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Identification of some industrially important *Actinoplanes* species

P.F. Long

Department of Biomolecular Screening, Glaxo Research and Development Ltd, Greenford Road, Greenford, Middx UB6 0HE, UK

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SUMMARY

Three actinomycetes designated A294, A385 and A448 all produced novel inhibitors of an enzyme involved in the regulation of mammalian cholesterol metabolism. The organisms were identified as members of the genus *Actinoplanes* using morphological and chemotaxonomic characters. Numerical phenetic data obtained for clusters defined at the 77.5% S_{sm} similarity level in the taxonomic study of *Actinoplanes* and related taxa by Goodfellow et al. [6], was used to construct a probabilistic identification matrix for the genus *Actinoplanes*. This was in an attempt to characterise the producing strains to species level. Identification scores for the three organisms, together with two type strains, were determined using the MATIDEN program of Sneath [36]. Strains A294 and A385 did not identify correctly with any cluster. Strain A448 was identified as *Actinoplanes derwentensis*. The two type strains identified within acceptable statistical limits to their correct clusters. Due to the absence of other taxonomic criteria with which to speciate members of the genus *Actinoplanes* we recommend that the two unidentified producing strains should be considered novel until such time as further techniques become available.

INTRODUCTION

Genera currently classified in volume 4 of Bergey's Manual to the family *Actinoplanaceae* [1] are *Actinoplanes*, *Ampullariella*, *Amorphosporangium*, *Dactylosporangium* and *Pilimelia* [49]. The family can be characterised on the basis that species produce motile spores within spore vesicles (sporangia), which are formed at the distal ends of aerial hyphae [3]. This morphological classification has been open to question [2,13] given that molecular systematic [45], chemical [16], and numerical phenetic data [31] suggest that *Ampullariellae* and *Amorphosporangium* be included within an amended description of the genus *Actinoplanes* [28,43]. Actinoplanetes can be classified chemotaxonomically, characteristic of which is an A1δ cell wall peptidoglycan [32,33]. The composition of the murein moiety of the peptidoglycan includes the *meso* and/or dihydroxy form of diaminopimelic acid and glycine (Wall chemotype II) [22]. The muramic acid moiety of the cell wall peptidoglycan is N-glycolated [14,48]. Whole cell hydrolysates contain arabinose and xylose [5], while phosphatidylethanolamine is present in the polar lipid pattern (phospholipid pattern II) [23]. The genus *Micromonospora* [27] shares the same chemotaxonomic profile as the actinoplanetes [14,22]; however, micromonosporae form single non-motile spores on substrate mycelium rather than motile spores within sporangia [3]. Molecular systematic data suggest the inclusion of the genus *Micromonospora* within the family *Actinoplanaceae* [44,45]. The numerical and chemical classification of Goodfellow et al. [6] was designed to investigate and clarify the taxonomy of the family *Actinoplanaceae* and related taxa. This study, which included a comparison of 75 phenetic characters, showed that the actinoplanetes encompassed organisms within the amended description of the genus *Actinoplanes*, and the genera *Dactylosporangium*, *Micromonospora* and *Pilmelia*. Given that the genus *Micromonospora* takes taxonomic preference over the other three genera within the family *Actinoplanaceae*, then *Actinoplanaceae* should be considered a synonym for the family *Micromonosporaceae* [15].

A logical progression from the data on the 74 phenetic characters determined in the numerical study of Goodfellow et al. [6] is the construction of a probability matrix for the identification of *Actinoplanes* species [11]. The theory of the probabilistic identification of bacteria has been reviewed [20,21,34,35,42,51]. Probabilistic schemes are composed of percentage positive scores of strains within each cluster using the minimum number of diagnostic characters that can discriminate between the clusters. Hence, no one unit character is essential for membership of a particular cluster [42]. This polythetic concept is important because the response of bacteria in phenetic tests can be variable. The data from the numerical classification of *Actinoplanes* [6] was used to construct a polythetic identification system for this genus. Here we report on the use of such a system for the probabilistic identification of three natural isolates that were found to produce novel inhibitors of an enzyme involved in the regulation of mammalian cholesterol metabolism.

MATERIALS AND METHODS

Isolation, maintenance and generic identification of isolates

The source material and isolation techniques used to culture the three strains designated A294, A385 and A448

Correspondence to: P.F. Long, Department of Biomolecular Screening, Glaxo Research and Development Ltd, Greenford Rd, Greenford, Middx UB6 0HE, UK.

are shown in Table 1. All strains, including the two type strains, *Actinoplanes philippinensis* DSM 43019 and *Actinoplanes utahensis* DSM 43147, were maintained on modified Bennett's agar [2] and stored as spore/mycelial suspensions in 15% (v/v) glycerol plus 0.001% (v/v) Tween 80 solution at −20 °C [50] until required. Morphology of the isolates was determined microscopically using a 40× long working-distance objective after the strains had been grown for 14 days at 28 °C on M3 agar [30] supplemented with 0.1% (w/v) fructose. Several plates of each isolate were also flooded with sterile distilled water. The water was examined microscopically after 30 min using a 100× objective. Some chemotaxonomic markers diagnostic for the genus *Actinoplanes* were determined using the methods outlined by Long et al. [25].

Selection of most diagnostic characters in the data set

The percentage of strains in all of the 15 clusters defined by Goodfellow et al. [6] which gave positive reactions for each of the 75 unit characters tested were calculated. Percentages of 0 were changed to 1% and 100 to 99% [21]. These percentage positive values were used to run the CHARSEP program of Sneath [36] to determine the minimum number of characters necessary to discriminate between the clusters. Characters with a VSP separation index [41] of >25% were selected [36].

Practical determination of characters to be used in the identification matrix

Listed below are the practical details for determining the 53 characters selected using CHARSEP. Further details can be found elsewhere [6]. Glycerol/Tween suspensions were used to inoculate all the tests except for carbon utilization. For the carbon utilization tests, inocula were prepared in sterile distilled water from cultures grown for 14 days on modified Bennett's agar. All tests were incubated for 24 days at 25 °C unless otherwise stated. Carbon utilization, antibiotic resistance and tolerance tests were all performed in repli dishes (Sterilin, Teddington, Middlesex, UK). All remaining tests were carried out in 90-mm petri dishes except for arbutin degradation, urease activity, nitrate reduction and hydrogen sulphide production which were performed in tubes. Each test was replicated five times.

TABLE 1

Description of actinomycete isolation

Organism	Soil, origin and description	Isolation technique	Isolation medium	Incubation time (days)
A294	Crete, beach	Chemotaxis	Oatmeal soil extract agar	23
A385	USA, Semi-desert	Dilution plating	Soil agar	13
A448	Greece, Sandy heathland	Chemotaxis	Oatmeal soil extract agar	28

All organisms were isolated at 28 °C. The chemotaxis technique used was Palleroni's KCl procedure (see [49] for details).

Diffusible pigment production

The production of diffusible exopigments was recorded after 14 days growth on *Micromonospora* maintenance agar [24] and tryptic soy agar (Bacto) supplemented with RNA [4].

Antimicrobial activity [53]

The *Actinoplanes* test strains were spot inoculated onto the surface of modified Bennett's agar in glass petri dishes. The plates were incubated for 24 h before being inverted for 40 min over 1.5 ml chloroform in a fume hood. The killed colonies were then overlayed with modified Bennett's agar containing agar at 0.6% (w/v). The overlayed agar had been previously seeded with one of two test organisms, *Staphylococcus aureus* NCTC 8532 and *Streptomyces murinus* ISP 5091. Zones of inhibition were recorded as positive reactions after 24 h. The incubation temperature for *S. aureus* was 37 °C and for *S. murinus* it was 25 °C.

Biochemical tests

Arbutin hydrolysis [53] was determined after 21 days growth in the basal medium of Kutzner [18] supplemented with 1.0 g L^{-1} arbutin. Brown-black pigmentation of the medium indicated a positive reaction, however, comparison with controls (basal medium without arbutin) was necessary to avoid confusion with melanin production. Nitrate reduction [8] was determined after 14 days by the addition of 0.2 ml of Griess Ilosvay reagents I and II to tubes of nutrient broth (Oxoid, Basingstoke, UK) that contained 2.0 g L^{-1} KNO_3 and 6.0 g L^{-1} agar (pH 7.0). Development of a red colour after 30 min indicated a positive reaction. The presence of nitrate was confirmed in tubes with a negative reaction by formation of a red colour after addition of a trace amount of zinc dust. Blackening of sterile lead acetate paper strips placed into the mouth of the tubes used for the nitrate reduction test indicated the production of hydrogen sulphide after 14 days [17]. Urease activity was detected by the addition of 10 ml 15% (w/v) filter-sterilised urea to the basal medium of Gordon [7] supplemented with 0.6% (w/v) agar and 0.001% (w/v) phenol red. Degradation of urea was scored positive by reddening of the medium.

Degradation tests

Chitin (0.5% w/v), elastin (0.3% w/v) and tyrosine (0.4% w/v) degradation were scored as positive for clearing of the insoluble compounds around the area of growth of the test strains using the basal medium of Gordon [7]. Pectin degradation was determined using the medium of Hankin et al. [10]. Spot inocula were grown for 10 days and then the plates were flooded with warm 1% (w/v) hexadecyltrimethylammonium bromide for 1 h. Hydrolysis zones were observed as clear areas around the spot inocula when excess reagent was decanted.

Carbon utilization

The ability of the test strains to grow on 15 different carbon sources (Table 2) was assessed using the basal medium of Stevenson [46]. The negative control was the basal medium alone, while the positive control was the basal medium plus 1% (w/v) glucose. A positive result was recorded when growth was greater than the negative control.

Tolerance tests

The ability to grow at 37 °C after 2 weeks on modified Bennet's agar and on modified Bennet's agar buffered to pH 8.0 after 6 weeks was assessed. Visible growth was scored positive. Modified Bennett's agar was used as the basal medium to determine growth in the presence of five chemical inhibitors (added to the basal medium before autoclaving) at one or more concentrations (Table 2). Absence of growth, or growth less than that on modified Bennett's agar alone, was recorded negative.

Antibiotic resistance

The ability to grow on modified Bennett's agar supplemented with nine antibiotics (Table 2) at one or more concentration was determined after 7 days incubation. Growth was scored as positive. Filter-sterilized solutions of the antibiotics were added after the basal medium had been autoclaved.

Theoretical evaluation of the identification matrix

The identification matrix was first assessed by checking the integrity of the characters selected from the VSP index using the DIACHAR program of Sneath [38]. Calculating the best identification score achievable for the hypothetical median organism of each cluster was then used to assess the identification matrix. This was performed using the MOSTTYP program of Sneath [39]. The matrix was further assessed by determining the identification scores of the nine single-member clusters from the numerical phenetic study of Goodfellow et al. [6] using the MATIDEN program [37]. The MATIDEN program provided an identification for unknown strains expressed as a probability based on the data in the matrix (q clusters $\times$ m characters). The percentages that made up the matrix were transposed by MATIDEN into proportions (Pij) where i was the character within a particular cluster j. Each character (m) for an unknown (u) was then compared with each proportion to give the best probable identification. Three identification coefficients were used in MATIDEN. These were:

1) Willcox probability [51]. This is expressed as:

$$\frac{Luj}{\sum_{q} Luj}$$

This is the probability that an unknown (u) is identified within a particular cluster (j) divided by the sum of the probabilities against all clusters (q) in the matrix. The closer the score is to 1.0 then the better the identification of an unknown with a cluster in the matrix.

2) Taxonomic distance (d) which is expressed as:

$$\sqrt{[\Sigma\,(ui - PiJ)^2/m^1]}$$

This gives the distance of an unknown from the centroid of the cluster with which it is compared. Hence, low scores indicate closer relatedness.

3) The standard error of the taxonomic distance (c) which is expressed as:

$$dj + cs_{dj}$$

where dj is the mean distance of members of a particular cluster (j) from the centroid and S_{dj} is the standard deviation of the distances.

Practical evaluation of the identification matrix

The criteria adopted for successful identification were those of Williams et al. [53]. These were:

1) Willcox probability score of greater than 0.85 with low scores for taxonomic distance and standard error of taxonomic distance.
2) First cluster scores better than the next two alternatives.
3) The number of characters against should be zero or few.

Finally, the identification matrix was assessed by re-determining the character states for the two type strains and the three natural isolates. Once determined, the identification scores were calculated using MATIDEN.

RESULTS

Identification of the producing strains designated A294, A385 and A448 (Table 1), to genus level is summarised in Table 3. Macroscopically, all the strains produced an orange, non-fragmenting substrate mycelium and brown aerial hyphae. The colour of the aerial and substrate mycelium of strain A294 blackened over the 14-day incubation period. Microscopic examination revealed the presence of sporangia at the distal end of the hyphae in all of the strains. The sporangia were cylindrical in shape in both A385 and A448 strains, but were irregular, or bottle-shaped in strain A294. Motile spores were released from the sporangia of all strains after the addition of water. The *meso*-isomer of 2,6-diaminopimelic acid (A_2pm) was detected in whole cell acid hydrolysates of each of the strains. Strain A385 also contained the hydroxylated form of A_2pm, while the LL-isomer was detected in addition to *meso*-A_2pm in strain A294. All of the producing strains contained the whole cell sugars galactose, xylose, glucose, rhamnose and ribose. Strains A385 and A448 also contained arabinose, which was absent in A294. Mannose was detected in strain A294. Phospholipids containing phosphotidylethanolamine were detected in all of the strains.

The five separation indices that formed the CHARSEP program of Sneath [36] indicated that some of the 74 characters used to define the 15 clusters in the numerical phenetic study of Goodfellow et al. [6] were of little

TABLE 2

Percentage positive matrix for the *Actinoplanes* clusters defined by Goodfellow et al. [6]

	Cluster strain no.	1	3	4	7	10	14	LA 178	LA 172	LA 179	LA 173	LA 182	LA 183	LA 174	LA 186	LA 177
	No. of strains	6	13	5	14	6	2	1	1	1	1	1	1	1	1	1
Diffusible pigment production on:																
Micromonospora maintenance agar		1	1	1	21.4	1	50	1	99	1	99	99	99	99	99	99
RNA agar		16.6	1	1	92.8	1	1	1	99	1	99	99	99	99	1	99
Antimicrobial activity against:																
Staphyloccus aureus NCTC 8532		66.6	46.1	99	28.6	99	1	1	99	1	1	1	1	1	1	1
Streptomyces murinus ISP 5091		1	15.4	1	14.3	1	50	1	99	1	1	1	1	99	99	1
Biochemical tests:																
Arbutin hydrolysis		99	99	99	99	5	1	1	99	99	99	99	99	99	99	99
H_2S production		50	46.1	1	64.3	1	99	99	1	1	1	1	99	1	1	99
Nitrate reduction		1	38.5	60	99	33.3	99	1	99	99	1	99	99	99	99	99
Urease production		1	15.4	1	50	1	99	1	1	99	99	1	1	99	1	99
Degradation of:																
Chitin		33.3	84.6	60	78.6	33.3	99	1	1	99	99	99	99	99	99	99
Elastin		99	99	40	85.7	1	99	1	99	1	1	99	99	99	1	99
Pectin		66.6	61.5	1	85.7	83.3	99	99	1	1	99	1	99	99	1	99
Tyrosine		50	38.5	40	85.7	33.3	99	1	99	1	99	99	99	99	1	1
Growth on sole carbon source at 1% (w/v):																
D-arabinose		33.3	61.5	40	99	1	1	99	1	1	1	1	1	1	1	1
meso-inositol		1	99	80	14.3	66.6	99	99	99	1	99	99	1	1	1	1
D-lactose		99	99	99	99	33.3	99	99	99	1	99	99	99	99	1	1
D-mannitol		99	99	99	99	50	99	99	99	99	99	1	1	99	1	99
D-melezitose		50	69.2	99	64.3	50	99	99	99	1	99	1	1	1	99	1
methyl-β-D-glucoside		99	99	99	99	1	99	99	99	99	99	1	99	99	1	99
Raffinose		99	61.5	99	28.6	33.3	1	99	1	1	1	1	1	1	1	1
L-rhamnose		99	61.5	99	28.6	33.3	99	99	99	99	99	99	1	99	99	99
D-ribose		1	1	40	57.1	1	99	99	1	99	99	1	99	1	99	99
D-sorbitol		50	84.6	99	99	16.6	99	99	1	99	99	1	99	1	99	99
At 0.1% (w/v)																
m-hydroxybenzoic acid		1	1	60	1	16.6	50	99	1	1	1	1	1	1	99	1
p-hydroxybenzoic acid		1	15.4	99	99	1	1	1	1	1	1	1	1	99	99	1
Sodium acetate		50	69.2	40	78.6	16.6	99	1	1	1	1	1	1	99	1	1
Sodium fumarate		50	99	1	85.7	1	99	1	1	1	1	1	1	99	1	1
Sodium succinate		66.6	76.9	1	85.7	1	99	1	1	1	1	1	1	1	1	1

TABLE 2

Continued

	Cluster strain no.	1	3	4	7	10	14	LA 178	LA 172	LA 179	LA 173	LA 182	LA 183	LA 174	LA 186	LA 177
	No. of strains	6	13	5	14	6	2	1	1	1	1	1	1	1	1	1
Growth at:																
37 °C		1	1	1	1	1	50	1	99	99	99	99	99	99	1	99
pH 6.0		99	38.5	40	92.8	99	99	99	99	99	99	1	99	99	99	99
Growth in the presence of (%, w/v):																
Brilliant green	(0.001)	1	1	1	1	1	1	1	1	99	1	1	99	99	1	1
Lysozyme	(0.005)	66.6	84.6	99	85.7	33.3	99	99	99	1	1	99	99	99	99	99
	(0.01)	50.0	76.9	99	71.4	1	99	99	99	1	1	99	1	99	1	99
Potassium tellurite	(0.001)	99	92.3	99	99	99	99	99	99	99	1	99	1	99	99	99
	(0.005)	1	92.3	99	35.7	1	99	99	99	99	1	99	1	99	1	99
Sodium azide	(0.0001)	83.3	76.9	40	71.4	66.6	99	99	99	99	99	99	99	99	99	99
	(0.001)	50	7.7	60	7.1	66.6	99	1	99	1	99	99	1	99	1	1
Sodium chloride	(1.0)	99	69.2	99	92.8	99	99	1	99	99	99	99	1	99	1	1
Antibiotic resistance (μg ml^{-1})																
Ampicillin	(0.5)	99	99	40	99	99	99	99	99	99	1	99	99	99	99	99
	(2.0)	99	1	1	85.7	33.3	99	99	99	99	1	1	99	99	99	99
	(8.0)	1	1	1	1	1	1	99	1	99	1	1	1	99	1	1
Cephaloridine hydrochloride	(1.0)	50	1	1	35.7	1	99	99	99	99	1	1	99	99	1	99
	(2.0)	33.3	1	1	14.3	1	1	99	1	99	1	1	1	1	99	1
Geotamycin sulphate	(0.5)	16.6	30.7	20	92.8	1	50	99	99	99	99	99	99	99	99	99
	(1.0)	1	1	99	7.1	1	50	1	99	1	1	99	1	99	1	1
Linomycin hydrochloride	(2.0)	83.3	84.6	40	85.7	66.6	99	99	99	99	99	99	99	99	99	99
	(8.0)	33.3	38.5	1	42.8	16.6	99	99	99	1	99	1	99	99	1	1
Methacycline hydrochloride	(2.0)	1	1	1	50	1	1	1	1	1	1	1	1	99	1	1
Neomycin sulphate	(0.5)	1	15.4	40	14.3	1	99	99	99	1	1	99	1	99	99	99
	(2.0)	1	1	1	1	1	1	1	99	1	1	99	1	99	1	1
Rifampicin	(0.25)	99	69.2	99	78.6	99	99	99	1	1	1	99	99	99	99	99
	(2.0)	1	1	1	7.1	1	1	1	1	1	1	1	99	99	99	1
Tobramycin sulphate	(0.5)	66.6	38.5	99	14.3	1	1	1	99	1	1	99	99	99	1	99
	(1.5)	16.6	7.7	40	1	1	1	1	99	1	1	99	99	99	99	1

Key for Table: Cluster strain number 1 = *A. consettensis*; 3 = *A. derwentensis*; 4 = *A. durhamensis*; 7 = *A. humidis*; 10 = *A. palleronii*; 14 = *A. phillippinensis*; LA178 = *A. auranticolor*; LA172 = *A. caeruleus*; LA179 = *A. campanulatus*; LA173 = *A. italicus*; LA178 = *A. digitatus*; LA183 = *A. lobatus*; LA174 = *A. missouriensis*; LA186 = *A. regularis*; LA177 = *A. utahensis*.

TABLE 3

Generic identification of the actinomycetes

Character	Strain		
	A294	A385	A448
Morphology:			
Sporangium formation	+	+	+
Sporangium shape	Irregular/bottle	Cylindrical	Cylindrical
Spore motility	+	+	+
Colour of aerial hyphae	Brown/black	Brown	Brown
Colour of substrate mycelium	Orange/black	Orange	Orange
Exopigment production	+(Black)	—	—
Chemotaxonomy:			
A_2pm isomer[1]	LL-/*meso*-	*meso*-/OH	*meso*-
Whole cell sugars	Galactose	Arabinose	Arabinose
	Xylose	Galactose	Galactose
	Glucose	Xylose	Xylose
	Rhamnose	Glucose	Glucose
	Ribose	Rhamnose	Rhamnose
	Mannose	Ribose	Ribose
Phospholipids	PII[2]	PII	PII

[1] A_2pm = 2,6-Diaminopimelic acid.
[2] See [23].

diagnostic value (Table 4). Characters with VSP index values of >25% [36] were selected to form the basis of a reduced data matrix for the probabilistic identification of *Actinoplanes* species. None of the tests with a VSP index of >25% were excluded from the matrix. The final identification matrix consisted of 15 clusters × 53 characters (Table 2).

The integrity of the character selection made using the VSP index was assessed using DIACHAR (Table 5). The sum of diagnostic scores for all 54 tests indicated that each cluster was well defined with a range of scores between 18.78 and 30.64. The best possible identification scores within each cluster was predicted using the MOSTTYP program (Table 6). The hypothetical mean organism for each cluster gave excellent results with Willcox probabilities of 1.000 for each cluster, low taxonomic distances (between 0.190 and 0.242) and negative values for the standard error of taxonomic distance. Identification scores were then calculated using the MATIDEN program for single member clusters using test data from the taxonomic study of Goodfellow et al. [6]. The identification scores are shown in Table 7. Again, these scores were excellent with Willcox probabilities of 1.000, scores for taxonomic distance between 0.013 and 0.45, and negative values for standard error of taxonomic distance.

Redetermination of the character states for the two cluster representatives, *A. philippinensis* DSM43019 and *A. utahensis* DSM 43147, indicated that both strains could be identified correctly using this probabilistic identification matrix (Table 8). With the exception of the score for standard error of taxonomic distance of *A. utahensis*, the remaining identification scores for both strains were within the criteria adopted for successful identification. Table 8 also shows the identification scores for the three unknown strains. Only one strain, A448, could be successfully identified to a cluster in the matrix within the criteria adopted. A448 was identified as *A. derwentensis*. Neither of the remaining two unknown isolates could be identified within acceptable limits from the identification scores.

DISCUSSION

The identification of actinomycetes which produce biologically active secondary metabolites is important for many reasons, not least to ensure patent security. The three actinomycetes used in this study (Table 1) that produced novel inhibitors of an enzyme involved in the regulation of mammalian cholesterol metabolism were identified as members of the genus *Actinoplanes* using morphological criteria and a selection of chemotaxonomic markers (Table 3). All three strains shared the same basic morphology, with sporangia formed at the distal ends of aerial hyphae. Although the shape of the sporangia were not the same for all three strains, the sporangia of each released motile spores when wet. The production of motile spores from aerial sporangia is a characteristic of the genus *Actinoplanes* [49]. Of particular interest was the black exopigment produced by strain A294 when grown to maturity on M3 agar + 0.1% (w/v) fructose. This colouration has not been reported in the literature [29].

The diaminoacid found in the cell wall peptidoglycan of *Actinoplanes* species is the *meso*-isomer and/or the hydroxylated form of 2,6-diaminopimelic acid (*meso*-A_2pm/OH-A_2pm). This is referred to as wall chemotype II [22,47]. The *meso*-isomer of A_2pm and OH-A_2pm were found in the whole cell acid hydrosylate of strain A385, while only *meso*-A_2pm was found in strain A448. Strain A294 was found to contain a mixture of the LL- and *meso*-isomers of A_2pm. In this respect strain A294 can be considered an atypical member of the genus *Actinoplanes* [29]. Also atypical was the whole cell sugar pattern for strain A294. Unlike the characteristic presence of arabinose and xylose in the whole cell hydrosylates of *Actinoplanes* species (Sugar Pattern D; [5,23]), strain A294 only contained xylose as a characteristic sugar. Both strains A385 and A448 showed a whole cell sugar pattern D. The polar lipid extracts of all three strains contained phosphatidylethanolamine. This phospholipid (Pattern II, [23]) is characteristic of the genus *Actinoplanes*.

Other chemotaxonomic tests could have been performed to confirm the generic status of the inhibitors. Such tests included menaquinone type and fatty acid pattern. These tests were not available in our laboratory at the time of this study. Another alternative to determine whether the three strains were members of the genus *Actinoplanes* and novel or not would have been to carry out molecular studies on the 16S rRNA gene. However, although a molecular

TABLE 4

Results showing the separation values of characters achieved using the CHARSEP program

Character	No of clusters the character is predominantly:-		Separation indices				
			Gyllenberg [9]				
	Negative	Positive	VSP%	CSP	Σc(i)	ΣR(i)	Niemela et al. [26] Index
Formation of Sporangia	2	13	14.40	0.39	14.31	372.06	4.65
Growth on:							
Micromonospora agar	8	7	85.18	0.85	14.15	792.40	8.77
RNA agar	8	7	90.55	0.91	14.63	819.28	8.77
Antimicrobial activity against:							
Aspergillus niger LIV 131	13	2	13.64	0.38	14.53	377.78	4.65
Bacillus subtilis NCIB 3610	13	2	22.73	0.46	13.17	342.42	4.65
Staphylococcus aureus NTCC 8532	11	4	52.46	0.66	13.80	607.20	7.22
Streptomyces murinus ISP 5091	12	3	45.70	0.62	14.09	507.24	6.12
Hydrolysis of arbutin	3	12	38.76	0.61	14.81	533.16	6.12
H_2S production	10	5	66.86	0.73	13.56	678.0	8.01
Nitrate reduction	5	10	55.90	0.68	13.76	688.10	8.01
Urease activity	10	5	75.86	0.81	14.22	711.00	8.01
Degradation of:							
Chitin	4	11	41.29	0.57	13.48	593.12	7.22
DNA	2	13	14.79	0.40	13.74	357.24	4.65
Elastin	6	9	77.29	0.82	14.33	773.82	8.52
Lecithin	2	13	22.40	0.47	14.71	382.46	4.65
Pectin	5	10	71.25	0.75	13.87	693.50	8.01
RNA	0	15	1.50	0.20	14.10	0	0
Tyrosine	8	7	66.10	0.68	13.14	735.84	8.77
Use of:							
D-arabinose	12	3	34.43	0.55	13.77	495.72	6.12
L-arabinose	2	13	13.64	0.38	14.53	377.78	4.65
Dextrin	1	14	0.04	0.22	14.69	205.66	2.71
D-galactose	2	13	17.10	0.42	14.69	381.94	4.65
meso-inositol	7	8	83.42	0.83	14.21	795.76	8.77
D-lactose	4	11	49.06	0.68	14.53	639.32	7.22
D-mannitol	4	11	45.30	0.65	14.36	631.84	7.22
D-melezitose	7	8	69.88	0.71	13.22	740.32	8.77
Methyl-α-D-glucoside	14	1	13.89	0.39	13.97	195.58	2.71
Methyl-β-D-glucoside	3	12	40.21	0.63	14.85	534.6	6.12
Raffinose	11	4	49.86	0.65	13.88	610.72	7.22
L-rhamnose	3	12	25.24	0.49	13.88	499.68	6.12
D-ribose	7	8	82.94	0.83	14.04	786.24	8.77
D-sorbitol	5	10	56.92	0.70	14.06	703.00	8.01
D-xylose	2	13	21.46	0.46	14.85	386.1	4.65
m-hydrobenzoic acid	12	3	34.26	0.55	12.81	497.16	6.12
p-hydroxybenzoic acid	11	4	59.94	0.75	14.71	647.24	7.22
Sodium acetate	11	4	47.30	0.60	13.31	585.64	7.22
Sodium fumarate	11	4	58.22	0.72	14.23	626.12	7.22
Sodium succinate	11	4	37.24	0.58	14.18	623.92	7.22
Syringic acid	15	0	0.01	0.17	14.23	0	0
Vanillin	13	2	21.46	0.46	14.85	386.10	4.65
Growth at:							
4 °C	2	13	21.46	0.46	14.85	386.10	4.65
37 °C	8	7	89.64	0.90	14.36	804.16	8.77
pH 6	4	11	36.07	0.57	14.02	616.88	7.22
pH 8	3	12	19.09	0.44	14.02	504.72	6.12

TABLE 4

Continued

Character	No of clusters the character is predominantly:-		Separation indices				
			Gyllenberg [9]				
	Negative	Positive	VSP%	CSP	Σc(i)	ΣR(i)	Niemela et al. [26] Index
Growth in the presence of (%, w/v):							
Brilliant green 0.001	12	3	40.21	0.63	14.85	534.6	6.12
Lysozyme 0.005	3	12	33.53	0.55	13.4	501.84	6.12
0.01	6	9	73.28	0.77	13.86	748.44	8.52
Potassium tellurite 0.001	2	13	21.87	0.47	14.78	384.28	4.65
0.005	6	9	78.50	0.84	14.43	779.22	8.52
0.01	8	7	93.26	0.94	14.78	827.68	8.77
Sodium azide 0.0001	1	14	0.05	0.29	13.48	188.72	2.71
0.001	8	7	73.66	0.74	13.52	757.12	8.77
Sodium chloride 1.0	4	11	60.25	0.74	14.49	637.56	7.22
2.0	9	6	84.74	0.87	14.56	786.24	8.52
3.0	14	1	0.07	0.29	14.69	205.66	2.71
Resistance to (μg ml^{-1})							
Ampicillin 0.5	2	13	12.39	0.36	14.46	375.96	4.65
2.0	5	10	65.77	0.77	14.40	720.00	8.01
8.0	12	3	40.21	0.63	14.85	534.6	6.12
Cephloridine HCl 1.0	8	7	83.64	0.84	14.02	785.12	8.77
2.0	13	2	24.95	0.49	14.4	374.40	4.65
Chlorotetracycline HCl 1.0	11	4	51.37	0.67	14.18	623.92	7.22
Gentamycin 0.5	5	10	41.31	0.58	13.65	682.50	8.01
1.0	11	4	62.60	0.75	14.3	629.2	7.22
4.0	12	3	40.21	0.63	14.85	534.6	6.12
Linomycin HCl 2.0	1	14	3.75	0.27	13.71	191.94	2.71
8.0	9	6	74.44	0.74	13.57	732.78	8.52
Methacycline HCl 0.25	5	10	49.01	0.65	13.95	697.50	8.01
2.0	14	1	10.85	0.34	14.36	201.04	2.71
Neomycin 0.5	8	7	83.73	0.84	14.19	794.64	8.77
2.0	12	3	40.21	0.63	14.85	534.6	6.12
Rifampicin 0.25	3	12	43.15	0.63	14.35	516.60	6.12
2.0	12	3	40.43	0.63	14.79	532.44	6.12
Tobramycin 0.5	8	7	81.09	0.81	14.02	785.12	8.77
1.5	10	5	74.06	0.80	14.23	711.50	8.01

CHARSEP describes the usefulness of each character in separating taxa using five separation indices that calculate results based on percentage or proportion of positive results. The indices are 1) VSP %, which estimates the effect of test error; 2) CPS, a similar index to %VSP which determines the number of taxa a particular character can separate with a score of close to 50% (i.e. poorly); 3) Σc(i), which describes the proportion of positive values per taxa; 4) ΣR(i), which gives the number of taxa a given character cannot separate between; and 5) Niemala Index, similar to ΣR(i) but based on $\log_e$ values.

study has been performed for *Actinoplanes* [44], sequence information with which to design genus-specific oligonucleotide probes, or to compare variable sequences to determine strain novelty were not generally available. For this reason, a probabilistic identification matrix was constructed in this study based on the numerical phenetic data of Goodfellow et al. [6] to determine whether the three organisms were novel strains of the genus *Actinoplanes*.

The first stage in the construction of the identification matrix was to determine which of the classification tests could be omitted, making identification simpler to perform without causing any cluster overlap. This was achieved using the CHARSEP program [36], and in particular the VSP separation index (Table 4). Characters with a VSP separation index of >25% were selected. This resulted in a final matrix of 15 clusters × 53 characters (Table 2). Testing to determine if the 15 clusters were well defined using these 53 characters would have been possible using the OVERMAT program of Sneath [40]. However, this program was not available. An alternative was to use the program DIACHAR [38] to

TABLE 5

Sum of diagnostic scores for all 54 characters achieved using the DIACHAR program

Cluster*	Sum of diagnostic scores
A. consettensis (6)	18.76
A. derwentensis (13)	19.91
A. durhamensis (5)	22.27
A. humidis (14)	18.85
A. palleronii (6)	22.85
A. philippinensis (2)	25.63
A. auranticolor (1)	30.64
A. caeruleus (1)	29.92
A. campanulatus (1)	29.94
A. italicus (1)	29.85
A. digitatus (1)	29.90
A. lobatus (1)	30.00
A. missouriensis (1)	31.90
A. regularis (1)	30.50
A. utahensis (1)	27.90

* The figures in parentheses are the number of strains in each cluster.

TABLE 6

Identification scores for the hypothetical median organism for each major cluster achieved using the MOSTTYP program

Cluster*	Identification scores		
	Willcox probability	Taxonomic distance	Standard error of taxonomic distance
A. consettensis (6)	1.000	0.242	−3.114
A. derwentensis (13)	1.000	0.213	−3.450
A. durhamensis (5)	1.000	0.209	−2.600
A. humidis (14)	1.000	0.190	−4.184
A. palleronii (6)	1.000	0.192	−3.676

* The figures in parentheses are the number of strains in each cluster.

calculate the sum of the diagnostic scores for the 53 characters for each cluster (Table 5). High scores would indicate that the clusters were well defined, which was seen in this study by comparison with the scores from other probabilistic matrices [19,53]. An added advantage from the use of DIACHAR would be for the design of selective isolation media for *Actinoplanes* based on this data set.

Further evaluation tests of the matrix confirmed that the 15 clusters × 53 characters identification matrix was well defined. For example, excellent scores were obtained for all coefficients of the hypothetical median organism (Table 6) against its own taxa using the MOSTTYP program [39] and

TABLE 7

Identification scores provided by the MATIDEN program for single member clusters using the classification test data

Cluster	Identification scores		
	Willcox probability	Taxonomic distance	Standard error of taxonomic distance
A. auranticolor	1.000	0.013	−8.956
A. caeruleus	1.000	0.045	−8.860
A. campanulatus	1.000	0.016	−8.710
A. italicus	1.000	0.017	−8.676
A. digitatus	1.000	0.018	−8.633
A. lobatus	1.000	0.019	−8.538
A. missouriensis	1.000	0.020	−8.296
A. regularis	1.000	0.021	−8.216
A. utahensis	1.000	0.027	−8.218

for the identification scores provided by the MATIDEN program [37] for single member clusters using the classification test data (Table 7). Only single-member cluster data were used because the original 0/1 data from the study of Goodfellow et al. [6] for clusters with more than one member strain were not available.

The final evaluation of the matrix re-determined character states of two cluster representatives, *A. philippinensis* DSM 43019 and *A. utahensis* DSM 43147, performed simultaneously with the three unknown isolates (Table 8). The two cluster representatives identified correctly to their clusters, with excellent scores for all coefficients in the MATIDEN program [37] in addition to zero characters against. This was with the exception of the standard error of taxonomic distance for *A. utahensis*. This score was unexplainably high at 17.91 given that the percentage positive scores for each unit character of the *A. utahensis* cluster (Table 2) were either 1% or 99%. The three organisms could all be assigned to a cluster based on the Willcox probability coefficient [51] in the MATIDEN program (Table 8). However, an identification based on just this one coefficient can be misleading because an apparent positive identification is possible if the cluster to which an unknown organism belongs is not included in the matrix. This is due to a normalising process during the calculation of the coefficient [52]. Values, therefore, of taxonomic distance and its standard error are important in highlighting these incorrect identifications. Also, the number of characters against will also show poor matching between the matrix and an unknown organism. This can be seen with strains A294 and A385 where the Willcox probabilities were high. However, so were the taxonomic distances, standard errors and numbers of characters against. Hence, strains A294 and A385 could not be identified. Strain A448 on the other hand gave a high Willcox probability, but low scores for taxonomic distance, standard error, and number of characters

TABLE 8

Identification scores for two cluster representatives and four unknown strains

Cluster number	Strain	Willcox probability	Taxonomic distance	Standard error of taxonomic distance	Characters against	Cluster identification
LA177	*A. utahensis* DSM 43147	1.00	0.27	17.91	0	LA177
14	*A. philipinensis* DSM 43019	1.00	0.22	1.33	0	14
	A294	1.00	0.51	5.82	13	7
	A385	0.95	0.52	6.04	13	7
	A448	0.99	0.42	2.90	5	3

against. Therefore, strain A448 could be identified from this matrix as *A. derwentensis*.

In the absence of 16S rRNA sequence information to further characterise the two actinomycetes not identified by this matrix, we recommend that strains A294 and A385 be considered novel until such times as alternative techniques to characterise the genus *Actinoplanes* become available.

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REFERENCES

1 Couch, J.N. 1955. A new genus and family of the *Actinomycetales*, with a revision of the genus *Actinoplanes*. Journal of the Elisha Mitchell Scientific Society 71: 148–155.

2 Couch, J.N. 1963. Some new genera and species of the *Actinoplanaceae*. Journal of the Elisha Mitchell Scientific Society 79: 53–70.

3 Goodfellow, M. and T. Cross 1984. Classification. In: The Biology of the Actinomycetes (Goodfellow, M., M. Mordarski and S.T. Williams, eds), pp. 7–164, Academic Press, London.

4 Goodfellow, M., G. Alderson and J. Lacey. 1979. Numerical taxonomy of *Actinomadura* and related actinomycetes. J. Gen. Microbiol. 112: 95–111.

5 Goodfellow, M., E. Stackebrandt and R.M. Kroppenstedt. 1988. Chemotaxonomy and actinomycete systematics. In: Biology of Actinomycetes '88 (Okami, Y., T. Beppu and H. Ogawara, eds), pp. 233–238, Japan Scientific Societies Press, Tokyo.

6 Goodfellow, M., L.J. Stanton, K.E. Simpson and D.E. Minnikin. 1990. Numerical and chemical classification of *Actinoplanes* and some related actinomycetes. J. Gen. Microbiol. 136: 19–36.

7 Gordon, R.E. 1968. The taxonomy of soil bacteria. In: The Ecology of Soil Bacteria (Gray, T.R.G. and D. Parkinson, eds), pp. 293–321, Liverpool University Press, Liverpool.

8 Gordon, R.E. and J.M. Mihm. 1957. A comparative study of some strains received as nocardiae. J. Bacteriol. 73: 15–27.

9 Gyllenberg, H.G. 1963. A general method for deriving determination schemes for random collections of microbial isolates. Annales Academiae Scientarum Fennicae, Series A (IV Biologica) 69: 1–23.

10 Hankin, L., M. Zucker and D.C. Sands. 1971. Improved solid medium for the detection and enumeration of pectinolytic bacteria. Appl. Microbiol. 22: 205–209.

11 Hill, L.R. 1974. Theoretical aspects of numerical identification. Int. J. Sys. Bacteriol. 24: 494–499.

12 Jones, K.L. 1949. Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. J. Bacteriol. 57: 141–145.

13 Kane, W.D. 1966. A new genus of *Actinoplanaceae, Pilimelia terevasa* and *Pilimelia anulata*. Journal of the Elisha Mitchell Scientific Society 82: 220–230.

14 Kawamoto, I., T. Oka and I. Nora. 1981. Cell wall composition of *Micromonospora olivasterospora, Micromonospora sagamiensis* and related organisms. J. Bacteriol. 146: 527–534.

15 Krassilnikov, N.A. 1938. Ray Fungi and Related Organisms, Actinomycetales. Izdatelstvo Akademii Nauk SSSR, Moscow.

16 Kroppenstedt, R.M. 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: Chemical Methods in Bacterial Systematics (Goodfellow, M. and D.E. Minnikin, eds), pp. 173–199, Academic Press, London.

17 Küster, E. and S.T. Williams. 1964. Production of hydrogen sulphide by streptomycetes and methods for its detection. Appl. Microbiol. 12: 46–52.

18 Kutzner, H.J. 1976. Methoden zur Untersuchung von Streptomyceten und einigen anderen Actinomyceten. Teilsammlung Darmstadt am Institut für Mikrobiologie der technischen Hochschule, Darmstadt.

19 Langham, C.D., S.T. Williams, P.H.A. Sneath and A.M. Mortimer. 1989. New probability matrices for identification of *Streptomyces*. J. Gen. Microbiol. 135: 121–133.

20 Lapage, S.P. 1974. Practical aspects of probabilistic identification of bacteria. Int. J. Sys. Bacteriol. 24: 500–507.

21 Lapage, S.P., S. Bascomb, W.R. Willcox and M.A. Curtis. 1970. Computer identification of bacteria. In: Automation, Mechanization and Data Handling in Microbiology (Baillie, A. and R.J. Gilbert, eds), pp. 1–22, Academic Press, London.

22 Lechevalier, M.P. and H.A. Lechevalier. 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Sys. Bacteriol. 20: 435–443.

23 Lechevalier, M.P., C. Debièvre and H. Lechevalier. 1977. Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem. Sys. Ecol. 5: 249–260.

24 Leudemann, G.M. 1971. Species concept and criteria in the genus *Micromonospora*. Trans. N.Y. Acad. Sci. 33: 203–218.

25 Long, P.F., N.R. Parekh, J.C. Munro and S.T. Williams.

1993. Isolation of actinophage that attack some maduromycete actinomycetes. FEMS Microbiol. Lett. 108: 195–200.

26 Niemala, S.I., J.W. Hopkins and C. Quadling. 1968. Selecting an economic binary test battery for a set of microbial cultures. Can. J. Microbiol. 14: 271–279.

27 Oerskov, J. 1923. Investigations into the Morphology of the Ray Fungi. Levin and Munksgaard, Copenhagen.

28 Palleroni, N.J. 1989. *Actinoplanes*. Couch 1950, 89 emend. mut. char. 1955, 153^{AL}. In: Bergey's Manual of Systematic Bacteriology, vol. 4 (Williams, S.T., M.E. Sharpe and J.G. Holt, eds), pp. 2419–2428, Williams and Wilkins, Baltimore.

29 Parenti, F. and C. Coronelli. 1979. Members of the genus *Actinoplanes* and their antibiotics. Ann. Rev. Microbiol. 33: 389–411.

30 Rowbotham, T.J. and T. Cross. 1977. Ecology of *Rhodococcus coprophilus* and associated actinomycetes in fresh water and agricultural habitats. J. Gen. Microbiol. 100: 231–240.

31 Ruan, J.S., L. Ziaotao, Z. Yamei and S. Yanlin. 1988. Numerical classification and chemotaxonomy of actinoplanetes and nocardiae. In: Biology of Actinomycetes, 88 (Okamie, Y., T. Beppu and H. Ogawara, eds), pp. 221–226, Japan Scientific Societies Press, Tokyo.

32 Schleifer, K.H. and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36: 407–477.

33 Schleifer, K.H. and P.H. Seidl. 1985. Chemical composition and structure of murein. In: Chemical Methods in Bacterial Systematics (Goodfellow, M. and D.E. Minnikin, eds), pp. 201–219, Academic Press, London.

34 Sneath, P.H.A. 1974. Test reproducibility in relation to identification. Int. J. Sys. Bacteriol. 24: 508–523.

35 Sneath, P.H.A. 1978. Identification of microorganisms. In: Essays in Microbiology (Norris, J.R. and M.H. Richmond, eds), pp. 10/1–10/32, Wiley, Chichester.

36 Sneath, P.H.A. 1979a. BASIC program for character separation indices from an identification matrix of percentage positive characters. Computer and Geosciences 5: 349–357.

37 Sneath, P.H.A. 1979b. BASIC program for identification of an unknown with presence–absence data against an identification matrix of percentage positive characters. Computers and Geosciences 5: 195–213.

38 Sneath, P.H.A. 1980a. BASIC program for the most diagnostic properties of groups from an identification matrix of percent positive characters. Computer and Geosciences 6: 21–26.

39 Sneath, P.H.A. 1980b. BASIC program for determining the best identification scores possible for the most typical example when compared with an identification matrix of percent positive characters. Computers and Geosciences 6: 27–34.

40 Sneath, P.H.A. 1980c. BASIC program for determining overlap between groups in an identification matrix of percent positive characters. Computers and Geosciences 6: 267–278.

41 Sneath, P.H.A. and R. Johnson. 1972. The influence on numerical taxonomic similarities of errors in microbiological tests. J. Gen. Microbiol. 72: 377–392.

42 Sneath, P.H.A. and R.R. Sokal. 1973. Numerical Taxonomy. The Principles and Practice of Numerical Classification. W.H. Freeman, San Francisco.

43 Stackebrandt, E. and R.M. Kroppenstedt. 1987. Union of the genera *Actinoplanes* Couch, *Ampullariella* Couch and *Amorphosporangium* Couch in a redefined genus *Actinoplanes*. Sys. Appl. Microbiol. 9: 110–114.

44 Stackebrandt, E. and C.R. Woese. 1981. Towards a phylogeny of the actinomycetes and related organisms. Curr. Microbiol. 5: 197–202.

45 Stackebrandt, E., B. Wunner-Fussl, V.J. Fowler and H.H. Schleifer. 1981. Deoxyribonucleic acid homology and ribosomal ribonucleic acid similarities among spore forming members of the order *Actinomycetales*. Int. J. Sys. Bacteriol. 31: 420–431.

46 Stevenson, I.K. 1967. Utilisation of aromatic hydrocarbons by *Arthrobacter* spp. Can. J. Microbiol. 13: 205–211.

47 Szaniszlo, P.J. and H. Gooder. 1967. Cell wall composition in relation to the taxonomy of some *Actinoplanaceae*. J. Bacteriol. 94: 2307–2347.

48 Uchida, K. and K. Aida. 1977. Acyl type of bacterial cell wall: its simple identification by colorimetric method. J. Gen. Microbiol. 23: 249–260.

49 Vobis, G. 1989. Actinoplanetes. In: Bergey's Manual of Systematic Bacteriology, vol. 4 (Williams, S.T., M.E. Sharpe and J.G. Holt, eds), pp. 2418–2419, Williams and Wilkins, Baltimore.

50 Wellington, E.M.H. and S.T. Williams. 1978. Preservation of actinomycete inoculum in frozen glycerol. Microbios Lett. 6: 151–157.

51 Willcox, W.B., S.P. Lapage, S. Bascomb and M.A. Curtis. 1973. Identification of bacteria by computer: theory and programming. J. Gen. Microbiol. 77: 317–330.

52 Willcox, W.R., S.P. Lapage and B. Holmes. 1980. A review of numerical methods in bacterial identification. Antonie van Leuwenhoek 46: 233–299.

53 Williams, S.T., M. Goodfellow, E.M.H. Wellington, J.C. Vickers, G. Alderson, P.H.A. Sneath, M.J. Sackin and A.M. Mortimer. 1983. A probability matrix for the identification of some streptomycetes. J. Gen. Microbiol. 129: 1815–1830.