Molecular-genetic and Chemotaxonomic Studies on *Actinomadura* and *Nocardiopsis*

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The relationships of 24 strains of 13 species of *Actinomadura* and 4 strains of *Nocardiopsis dassonvillei* were determined by nucleic acid hybridization studies. DNA–rRNA cistron similarity and DNA homology values reveal that *Actinomadura* is genetically heterogeneous. One cluster contained the type species *Actinomadura madurae*, *Actinomadura pelletieri*, *Actinomadura verrucosopora*, *Actinomadura malachitica*, *Actinomadura citrea* and ‘*Actinomadura kijaniata*’. A second cluster embraced *Actinomadura pusilla*, *Actinomadura roseoviolacea*, *Actinomadura libanotica*, *Actinomadura roseola* and *Actinomadura ferruginea*. The internal homogeneity of the two *Actinomadura* clusters was demonstrated by a high similarity in the menaquinone and fatty acid composition of the strains enclosed. *Actinomadura spadix*, *Actinomadura spiralis*, and two strains of *Actinomadura madurae* were found to be unrelated to each other and could not be allocated to one of the two major *Actinomadura* clusters. *Nocardiopsis dassonvillei* was genetically and phenotypically clearly separated from all *Actinomadura* species investigated.

INTRODUCTION

Some years ago we began to explore relatedness among various sporoactinomycete genera. The determination of DNA–DNA homologies allows the detection of relationships at the strain and species level, and DNA–rRNA cistron similarities determined by hybridization are valuable at the intrafamily level. Partial sequence analysis of 16S rRNA can be used to define orders or even lower ranks. As a result of our studies a hierarchic structure of the sporoactinomycetes emerged (Stackebrandt & Woese, 1981*a, b*; Stackebrandt *et al.*, 1981, 1983; Stackebrandt & Schleifer, 1983).

In most cases organisms in the clusters so obtained had a high similarity in characters of chemotaxonomic value, as seen with various streptomycete genera, the *Streptosporangium*–*Planomonospora*–*Planobispora* group and the *Actinoplanes* group of organisms (Stackebrandt *et al.*, 1981, 1983).

The genus *Actinomadura* is one of the sporoactinomycete genera rarely included in extensive chemotaxonomic and genetic investigations. This genus contains actinomycetes forming a stable mycelium, with or without an aerial mycelium bearing arthrospores of different morphology and having cell walls with *meso*-diaminopimelic acid (Lechevalier & Lechevalier, 1970; Williams & Wellington, 1981); most of the species are reported to contain madurose, identified as 3-*O*-methylgalactose (Lechevalier & Gerber, 1970). Contradictory results have been reported on the DNA G + C content of the type species *Actinomadura madurae*, ranging from 65 mol% (Mordarski *et al.*, 1977) to 74.5 mol% (Lechevalier *et al.*, 1971). In the few comparative chemotaxonomic investigations which have been done on *Actinomadura*, in most

cases only *A. madurae* and *A. pelletieri*, or only a small number of the more than 20 validly described species (Skerman *et al.*, 1980; Horan & Brodsky, 1982) were included in studies on isoprenoid quinones (Yamada *et al.*, 1977, 1982; Collins *et al.*, 1977; Collins & Jones, 1981; Aoki *et al.*, 1980), polar lipids (Minnikin *et al.*, 1977), and fatty acids (Agre *et al.*, 1975). Little information is therefore available about the homogeneity of *Actinomadura*. The first indication that this genus actually contains a heterogeneous collection of species came from the analysis of polar lipids (Agre *et al.*, 1975) and from numerical phenetic studies (Goodfellow & Pirouz, 1982).

In this communication we present data on a variety of chemotaxonomic characters, e.g. menaquinone composition, whole cell sugars, fatty acids of diagnostic value, and the DNA G + C content of 13 species of *Actinomadura* and of *Nocardiopsis dassonvillei*, which has recently been excluded from *Actinomadura* on phenotypic grounds (Meyer, 1976).

METHODS

Bacteria used and culture conditions. The strains investigated, their sources and their DNA G + C content are listed in Table 1. For the isolation of high-molecular weight DNA, organisms were cultivated in broth containing (% w/v): peptone (Oxoid), 0.5; yeast extract (Oxoid), 0.2; sucrose, 3.0; KNO₃, 0.2; K₂HPO₄, 0.1; MgSO₄ · 7H₂O, 0.05; KCl, 0.05; pH 7.3. For the isolation of *in vivo*-labelled DNA and ribosomal RNA, cells were grown in the same broth, but the organic compounds were reduced to half the amounts listed above. All organisms were cultivated aerobically by shaking for 2 to 4 d at 28 °C. Bacteria were harvested by filtering the suspension through a Whatman no. 1 filter paper.

Isolation of nucleic acids. Unlabelled and labelled DNAs were purified according to Marmur (1961), modified as described by Meyer & Schleifer (1978) and Stackebbrandt *et al.* (1981). Ribosomal RNA (23S) was isolated as described by Stackebbrandt *et al.* (1981). *In vivo* labelling of DNA and rRNA with [³H]adenine (New England Nuclear, 14.1 Ci mmol⁻¹, 522 GBq mmol⁻¹), using 0.5 mCi and 1 mCi, respectively, was done as described by Stackebbrandt *et al.* (1981). *In vitro* labelling of DNA by nick-translation with 50 µCi [³²S]dATP (New England Nuclear, 494 Ci mmol⁻¹, 18 300 GBq mmol⁻¹) was done using the nick-translation kit of Bethesda Research Laboratories (Cambridge, U.K.). The specific activities for DNA ranged between 8 × 10³ and 2.5 × 10⁴ c.p.m. (µg [³H]DNA)⁻¹, and 1 × 10⁵ and 7 × 10⁵ c.p.m. (µg [³⁵S]DNA)⁻¹, respectively; that for 23S rRNA was 6 × 10⁴ c.p.m. (µg [³H]RNA)⁻¹.

Hybridization procedures. DNA-DNA hybridization experiments were carried out in two different ways. The DNA-filter method was done as originally described by McConaughy *et al.* (1969) and Denhardt (1966), modified as described by Stackebbrandt & Kandler (1979). A sample (0.1 µg) of fragmented and denatured [³H]DNA or [³⁵S]DNA in 0.2 ml 3 × SSC (1 × SSC is 0.15 M-sodium chloride, 0.015 M-trisodium citrate, pH 7.0), adjusted to 33% formamide, was hybridized against 7 to 10 µg filter-bound DNA for 20 h at 60 °C, corresponding to slightly 'stringent' reassociation conditions (20 °C below *T_m* of the DNA). When [³⁵S]DNA was used as a reference DNA, the hybridization buffer contained 10 mM-dithiothreitol.

Alternatively, in some cases the DNA homology was determined spectrophotometrically by measuring the reassociation kinetics of DNA solutions (De Ley *et al.*, 1970) in a recording spectrophotometer (Gilford, Model 2600). Purified DNA, dialysed twice against 2 × SSC was fragmented by passing three times through a French pressure cell at 1.7 GPa and adjusted to 50 µg DNA ml⁻¹. The DNA of *Actinomadura* and *Nocardiopsis* strains have an average G + C content of 68 mol%, from which it follows that the melting point of the DNA in 2 × SSC is 101.5 °C (Frank-Kamenetskii, 1971). The melting point of the DNA was decreased to 92 °C by the addition of 14% formamide to the DNA solution. Three DNA solutions (equal concentrations of DNAs of strain A and of strain B and a mixture of equal volumes of both DNA samples) and a buffer blank (2 × SSC adjusted to 14% formamide) were heated at 97 °C for 10 min and adjusted to a temperature of 82 °C within 2–3 min. The hybridization temperature was calculated according to Gillis *et al.* (1970). Owing to the presence of 14% formamide in the reassociation solution, hybridization was carried out under stringent conditions (10 °C below *T_m* of the DNA).

DNA-23S rRNA hybridization and the determination of the melting points of the DNA-rRNA duplexes followed the method of De Ley & De Smedt (1975), with the slight modifications described by Döpfer *et al.* (1982).

Determination of the G + C mol% of the DNA. The guanine (G) plus cytosine (C) content of the DNA was calculated from its thermal denaturation temperature (Mandel & Marmur, 1968). The melting profiles were determined in a Gilford 2600 spectrophotometer.

Cell wall analysis. Amino acids and sugars of cell material were released by acid hydrolysis (Becker *et al.*, 1965; Lechevalier *et al.*, 1971). The whole cell hydrolysates were separated by cellulose TLC with methanol/water/10 M-HCl/pyridine (80:17.5:2.5:10, by vol.) as developing solvent in the case of the amino acids and with ethyl acetate/pyridine/water (100:35:25, by vol.) in the case of the sugars. The separated amino acids and sugars were identified with specific spraying reagents (Stanek & Roberts, 1974).

Extraction and analysis of fatty acid methyl esters. Freeze-dried cells were subjected to acid methanolysation (Minnikin *et al.*, 1975). The fatty acid methyl esters were extracted with petrol ether, and purified by TLC on silica gel PF₂₅₄₊₃₆₆ of 0.4 mm thickness. The developing solvent was petroleum ether (b.p. 60–80 °C)/diethylether (85:15, v/v). Under UV₃₆₆-irradiation the fatty acid methyl esters are visible as light blue spots. The fatty acid methyl esters were eluted with chloroform and analysed with a Varian 2700 and a Hewlett Packard 5700 gas chromatograph. Both chromatographs were fitted with a splitter and auxiliary gas supply to run capillary columns. The conditions are the following: Varian fused silica capillary column 25 m × 0.32 mm (WCOT), OV101, oven temperature 180 °C, inlet pressure 0.3 bar (30 kPa), split 1:40; Hewlett Packard, fused silica capillary column 25 m × 0.32 mm (WCOT), FFAP, oven temperature 180 °C, inlet pressure 1 bar, split 1:20, detector FID. The fatty acid methyl esters were identified by comparing their retention times with that of standard mixtures (Kroppenstedt & Kutzner, 1978; Kroppenstedt, 1982a).

Extraction, purification and identification of isoprenoid quinones. Samples (100–200 mg) of freeze-dried cells were suspended in 2 ml acetone, cooled in an ice bath, disrupted by ultrasonication for 2 min and separated by centrifugation. This procedure was repeated three times, and the acetone extracts were dried on a rotary evaporator at 40 °C (Kroppenstedt *et al.*, 1981). After the isolation of menaquinones with adsorption TLC on silica gel (Dunphy *et al.*, 1971), the menaquinones were separated by HPLC on two different stationary phases (RP 18 and silver-loaded ion exchanger) and identified on their retention behaviour according to their isoprenylic chainlength and their degree of saturation (Kroppenstedt, 1982b).

RESULTS

The G + C mol% of the DNA of 24 strains of 13 species of *Actinomadura* and 4 strains of *Nocardiopsis dassonvillei* ranged between 65.5 and 69.7% and 64 and 69%, respectively (Table 1).

DNA–rRNA hybridization

Information about the phylogenetic coherence of the genus *Actinomadura* and about the relationship of this genus to *Nocardiopsis*, *Streptomyces* and *Streptosporangium* was achieved by the determination of the melting point of DNA–rRNA duplexes [$T_{m(e)}$ values], obtained with ³H-labelled 23S rRNA of *Actinomadura madurae* DSM 43067 and DNA from representatives of the various genera (Table 2). In respect of the thermal stability of DNA–rRNA duplexes, strains of *Actinomadura* form two clusters. Organisms of the first cluster (here designated as *A. madurae*-cluster) show high $T_{m(e)}$ values (79.0 to 84 °C) with reference rRNA of *A. madurae* DSM 43067, i.e. three strains of *A. madurae*, all strains of *A. pelletieri* investigated, *A. verrucosospora* DSM 43358, *A. malachitica* DSM 43462, *A. citrea* DSM 43661 and '*A. kijaniata*' ATCC 31588. The second cluster embraces all other strains of *Actinomadura* investigated, including two strains classified as *A. madurae*. The thermal stability of the duplexes formed with DNA of strains of this second cluster and rRNA of *A. madurae* DSM 43067 is markedly lower (73.1–75.8 °C) than that found for members of the *A. madurae*-cluster. The $T_{m(e)}$ values of members of this second *Actinomadura* rRNA-cluster are in the same range as those found for duplexes formed by *A. madurae* DSM 43067 and four strains of *Nocardiopsis dassonvillei* (72–73.7 °C), *Streptosporangium roseum* DSM 43021, and *Streptomyces griseus* DSM 43236 (73.8 and 73.2, respectively).

DNA–DNA hybridization

Since the determination of relationships by rRNA cistron similarity experiments does not allow the detection of relationships between closely related species, organisms of the two *Actinomadura* rRNA-clusters were subjected to DNA–DNA hybridization experiments. The average binding efficiency in the homologous system was 28%. DNA, labelled *in vivo* with [³H]adenine or *in vitro* with [³⁵S]dATP by nick-translation showed the same binding efficiency. Homology values obtained by the spectrophotometrical method showed a high correspondence to those values obtained by the filter-method. The homology values are listed in Tables 3–6. Organisms of the *A. madurae*-rRNA cluster share homology values ranging from 18 to 100% (Tables 3 and 6). The highest values are found between *A. madurae* strains DSM 43067 and DSM 43380 (96%), between various strains of *A. pelletieri* (85–100%) (Table 3), and between the two strains of *A. verrucosospora* (96%) (Table 6). Except for *A. madurae* DSM 43122 and *A.*

Table 1. *Source and DNA G+C content of the organisms investigated*

Strain	Source*	DNA G+C content (mol %)
<i>Actinomadura madurae</i>	DSM 43067† (ATCC 19425, CCM 136, NCTC 5654)	68.2
<i>Actinomadura madurae</i>	DSM 43380 (M. Goodfellow strain A32)	68.2
<i>Actinomadura madurae</i>	DSM 43381 (IMRU 1136)	67.7
<i>Actinomadura madurae</i>	DSM 43121 (IMRU 754)	66.0
<i>Actinomadura madurae</i>	DSM 43122 (IMRU 780)	68.0
<i>Actinomadura madurae</i>	DSM 43123 (IMRU 1260)	68.0
<i>Actinomadura pelletieri</i>	DSM 43383	66.0
<i>Actinomadura pelletieri</i>	DSM 43382 (C. Philpot strain 1065)	67.3
<i>Actinomadura pelletieri</i>	DSM 43384 (M. Goodfellow strain A35)	65.5
<i>Actinomadura pelletieri</i>	DSM 43117 (IMRU 513)	67.2
<i>Actinomadura pelletieri</i>	DSM 43118 (IMRU 755)	65.5
<i>Actinomadura pusilla</i>	DSM 43357† (ATCC 27296)	68.3
<i>Actinomadura roseoviolacea</i>	DSM 43144† (ATCC 27297)	68.5
<i>Actinomadura verrucosospora</i>	DSM 43358† (ATCC 27299)	69.0
<i>Actinomadura verrucosospora</i>	DSM 43550	69.0
<i>Actinomadura spadix</i>	DSM 43459† (ATCC 27298)	66.4
<i>Actinomadura citrea</i>	DSM 43461† (ATCC 27887)	67.6
<i>Actinomadura malachitica</i>	DSM 43462† (ATCC 27888)	68.1
<i>Actinomadura roseola</i>	DSM 43551†	66.2
<i>Actinomadura roseola</i>	DSM 43552†	66.2
<i>Actinomadura ferruginea</i>	DSM 43553†	68.1
<i>Actinomadura libanotica</i>	DSM 43556	66.2
<i>Actinomadura spiralis</i>	DSM 43555†	68.1
' <i>Actinomadura kijaniata</i> '	ATCC 31588†	69.7
<i>Nocardiopsis dassonvillei</i>	DSM 43111† (ATCC 23218, NCTC 10488)	69.0
<i>Nocardiopsis dassonvillei</i>	DSM 43235 (ATCC 23219, NCTC 10489)	68.5
<i>Nocardiopsis dassonvillei</i>	DSM 43377 (M. Goodfellow, strain A92)	64.0
<i>Nocardiopsis dassonvillei</i>	DSM 43378 (IMRU 714)	64.5
<i>Streptosporangium roseum</i>	DSM 43021† (ATCC 12428)	70.3
<i>Streptomyces griseus</i>	DSM 40236† (ATCC 23345, ISP 5236)	71.0
<i>Arthrobacter globiformis</i>	DSM 20124† (ATCC 8010, NCTC 8907)	63.4

* Abbreviations: ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; CCM, Czechoslovak Collection of Micro-organisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Gottingen, F.R.G.; M. Goodfellow, Department of Microbiology, Newcastle upon Tyne, U.K.; IMRU, Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J., U.S.A.; ISP, International *Streptomyces* Project; NCTC, National Collection of Type Cultures, London, U.K.; C. Philpot, London School of Hygiene and Tropical Medicine, London, U.K.

† Type strain.

pelletieri DSM 43382, which are specifically related (75%), homology values found between strains of *A. madurae*, *A. pelletieri*, *A. verrucosospora*, and *A. citrea* DSM 43061 range between 25 and 44%. *Actinomadura malachitica* DSM 43462, and '*A. kijaniata*' ATCC 31588 show homology values of only 18 to 25% to the reference DNAs, while representatives of the second *Actinomadura* rRNA-cluster, as well as strains of *Nocardiopsis* and *Streptosporangium* show even lower homology values (7–12%). Those *Actinomadura* strains which did not group with *A. madurae* DSM 43067 in the DNA-rRNA cistron similarity study do not form a genetically coherent group. Using reference [³H]DNA of *A. pusilla* DSM 43357 and *A. roseoviolacea* DSM 43144 and [³⁵S]DNA of *A. libanotica* DSM 43554, these three species, together with *A. roseola* DSM 43551 and DSM 43552 and *A. ferruginea* DSM 43553 form a distinct cluster in which DNA homology values are as high (Table 4) as those found among members of the *A. madurae* rRNA-cluster (Table 3). Four strains of *Actinomadura*, namely *A. spadix* DSM 43459, *A. spiralis* DSM 43555, *A. madurae* DSM 43121 and *A. madurae* DSM 43123, show little DNA homology to members of the two clusters formed around *A. madurae* and *A. pusilla*, or to each other (Table 4).

Table 2. Melting points ($T_{m(e)}$) of DNA–23S rRNA duplexes of 3H -labelled 23S rRNA from *Actinomadura madurae* DSM 43067, and DNA from representatives of *Actinomadura*, *Nocardiopsis* *Streptosporangium*, *Streptomyces* and *Arthrobacter*

Filter-bound DNA from strain:		$T_{m(e)}$
<i>Actinomadura madurae</i>	DSM 43067	83.7
<i>Actinomadura madurae</i>	DSM 43380	84.0
<i>Actinomadura madurae</i>	DSM 43381	80.8
<i>Actinomadura madurae</i>	DSM 43122	81.1
<i>Actinomadura pelletieri</i>	DSM 43382	79.8
<i>Actinomadura pelletieri</i>	DSM 43383	79.1
<i>Actinomadura pelletieri</i>	DSM 43118	79.6
<i>Actinomadura verrucosospora</i>	DSM 43358	81.0
<i>Actinomadura malachitica</i>	DSM 43462	81.2
' <i>Actinomadura kijaniata</i> '	ATCC 31588	79.0
<i>Actinomadura citrea</i>	DSM 43461	79.2
<i>Actinomadura ferruginea</i>	DSM 43553	73.9
<i>Actinomadura roseola</i>	DSM 43551	74.8
<i>Actinomadura pusilla</i>	DSM 43357	74.0
<i>Actinomadura roseoviolacea</i>	DSM 43144	75.2
<i>Actinomadura libanotica</i>	DSM 43556	74.4
<i>Actinomadura spiralis</i>	DSM 43555	74.4
<i>Actinomadura spadix</i>	DSM 43459	75.8
<i>Actinomadura madurae</i>	DSM 43121	73.1
<i>Actinomadura madurae</i>	DSM 43123	73.0
<i>Nocardiopsis dassonvillei</i>	DSM 43111	73.7
<i>Nocardiopsis dassonvillei</i>	DSM 43235	71.7
<i>Nocardiopsis dassonvillei</i>	DSM 43377	73.7
<i>Nocardiopsis dassonvillei</i>	DSM 43378	72.5
<i>Streptosporangium roseum</i>	DSM 43021	73.8
<i>Streptomyces griseus</i>	DSM 40236	73.2
<i>Arthrobacter globiformis</i>	DSM 20124	63.9

The homology values for four strains of *Nocardiopsis dassonvillei*, obtained with the DNA-filter method and the spectrophotometrical method, are shown in Tables 5 and 6. Strains of *N. dassonvillei* group into two pairs of highly related organisms, which are moderately related to each other (36–38%). No relationship could be detected between strains of *N. dassonvillei* and representatives of *Actinomadura*.

Biochemical characteristics

We could reconfirm that *meso*-diaminopimelic acid is the diagnostic amino acid in cell walls of all *Actinomadura* and *Nocardiopsis* strains investigated (Lechevalier & Lechevalier, 1970). Tables 7 and 8 show the menaquinone, whole cell sugar composition and the fatty acid composition of all organisms tested.

The analysis of the menaquinones proved difficult. Using Li-Chrosorb RP 18 as a stationary phase, menaquinones of *A. madurae* DSM 43067 were found to be of the MK-9(H4)-, MK-9(H6)-, and MK-9(H8)-types, similar to those found in *Streptomyces* (Collins & Jones, 1981; Batrakov & Bergelson, 1978). However, using the silver-loaded ion exchanger as a stationary phase (Kroppenstedt, 1982*b*), the retention times were different. These differences in the separation behaviour of menaquinones of the same number of isoprenoid units and double bonds depends upon the position at which the saturation of double bonds occur. While menaquinones of the MK-9(H6)-type in *Streptomyces olivaceus* are saturated at positions II, III, and IX (Batrakov & Bergelson, 1978), that of *A. madurae* is saturated at positions II, III, and VIII (Aoki *et al.*, 1980; Yamada *et al.*, 1982). Our analysis of the menaquinones of the MK-9(H4)-, and MK-9(H8)-types in strains of *Actinomadura* and *Nocardiopsis* also show retention times different to those of the same types found in *S. olivaceus* (Batrakov & Bergelson, 1978), indicating differences in the position of saturation in these isomers too. The exact position of

Table 3. *Percentage DNA homologies among strains of the Actinomadura madurae rRNA-cluster and other Actinomadura strains*

Filter-bound DNA from strain:		[³⁵ S]DNA from strain:				
		DSM 43067	DSM 43122	DSM 43383	DSM 43384	DSM 43118
<i>Actinomadura madurae</i>	DSM 43067	100	44	29	28	30
<i>Actinomadura madurae</i>	DSM 43380	96	48	32	31	33
<i>Actinomadura madurae</i>	DSM 43122	46	100	42	44	45
<i>Actinomadura madurae</i>	DSM 43381	33	58	37	37	40
<i>Actinomadura pelletieri</i>	DSM 43383	26	32	100	96	100
<i>Actinomadura pelletieri</i>	DSM 43384	25	33	98	100	100
<i>Actinomadura pelletieri</i>	DSM 43118	—	32	—	98	100
<i>Actinomadura pelletieri</i>	DSM 43117	29	38	90	85	89
<i>Actinomadura pelletieri</i>	DSM 43382	39	75	31	31	33
<i>Actinomadura verrucosospora</i>	DSM 43358	32	35	—	31	—
<i>Actinomadura verrucosospora</i>	DSM 43550	35	39	—	30	32
<i>Actinomadura citrea</i>	DSM 43461	38	42	—	30	33
<i>Actinomadura malachitica</i>	DSM 43462	20	22	—	18	20
<i>Actinomadura kijaniata</i>	ATCC 31588	20	25	—	18	19
<i>Actinomadura pusilla</i>	DSM 43357	7	11	9	11	11
<i>Actinomadura roseoviolacea</i>	DSM 43144	7	7	8	—	9
<i>Actinomadura spadix</i>	DSM 43459	11	8	7	—	10
<i>Actinomadura roseola</i>	DSM 43551	8	—	8	—	9
<i>Actinomadura madurae</i>	DSM 43121	8	9	10	10	9
<i>Actinomadura madurae</i>	DSM 43123	11	12	11	11	12
<i>Nocardopsis dassonvillei</i>	DSM 43111	8	—	9	9	7
<i>Nocardopsis dassonvillei</i>	DSM 43235	7	10	9	—	8
<i>Streptosporangium roseum</i>	DSM 43021	8	—	7	—	8

—, Not determined.

Table 4. *Percentage DNA homologies among Actinomadura pusilla, Actinomadura roseoviolacea, Actinomadura libanotica, Actinomadura spadix and Actinomadura spiralis and other Actinomadura strains*

Filter-bound DNA from strain:		[³ H]DNA from strain:		[³⁵ S]DNA from strain:		
		DSM 43357	DSM 43144	DSM 43556	DSM 43459	DSM 43555
<i>Actinomadura pusilla</i>	DSM 43357	100	27	22	7	7
<i>Actinomadura roseoviolacea</i>	DSM 43144	31	100	26	9	10
<i>Actinomadura libanotica</i>	DSM 43556	25	31	85	9	11
<i>Actinomadura roseola</i>	DSM 43551	29	33	24	12	12
<i>Actinomadura roseola</i>	DSM 43552	29	29	25	12	14
<i>Actinomadura ferruginea</i>	DSM 43553	26	26	24	10	11
<i>Actinomadura spadix</i>	DSM 43459	11	9	9	100	10
<i>Actinomadura spiralis</i>	DSM 43555	10	12	8	10	100
<i>Actinomadura madurae</i>	DSM 43121	7	7	7	8	8
<i>Actinomadura madurae</i>	DSM 43123	10	10	7	5	5
<i>Actinomadura madurae</i>	DSM 43067	7	7	7	12	11
<i>Actinomadura madurae</i>	DSM 43381	9	11	8	13	13
<i>Actinomadura pelletieri</i>	DSM 43384	9	8	8	13	12
<i>Nocardopsis dassonvillei</i>	DSM 43235	7	6	10	9	10
<i>Streptosporangium roseum</i>	DSM 43021	13	13	15	9	8

Table 5. Percentage DNA homologies among strains of *Nocardiopsis dassonvillei* DSM 43235 and DSM 43377 and various *Actinomadura* strains

Filter-bound DNA from strain:		[³⁵ S]DNA from strain:	
		DSM 43235	DSM 43377
<i>Nocardiopsis dassonvillei</i>	DSM 43111	100	38
<i>Nocardiopsis dassonvillei</i>	DSM 43235	100	38
<i>Nocardiopsis dassonvillei</i>	DSM 43377	38	100
<i>Nocardiopsis dassonvillei</i>	DSM 43378	36	98
<i>Actinomadura madurae</i>	DSM 43067	8	11
<i>Actinomadura madurae</i>	DSM 43381	9	—
<i>Actinomadura pelletieri</i>	DSM 43384	9	—
<i>Actinomadura verrucosospora</i>	DSM 43358	11	—
<i>Actinomadura verrucosospora</i>	DSM 43550	11	—
<i>Actinomadura citrea</i>	DSM 43461	12	—
<i>Actinomadura malachitica</i>	DSM 43462	11	—
' <i>Actinomadura kijaniata</i> '	ATCC 31588	12	—
<i>Actinomadura pusilla</i>	DSM 43357	7	—
<i>Actinomadura roseoviolacea</i>	DSM 43144	10	—
<i>Actinomadura roseola</i>	DSM 43551	12	—
<i>Actinomadura roseola</i>	DSM 43552	12	—
<i>Actinomadura ferruginea</i>	DSM 43553	11	—
<i>Actinomadura libanotica</i>	DSM 43556	9	—
<i>Actinomadura spiralis</i>	DSM 43555	11	—
<i>Actinomadura spadix</i>	DSM 43459	10	11

—, Not determined.

Table 6. Percentage DNA homologies among various strains of *Nocardiopsis dassonvillei* and *Actinomadura* using the spectrophotometric reassociation method and comparison of homologies with those obtained by the DNA-filter method

DNA from strain:	DNA homology (%)	
	Spectrophotometric method	DNA-filter method
<i>Nocardiopsis dassonvillei</i> :		
DSM 43235* × DSM 43111	99	100
DSM 43111 × DSM 43378	35	—
DSM 43235* × DSM 43378	35	36
<i>Actinomadura roseola</i> :		
DSM 43551 × DSM 43552	100	—
<i>Actinomadura roseola</i> DSM 43551 ×		
<i>Actinomadura ferruginea</i> DSM 43553	26	—
<i>Actinomadura roseoviolacea</i> DSM 43144* ×		
<i>Actinomadura roseola</i> DSM 43552	29	33
<i>Actinomadura pusilla</i> DSM 43357 ×		
<i>Actinomadura roseoviolacea</i> DSM 43144	40	3
<i>Actinomadura verrucosospora</i> :		
DSM 43358 × DSM 43550	96	—

—, Not determined.

* Strains were labelled for the filter hybridization.

Table 7. *Menaquinone isoprenologue and whole cell sugar composition*

Strain		Menaquinone isoprenologue and degree of hydrogenation†											
		MK-9							MK-10				
		H ₀	H ₂	H ₄ *‡	H ₆ *§	H ₆	H ₈ *¶	H ₈ ††	H ₀	H ₂	H ₄	H ₆	H ₈
1. <i>Actinomadura madurae</i>	DSM 43067				75		12	11					
2. <i>Actinomadura madurae</i>	DSM 43380				74		10	8					
3. <i>Actinomadura madurae</i>	DSM 43381			8	54		11	10			11		
4. <i>Actinomadura madurae</i>	DSM 43122				30		64						
5. <i>Actinomadura pelletieri</i>	DSM 43382			11	75		6	8					
6. <i>Actinomadura pelletieri</i>	DSM 43384			14	80								
7. <i>Actinomadura pelletieri</i>	DSM 43117			20	74								
8. <i>Actinomadura pelletieri</i>	DSM 43118			20	44		21						
9. <i>Actinomadura pelletieri</i>	DSM 43383			7	65		9	16					
10. <i>Actinomadura verrucosospora</i>	DSM 43358			11	85								
11. <i>Actinomadura verrucosospora</i>	DSM 43550				65		16	18					
12. <i>Actinomadura malachitica</i>	DSM 43462			10	74		8	6					
13. <i>Actinomadura citrea</i>	DSM 43461				79		8						
14. <i>Actinomadura pusilla</i>	DSM 43357	15	20	60									
15. <i>Actinomadura roseoviolacea</i>	DSM 43144		18	75									
16. <i>Actinomadura libanotica</i>	DSM 43556		19	75									
17. <i>Actinomadura roseola</i>	DSM 43551		26	68									
18. <i>Actinomadura roseola</i>	DSM 43552		29	62									
19. <i>Actinomadura ferruginea</i>	DSM 43553	13	35	40									
20. <i>Actinomadura spadix</i>	DSM 43459		6	50			40						
21. <i>Actinomadura spiralis</i>	DSM 43555		15	80									
22. <i>Actinomadura madurae</i>	DSM 43121		10	82	8								
23. <i>Actinomadura madurae</i>	DSM 43123				ND								
24. <i>Nocardiopsis dassonvillei</i>	DSM 43111			9					6	7	39	27	
25. <i>Nocardiopsis dassonvillei</i>	DSM 43235									20	27	28	10
26. <i>Nocardiopsis dassonvillei</i>	DSM 43377			32	18						18	15	
27. <i>Nocardiopsis dassonvillei</i>	DSM 43378			33	17						24	20	

ND, Not determined.

(+), Trace only.

* Menaquinones found to be different from those of streptomycetes.

† Only those menaquinones are shown that are present in more than 5% of total menaquinones.

‡ Menaquinones show other retention times on both columns as MK-9(H₄) of *Streptomyces olivaceus* (Batrakov & Bergelson, 1978).§ Menaquinones show the same retention times on both columns as MK-9(II,III,VIII-H₆) of *Actinomadura madurae* (Yamada *et al.*, 1982).|| Menaquinones show the same retention times on both columns as MK-9(II,III,IX-H₆) of *Streptomyces olivaceus* (Batrakov & Bergelson, 1978).¶ Menaquinones show other retention times on both columns as MK-9(II,III,VII,IX-H₈) of *Streptomyces olivaceus* (Batrakov & Bergelson, 1978).†† Menaquinones show the same retention times on both columns as MK-9(II,III,VII,IX-H₈) of *Streptomyces olivaceus* (Batrakov & Bergelson, 1978).

saturation, however, can only be detected by mass spectroscopy, which has not been done in this study. The menaquinones found to be different from those of streptomycetes are marked with an asterisk in the text and in Table 7.

Members of the *A. madurae* rRNA-cluster show the following characteristics: menaquinones are predominantly of the MK-9(H₆)*-type, as characterized by Aoki *et al.* (1980) and Yamada *et al.* (1982). In some strains additional menaquinones of the MK-9(H₄)*-, MK-9(H₈)*- and MK-9(H₈)-types occur. Ribose, mannose, glucose, galactose and madurose are almost always present as whole cell sugars, though in some strains the latter sugar is present in very small amounts only. Arabinose and galactose are always missing. The fatty acid composition of all members of this cluster is uniform. The percentage of hexadecanoic acid (16:0) is high (11–50%), exceeding in most strains that of 14-methylpentadecanoic acid (i-16:0). The exceptions are *A. pelletieri* strains DSM 43118, DSM 43383 and DSM 43384 and *A. citrea* DSM 43461. Iso- and anteiso-

Table 7.—*continued*

	Whole cell wall sugars						
	Madurose	Xylose	Arabinose	Galactose	Glucose	Mannose	Ribose
1.	+	—	—	—	+	—	+
2.	+	+	+	+	+	+	+
3.	(+)	—	—	+	++	(+)	+
4.	+	—	—	—	++	—	+
5.	+	—	+	(+)	+	—	+
6.	(+)	—	—	(+)	++	(+)	+
7.	+	—	—	+	+	+	+
8.				ND			
9.	+	—	—	(+)	++	(+)	+
10.	(+)	—	—	(+)	++	(+)	+
11.				ND			
12.	+	—	—	+	++	(+)	+
13.	(+)	—	—	+	++	+	+
14.	(+)	—	—	(+)	++	+	+
15.	(+)	—	—	(+)	++	+	+
16.	(+)	—	—	+	++	(+)	+
17.	+	—	—	++	+	—	—
18.	(+)	—	—	+	+	—	+
19.	(+)	—	—	+	++	—	+
20.	+	—	—	—	+	+	(+)
21.	+	+	—	—	+	+	+
22.	+	—	—	++	++	—	+
23.	+	+	+	+	++	—	+
24.	—	—	—	—	+	—	+
25.	—	—	—	(+)	+	—	—
26.	—	—	—	—	+	+	+
27.	—	—	—	—	+	+	+

branched fatty acids with an odd number of carbon atoms (iso/anteiso, 15/17) are almost missing. Among the 10-methyl-branched fatty acids, 10-methyloctadecanoic acid (18-me, tuberculostearic acid) is predominant over 10-methylheptadecanoic acid, which occurs mostly in less than 10% of total fatty acids.

All members of the *A. pusilla*-cluster have a similar menaquinone composition with the MK-9(H4)*-type dominating. Menaquinone of the MK-9(H2)-type is present in all organisms. Ribose, glucose, galactose and madurose are present, though the latter sugar mostly in trace amounts. Mannose is missing in strains of *A. roseola* and *A. ferruginea*, while xylose and arabinose are missing in all strains. The most predominant feature of the other strains of this cluster is the high amount of 10-methylheptadecanoic acid (17-me) (17–33%). Hexadecanoic acid (16:0) (1–15%) is less common than in the *A. madurae*-rRNA cluster. *Actinomadura spadix*, *A. spiralis*, and the two *A. madurae* strains DSM 43121 and DSM 43123 are chemotaxonically diverse and none of them show a specific similarity to members of the *A. madurae*- and *A. pusilla*-clusters.

Strains of *Nocardiopsis* synthesize menaquinones with a long isoprenylic side chain [MK10(H₂,H₄,H₆)]. Strains DSM 43377 and DSM 43378 in addition have remarkably high amounts of menaquinones of the MK9(H₄*,H₆*)-types. Madurose is not present in whole cell hydrolysates, whereas ribose is present in all strains. The other sugars are not uniformly distributed. All strains have the same fatty acid composition, resembling in some aspects that of members of the *A. pusilla*-cluster. Hexadecanoic acid is present in small amounts only (1–2%), while 14-methylpentadecanoic acid (15–40%), 14-methylhexadecanoic acid (10–25%), and 10-methyloctadecanoic acid (11–18%) are predominant. This fatty acid pattern can easily be distinguished from the two patterns found in members of the two *Actinomadura*-clusters by its high amount of terminally branched fatty acids with an odd number of carbon atoms in the straight chain residues [iso- and anteiso C15 and C17 fatty acids (17–44%)].

Table 8. *Percentage fatty acid composition of the tested strains*

Abbreviations for fatty acids are illustrated by the following examples: straight-chain saturated: hexadecanoic acid (16:0), monounsaturated: octadecanoic acid (oleic acid, 18:1), iso-branched: 14-methylheptadecanoic acid (iso-16), anteiso-branched: 14-methylhexadecanoic acid (anteiso-17), 10-methyl-branched: 10-methyloctadecanoic acid (tuberculostearic acid, 18-me).

Strain		Straight chain					Branched chain					
		14:0	16:0	18:0	15:0	17:0	i-14	i-16	i-18	i-15	i-17	
1.	<i>Actinomadura madurae</i>	DSM 43067	1	30	4	1	3		4			
2.	<i>Actinomadura madurae</i>	DSM 43380	2	38	7	1	3		7	1		
3.	<i>Actinomadura madurae</i>	DSM 43381	3	39	7	1	2		7			
4.	<i>Actinomadura madurae</i>	DSM 43122		17	1	6	7		14	2		
5.	<i>Actinomadura pelletieri</i>	DSM 43382	2	28	3	7	16		5			
6.	<i>Actinomadura pelletieri</i>	DSM 43384		31	5		3		35	6		
7.	<i>Actinomadura pelletieri</i>	DSM 43117		33	1	3	11		16	2		
8.	<i>Actinomadura pelletieri</i>	DSM 43118		24		2	4		45	4		
9.	<i>Actinomadura pelletieri</i>	DSM 43383		14	1	1	2		60	3		
10.	<i>Actinomadura verrucosospora</i>	DSM 43358	4	50	5		1		3			
11.	<i>Actinomadura verrucosospora</i>	DSM 43550	2	39	3	1	1		5			
12.	<i>Actinomadura malachitica</i>	DSM 43462	2	34	4	1	1		8			
13.	<i>Actinomadura citrea</i>	DSM 43461	1	11	4	2	2	1	40	2		
14.	<i>Actinomadura pusilla</i>	DSM 43357	2	15	1	11	13	3	20		5	1
15.	<i>Actinomadura roseoviolacea</i>	DSM 43144		5	1	6	5	3	41		4	
16.	<i>Actinomadura libanotica</i>	DSM 43556	2	4	1	5	2	2	27		4	1
17.	<i>Actinomadura roseola</i>	DSM 43551	2	2	1	5	1	5	26		5	1
18.	<i>Actinomadura roseola</i>	DSM 43552	2	4		11	11	3	21		6	
19.	<i>Actinomadura ferruginea</i>	DSM 43553	1	1	1	3	3	1	36	3		
20.	<i>Actinomadura spadix</i>	DSM 43459		2		1	1	1	50	2		2
21.	<i>Actinomadura spiralis</i>	DSM 43555	1	17	7	2	2		7		6	1
22.	<i>Actinomadura madurae</i>	DSM 43121	1	38	8	1	3		5	1		
23.	<i>Actinomadura madurae</i>	DSM 43123		12	5	2	5		27	9		
24.	<i>Nocardiopsis dassonvillei</i>	DSM 43111	1	1	7		2	1	15	3	9	4
25.	<i>Nocardiopsis dassonvillei</i>	DSM 43235		2	19		2	7	26	4		
26.	<i>Nocardiopsis dassonvillei</i>	DSM 43377		1	6	1	3		6		2	4
27.	<i>Nocardiopsis dassonvillei</i>	DSM 43378		1	3			2	40	10		3

DISCUSSION

The DNA G + C content of *Actinomadura* strains is similar to that reported by Bradley *et al.* (1973) and intermediate between the values of 74 mol% reported by Lechevalier *et al.* (1971) and 65 mol% reported by Mordarski *et al.* (1977) and Bradley & Mordarski (1976) for *A. madurae*. The G + C values of *Nocardiopsis dassonvillei* show a broader range, separating the strains into two groups.

Our data on the fatty acid composition of *Actinomadura* and *Nocardiopsis* strains is only partially in accord with those published by Agre *et al.* (1975), although *A. madurae* and *A. pusilla* were also clearly separated from strains of *Nocardiopsis*, *A. pusilla* and *A. roseoviolacea* in the latter study. Qualitative and quantitative differences in the fatty acid composition are obvious. To give some examples, palmitoleic acid, which in our study is present only in small amounts in strains of *A. madurae* and *A. pusilla* (0–6%), is a dominant fatty acid (13–56%) in strains of these two species in the study of Agre *et al.* (1975). While we found high amounts of 10-methyl branched fatty acids in strains of *N. dassonvillei*, *A. pusilla* and *A. roseoviolacea*, this component was not listed among the fatty acids in these strains by Agre *et al.* (1975); oleic acid, on the other hand, was reported to be present in *A. pusilla* and *A. roseoviolacea*, while we were unable to detect this component in these strains. Some of the contradicting results of the two studies may be explained by differences in the age of the organisms investigated, which may influence their fatty acid composition (Kroppenstedt, 1977). It is now established that a good resolution of iso- and anteiso-branched fatty acids require at least a 6 m polar column (Kaneda, 1977) and that the very complicated mixtures of fatty acids occurring in some genera of the *Actinomycetales* (Ballio

Table 8.—*continued*

	Branched chain		Unsaturated fatty acids							10-Methyl-branched chain		
	ai-15	ai-17	i-16:1	i-17:1	i-18:1	15:1	16:1	17:1	18:1	16	17	18
1.				2			2		24	3	3	23
2.							2	1	8		2	28
3.									12	1		28
4.					1		6	9	6		14	17
5.							2	6	14		4	13
6.									12		2	6
7.							2	4	18		2	8
8.					3		2	2	8			6
9.					2			1	10	1	1	4
10.							2		15			20
11.							4		13	1		31
12.							3		3	3	2	39
13.					3		2	2	5		8	16
14.	1									7	18	3
15.						5				5	23	2
16.	20		2				2	8		2	17	1
17.	3		7				2	2		2	33	3
18.			2					10			30	
19.	2		1		9			14	3		17	2
20.	2	15	10		4			1	1	3	5	
21.	2	3	4				2	6	34		6	
22.							2	1	13	2	1	24
23.				8				3	9	2	3	16
24.	6	25		4				2	2		3	15
25.	7	12						2	6			11
26.	4	15			1			2	8		3	18
27.	4	10			1			1	4	1	4	16

& Barcellona, 1968; Ballio *et al.*, 1968) can only be resolved and identified by gas chromatography using capillary columns with different separation behaviours (Kroppenstedt, 1982a). In the light of these results it might be that Agre *et al.* (1977) misinterpreted 10-methylheptadecanoic acid for oleic acid because both fatty acids show nearly the same retention time on a non-polar packed column.

The analysis of 13 species of *Actinomadura* by nucleic acid reassociation and rRNA cistron similarity studies points towards a phylogenetic heterogeneity of this genus. Using the 23S rRNA of *A. madurae* DSM 43067 as a phylogenetic marker, six species of *Actinomadura* are clearly separated from the other *Actinomadura* strains investigated. Within the *Actinomadura madurae* rRNA-cluster, *A. madurae*, *A. pelletieri*, *A. verrucosospora* and *A. citrea* form a centre of moderately related species, as seen from the DNA homology values above 30%, while '*A. kijaniata*' and *A. malachitica* are more peripherally related. The specific relationship between *A. madurae* and *A. pelletieri* has been noticed in several earlier investigations. Strains of both species produce a prodigiosin-like pigment (Gerber & Lechevalier, 1964, 1965) and they resembled each other in the composition of fatty acids (Agre *et al.*, 1975), phospholipids (Lechevalier *et al.*, 1977), polar lipids (Minnikin *et al.*, 1977), and menaquinones (Yamada *et al.*, 1977; Collins *et al.*, 1977; Collins & Jones, 1981). On the other hand, Goodfellow & Pirouz (1982) in a numerical phenetic analysis found strains of *A. madurae* and *A. pelletieri* to cluster separately from each other, thus bringing into question the classification of these two species in one genus. In the same investigation *A. citrea* and *A. verrucosospora* clustered with *A. madurae*, which is consistent with our finding concerning the genetic and phenotypic relationship of these

organisms. In contrast to our results, *A. spadix*, *A. pusilla* and *A. roseoviolacea* were also found to be members of the phenetically defined *A. madurae*-cluster. As mentioned by Goodfellow & Pirouz (1982), the relationships determined by numerical phenetic analysis may be distorted by several factors involved in the tests and statistics used.

Actinomadura strains which do not belong to the *A. madurae* rRNA-cluster constitute a collection of moderately to slightly related organisms. Among these, *A. pusilla*, *A. roseoviolacea*, *A. roseola*, *A. libanotica* and *A. ferruginea* are geno- and phenotypically related. This has been shown before for *A. pusilla* and *A. roseoviolacea* by similarities in the fatty acid composition (Agre *et al.*, 1975), which differs from that of *A. madurae* and relatives on the one hand, and from that of *N. dassonvillei* on the other. *Actinomadura helvata*, not included in these studies, is phenotypically very similar to members of the *A. pusilla*-cluster (R. M. Kroppenstedt, unpublished) suggesting that it is probably a member of this group.

Nocardiopsis, originally excluded from *Actinomadura* on the basis of morphological and biochemical grounds (Meyer, 1976), bears little DNA and rRNA similarity to members of *Actinomadura*. Despite a superficial resemblance in the peptidoglycan type and the DNA G + C content, the low degree of genetic relatedness of these two taxa is also reflected in differences in the composition of menaquinones, polar lipids, fatty acids, whole cell sugars and in spore-chain morphology. Our data indicate that the species *N. dassonvillei* contains two groups of remotely related strains, separated from each other not only by DNA homologies of about 30% but also in the menaquinone composition and the DNA G + C content.

We believe the genus *Actinomadura* should be restricted to members of the *A. madurae* rRNA-cluster. The $\Delta T_{m(e)}$ values of the DNA-rRNA duplexes found among members of this cluster cover a narrow range of 5.4 °C. Similar values have been found among *Streptomyces*, *Streptosporangium* and relatives, and *Actinoplanes* and relatives (Stackebrandt *et al.*, 1981), *Microbacterium* and *Curtobacterium* (Döpfer *et al.*, 1982), *Staphylococcus* (Kilpper-Bälz & Schleifer, 1981) and various Gram-negative genera (De Smedt & De Ley, 1977; Gillis & De Ley, 1980). All other *Actinomadura* species show $\Delta T_{m(e)}$ values which are on average 6.5 °C lower than the average $\Delta T_{m(e)}$ values of members of the *A. madurae* rRNA-cluster. These values are in the same order of magnitude as those found to separate members of different genera, e.g. *Streptomyces* from *Streptosporangium* and *Actinoplanes* (Stackebrandt *et al.*, 1981), *Curtobacterium* and *Microbacterium* from *Cellulomonas* (Döpfer *et al.*, 1982), *Staphylococcus* from *Bacillus* (Kilpper-Bälz & Schleifer, 1981) and *Actinomadura* (*A. madurae* rRNA-cluster) from *Streptomyces* (this study).

The taxonomic rank of the remaining *Actinomadura* species is not as clearcut. Whether or not the genotypically and phenotypically coherent cluster of *A. pusilla*, *A. roseoviolacea*, *A. roseola*, *A. libanotica* and *A. ferruginea* together constitute an individual genus cannot be deduced from the results presented here. However, the analysis of oligonucleotides of the 16S rRNA reveal that this group of organisms and *Nocardiopsis* are not phylogenetically closely related either to each other or to any genus of *Actinomycetales* so far investigated by this method (unpublished results).

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