

Impact of cultivation on characterisation of species composition of soil bacterial communities

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Abstract

The species composition of culturable bacteria in Scottish grassland soils was investigated using a combination of Biolog and 16S rDNA analysis for characterisation of isolates. The inclusion of a molecular approach allowed direct comparison of sequences from culturable bacteria with sequences obtained during analysis of DNA extracted directly from the same soil samples. Bacterial strains were isolated on *Pseudomonas* isolation agar (PIA), a selective medium, and on tryptone soya agar (TSA), a general laboratory medium. In total, 12 and 21 morphologically different bacterial cultures were isolated on PIA and TSA, respectively. Biolog and sequencing placed PIA isolates in the same taxonomic groups, the majority of cultures belonging to the *Pseudomonas* (sensu stricto) group. However, analysis of 16S rDNA sequences proved more efficient than Biolog for characterising TSA isolates due to limitations of the Microlog database for identifying environmental bacteria. In general, 16S rDNA sequences from TSA isolates showed high similarities to cultured species represented in sequence databases, although TSA-8 showed only 92.5% similarity to the nearest relative, *Bacillus insolitus*. In general, there was very little overlap between the culturable and uncultured bacterial communities, although two sequences, PIA-2 and TSA-13, showed >99% similarity to soil clones. A cloning step was included prior to sequence analysis of two isolates, TSA-5 and TSA-14, and analysis of several clones confirmed that these cultures comprised at least four and three sequence types, respectively. All isolate clones were most closely related to uncultured bacteria, with clone TSA-5.1 showing 99.8% similarity to a sequence amplified directly from the same soil sample. Interestingly, one clone, TSA-5.4, clustered within a novel group comprising only uncultured sequences. This group, which is associated with the novel, deep-branching *Acidobacterium capsulatum* lineage, also included clones isolated during direct analysis of the same soil and from a wide range of other sample types studied elsewhere. The study demonstrates the value of fine-scale molecular analysis for identification of laboratory isolates and indicates the culturability of approximately 1% of the total population but under a restricted range of media and cultivation conditions. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Microbial diversity in natural environments has been studied traditionally by the cultivation of bacteria on laboratory media and the subsequent characterisation of pure isolates. This approach has several limitations, including selective enrichment of bacteria that grow efficiently on the media and under the cultivation conditions used, insufficient information on the cultivation requirements of most bacteria and the inability to recover starving, dormant and

viable but non-culturable cells. Torsvik et al. [1] using microscopic techniques demonstrated that, while the majority of bacteria in soil samples are actively respiring, only about 1% of the total number of cells can be cultured on laboratory media. In addition, it is generally accepted that less than 1% of bacterial species have been isolated and characterised [1–4].

Advances in molecular ecology, in particular development of 16S rRNA-based methodologies, enable cultivation-independent analysis of bacterial community composition through the detection and characterisation of bacterial nucleic acid sequences within environmental samples [4–6]. The most common approach involves polymerase chain reaction (PCR) amplification, cloning and sequence analysis of 16S rRNA genes and has been used

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to investigate the diversity and community composition of a range of terrestrial environments ([7–14] and others). These studies have produced similar findings despite the soils having different physical and chemical properties, geographical locations and climates and application of different DNA extraction regimes and PCR primers. Firstly, all of the soil bacterial communities studied so far are extremely diverse. Secondly, the majority of sequences in clone libraries are different from sequences from cultured bacterial species represented on the databases, with identical clone and culture sequences occurring very rarely. Finally, a significant number of clone sequences, from around 10% [11,15] to 64% [9] of the libraries analysed in these studies, belong to novel bacterial clades that do not currently have cultured representatives or that are only distantly related to culturable species.

Increasingly, bacterial community analyses concentrate exclusively on molecular data, as this approach is considered to allow analysis of a considerably larger section of the community than cultivation-based techniques. Sequence databases consequently contain much data from such studies but, in contrast, comparatively few culture-based studies have employed sequencing. While it is clear that culturable methods impose selection on the types of bacteria recovered, it is important to note that considerable bias may also be inherent to molecular methodologies, for example, through differences in cell lysis efficiencies, PCR primer bias and variable rRNA or rDNA copy numbers. The relative importance of the culturable population within the total community is an important question in microbial ecology but few studies have attempted to address this issue by directly comparing the culturable and total bacterial communities. Øvreås and Torsvik [3] reported considerable diversity of cultured bacteria using a combination of Biolog, REP-PCR, amplified ribosomal DNA restriction analysis (ARDRA) and probe hybridisation. Differences observed between the total community diversity of organic and sandy soils, determined by reassociation of DNA isolated from the bacterial fraction of environmental samples, combined with ARDRA and denaturing gradient gel electrophoresis (DGGE) analysis, however, were significantly higher than differences in the culturable fractions of these two different soil types. In addition, using restriction fragment length polymorphism patterns of clones and isolates from four arid soils, Dunbar et al. [16] found that cloning and cultivation generally described similar relationships between communities, with both methods consistently indicating that one environment was distinct from the other three. Significant differences, however, were also apparent between the cloning and cultivation data. While both of these studies demonstrated significant differences between culturable and uncultured communities, the relative importance of each fraction remains unclear.

Land use in the UK has undergone considerable change over the last decade due to both economic and political

pressure and increasing public concern regarding the quality of the environment. This has led to more extensive grazing regimes in the uplands of Britain with a reduction in fertiliser application. While considerable information is available on the effect of extensification on the vascular plant community, the effect on soil microbes, which are central to nutrient cycling, soil fertility and plant productivity, are not understood. Plant and bacterial activity are closely linked through microbial utilisation of root exudates, dead cells and litter and soil bacterial diversity may therefore be influenced by plant diversity and community structure.

In a study of soil microbial communities across a range of upland grasslands, Grayston et al. [17] showed that soil microbial biomass was highest in unimproved, extensive grasslands and lowest in improved, intensively managed grasslands. Accompanying these differences in biomass, microbial community structure, measured by phospholipid fatty acid profiling, community level physiological profiles (CLPP) and plating, shifted from one dominated by fungi in unimproved grasslands to one dominated by bacteria in improved grasslands. This was attributed to differences in substrate supply between grasslands, which was reflected in differences in the CLPP of the communities. McCaig et al. [11] compared sequences of 275 clones from soils sampled from the same upland pastures and the aim of the present study was to compare the composition of the 16S rDNA libraries in McCaig et al. [11] to culturable bacteria isolated from the same soil samples. Isolates were obtained on both selective and non-selective media and were characterised using 16S rDNA sequence analysis, to allow direct comparison of clones and cultures, and using Biolog, to provide information on the physiology of isolates. Use of sequence analysis, rather than DNA fingerprinting techniques, provided greater discrimination than earlier studies and better characterisation of clones and isolates.

2. Materials and methods

2.1. Sample collection

Rhizosphere soil samples from three characteristic grassland types, designated unimproved, semi-improved and improved (U4a, U4b and MG6 of the National Vegetation Classification, respectively) [18], were collected from Sourhope Research Station (55°28'30"N/2°14'W) in the Borders Region, Scotland, as part of the MICRONET programme [17]. Grassland and soil characteristics are described elsewhere [17]. At each sampling site, three 5 × 5-m quadrats were randomly located and 50 cores (diameter, 3.5 cm; depth, 5 cm) were collected, combined and sieved (mesh size, < 2 mm) to remove plant material. Due to the density of the grass root systems, all soil was assumed to be in contact with plant roots and was considered rhizo-

sphere. Subsamples of soil used for molecular analyses were stored at -70°C .

2.2. Isolation of bacterial strains

Soil samples (10 g) were shaken in 100 ml 1/4-strength Ringer's solution (Oxoid, Basingstoke, UK) for 10 min to extract rhizosphere microorganisms. Soil slurries were then serially diluted in 1/4-strength Ringer's solution and suspensions (0.1 ml) spread, in duplicate, on 1/10th strength tryptone soya agar (TSA; Oxoid) plus cycloheximide (50 mg l^{-1}) and *Pseudomonas* isolation agar (PIA; Oxoid). TSA was selected as it supports growth of a wide range of soil bacteria, and PIA, a selective medium, was used because stimulation of growth of pseudomonads in the rhizosphere has been demonstrated by both phenotypic [19] and molecular techniques [20]. Cultures were incubated at 25°C for 21 days and, on the basis of pigment, shape, size, surface texture and, opacity, morphologically distinct colonies were isolated by the transfer of single colonies to the agar medium from which they were obtained. Isolates were examined for purity by microscopy, Gram-stained and characterised using the Biolog[®] system [21] and 16S rDNA sequence analysis (see below).

2.3. Biolog analysis

Isolates were grown on appropriate Biolog agar media, BUGM (Biolog Universal Growth Medium [21] for Gram-negative (GN) and BUGM plus 1% (w/v) glucose for Gram-positive (GP) bacteria, for 18 h to ensure viability and metabolic vigour. One to two colonies were then removed from the plates, suspended in tubes of sterile 0.85% (w/v) NaCl solution (20 ml) and the turbidity measured using a turbidimeter (Biolog, Hayward, CA, USA). With reference to the Biolog GN and GP turbidity standards (Biolog, Hayward, CA, USA), the cell density was adjusted to approximately $3\text{--}4.5 \times 10^8$ cells ml^{-1} for the GN and GP isolates, respectively, by addition of sterile saline or further colonies. Biolog GN or GP plates (Biolog, Hayward, CA, USA), as appropriate, were then immediately inoculated with 150 μl of cell suspension per well. The microplates were incubated at 25°C and colour development measured as optical density at 590 nm using a microplate reader (Emax, Molecular Devices, Oxford, UK) after incubation for 4, 24 and 48 h. MicroLog software (Biolog, Hayward, CA, USA) was used to identify cultures based on their metabolic profiles.

2.4. PCR amplification, cloning and sequencing

DNA was extracted from bacterial cultures using either method A (T.M. Embley, personal communication), a modification of the small-scale extraction method of Pitcher et al. [22], or method B, involving lysis with glass beads

in the presence of phenol. For method A, a loopful of each bacterial isolate was suspended in a fresh solution of 50 mg ml^{-1} lysozyme in TE buffer (100 mM Tris-HCl, 10 mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, pH 8) by vortexing. After incubation at 37°C for 15 min, 450 μl of GE reagent (5 M guanidine thiocyanate, 0.1 M $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, pH 8) was added and mixed by vortexing. Lysed cells were mixed with an equal volume of ice-cold 7.5 M ammonium acetate and incubated on ice for a further 15 min, followed by two chloroform/isoamyl alcohol (24:1) extractions. DNA in the final aqueous phase was precipitated with propan-2-ol and pellets were washed twice with 70% ethanol, air-dried and resuspended in 50 μl of sterile double deionised water. Method B, although adopted initially for those bacterial cultures that did not lyse efficiently with method A, was found to be simpler and faster for routine extraction from all bacterial isolates. A loopful of bacterial cells from each culture was added to a 2-ml screw-capped tube containing 200 μl 1 M Tris-HCl (pH 8), 200 μl liquified phenol (in Tris buffer) and ca. 0.1 g Ribolyser[™] matrix (Hybaid, Teddington, UK). Tubes were vibrated in a Hybaid Ribolyser[™] Cell Disrupter (Hybaid) for 5 s at 4.0 m s^{-1} , chilled on ice for 1 min and centrifuged in a microfuge for 10 min at $11\,000 \times g$. Two chloroform/isoamyl alcohol extractions were carried out. DNA was precipitated with sodium acetate and ethanol pellets were washed twice in 70% ethanol, dried and resuspended in 100 μl sterile water. DNA yields and quality for both methods were assessed by standard electrophoresis through a 1% (w/v) ethidium bromide-stained agarose gel.

PCR amplification of bacterial 16S rRNA genes was carried out using the primers Bf [23] and 1541r [24]. Amplification was performed in a total reaction volume of 50 μl , containing 1 μl of undiluted DNA (or 1 μl of a 10^{-1} dilution), 1 U of *Taq* DNA polymerase (Promega UK, Southampton, UK), an appropriate dilution of manufacturer's buffer, 250 μM dNTPs and 0.4 μM primers. Thirty cycles of amplification were carried out on a Hybaid Omne-E Thermal Cycler (Hybaid) as follows: 95°C for 10 min, 50°C for 1 min, 72°C for 2 min (one cycle); 94°C for 30 s, 50°C for 30 s, 72°C for 2 min (nine cycles); 92°C for 30 s, 50°C for 30 s, 72°C for 2 min 30 s (20 cycles); followed by a final incubation at 72°C for 10 min. Products were visualised on an ethidium bromide-stained 1% (w/v) agarose gel and were sequenced using the Thermo Sequenase cycle sequencing kit (Amersham International, Slough, UK), or using an automated sequencing facility (NCIMB, Aberdeen, UK), using the primers 537r [24]. Products from TSA-5 and TSA-14, which consistently produced unreadable sequence, were purified using 500- μl Vivaspinn concentrators (Vivascience Limited, Binbrook, Lincoln, UK) and cloned using the pGEM[®]-T Easy Vector System I (Promega UK, Southampton, UK) and Epicurian Coli[®] XL1-Blue MRF' Kan supercompetent *Escherichia coli* (Stratagene, Cambridge, UK). Positive clones were identified by direct PCR from white colonies using the vector primers

Table 1
Identification of cultures isolated on PIA using 16S rDNA sequence analysis and Biolog

Isolate No.	16S rDNA sequence analysis			Biolog	
	nearest relative in GenBank	phylogenetic group	% similarity	nearest relative in MicroLog database ^a	% similarity
PIA-1	<i>Pseudomonas</i> sp. NKB1	γ-proteobacteria	99.4	<i>Pseudomonas fragi</i>	86.7
PIA-2	<i>Pseudomonas</i> sp. NKB1	γ-proteobacteria	99.1	<i>Pseudomonas tolaasii</i>	81.7
PIA-3	<i>Pseudomonas</i> clone NB1-f	γ-proteobacteria	99.5	NA	
PIA-4	<i>Bordetella</i> sp. KP22	β-proteobacteria	99.2	<i>Alcaligenes xylosoxydans</i>	85.5
PIA-5	<i>Pseudomonas</i> sp. NKB1	γ-proteobacteria	97.7	<i>Pseudomonas tolaasii</i>	64.2
PIA-6	<i>Pseudomonas</i> clone NB1-f	γ-proteobacteria	100.0	<i>Pseudomonas fluorescens</i> type A	74.3
PIA-7	<i>Pseudomonas veronii</i>	γ-proteobacteria	99.5	<i>Pseudomonas corrugata</i>	59.5
PIA-8	<i>Pseudomonas</i> clone NB0.1-H	γ-proteobacteria	99.8	<i>Pseudomonas fluorescens</i> type C	78.8
PIA-9	<i>Pseudomonas veronii</i>	γ-proteobacteria	99.4	<i>Pseudomonas tolaasii</i>	51.6
PIA-10	<i>Pseudomonas</i> clone NB1-f	γ-proteobacteria	100.0	<i>Pseudomonas corrugata</i>	55.8
PIA-11	<i>Pseudomonas</i> sp. NKB1	γ-proteobacteria	97.9	<i>Pseudomonas marginalis</i>	54.6
PIA-12	Uncultured <i>Drosophila</i> pathogen	γ-proteobacteria	97.3	<i>Enterobacter gergoviae</i>	57.2

Nearest relatives on GenBank and MicroLog databases and percentage similarities are listed.

^aNA, not available; isolates show < 50% similarity to reference species on MicroLog database.

SP6 and T7 and eight products for each isolate sequenced as above. The complete sequence of clone TSA-5.4 was obtained using the primers pC*, pD*, pE, pE* and pF* [25]. Sequences have been deposited in GenBank under the consecutive accession numbers AF240117 to AF240153.

2.5. Data analysis

Dendrograms were produced for Biolog data to show the similarities between bacteria based on their metabolic profiles using Genstat 5.3 (NAG Ltd, Oxford, UK). Partial 16S rDNA sequences (300–350 bp) and near full-

Table 2
Identification of cultures isolated on TSA using 16S rDNA sequence analysis and Biolog

Isolate No. ^a	16S rDNA sequence analysis			Biolog	
	nearest relative in GenBank	phylogenetic group	% similarity	nearest relative in MicroLog database ^b	% similarity
TSA-1	<i>Staphylococcus arlettae</i>	Gram +ve	98.6	<i>Bacillus megaterium</i>	88.9
TSA-2	Soil clone LRE9	γ-proteobacteria	96.1	NA	
TSA-3	<i>Mycobacterium</i> sp. FI-25796	actinomycete	96.3	NA	
TSA-4	<i>Streptomyces griseus</i>	actinomycete	98.4	NA	
TSA-5	mixed culture			NA	
TSA-7	<i>Cellulomonas turbata</i>	actinomycete	100.0	<i>Cellulomonas turbata</i>	64.0
TSA-8	<i>Bacillus insolitus</i>	Gram +ve	92.5	<i>Bacillus brevis</i>	83.3
TSA-9	<i>Streptomyces griseus</i>	actinomycete	98.6	<i>Bacillus brevis</i>	83.3
TSA-10	<i>Rhodococcus opacus</i>	actinomycete	98.0	<i>Actinobacter</i> genospecies 9	59.8
TSA-11	<i>Methylobacterium</i> sp.	α-proteobacteria	98.1	<i>Vibrio tubiashi</i>	77.2
TSA-12	<i>Arthrobacter globiformis</i>	actinomycete	94.3	<i>Bacillus brevis</i>	54.5
TSA-14	mixed culture			NA	
TSA-13	<i>Pseudomonas pictorum</i> .	γ-proteobacteria	98.1	NA	
TSA-15	<i>Staphylococcus arlettae</i>	Gram +ve	99.8	<i>Bacillus megaterium</i>	68.2
TSA-16	<i>Xanthomonas sacchari</i>	γ-proteobacteria	98.5	NA	
TSA-17	<i>Rhodococcus erythropolis</i>	actinomycete	98.3	NA	
TSA-18	<i>Zoogloea ramigera</i>	α-proteobacteria	95.8	NA	
TSA-19	<i>Bacillus macroides</i>	Gram +ve	99.1	NA	
TSA-20	<i>Janthinobacterium lividum</i>	β-proteobacteria	99.5	<i>Janthinobacterium lividum</i> A	54.3
TSA-21	<i>Streptomyces lavendulae</i>	actinomycete	99.1	<i>Bacillus megaterium</i>	88.0
TSA-5.1	Sourhope clone sl2_505	γ-proteobacteria	99.8		
TSA-5.2	Star-like microcolony	α-proteobacteria	100.0		
TSA-5.3	unidentified eubacterium	α-proteobacteria	97.9		
TSA-5.4	sludge clone 2951	<i>Acidobacterium</i>	95.6		
TSA-14.1	uncultured organism BHA7	γ-proteobacteria	91.4		
TSA-14.2	unidentified β-proteobacterium	β-proteobacteria	99.3		
TSA-14.3	uncultivated soil bacterium	α-proteobacteria	97.8		

Nearest relatives on GenBank and MicroLog databases and percentage similarities are listed.

^aSequences TSA-5.1 to 5.4 and TSA-14.1 to 14.3 represent 16S rDNA clones from mixed isolates for TSA-5 and TSA-14, respectively.

^bNA, not available; isolates show < 50% similarity to reference species on MicroLog database.

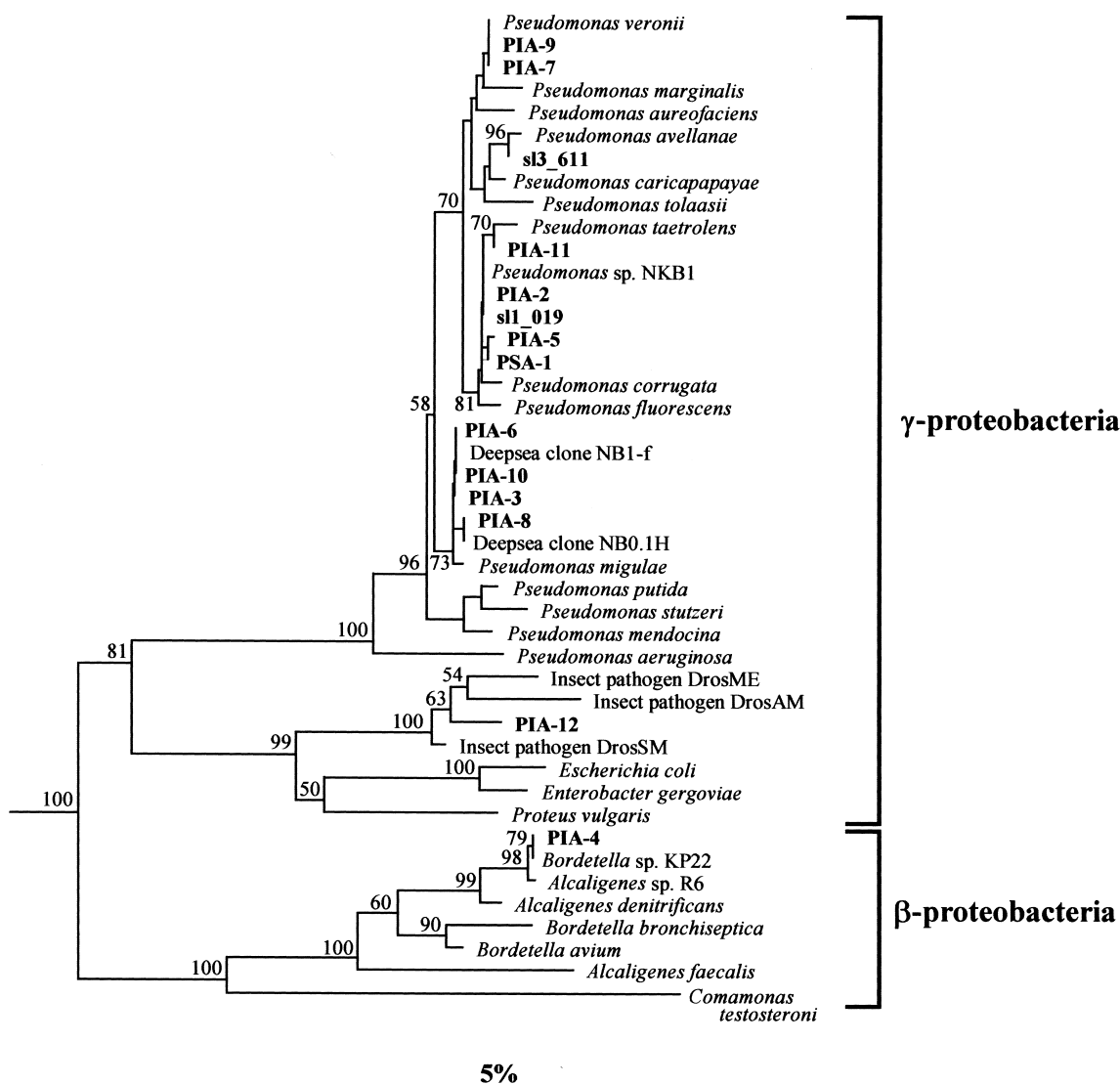


Fig. 1. Neighbor-joining tree showing the relationship of PIA isolates to reference proteobacteria and grassland rhizosphere clones obtained from the same soil samples [11]. Analysis is based on 352 bp of aligned 16S rDNA sequence data. Bootstrap values are given for nodes with > 50% support in bootstrapping analysis of 100 replicates. Isolate sequences are prefixed 'PIA'. Clone sequences obtained from grassland soil samples are designated 'saf' and 'sl' for unimproved and improved grassland, respectively. Sequences obtained during other direct analyses of environmental samples are NB1-f and NB0.1H [39] and sequences prefixed 'Dros' [40]. For convenience the tree has been pruned from a larger tree containing additional sequences from reference bacteria. The scale bar represents 5% estimated change.

length sequences (1325–1335 bp) were compared against sequences held in the Ribosomal Database Project (RDP) [26] using the SEQUENCE_SIMILARITY function and against sequences held in GenBank by FastA searches using Genetics Computer Group software [27] mounted at the Seqnet node of the BBSRC Daresbury Laboratory (Warrington, UK) or at the Human Genome Mapping Project Research Centre (Hinxton, Cambridge, UK). On the basis of results from database searches, sequences were aligned on the Genetic Data Environment, running within ARB [28], against representative bacterial sequences from the RDP and GenBank, in addition to clone sequence data obtained from the same soil samples [11]. Phylogenetic trees of short alignments were constructed by distance

matrix analysis using the Jukes and Cantor model [29] and neighbour-joining [30] in PHYLIP 3.5 [31]. A more detailed analysis of complete sequences for clones belonging to a deep-branching uncultured bacterial group was also carried out. This alignment comprised 1149 sites, which could be unambiguously aligned for all sequences including representatives from a range of phylogenetic groups. Distance matrix calculations were carried out as described above. Maximum parsimony analyses were done for 461 informative positions only and used the heuristic search option in PAUP 4.0b4a [32]. Bootstrapping (100 replicates) was used to investigate support for groups in maximum parsimony analyses, with one random addition sequence per replicate. Programs in PHYLIP 3.5 [31]

were used for bootstrapping in all distance matrix analyses.

3. Results

Bacterial isolates obtained on both selective and general laboratory media were characterised using both Biolog and 16S rDNA sequence analysis, the latter allowing comparison of isolates with cloned 16S rDNA sequences obtained from the same soil samples. In total, 12 and 21 morphologically different bacterial cultures were isolated on PIA and TSA, respectively. A single isolate, TSA-6, could not be subcultured and only 20 TSA isolates were characterised using Biolog and 16S rDNA sequencing. TSA-5 and TSA-14 were mixed cultures and an additional cloning step of 16S rDNA amplification products was included prior to sequencing. The closest relatives available on the databases for both methods are given in Tables 1 and 2 for PIA and TSA isolates, respectively, along with

the distribution of each morphotype on the different grassland types.

3.1. PIA isolates

All 12 cultures isolated on PIA were characterised using 16S rDNA analysis and 11 of these cultures were successfully analysed using Biolog. Initial subcultures of PIA-3 could not be characterised using Biolog but after several rounds of culturing this isolate clustered most closely with *Serratia marcescens*. This is a laboratory contaminant found to be present in many isolates after several subcultures, particularly in those isolates that are slow-growing. 16S rDNA analysis of the original isolate, however, indicates that PIA-3 was a *Pseudomonas* sp. (Table 1). For all other PIA isolates, Biolog and sequence analysis gave similar results. PIA isolates comprised mainly strains belonging to the genus *Pseudomonas* (sensu stricto) although two non-pseudomonads (PIA-4 and PIA-12) were also obtained, placed within the *Alcaligenes/Bordetella* group (β -proteobacteria) and the *Enterobacteraceae* (γ -proteobacteria), respectively. A phylogenetic tree showing the relationship between PIA isolates based on 16S rDNA sequence analysis and a dendrogram produced by cluster analysis of metabolic profile data from each isolate are shown in Figs. 1 and 2. While both methods placed all PIA isolates into the same genera, groupings at the species level varied. For example, PIA-7 clustered with PIA-9 when comparing sequence data but PIA-7 clustered with PIA-10 and PIA-6, and PIA-9 with PIA-5, when considering information obtained from Biolog. Sequence similarity with database sequences for most isolates was high ($\geq 97.7\%$, i.e. < 7 mismatched bases) and isolates were generally closely related to cultures sequenced previously. PIA-12, however, clustered within a group of clone sequences representing uncultured bacterial pathogens of *Drosophila paulistorum* (100% bootstrapping support) and was only 92.9% similar to *Proteus vulgaris*, the most closely related organism on the database. Other isolates, for example the cluster containing PIA-6, also shared high sequence similarity with environmental clones. In addition, the two clones sl1_019 and sl3_611, obtained during a direct analysis of the same soil samples [11], showed high similarity (99.5 and 98.3%, respectively) to isolates obtained during this study (Fig. 1).

3.2. TSA isolates

All 20 TSA isolates were characterised by 16S rDNA sequence analysis. Eighteen cultures were analysed by direct sequencing of PCR amplification products and the remaining two isolates, TSA-5 and TSA-14, produced mixed sequence and a cloning step, prior to sequencing, was included. This confirmed that TSA-5 and TSA-14 comprised at least four and three sequence types, respectively (Table 2, Figs. 3 and 4). All but one clone type (TSA-5.4, discussed below) fell within the proteobacteria

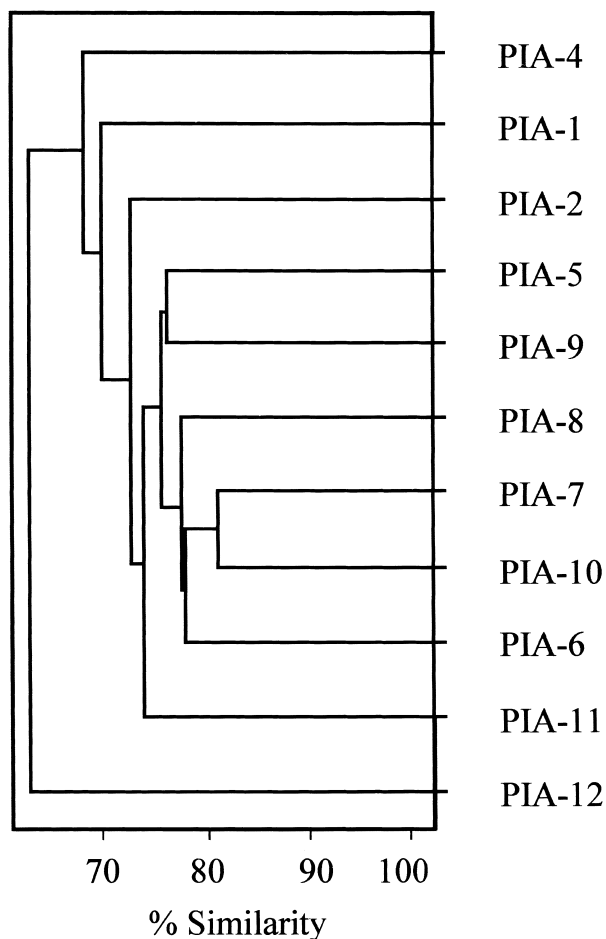


Fig. 2. Dendrogram showing clustering of PIA isolates on the basis of sole carbon source utilisation profiles [21]. Analysis is based on the utilisation of 95 different carbon sources (measured as optical density at 590 nm) contained within Biolog GN plates after 24 h incubation at 15°C.

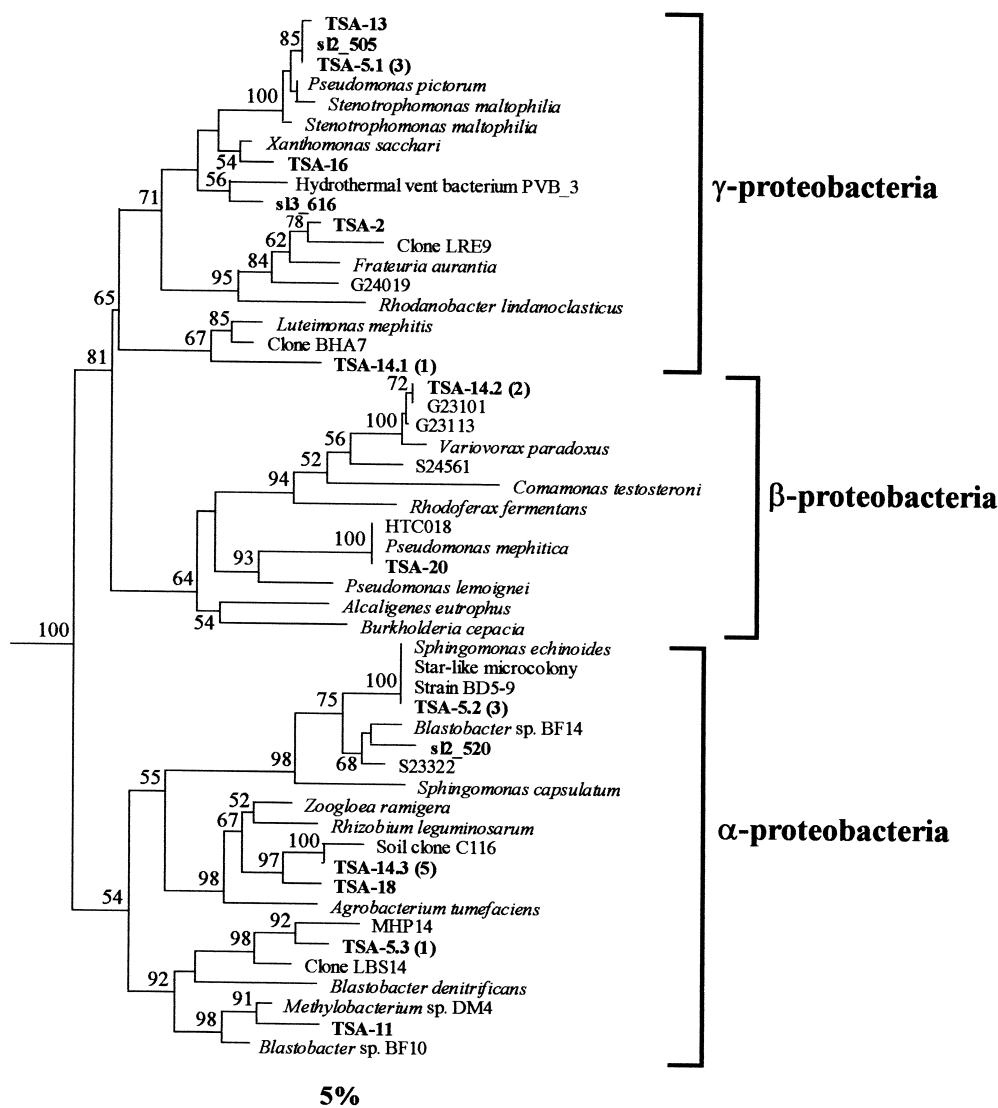


Fig. 3. Neighbor-joining tree showing the relationship of proteobacteria isolated on TSA and sequences cloned from mixed TSA isolates to reference bacteria and grassland rhizosphere clones obtained from the same soil samples [11]. Analysis is based on 302 bp of aligned 16S rDNA sequence data. Isolates obtained during this study are prefixed 'TSA'. Sequences cloned from mixed TSA isolates are prefixed 'TSA-5' or 'TSA-14'. Numbers in parentheses following these sequences indicate the number of times each clone type was observed. Sequences obtained during other direct analyses of environmental samples are PVB_3 [41], LRE9 and LBS14 [20], G24019, G23101 and G23113 (GenBank accessions AB011750, AB011735 and AB011736, respectively), HTC018 [42], star-like microcolony [43], BD5–9 [44], S24561 and S23322 [38], C116 [9] and MHP14 [45]. For other sequence nomenclature and branch labelling, see the legends to Fig. 1.

(Fig. 3). TSA-5.1 and TSA-14.3 were similar to other TSA isolates (TSA-13 and TSA-18, respectively) but all clones with the exception of TSA-5.2, were most similar to clone sequences isolated in other soil analyses or to clones isolated directly from soil in this study (e.g. TSA-5.1 and sL2_505). Interestingly, a single clone, TSA-5.4 clustered with high bootstrapping support (98%) within a group of environmental clones which included several clones isolated by direct amplification from the soil samples used in this study (Fig. 4). This cluster of uncultured sequences, designated 'Sourhope 1' [11], belongs to a sub-cluster of Group Y [9] and seems to be associated with the *Acidobacterium/Holophaga* lineage [9,33] (86% bootstrapping

support). While Fig. 4 shows the neighbour-joining tree produced from distance matrix analysis, the branching topology and high bootstrap values shown on this tree are supported by maximum parsimony analysis.

Only 10 isolates could be characterised using Biolog (Table 2). The Biolog system was originally developed for identification of medically important bacteria [34] and as such the MicroLog database is limited for soil bacteria, for example containing only four representatives from the actinomycetes. The Biolog system classified six TSA isolates as *Bacillus* spp. but only one (TSA-8) clustered with bacilli on the basis of 16S rDNA sequence data (92.5% similarity; Table 2, Fig. 5). Inconsistency between

the two data sets for these isolates may be due to *Bacillus* sporulation, which interferes with the Biolog analysis, specifically leading to false positive reactions in the blank (C free) wells, leading to anomalous results. Four isolates (TSA-7, TSA-8, TSA-10 and TSA 20) were placed in the same bacterial groups by both methods and all except isolate TSA-10 were most closely related to the same species.

Phylogenetic trees showing the relationship between 16S rDNA sequences of TSA isolates to reference bacteria and clones from the same soil samples are shown in Figs. 3, 5 and 6. TSA isolates from a range of phylogenetic groups were obtained including the low G+C Gram-positive bacteria (Fig. 5), the α -, β - and γ -proteobacteria (Fig. 3) and several actinomycete groups (Fig. 6). In general, the bacterial species isolated on PIA and TSA were very different. As with PIA isolates, the percentage similarity of TSA isolates to the nearest relatives on sequence databases was generally high, with >97% sequence similarity in 13 of the 18 pure isolates. TSA-8, however, showed only 92.5% similarity to the Gram-positive bacterium *Bacillus insolitus* (Table 2, Fig. 5) and, therefore, possibly repre-

sents a new bacterial species. *Bacillus* spp. and *Staphylococcus* spp. were represented within both the culturable population and clone sequences but showed only 94.6% (TSA-8 and sl3_807) and 97.5% (TSA-1, TSA-15 and saf3_107) similarity, respectively, between isolates and clones (Fig. 3). Similarly, there was very little overlap between the cultured and uncultured proteobacterial populations, although high sequence similarity (99–100%) was observed between TSA-13, TSA-5.1 and sl2_505, which fall within the xanthomonads (Fig. 3).

4. Discussion

Both Biolog and 16S rDNA sequencing were used in this study to characterise a range of soil bacterial isolates. In general, DNA extraction followed by PCR amplification and 16S rDNA sequence analysis was a more efficient strategy for identification than the Biolog system, where several cultures were identified wrongly or not at all, mainly through limitations in the MicroLog database for identification of environmental bacteria. Both Biolog and

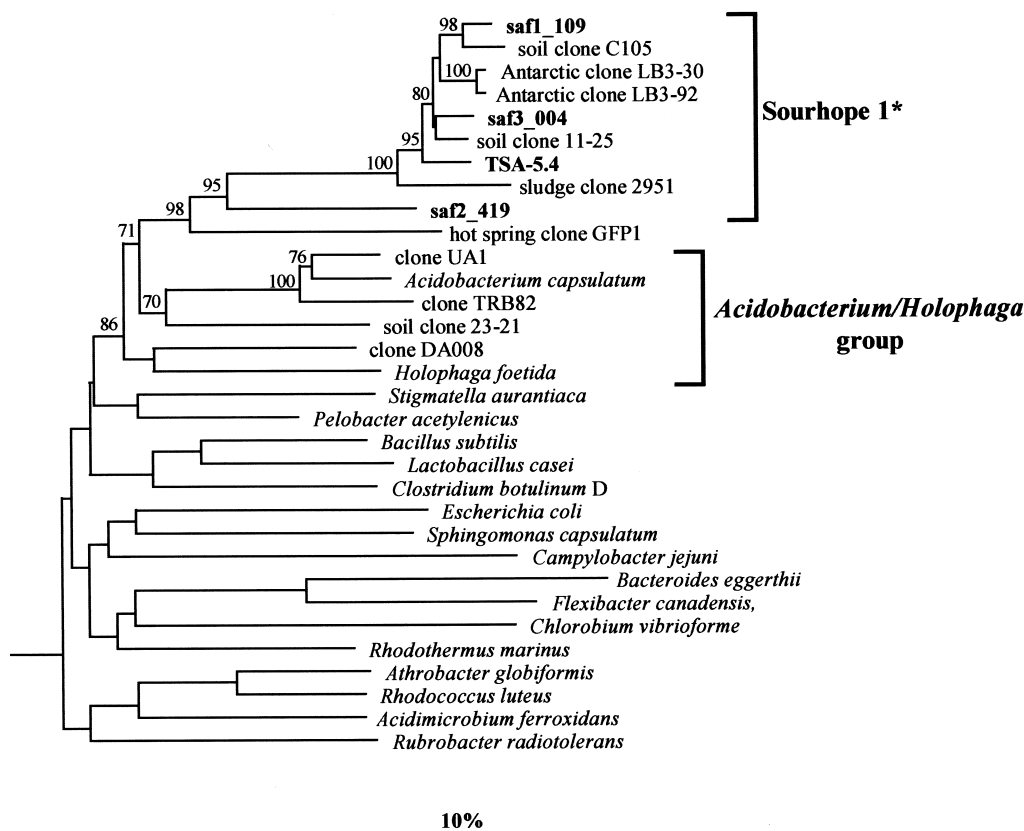


Fig. 4. Neighbor-joining tree showing the relationship of a sequence cloned from a mixed TSA isolate (TSA-5.4) to reference bacteria and grassland rhizosphere clones obtained from the same soil samples [11]. Analysis is based on 1149 bases of aligned 16S rDNA sequence data. Sequences obtained during other direct analyses of environmental samples are Antarctic clones LB3–30 and LB3–92 and clone TRB82 (GenBank accessions af173822, af173824 and af047646, respectively), soil clones 11–25 and 23–21 [46], clone DA008 [47], sludge clone 2951 [40], hot spring clone GFP1 [39] and clone UA1 [48]. Bootstrap values for the distance matrix analysis are given for nodes within the *Acidobacterium/Holophaga* lineage with >50% support in bootstrapping analysis of 100 replicates. *Sourhope 1 is a cluster of sequences defined previously in McCaig et al. [11]. For other sequence nomenclature and branch labelling, see the legends to Figs. 1 and 3.

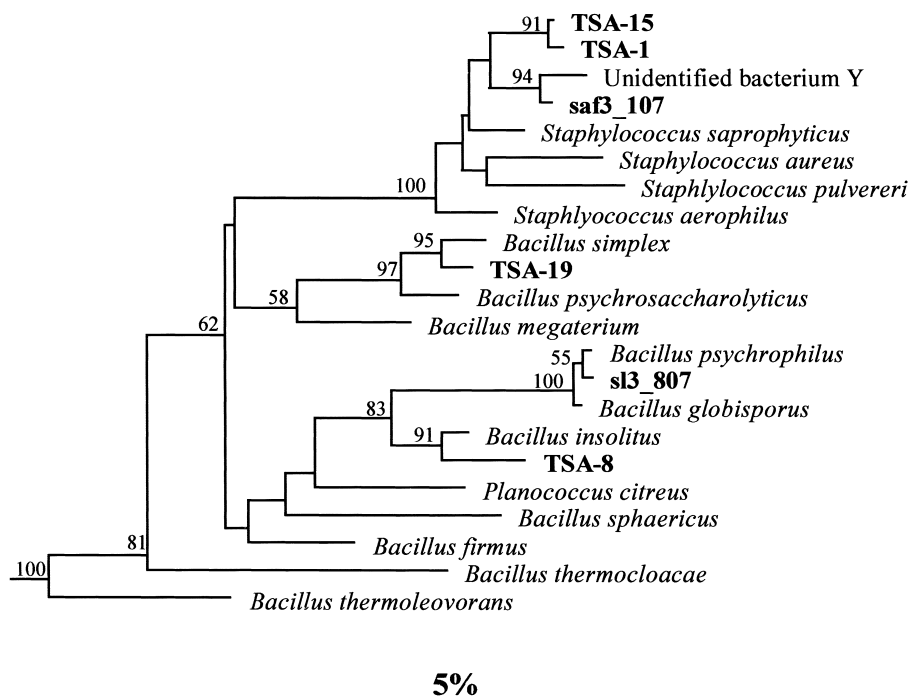


Fig. 5. Neighbor-joining tree showing the relationship of Gram-positive bacteria isolated on TSA to reference bacteria and grassland rhizosphere clones obtained from the same soil samples [11]. Analysis is based on 296 bp of aligned 16S rDNA sequence data. Unidentified bacterium Y was obtained from another environmental study [49]. For other sequence nomenclature and branch labelling, see the legend to Figs. 1 and 3.

16S rDNA analyses have also been used to analyse total microbial community structure in the same three grasslands used in this study [11,17]. While Biolog was capable of distinguishing between total soil communities experiencing different soil management regimes [17], the resolution of 16S rDNA sequence analysis of clone libraries was too high, given the limited number of clones for analysis [11]. Molecular approaches, however, can potentially address a larger proportion of the soil bacterial community than Biolog, which is limited to culturable bacteria and organisms capable of responding rapidly to the narrow range of substrate types and concentrations assayed. Application of other 16S rDNA-based approaches, such as ARDRA, DGGE or temperature gradient gel electrophoresis (TGGE), can be used to increase the speed of data collection, by allowing analysis of total amplified rDNA products, or by simplifying screening of clones.

Several factors may have influenced the types of bacteria characterised in this study. Firstly, cell extraction techniques require a balance between cell removal and cell death and the proportion of bacteria recovered may have been small, influencing diversity. Secondly, it is generally accepted that culturing imposes considerable bias on bacterial communities. Although a relatively non-selective medium (TSA) was used to isolate bacteria, it may have favoured growth of fast-growing aerobic bacteria with neutral pH optima. Selection of isolates on the basis of colony morphotype was chosen to obtain a wide range of bacteria but this may also have imposed bias. Hahn and

Höfle [35] found morphologically indistinguishable colony types that comprised taxonomically different bacteria but in only two out of 18 colony types tested. Conversely, a single bacterial species may possess several distinguishable colony morphologies [35]. Indeed, Biolog and sequence data in this study indicate that many of the PIA isolates, while representing different colony morphotypes, were physiologically and/or phylogenetically related. Finally, both culturability and diversity of soil bacteria may have been influenced by time of sampling. It is worth noting that the soils used in this study were sampled in January and many additional isolated species, as determined by Biolog, were observed in soils sampled during summer months (data not shown).

A significant advantage of using 16S rDNA sequence analysis to identify bacterial isolates in this study is the ability to compare directly culturable sequences and uncultured community sequences present within the same samples, reported previously by McCaig et al. [11]. The phylogenetic distribution of soil clones and cultures differed greatly. For example, while 40% of clones belonged to the α -proteobacteria, only two isolates (TSA-11 and TSA-18) fell within the α -proteobacteria. Interestingly, clones from both mixed colony types fell within the α -proteobacteria. Mitsui et al. [36] studied the incubation time and media requirements of culturable bacteria from different phylogenetic groups and found that strains from the α -proteobacteria were oligotrophic and slow colony formers. It is possible, however, that the medium used in

this study did not favour growth of these populations. McCaig et al. [11] also showed that a large number of clones (13%) clustered within the actinomycete clade, particularly within the *Arthrobacter* and *Nocardioideis* groups, but few clone sequences fell within the actinomycete groups represented by the TSA isolates. None of the isolates was found in both types of medium, reflecting their selective nature. In addition, only three operational taxonomic units (OTUs) defined here as groups of sequences with >97% similarity, contained both isolates and clones (i.e. all sequences belonging to the *Pseudomonas* clade of the γ -proteobacteria in Fig. 3 and the groups TSA-13/sl2_505/TSA-5.1 and TSA-16/sl3_616 which fell within the *Xanthomonas/Stenotrophomonas* clade of the γ -proteobacteria in Fig. 3). Surprisingly, two isolates were identical to clone sequences, consistent with the 1% figure generally quoted for culturability of environmental bacteria. Approximately 15% of clone sequences showed high similarity (>97%) to cultured bacteria represented on the databases but not isolated during this study, suggesting that

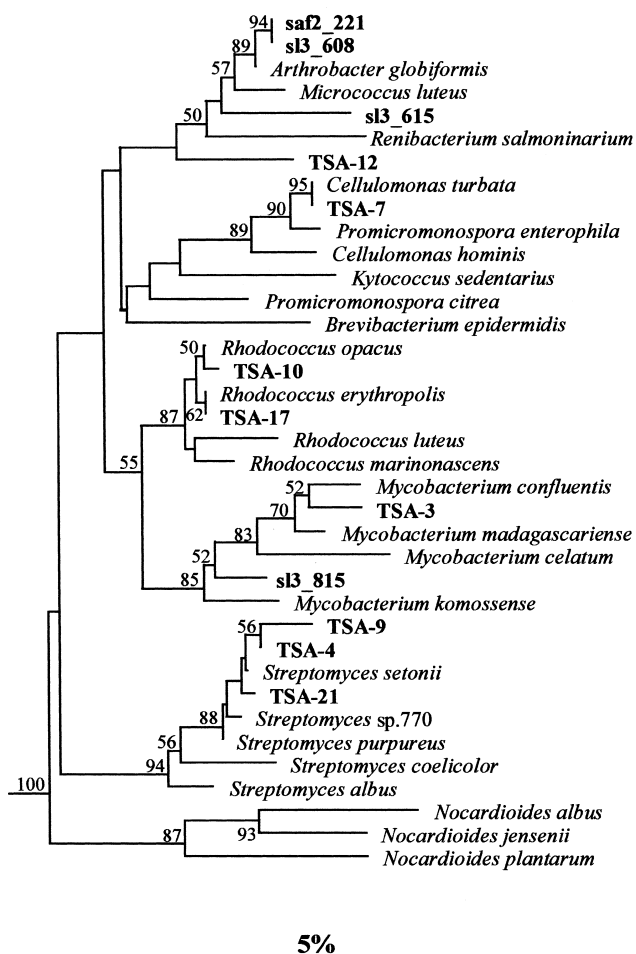


Fig. 6. Neighbor-joining tree showing the relationship of actinomycetes isolated on TSA to reference bacteria and grassland rhizosphere clones obtained from the same soil samples [11]. Analysis is based on 304 bp of aligned 16S rDNA sequence data. For sequence nomenclature and branch labelling, see the legends to Figs. 1 and 3.

the culturability of the grassland soils used in this study may be considerably higher than 1% if a wider range of isolation strategies are adopted. In addition, some isolates shared only low sequence similarity to other cultured bacteria represented on the databases. This implies that many more bacteria that are culturable using standard isolation techniques may not yet have been included on freely available sequence databases.

An important finding was the cloning of a sequence from a mixed colony, which belongs to a group of environmental sequences with no cultured representative and only distant cultured relatives. These sequences have been found in direct analyses of several environmental samples, ranging from soil to sewage sludge and Antarctic ice ([9,11,37,38] and GenBank accession numbers af173822 and af173824) and probably fall within the novel phylogenetic branch containing *Acidobacterium capsulatum* and *Holophaga foetida*. Several other similarly deep-branching clusters of cloned sequences from these samples have been found associated with this lineage [11]. The detection of such an organism within a bacterial colony on laboratory media using standard isolation techniques is the first indication that representatives of these organisms can be isolated, albeit in mixed culture. The implementation of more innovative strategies for characterising and/or isolating such organisms may be necessary.

In conclusion, most of the bacteria characterised in this study are different from organisms present within both the Biolog and 16S rDNA databases. Several novel bacteria were isolated as either pure or mixed cultures, suggesting that many novel culturable bacteria exist. These may account, at least in part, for the generally accepted discrepancy between sequence data from cultured and uncultivated bacterial communities. Furthermore, characterisation of laboratory isolates by sequence analysis may provide data complementary to the large amount of cloned sequence data now available for which little or no phenotypic information is available.

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