extracted from strain Delta1^T was confirmed by comparison with the same fatty acid obtained from *Humibacter albus* DSM 18994^T (Vaz-Moreira *et al.*, 2008). In addition, the electron impact mass spectrum of cyclohexyl C_{17:0} methyl ester (methyl 11-cyclohexyl undecanoate) extracted from strain Delta1^T showed a molecular ion at *m/z* 282 as well as fragment ions at *m/z* 239, 199, 143, 87, 83, 74 and 55, and was almost identical to the published spectrum of the authentic compound (<http://www.lipidlibrary.co.uk/>). Fatty acid analysis was repeated under different conditions (see Table 2), including growth at suboptimal pH (7.2), giving consistent results. Cyclohexyl C_{17:0} was a major component in the fatty acid profile of strain Delta1^T even under different cultivation conditions, while this fatty acid was never detected in parallel cultivations of strain Delta3^T. The approx. 50 % higher content of ai-C_{17:0} in Delta3^T was confirmed under the different cultivation conditions (see Table 2). Cyclohexyl C_{17:0} has been found in major amounts in some representatives of the family *Microbacteriaceae* (Suzuki & Komagata, 1983; Aizawa *et al.*, 2007; Qiu *et al.*, 2007; Vaz-Moreira *et al.*, 2008), but not in the family *Micromonosporaceae*. We are aware that the difference in fatty acid patterns between the two strains is striking, especially when considering their high 16S rRNA gene sequence relatedness. The cyclohexyl fatty acid may functionally replace the branched chain fatty acid ai-C_{17:0} in the membrane of strain Delta1^T. We can only

speculate on the possibility that this difference may be related to the different environmental conditions (in particular the mean temperature) of the samples from which the two strains originated.

The DNA G + C contents of strains Delta1^T and Delta3^T are 72.7 and 71.9 mol%, respectively.

Despite the high similarity in 16S rRNA gene sequences, differences in chemotaxonomic markers suggested that the two strains could belong to different species. This finding is consistent with the situation in other genera of the *Micromonosporaceae*, e.g. the type strains of the species *Salinispora tropica* and *Salinispora arenicola* share >99.5 % 16S rRNA gene sequence identity (Jensen & Mafnas, 2006). Overall genome similarity between strains Delta1^T and Delta3^T was measured by DNA–DNA hybridization. For this purpose, DNA was isolated and purified on hydroxyapatite (Cashion *et al.*, 1977) and reassociation rates were measured by spectrophotometry (De Ley *et al.*, 1970; Huß *et al.*, 1983). The suggested threshold value for species delineation is 70 % DNA–DNA relatedness (Wayne *et al.*, 1987), while the determined value for the two strains was <10 %, supporting their classification as members of two different species.

Although the two strains are members of the family *Micromonosporaceae*, they are clearly different from established genera of this family. Among the described genera, only *Micromonospora*, *Salinispora* and *Verrucosipora* form single spores (formed also by *Planosporangium*, which in addition forms sporangia), while the remaining genera either form sporangia (*Actinoplanes*, *Dactylosporangium*, *Pilimelia*, *Virgisporangium* and *Luedemannella*) or spore

Table 2. Fatty acid profiles of strains Delta1^T and Delta3^T

Values are percentages of total fatty acids. Experiment A consisted of cultivation in liquid BTT medium supplemented with 25 mM MES (pH 6.0) at 30 °C at 200 r.p.m. for 10 days. Experiment B consisted of cultivation on BTT agar at 28 °C for 9 days. Experiment C consisted of cultivation in liquid ISP2 medium (medium 987; <http://www.dsmz.de>) (4.0 g glucose, 4.0 g yeast extract and 10.0 g malt extract per litre distilled water; pH 7.2) at 28 °C for the number of days indicated. —, Not detected.

Fatty acid	Delta1 ^T			Delta3 ^T		
	Expt A	Expt B	Expt C (7 days)	Expt A	Expt B	Expt C (4 days)
C _{13:0}	—	—	—	0.09	—	—
C _{15:0}	0.25	—	0.19	1.07	0.15	0.38
C _{16:0}	0.25	0.13	0.19	0.16	0.49	0.60
C _{17:0}	0.47	—	—	1.57	0.49	0.86
C _{18:0}	0.53	0.33	0.40	0.23	0.64	0.60
i-C _{13:0}	0.38	0.19	0.17	0.24	0.33	0.29
i-C _{14:0}	2.02	2.43	1.88	1.51	2.55	1.78
i-C _{15:0}	6.04	2.19	4.45	9.31	8.66	10.05
i-C _{16:0}	28.10	26.29	36.55	28.00	37.28	27.58
i-C _{17:0}	5.12	2.35	5.25	3.06	3.32	2.93
i-C _{18:0}	0.90	2.20	1.35	0.24	0.29	0.20
ai-C _{13:0}	—	—	—	0.09	0.16	0.20
ai-C _{15:0}	1.88	1.18	2.36	8.21	7.11	10.97
ai-C _{17:0}	14.08	9.57	17.42	36.42	28.43	34.73
C _{16:1} ω11c	2.50	2.96	1.17	—	—	—
C _{17:1} ω8c	—	—	—	0.90	—	—
C _{18:1} ω9c	0.14	—	—	0.07	—	—
Cyclohexyl C _{17:0}	35.71	46.19	24.42	—	—	—
i-C _{15:1}	—	—	—	—	0.33	0.24
i-C _{16:1}	0.84	2.73	2.13	1.15	4.75	3.10
i-C _{17:1}	0.21	—	—	0.31	—	—
ai-C _{17:1}	0.32	0.62	0.60	1.80	4.46	4.50
C _{12:0} 2-OH	—	0.21	—	—	—	—
C _{16:1} 2-OH	—	0.20	1.46	5.33	0.55	1.00
i-C _{14:0} 3-OH	0.13	—	—	0.25	—	—
i-C _{16:0} 3-OH	—	0.24	—	—	—	—

chains, with *Pseudosporangium* forming false sporangia which are derived from highly aggregated spores or spore chains (Ara *et al.*, 2008a). Taken together, the morphological, chemotaxonomic and phylogenetic data indicate that the two strains represent a new genus within the family *Micromonosporaceae*, for which we propose the name *Rugosimonospora* gen. nov. On the basis of differences in the polar lipid and menaquinone patterns and fatty acid profiles as well as the low DNA–DNA relatedness, we propose two different species to accommodate the two strains.

Description of *Rugosimonospora* gen. nov.

Rugosimonospora (Ru.go'si.mo.no.spo'ra. L. adj. *rugosus* rugose, wrinkled; Gr. adj. *monos* single; Gr. fem. n. *spora* a seed and, in bacteriology, a spore; N.L. fem. n. *Rugosimonospora* a bacterium forming single, rugose spores).

Gram-positive-staining, non-acid-fast, aerobic actinomycetes that form non-fragmenting substrate mycelium. Spores develop individually from substrate hyphae. Spores

are spherical, 0.7–0.8 µm in diameter, present a rugose surface and are non-motile. Colonies are colourless to yellow to orange, depending on the growth medium, and can develop spots of green where sporulation has occurred. Optimum temperature for growth is 22–28 °C. Optimum pH for growth is pH 5–6. Tolerant of 10–15 µg nalidixic acid ml⁻¹. Sensitive to 1–2 % NaCl and 10 µg lysozyme ml⁻¹. Gelatin, casein and starch are hydrolysed. H₂S is produced. Nitrate is not reduced. Good growth using xylose, mannose and glycerol as sole carbon source. Pink to red–brown soluble pigments are produced on tyrosine-containing media. The diagnostic diamino acid of the peptidoglycan is 3-hydroxydiaminopimelic acid. The whole-cell sugars are galactose, arabinose and xylose. The polar lipid pattern consists of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine and methyl phosphatidylethanolamine (phospholipid pattern type PII). Additional unknown phospholipids and aminophospholipids may occur. The acyl type of the peptidoglycan is *N*-glycolyl. Major menaquinone is MK-9(H₈); MK-9(H₆) occurs in minor

amounts. The DNA G + C content is approx. 72–73 mol%. Strains have been isolated from soil. The type species is *Rugosimonospora acidiphila*.

Description of *Rugosimonospora acidiphila* sp. nov.

Rugosimonospora acidiphila (a.ci.di.phi'la. N.L. neut. n. *acidum* acid; Gr. adj. *philos* loving; N.L. fem. adj. *acidiphila* acid-loving).

The chemotaxonomic and general characteristics are the same as given above for the genus. Grows between 17 and 32 °C and at pH 4.5–7.2. Does not grow on 1 % NaCl. In addition to MK-9(H₈) and MK-9(H₆), also contains significant amounts of MK-9(H₄). Predominant fatty acids are cyclohexyl C_{17:0}, i-C_{16:0} and ai-C_{17:0}. The G + C content of DNA of the type strain is 72.7 mol%.

The type strain, Delta1^T (=DSM 45227^T =NBRC 104874^T), was isolated from a temperate forest soil from Gerenzano, Italy.

Description of *Rugosimonospora africana* sp. nov.

Rugosimonospora africana (a.fri.ca'na. L. fem. adj. *africana* African, referring to the origin of the type strain).

The chemotaxonomic and general characteristics are the same as given above for the genus. Grows between 15 and 37 °C and at pH 4.5–7.2. Does not grow on 2 % NaCl. Predominant fatty acids are ai-C_{17:0} and i-C_{16:0}. The G + C content of DNA of the type strain is 71.9 mol%.

The type strain, Delta3^T (=DSM 45228^T =NBRC 104875^T), was isolated from a soil sample from an undefined location in Cameroon.

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