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Actinomycete diversity associated with foaming in activated sludge plants

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Large numbers of mycolic acid-containing actinomycetes were isolated from foam and scum samples taken from three activated-sludge sewage-treatment plants using several selective isolation media. Organisms presumptively identified as gordonae formed the dominant population in all of the samples. A representative set of these strains have chemical properties consistent with their classification in the genus *Gordona*. Forty-eight of the *Gordona* strains were compared through 165 unit characters with the type strains of validly described species of *Gordona*. The resultant data were examined using the Jaccard and simple matching coefficients and clustering achieved using the unweighted pair group method with arithmetic averages algorithm. The numerical classification was only marginally affected by the statistics used or by test error, estimated as 3.92%. The isolates were assigned to five multi-membered and 28 single-membered clusters defined by the simple matching coefficient at the 89% similarity level. With few exceptions, the isolates were sharply separated from the *Gordona* marker strains. Essentially the same classification was obtained when the test strains were examined using a Curie-point pyrolysis mass spectrometric procedure. It can be concluded that the gordonae form a heterogeneous taxonomic group, the members of which can be distinguished from representatives of validly described species of *Gordona*.

Keywords: actinomycete diversity; chemotaxonomy; numerical taxonomy; pyrolysis mass spectrometry; activated sludge foam

Introduction

Biological wastewater treatment, probably the single most important use of microorganisms, is central to the protection of the environment. The activated sludge process is the most commonly employed method for the treatment of wastewater. Typically, this is a continuous flow process where a liquid organic waste, usually sewage, is aerated in the presence of an inoculum composed of undefined, but complex, bacterial consortia. The waste is converted into sludge and carbon dioxide; the sludge is allowed to settle and a portion fed back into the system to form the inoculum, that is, the activated sludge.

Little is known about the numbers, kinds and activities of organisms which form bacterial consortia or flocs. This ignorance can be partly attributed to pragmatism as many activated sludge process developments have been achieved by heuristic improvements guided by a vague understanding of the microbial processes. The dearth of knowledge also has a methodological basis as microbiologists, until recently, lacked adequate tools to investigate complex microbial communities. It is necessary to determine the extent of microbial diversity in order to monitor the microbiology of the activated sludge process and to prevent the formation of activated sludge foams.

The formation of stable, often chocolate-coloured, viscous foams or scums on the surfaces of activated sludge aeration tanks was first reported in 1969 [2]. The occurrence of foam is usually associated with mycolic acid-containing actinomycetes and '*Microthrix parvicella*' [9,14,33,60]. Mycolic acid-containing organisms have hydrophobic cell surfaces, a property which has been related to the presence of free long-chain mycolic acids [3], and when present in sufficient numbers they render flocs hydrophobic and hence amenable to the attachment of air bubbles. The air bubble-floc aggregates are less dense than water and hence float to the surface of activated sludge where they accumulate as foam.

Activated sludge foams cause a number of problems which include a reduction in oxygen transfer at the surfaces of mechanically aerated basins, poorer effluent quality, carriage and dispersal of potential microbial pathogens in wind-blown scum, and drying out of foam with resultant cleaning and odour problems [33,60]. Sewage treatment plants designed to remove phosphorus seem particularly prone to foaming [67]. There are no clear guidelines to prevent or control the accumulation of foam; strategies which work in one location may not work in another. The failure of a single heuristic solution to prevent the formation of foam implies that the underlying causes of foaming are multifactorial and involve taxonomically diverse bacteria predominated by actinomycetes.

Engineering solutions to the problem of foaming require an understanding of the ecology of the causal organisms, this in turn presupposes an appreciation of their taxonomic diversity and identity. Early work relied heavily on the identification of causal organisms by simple morphological examination [13]. According to this scheme, bacteria were assigned to groups based on a few, subjectively weighted morphological and staining properties. The reliance placed on such artificial groups has been the source of confusion

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in actinomycete systematics, not least in the classification and identification of mycolic acid-containing actinomycetes [18,60]. These organisms have many phenotypic properties in common [18], form a distinct phyletic line [10,46,50], and can be assigned to seven genera, namely *Corynebacterium*, *Dietzia*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Tsukamurella*, using a combination of chemical, molecular and morphological features [10,18,31,47].

The first comprehensive attempt to isolate and characterise actinomycetes associated with activated sludge foaming was made by Lechevalier *et al* [36] who examined 21 plants located in nine states across the USA. The predominant species were found to be *Nocardia amarae*, which was isolated from 15 plants, and '*Nocardia rhodochrous*' which was recovered from seven plants. *Nocardia amarae* has subsequently been isolated from foaming sludge in various parts of the world [6,8,9,12,30,40,52,62]. Lemmer and Kroppenstedt [37] studied the distribution of actinomycetes in scum samples taken from sewage treatment plants in Germany and Switzerland; they isolated 11 strains of *Rhodococcus*, two of *Tsukamurella* and a single strain of *Nocardia amarae*, but members of only one species were associated with any one plant. In contrast, Sezgin *et al* [56] found that the most common isolates in activated sludge scum taken from two sewage treatment plants in the USA were *Nocardia amarae* and *Nocardia asteroides*. A novel actinomycete with a pine-tree-like micromorphology, *Nocardia pinensis*, is the cause of extensive foaming on the surface of aeration tanks in inactivated-sludge sewage-treatment plants in Australia [6,55,61].

It has been postulated that activated sludge foams can be attributed mainly to members of the genus *Nocardia* [33]. However, given the reclassification of *Nocardia amarae* in the genus *Gordona* as *Gordona amarae* [26,35,50] and the discovery that *Nocardia pinensis* needs to be reclassified [8], it is clear that mycolic acid-containing actinomycetes other than nocardiae are involved. Similarly, organisms either labelled '*Nocardia rhodochrous*' or simply referred to as nocardioform actinomycetes [36,62] undoubtedly included members of the genera *Gordona*, *Rhodococcus* and *Tsukamurella*.

The primary aim of the present study was to determine the taxonomic diversity of mycolic acid-containing actinomycetes in foam collected from three activated-sludge sewage-treatment plants.

Materials and methods

Selective isolation and enumeration

Collection of samples: Samples of activated sludge foam or scum were collected from three sewage treatment works between April and July 1995. Grab samples of surface scum and underlying mixed liquor were taken from the aeration zones of the mainstream (University of Capetown configuration) and the sidestream Tetra Phostrip® processes at Milcote Pilot Sewage Treatment Plant, Stratford-upon-Avon, UK (UTM Zone 30 [3 degrees West] 586490 mE 5781270 mN); additional samples were taken from the secondary clarifiers. Similarly, grab samples of foam were collected from selected aeration lanes and the corresponding secondary clarifiers at Wanlip Sewage Treatment Works, Leics, UK (UTM Zone 30 [3 degrees West] 627280 mE 5840450 mN). Foam samples were also taken from the corresponding sites at the northern and southern sectors of the Stoke Bardolph Water Reclamation Works, Notts, UK (UTM Zone 30 [3 degrees West] 630460 mE 5870800 mN), namely from the mixed liquor aeration tanks and corresponding secondary clarifier still boxes. Part of the individual samples from each of the plants were bulked to give three composite samples; both individual and composite samples were made up to 10 ml with quarter strength Ringer's solution. Individual wastewater samples taken from the inlet streams at the Milcote and Wanlip plants were treated in the same way. All of the samples were stored at 4°C and were examined within 24 h of collection.

Direct enumeration of bacterial populations: The total bacterial counts in the scum and foam samples taken from the Milcote and Wanlip sites were estimated using epifluorescence microscopy. Membrane filtration and staining of the microbial cells was carried out using the nucleic acid-specific fluorochrome 4′,6-diamino-2-phenylindole (DAPI), as described previously [66]. Twenty graticules were examined for each slide preparation and the total bacterial counts calculated using the formula of Kepner and Pratt [34].

Selective isolation: Mycolic acid-containing actinomycetes belonging to the genera *Gordona*, *Nocardia*, *Rhodococcus* and *Tsukamurella* were sought from both individual and composite samples using appropriate selective isolation procedures. Aliquots (0.1 ml) from each of the 10^{-4} to 10^{-6} dilutions, prepared from both the individual and composite samples, were spread over the surface of glucose yeast extract agar plates (GYEA; [28]) supplemented with cycloheximide (50 μg ml^{-1}; Sigma, Poole, Dorset, UK) and GYEA plates supplemented with cycloheximide (50 μg ml^{-1}) and nalidixic acid (20 μg ml^{-1}). Similarly, 0.1-ml aliquots from the 10^{-4} to 10^{-6} dilutions, prepared from the three composite samples, were plated out onto modified Czapek's [29] and tryptone yeast extract agars (TYEA; [6]). The Stoke Bardolph composite samples, which were examined in an initial survey, were also plated out onto Diagnostic Sensitivity Test agar (Oxoid, Basingstoke, Hants, UK) supplemented with cycloheximide (50 μg ml^{-1}), chlortetracycline (45 μg ml^{-1}), methacycline (10 μg ml^{-1}) and nystatin (50 μg ml^{-1}) to detect norcardiae [44], and onto M3 [49] and Münz paraffin agars [42] for the isolation of rhodococci. In all cases, isolation plates, five per dilution, were incubated at 30°C for 14 days. The numbers of gordonae, rhodococci and tsukamurellae growing on the GYEA plates supplemented with cycloheximide were expressed as the number of colony-forming units (CFUs) per ml sample.

Selection of isolates: One hundred and twenty-seven isolates, presumptively identified as gordonae, were subcultured onto GYEA plates [28] and incubated at 30°C for 5 days. The strains were checked for purity by microscopic examination of Gram-stained [48] and acid-alcohol fast-

stained [27] smears. Approximately equal numbers of isolates were taken from the GYEA, TYEA and modified Czapek's agar plates prepared using the individual and composite samples. The isolates were maintained as pure cultures on GYEA plates held at 4°C and as glycerol suspensions (20%, v/v) at −20°C [68].

Taxonomic studies

Lipid analyses: Both isolates and control strains were grown on GYEA plates for 5 days at 37°C. Biomass (*ca* 50 mg) was degraded by acid methanolysis and hexane extracts of the resultant methanolysates examined for mycolic acid methyl esters (MAMES) by single-dimensional thin-layer chromatography (TLC), as described previously [38]. The mobility of the MAMES extracted from the isolates were compared with those from the control strains, namely *Gordona bronchialis* N654^T (^{T}, type strain), *Nocardia asteroides* N317^T, *Rhodococcus rhodochrous* N54^T and *Tsukamurella paurometabola* JC7^T.

The small-scale procedure of Minnikin *et al* [39] was used to extract isoprenoid quinones from representative isolates (Table 1). Purified isoprenoid quinones were separated by HPLC using a Former 425 instrument (Kontron Instruments Ltd, Watford, Herts, UK) fitted with a Spherisorb (ODS) analytical HPLC column (25 cm long; particle size 5 μm; Jones Chromatography, Hengoed, Mid-Glamorgan, UK); acetonitrile-isopropanol (75 : 25, v/v; HPLC grade, Fisons, Loughborough, Leics, UK), with a flow rate of 1.0 ml min^{-1} at room temperature, was used as the mobile phase. The isoprenoid quinones were detected at 254 nm and their retention times compared with those of standards extracted from *Gordona bronchialis* N654^T, *Rhodococcus rhodochrous* N54^T and *Tsukamurella paurometabola* JC7^T.

Numerical classification: Forty-eight representative isolates assigned to the genus *Gordona* on the basis of chemical data were compared with the type strains of the validly described species of *Gordona* for 179 unit characters derived from the application of standard procedures [17,21,23]. Test results were recorded after 14 days incubation at 30°C, apart from the temperature tests. The resultant binary data were examined using the CLUSTAN IC program on an IBM-PC compatible computer using the simple matching (S_{SM}; [63]), which includes both positive and negative matches, and the Jaccard coefficient (S_J; [57]), which only includes positive matches. Clustering was achieved using the unweighted pair group method with arithmetic averages algorithm (UPGMA; [59]). Cophenetic correlation values [64] were determined for each analysis using the CLUSTAN procedure 'COMPARE' in order to estimate how well the structure inherent in the similarity matrices was preserved by the clustering process. Three strains were examined in duplicate (Table 1) and an estimate of test variance calculated (formula 15; [58]) and used to determine the average probability (P) of an erroneous test result (formula 4; [58]).

Curie-point pyrolysis mass spectrometry: All of the organisms included in the numerical taxonomic study were analysed by Curie-point pyrolysis mass spectrometry (PyMS) using an established procedure [11,53]. Triplicate samples of each organism were analysed as a single batch on a Horizon Instruments RAPyD-400X pyrolysis mass spectrometer and the resultant data analysed using the GENSTAT statistical package [41]. Five strains (Table 1) were examined in duplicate to determine test reproducibility.

Integrated ion counts for each sample at unit mass intervals from 51 to 400 were recorded and stored on hard discs together with total ion counts and the sample pyrolysis sequence. Variation between spectra due to inoculum size was normalised by iterative re-normalisation [32] and individual masses ranked according to their characteristicity values [15] prior to principal component analysis. Principal components (PCs) accounting for less than 0.1% of the total variance were deleted from the data set. Canonical variate analysis (CVA) was then used to generate sample groups using the retained PCs while taking into account the triplicate sets [69]. The products of the PC-CVA analyses were displayed as ordination plots where strains were positioned in multidimensional space according to the mid-point between each of the triplicate samples. Further details on the data-handling procedure can be found elsewhere [19].

Results

Selective isolation and enumeration

Relatively large numbers of isolates growing on GYEA, TYEA and modified Czapek's agar plates, seeded with either individual or composite foam or scum samples, were presumptively identified as gordonae as they formed rough greyish-pink, dry colonies. Smaller numbers of orange and pink mucoid colonies were presumptively identified as rhodococci while relatively large, orange to red colonies with irregular edges were considered to be tsukamurellae. The highest numbers of presumptive gordonae, rhodococci and tsukamurellae were detected on the GYEA plates supplemented with cycloheximide (Table 2). The highest numbers of presumptive gordonae and rhodococci were found at the Stoke Bardolph plant where foaming was most pronounced whereas the lowest counts were recorded from scum samples collected at the Milcote site. The highest actinomycete counts, notably those for presumptive gordonae, were recorded for samples collected at Stoke Bardolph in July when relatively high levels of foam were apparent. In contrast, significant numbers of actinomycetes were not isolated from the samples taken from the inlet streams at the Milcote or Wanlip plants or on the GYEA plates supplemented with nalidixic acid. Nocardiae were not detected on the DST agar plates seeded with dilutions of foam from the Stoke Bardolph plant, neither were rhodococci recovered on the corresponding M3 and Münz paraffin agar plates.

It is evident from Table 3 that the viable counts only represent a tiny fraction of a total bacterial counts.

Detection of chemical markers

Mycolic acid: Thin-layer chromatographic analysis of whole-organism methanolysates of the presumptive *Gordona* isolates revealed the presence of two spots which cor-

Table 1 Designation, source and history of strains assigned to clusters defined at the 89% similarity level in the S_{SM}, UPGMA analysis

Laboratory number	Designation	Source
Multi-membered clusters		
Cluster 1		
SB04, SB011, SB029	*Gordona* isolates	Activated sludge foam, aeration basins, Stoke Bardolph
Cluster 2		
SB017†, SB019, SB020, SB022, SB025, SB026, SB027	*Gordona* isolates	Activated sludge foam, aeration basins and final clarifiers, Stoke Bardolph
Cluster 3		
SB044, SB050, SB061, SB062, SB063	*Gordona* isolates	Activated sludge foam, aeration basins and final clarifiers, Stoke Bardolph
Cluster 4		
SB016†, SB021†	*Gordona* isolates	Activated sludge foam, aeration basins and final clarifiers, Stoke Bardolph
Cluster 5		
WA018†, WA020, WA021	*Gordona* isolates	Activated sludge foam, aeration basins, Wanlip
Cluster 6		
N667^{T‡}	*Gordona amarae*	MP Lechevalier, Rutgers University, New Brunswick, USA, Se6; foam, sewage treatment plant, Andover, Florida, USA
N1233^{T}	*Gordona hydrophobica*	DSM44015, Braunschweig, Germany; biofilter, waste gas treatment
Single-membered clusters		
Marker strains		
N934^{T‡}	*Gordona aichiensis*	M Tsukamura, Chubu Chest Hospital, Obu, Aichi-chen 474, Japan, E9028; sputum
N654^{T†§‡}	*Gordona bronchialis*	NCTC 10677; M Tsukamura, 3410; H Kondo; sputum, pulmonary lesion
N935^{T}	*Gordona obuensis*	M Tsukamura, Japan, E8183; sputum
N4^{T‡}	*Gordona rubropertincta*	NCIB 9664; ATCC 14352; RS Breed; RE Gordon, 154; soil
N930^{T}	*Gordona sputi*	M Tsukamura, Japan, E 3884; sputum
N659^{T‡}	*Gordona terrae*	NCTC 10669; M Tsukamura, 3612; soil
Test strains		
SB02, SB06, SB08, SB010, SB013, SB014, SB023, SB035, SB036	*Gordona* isolates	Activated sludge foam, aeration basins, Stoke Bardolph
SB048, SB052, SB053	*Gordona* isolates	Foam, final clarifiers, Stoke Bardolph
SB057, SB068†	*Gordona* isolates	Composite samples of activated sludge foam, aeration basins and final clarifiers, Stoke Bardolph
ML02§, ML03§, ML04, ML07, ML09, ML012	*Gordona* isolates	Activated sludge foam, aeration basins, Milcote
ML016, ML020, ML022	*Gordona* isolates	Foam, final clarifiers, Milcote
WA06, WA015	*Gordona* isolates	Inlet stream, Wanlip
WA016, WA023	*Gordona* isolates	Activated sludge foam, aeration basins, Wanlip
WA031	*Gordona* isolates	Foam, final clarifier, Wanlip

^{T}Type strain; †strains examined for isoprenoid quinones; §duplicated strains included in the numerical taxonomic study; ‡duplicated strains included in the pyrolysis mass spectrometric analysis.
ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikrorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratories, London, UK.

Table 2 Mean total viable counts of bacteria, and the proportion of the total counts attributed to gordonae, rhodococci and tsukamurellae, grown on glucose-yeast extract agar supplemented with cycloheximide (50 μg ml^{-1}) after seeding with dilutions of activated sludge foam and clarifier foam samples collected from Milcote, Stoke Bardolph and Wanlip Sewage Treatment Works and incubation at 30°C for 14 days

Sewage treatment works	Month	Mean total viable count of bacteria $\times 10^8$ (CFU ml^{-1})	Mean percentage of total count			Degree of foaming
			presumptive gordonae	presumptive rhodococci	presumptive tsukamurellae	
Milcote	April	0.13 ± 0.17	4	1	0	+
Stoke Bardolph	April	0.27 ± 0.19	20	8	0	+++
	July	1.31 ± 0.26	40	11	3	++++
Wanlip	April	0.16 ± 0.22	10	2	0	++

CFU, colony forming units.
+ Surface scum, no stable foam; ++ moderate surface foam; +++ thick stable surface foam; ++++ extensive stable foam.

Table 3 Total viable counts of bacteria obtained using the dilution plate technique compared to the total direct bacterial counts based on epifluorescence microscopy

Sewage treatment works	Sample description	Total viable count $\times 10^7$ (CFU ml^{-1})	Total count $\times 10^7$ (ml^{-1})	Percentage of the total count represented by the total viable count (%)
Milcote	UCT, aeration basin (1)	0.17 ± 0.05	133.3 ± 78.5	0.13
	UCT, aeration basin (2)	0.52 ± 0.11	301.3 ± 133.1	0.17
	Phostrip® aeration basin	0.74 ± 0.13	260.1 ± 119.5	0.29
Wanlip	Aeration pocket 9, lane 8	0.46 ± 0.12	204.6 ± 174.4	0.23
	Aeration pocket 9, lane 9	0.51 ± 0.19	315.6 ± 125.9	0.16

CFU, colony forming units; UCT, University of Capetown configuration.

responded with MAMES (R_f values 0.68–0.71) and non-hydroxylated fatty acid methyl esters (R_f values 0.8–0.9; [38]). A MAME spot with an R_f value equivalent to those recorded for the isolates was detected in the methanolysate of *Gordona bronchialis* N654^T (R_f value 0.68). In contrast, the remaining control strains gave MAME spots with distinctly lower (*Nocardia asteroides* N317^T [R_f value 0.64] and *Rhodococcus rhodochrous* N54^T [R_f value 0.61]) or markedly higher (*Tsukamurella paurometabola* JC7^T [R_f value 0.76]) R_f values.

Isoprenoid quinones: Lipid extracts of all of the test strains contained menaquinones which co-chromatographed with the vitamin K standard (Sigma). The five representative *Gordona* isolates (Table 1) contained dihydrogenated menaquinones with nine isoprene units (MK-9 [H_2]) as the major isoprenologue, as did *Gordona bronchialis* N654^T. The corresponding components extracted from *Rhodococcus rhodochrous* N54^T and *Tsukamurella paurometabola* JC^T were MK-8 [H_2] and MK-9, respectively.

Numerical classification

Test error and final database: Experimental test error was estimated from data collected from the three duplicated cultures (Table 1). The average probability (P) of an erroneous test result, calculated from the pooled variance ($S_i^2 = 0.03$) of the unit characters, was 3.92%. Fourteen tests were deleted from the raw data matrix as they showed little, if any, separation value. All of the strains grew at 15°C and 20°C, were resistant to capreomycin sulphate (8 μg ml^{-1}) and novobiocin (2 μg ml^{-1}) and used octanoic acid (1.0%, w/v) as a source of carbon for energy and growth. In contrast, none of the organisms grew in the presence of demeclocyline (32 μg ml^{-1}) or doxycycline (100 μg ml^{-1}), neither did they use butan-2,3-diol (1.0%, v/v), coumarin (1.0%, v/v), malonic acid (Na salt; 1.0%, w/v), oxalic acid (1.0%, w/v), propan-1,2-diol (1.0%, v/v), propan-1-ol (1.0%, v/v), propan-2-ol (1.0%, v/v) as sole carbon sources.

The final database contained information on 56 strains, excluding the duplicated strains, and 165 unit characters.

Clustering of strains: The classification based on the S_{SM}, UPGMA analysis was examined in detail as it gave the most compact clusters together with the relatively high cophenetic correlation value of 0.765. The *Gordona* isolates were assigned to five multi-membered and 28 single-membered clusters defined at the 89% similarity level (Figure 1). The remaining multi-membered taxon, cluster 6, encompassed the type strains of *Gordona amarae* and *Gordona hydrophobica*. The remaining type strains of validly described *Gordona* species formed single-membered clusters. The majority of the *Gordona* isolates were sharply separated from the type strains. Clusters 1 to 4 contained organisms isolated from the Stoke Bardolph plant and cluster 5 strains from the Wanlip site. The same clusters were recognised in the S_J, UPGMA analysis albeit at a lower similarity level.

Characterisation of taxa: The properties of the multi-membered clusters and the type strains of six validly described *Gordona* species are given in Table 4.

Curie-point pyrolysis mass spectrometry

Excellent agreement was found between the results of the triplicate analyses of each strain (data not shown), that is, the members of each triplicate set were either superimposed or occupied adjacent positions in the three-dimensional ordination plot. Similarly, the duplicated cultures were clustered together as anticipated. Once the outlying strains had been removed from the dataset and the remaining data re-analysed to determine the finer taxonomical detail it was clear that the balance of the strains formed two broad groups (Figure 2). One group encompassed the type strains of the validly described *Gordona* species, two Milcote and four Wanlip isolates. The second group contained all of the Stoke Bardolph isolates and the remaining Milcote and Wanlip strains. When the PyMS data were re-examined, after the deletion of the results from the type strains, groups of *Gordona* isolates corresponding to the multi-membered clusters recovered in the numerical taxonomic study were detected. The cluster 3 isolates formed a particularly tight group and the remaining Wanlip isolate WAO31, was closely associated with the strains assigned to cluster 2.

Discussion

Surprisingly few taxonomic studies have been carried out on mycolic acid-containing actinomycetes which cause or are associated with foaming in activated-sludge sewage-treatment plants. Actinomycetes isolated from activated sludge foams have usually been characterised using a few phenotypic properties with little attempt made to determine

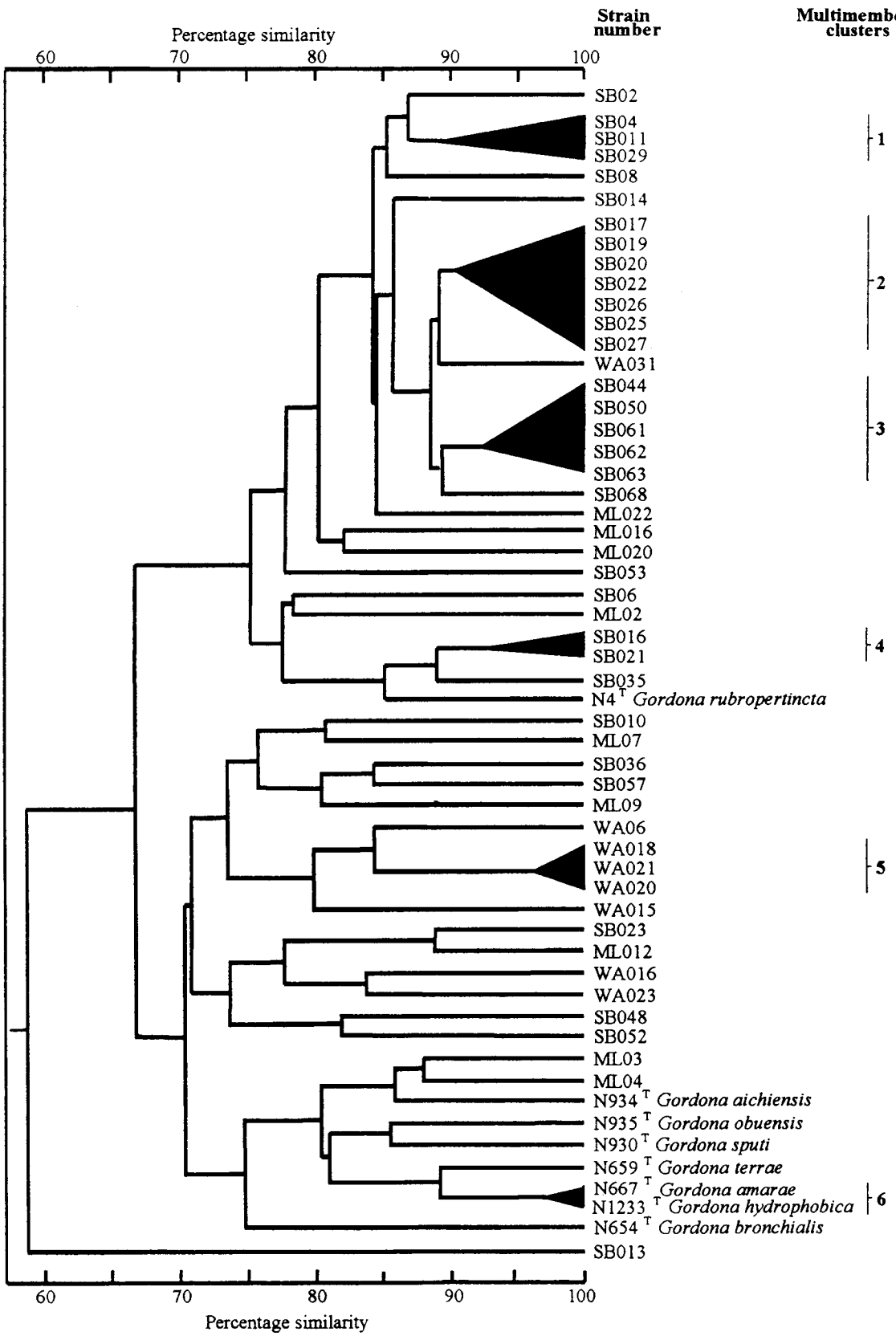

Figure 1 Abridged dendrogram showing relationships between clusters defined at the 89% similarity level in the S_{SM}, UPGMA analysis. TDenotes type strains.

Table 4 Distribution of positive characters to *Gordona* clusters defined at the 89% similarity level in the S_{SM}, UPGMA analysis*

Unit character								Clusters					
								1	2	3	4	5	6
		G.aichiensis	*G.bronchialis*	*G.obuensis*	*G.rubropertincta*	*G.sputi*	*G.terrae*	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*G.amarae* *G.hydrophobica*
	Number of strains/strain number	N934^T	N654^T	N935^T	N4^T	N930^T	N659^T	3	7	5	2	3	2
(a) BIOCHEMICAL TESTS													
Allantoinase production		1	1	1	0	1	1	0	0	2	1	3	2
Nitrate reduction		1	1	1	0	0	1	1	1	2	0	3	1
Urease production		1	0	1	0	1	0	0	0	0	0	3	0
(b) DEGRADATION TESTS (%,w/v)													
DNA (0.2)		0	0	0	0	0	0	0	1	1	0	0	0
Starch (1.0)		0	0	0	0	0	0	0	0	2	0	0	0
Tributyrin (0.1)		1	1	1	1	0	1	3	7	5	2	3	2
(c) NUTRITIONAL TESTS													
(i) Sole carbon sources at 1.0%, w/v or v/v													
Monosaccharides:													
Deoxyhexoses:													
L-Rhamnose		1	0	0	1	1	1	0	2	0	0	3	2
D(+) Turanose		1	1	1	1	1	1	0	7	2	2	3	2
Hexoses:													
D(+) Galactose		0	1	1	1	1	1	0	0	0	0	3	2
D(+) Xylose		0	1	1	1	0	0	0	1	1	0	2	0
Pentoses:													
D(−) Arabinose		0	0	0	1	0	1	0	0	0	0	0	2
D(+) Arabinose		0	0	0	0	0	1	0	0	1	0	0	2
L(−) Arabinose		0	1	0	0	0	0	2	0	2	0	3	0
Disaccharides:													
D(+) Lactose		0	1	0	0	0	0	3	0	0	0	3	0
D(+) Melibiose		0	1	1	1	1	1	0	6	1	2	3	2
D(+) Trehalose		0	1	0	0	0	0	2	7	5	2	3	0
Polysaccharides:													
Glycoside:													
Salicin		0	1	0	0	0	0	0	0	0	0	0	2
Polyglucosides:													
Glycogen		0	1	0	0	0	0	1	0	0	0	0	0
Inulin		0	1	0	0	0	0	0	0	0	0	0	0
Trisaccharide:													
D(+) Melezitose		0	1	0	0	0	0	0	2	0	1	0	2
Sugar alcohols:													
Hexitols:													
D-Mannitol		1	1	1	1	1	1	0	3	1	1	1	2
D-Sorbitol		0	0	0	0	0	0	1	0	0	0	0	0
Tetritols and pentitols:													
Adonitol		1	0	1	1	0	0	0	0	0	0	0	2
D(+) Arabitol		1	0	1	1	1	1	0	0	0	0	0	2
Xylitol		0	0	0	1	0	0	0	0	0	0	0	1
(ii) Sole carbon sources at 0.1%, w/v or v/v													
Alcohols:													
Dihydric:													
Butane-1,4-diol		0	1	0	0	0	0	0	0	0	0	0	0
Polyhydric:													
Glycerol		1	1	1	1	1	1	3	7	0	2	3	2
Aliphatic amino acid:													
L-Aspartate		0	1	0	0	0	0	0	0	0	0	0	2
Aromatic/heterocyclic acids:													
Benzoic acid (Na salt)		1	1	1	1	0	1	1	0	1	0	0	1
m-Hydroxybenzoic acid		0	1	0	0	0	0	0	0	0	0	0	1
p-Hydroxybenzoic acid		1	1	1	1	1	1	3	7	0	2	0	2

Table 4 Continued

Unit character								Clusters					
								1	2	3	4	5	6
		G.aichiensis	*G.bronchialis*	*G.obuensis*	*G.rubropertincta*	*G.sputi*	*G.terrae*	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*G.amarae* *G.hydrophobica*
	Number of strains/strain number	N934^T	N654^T	N935^T	N4^T	N930^T	N659^T	3	7	5	2	3	2
Carboxylic acid:													
Valeric acid		1	1	1	0	1	1	0	0	0	0	0	2
Dicarboxylic acids:													
Adipic acid		1	1	1	1	1	1	0	0	0	0	3	2
Fumaric acid (Na salt)		1	1	1	1	1	1	1	7	5	2	3	2
Malonic acid (Na salt)		1	0	1	1	0	1	0	0	0	0	0	2
Pimelic acid		1	0	1	1	1	1	0	0	0	0	0	2
Sebacic acid		0	0	0	0	0	0	0	0	0	0	0	1
Suberic acid		1	1	1	1	1	1	0	0	0	0	3	2
Succinic acid (Na salt)		1	1	1	1	1	0	3	7	0	2	3	2
Hydroxy acids:													
Aliphatic:													
Citric acid (Na salt)		0	1	1	1	0	1	1	7	5	2	3	2
Lactic acid (Na salt)		0	1	1	1	0	1	1	7	0	2	0	2
Malic acid (Na salt)		1	1	1	1	1	1	1	1	0	1	1	2
Miscellaneous nitrogenous compound													
Betaine		1	0	1	0	0	1	0	0	0	0	0	2
Sterol:													
Testosterone		0	1	0	0	0	0	1	7	0	2	3	0
(d) TOLERANCE TESTS													
(i) Growth at:													
4°C		1	0	0	0	0	1	0	0	0	0	0	0
37°C		0	1	1	1	1	1	1	2	4	1	0	2
55°C		0	0	0	0	0	0	1	0	0	0	0	0
(ii) Growth at:													
pH 4.5		0	0	1	0	1	1	0	1	1	0	0	2
pH 5.0		1	1	1	1	1	1	1	1	1	0	2	2
pH 5.5		1	1	1	1	1	1	2	0	0	2	3	2
pH 10.0		1	0	1	1	1	1	3	6	5	2	1	2
(iii) Resistance to antibiotics (μg ml^{-1})													
Aminoglycosides and aminocyclitols:													
Amikacin (5)		1	1	0	1	0	1	0	0	0	2	0	2
Bekanamycin sulphate (32)		1	1	0	0	0	0	0	1	0	0	0	2
Gentamycin sulphate (4)		1	1	0	1	1	1	0	1	0	2	3	2
Gentamycin sulphate (8)		1	1	0	0	0	1	0	1	0	0	0	2
Gentamycin sulphate (16)		1	1	0	0	0	0	0	0	0	0	0	2
Kanamycin sulphate (10)		1	1	0	1	1	1	0	0	0	2	0	2
Lividomycin sulphate (32)		0	1	0	0	1	1	2	0	0	0	0	2
Neomycin sulphate (4)		1	1	1	1	1	1	0	1	3	2	3	2
Neomycin sulphate (8)		1	1	0	1	1	1	0	0	0	1	0	2
Paromomycin sulphate (32)		0	1	0	0	0	1	0	0	0	0	0	2
Streptomycin sulphate (4)		1	1	1	0	1	1	2	7	5	2	3	2
Tobramycin sulphate (4)		1	1	0	1	1	1	0	0	0	2	0	2
Tobramycin sulphate (8)		1	1	0	1	0	1	0	0	0	2	0	2
Antifungal agents:													
Fusidic acid (Na salt) (100)		0	0	0	0	0	0	0	1	0	0	0	0
5-Fluorouracil (20)		1	1	1	1	1	1	3	1	0	2	3	2
5-Fluorouracil (40)		1	1	1	1	1	1	1	1	0	2	3	2

Table 4 Continued

Unit character								Clusters					
								1	2	3	4	5	6
		G.aichiensis	*G.bronchialis*	*G.obuensis*	*G.rubropertincta*	*G.sputi*	*G.terrae*	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*G.amarae* *G.hydrophobica*
	Number of strains/strain number	N934^T	N654^T	N935^T	N4^T	N930^T	N659^T	3	7	5	2	3	2
Antitubercular drugs:													
Ethambutol (5)		1	1	1	0	1	1	3	7	5	2	3	2
Ethambutol (10)		0	1	1	0	1	1	3	7	5	2	3	2
Isoniazid (10)		1	0	1	1	1	1	3	7	5	2	3	2
Isoniazid (40)		1	0	1	1	1	1	3	7	5	2	3	2
Isoniazid (160)		1	0	1	1	1	1	3	7	5	2	3	2
Isoniazid (200)		1	0	1	1	1	1	3	7	5	2	3	2
Cephalosporins:													
Cefoxitin (Na salt) (16)		1	1	1	1	1	1	0	0	0	0	3	2
Cephaloridine (100)		0	1	0	0	0	1	0	0	0	0	0	2
Cephradine (32)		1	1	1	1	1	1	0	1	0	2	3	2
Cephapirin (Na salt) (32)		1	1	0	0	1	1	0	0	0	0	3	2
Glycopeptides and peptides:													
Bacitracin (32)		1	0	0	1	1	1	0	1	0	2	3	2
Vancomycin (2)		1	1	1	0	1	1	0	0	0	0	0	2
Vancomycin (4)		0	1	1	0	1	1	0	0	0	0	0	2
Vancomycin (16)		0	1	0	0	1	1	0	0	0	0	0	2
Vancomycin (2) + Erythromycin (6)		0	1	0	0	1	1	0	0	0	0	0	2
Vancomycin (4) + Erythromycin (12)		0	1	0	0	1	1	0	0	0	0	0	2
Vancomycin (8) + Erythromycin (24)		0	1	0	0	0	1	0	0	0	0	0	2
Macrolides:													
Erythromycin (5)		1	1	1	1	1	1	3	7	5	2	0	2
Erythromycin (10)		1	1	1	1	1	1	3	7	5	2	0	2
Oleandomycin phosphate (32)		1	1	1	1	1	1	3	7	5	2	0	2
Speramycin (16)		0	1	1	1	1	1	0	1	0	2	3	2
Penicillins:													
Amoxicillin (500)		1	1	1	0	1	1	0	1	2	1	3	2
Ampicillin (10)		1	1	0	0	1	1	0	0	0	1	3	2
Ampicillin (20)		1	1	0	0	1	1	0	0	0	0	0	2
Carbenicillin (Na salt) (16)		1	1	1	1	1	1	0	1	0	2	3	2
Carbenicillin (Na salt) (32)		1	1	1	1	1	1	0	0	0	2	3	2
Methicillin (32)		1	1	1	1	1	1	3	6	5	2	3	2
Penicillin G (16)		1	1	0	0	1	1	1	1	0	1	3	2
Penicillin G (32)		1	1	0	0	1	1	0	0	0	0	1	2
Ticarcillin (16)		1	1	1	1	1	1	0	0	0	2	3	2
Rifampicins:													
Carbomycin (32)		0	1	0	0	0	1	0	0	0	0	0	2
Rifampicin (2)		1	1	1	0	1	1	0	0	0	0	0	2
Rifampicin (4)		1	1	1	0	1	0	0	0	0	0	0	2
Rifampicin (8)		0	1	1	0	1	0	0	0	0	0	0	2
Tetracyclines:													
Chlortetracycline hydrochloride (4)		1	0	0	0	0	0	0	0	0	0	3	2
Chlortetracycline hydrochloride (8)		0	0	0	0	0	0	0	0	0	0	2	2
Methacycline hydrochloride (8)		0	0	1	0	1	1	0	0	0	0	2	2
Tetracycline (5)		0	0	0	0	0	0	0	0	0	0	3	2
Tetracycline (10)		0	0	0	0	0	0	0	0	0	0	3	2
Tetracycline (20)		0	0	0	0	0	0	0	0	0	0	0	2
Miscellaneous:													
Chloramphenicol (8)		1	1	1	0	1	1	0	0	0	0	0	2
Chloramphenicol (32)		0	1	0	0	1	1	0	0	0	0	0	2
Tyrothricin (32)		1	0	1	0	1	1	0	0	0	1	2	2

Table 4 Continued

Unit character							Clusters					
							1	2	3	4	5	6
	G.aichiensis	*G.bronchialis*	*G.obuensis*	*G.rubropertincta*	*G.sputi*	*G.terrae*	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*Gordona* sp	*G.amarae* *G.hydrophobica*
Number of strains/strain number	N934^T	N654^T	N935^T	N4^T	N930^T	N659^T	3	7	5	2	3	2
(iv) Growth in the presence of chemical inhibitors (% w/v or v/v)												
Malachite green (0.001)	1	1	1	1	1	1	3	0	3	1	1	2
Phenol (0.1)	1	0	1	1	1	1	3	7	5	2	3	2
Phenolphthalein diphosphate (0.001)	1	0	1	1	1	1	3	7	5	2	3	2
Tetrazolium salt (0.01)	1	1	1	1	1	1	2	1	0	2	3	2
Tetrazolium salt (0.05)	1	1	1	0	1	1	0	0	0	0	0	2
Thallium acetate (0.01)	0	1	1	0	1	1	0	0	0	0	0	1
Toluidine blue (0.005)	1	1	1	1	1	1	3	1	0	2	3	2
(v) Growth in the presence of metal salts (% w/v or v/v)												
Cobalt chloride (0.005)	1	1	1	1	1	1	3	0	1	2	3	2
Cobalt chloride (0.01)	1	1	1	1	1	1	3	0	0	2	3	2
Ferrous sulphate (0.05)	1	1	1	1	1	1	2	0	0	2	3	2
Ferrous sulphate (0.1)	1	1	1	1	1	1	1	0	0	0	3	2
Sodium chloride (5.0)	1	1	1	1	1	1	0	2	1	0	0	2
Sodium chloride (7.0)	0	0	1	0	1	1	0	0	1	0	0	2
Sodium selenite (0.05)	1	1	1	1	1	1	1	5	1	2	0	2
Zinc chloride (0.01)	1	1	1	1	1	1	1	0	0	1	3	2
Zinc sulphate (0.05)	1	1	1	1	1	1	0	0	0	2	3	2

*Properties of isolates forming single-membered clusters are omitted.
All of the strains were positive for the following characters: catalase; D(−) fructose (1.0%, w/v), D(+) glucose (1.0%, w/v), D(+) mannose (1.0%, w/v), sucrose (1.0%, w/v), acetic acid (Na salt; 0.1%, w/v), butan-1-ol (0.1%, v/v), butyric acid (Na salt; 0.1%, w/v), pyruvic acid (Na salt; 0.1%, w/v) as sole carbon sources; growth at pH 6.0; resistance to amikacin (1 μg ml^{-1}), nalidixic acid (32 μg ml^{-1}), novobiocin (Na salt; 4 μg ml^{-1}), oleandomycin phosphate (1 and 16 μg ml^{-1}), polymixin B sulphate (32 μg ml^{-1}) and streptomycin sulphate (2 μg ml^{-1}), and growth in the presence of bismuth citrate (0.01%, v/w), cobalt chloride (0.0005%, w/v), cupric chloride (0.01%, w/v), oleic acid (0.1%, w/v), sodium chloride (10.0%, w/v), sodium selenite (0.1%, w/v), teepol (0.01%, v/v) and zinc chloride (0.001% and 0.005%, w/v).
All of the strains were negative for the following characters: gelatin hydrolysis (4.0%, w/v), nitrite reduction, xanthine degradation (0.4%, w/v); butane-1,3-diol (0.1%, v/v), D-mandelic acid (0.1%, w/v) sorbitol (1.0%, w/v), tartaric acid (Na salt; 0.1%, w/v) and vanillic acid (0.1%, w/v) as sole carbon sources; growth at 42°C and resistance to chloramphenicol (64 μg ml^{-1}), chlortetracycline hydrochloride (16 μg ml^{-1}), methacycline hydrochloride (32 μg ml^{-1}) and oxytetracycline hydrochloride (32 and 64 μg ml^{-1}).

the extent of taxonomic diversity found amongst the isolated mycolic acid-containing microflora [6,12,37,52,56]. In the present investigation, actinomycetes isolated from foams and scums taken from three activated-sludge sewage-treatment plants were presumptively identified as members of the genera *Gordona*, *Rhodococcus* and *Tsukamurella* on the basis of colony morphology and pigmentation. Representatives of the *Gordona* strains were found to contain mycolic acids which co-chromatographed with those from the type strain of *Gordona bronchialis* and predominant amounts of dihydrogenated menaquinones with nine isoprene units. This combination of properties is consistent with the organisms being assigned to the genus *Gordona* [31].

Large numbers of gordonae were isolated from foam and scum samples taken from the three activated-sludge sewage-treatment plants. A correlation was observed between the extent of foaming and the numbers of gordonae. The highest count, $0.14 \times 10^8 \pm 1.81$ ml^{-1} foam, was recorded for a sample collected in July at Stoke Bardolph (data not shown), when foaming was most extensive, and when gordonae accounted for 40% of the mean total viable count. Relatively large numbers of presumptive rhodococci were found in foam samples taken from Stoke Badolph in April and July whereas tsukamurellae were only detected in small numbers in foam samples collected from the Stoke Bardolph plant in July. Nocardiae were not isolated from any of the Stoke Bardolph samples. The failure to isolate significant numbers of actinomycetes from the inlet samples taken at the Milcote and Wanlip sites suggests that mycolic acid-containing actinomycetes form part of the resident flora of activated sludge.

The numerical taxonomic groupings of the representative gordonae were only marginally affected by the statistics used or by the test error (*P*) of 3.92%. Experimental test error of this nature is comparable to that found in previous

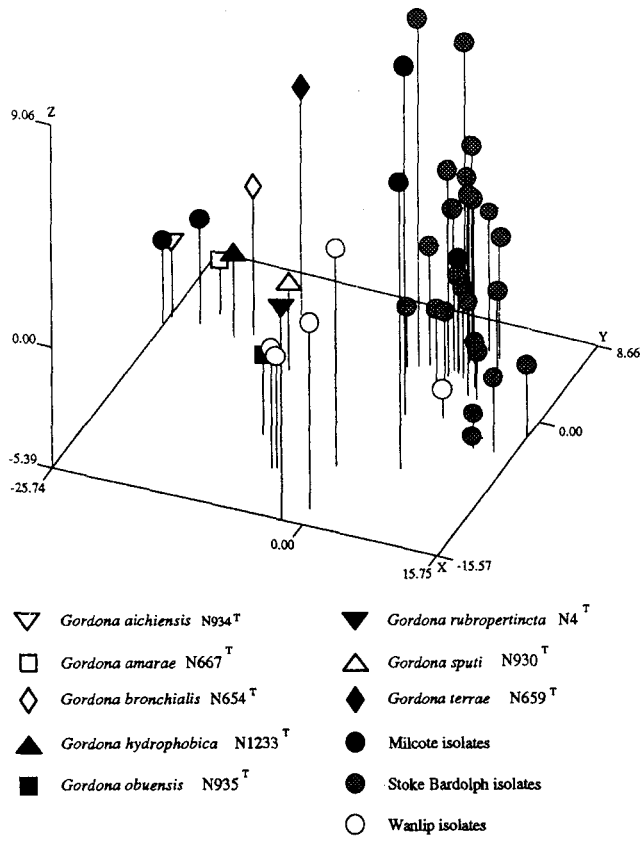

Figure 2 Three-dimensional representation of relationships between 39 isolates from foam and the *Gordona* type strains based on the first three canonical variates of the principal component-canonical variate analysis. The first three axes account for 90.90%, 3.59% and 1.81% of the total variation between strains, respectively.

numerical phenetic surveys involving gordonae [20,22,24] and is well within the 10% guideline recommended by Sneath and Johnson [58]. It can, therefore, be concluded that the failure of the isolates to show high overall similarities with the representatives of the validly described species of *Gordona* is taxonomically meaningful. It is also evident that the *Gordona* isolates form a diverse group of organisms with some strains only associated with a particular plant. However, further comparative taxonomic studies, involving nucleic acid relatedness and sequencing analyses, are needed to establish whether the multi- and single-membered clusters composed of *Gordona* isolates merit species recognition.

Clusters defined in numerical taxonomic surveys are 'operator unbiased' representations of natural relationships between strains though group composition may be influenced by the choice of strains and tests, experimental procedures, test error and statistics [51]. It is, therefore, essential to evaluate the taxonomic integrity of clusters by examining representative strains using independent taxonomic criteria derived from the application of chemotaxonomic and/or molecular systematic techniques. Curie-point pyrolysis mass spectrometry provides a rapid and effective way of evaluating relationships established in numerical taxonomic studies [25].

In the present study good congruence was found between the classifications derived from the application of the numerical taxonomic and PyMS methods. In particular, the PyMS data underpin the separation between the gordonae isolated from the foam and scum samples and the type strains of the validly described species of *Gordona*. They also help to confirm the heterogeneity found amongst the *Gordona* isolates, especially their assignment to multimembered clusters. It is also interesting, though surprising, that the type strains of *Gordona amarae* and *Gordona hydrophobica* were closely related in each of the analyses; these strains have been distinguished on the basis of 16S rRNA sequence data [4].

The results of the present investigation provide further evidence that some members of the genus *Gordona* cause or are associated with foaming in activated-sludge sewage-treatment plants [5,7,12,36,52]. It is also clear from the chemical and numerical data that the gordonae isolated from the foam and scum samples taken from Milcote, Stoke Bardolph and Wanlip plants form several new centres of taxonomic variation. An improved understanding of the taxonomy, and subsequently the biology, of these and related mycolic acid-containing actinomycetes in activated sludge will help to promote effective strategies for controlling their growth in activated-sludge sewage-treatment plants.

The large discrepancies found between the viable plate and total direct bacterial counts provide a further reminder that culture-based techniques can grossly underestimate bacterial diversity in complex microbial communities [1,66]. Unexpectedly high levels of microbial diversity have been detected in several natural microbial communities by comparative 16S rRNA sequence analysis [16,43,45]. The great advantage of rRNA analysis and rRNA-based hybridisation probes is that microorganisms present in natural communities can be detected and characterised without prior culture. These molecular methods were used by Schuppler *et al* [54] who discovered several novel gordonae, mycobacteria and rhodococci in sludge taken from a normally functioning municipal wastewater treatment plant. In parallel experiments they demonstrated that cultured isolates only represented a small fraction of the organisms present in the activated sludge.

The results of the present study provide further evidence that there is immense microbial diversity in activated sludge [54,65,66] and thereby cast doubt on the view that foaming can be attributed to members of one or a few species [5,6,7,12,30,40,52,62]. It seems more likely that foaming is caused by a diverse array of mycolic acid-containing actinomycetes some which may be site specific. It is also clear that little meaningful ecological work can be carried out until the taxonomy of the foaming organisms has been determined. Further extensive studies are required to unravel the full extent of the taxonomic variation encompassed by mycolic acid-containing actinomycetes associated with or causing foaming in activated-sludge sewage-treatment plants. Such studies should employ a two-pronged strategy, one approach making use of molecular ecological methods and the other based upon selective isolation and rapid characterisation procedures.

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