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The rapid differentiation of *Streptomyces* isolates using Fourier transform infrared spectroscopy

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Abstract

Fifteen putative *Streptomyces* spp. isolated from soil were selected to be analysed using Fourier transform infrared (FT-IR) spectroscopy and 16S rRNA gene sequencing. Four colour groupings (groups 1–4) were obtained and described according to the colour of their substrate mycelia, aerial mycelia, spore mass and pigmentation. The dendrogram constructed using unsupervised cluster analysis of the FT-IR data was in good congruence with the four colour groups and the neighbour-joining phylogenetic tree for 16S rDNA sequencing. In particular, those isolates having 100% 16S rDNA similarities which are supposed to be from the same species can be separated from each other using FT-IR analysis. This high throughput method only takes 1–10 s to collect a FT-IR spectrum from each sample, and both 96- and 384-well microplates are available for automated analysis. FT-IR therefore presents itself as a rapid, whole-organism fingerprinting approach which can be used for preliminary differentiation of *Streptomyces* spp. at sub-species or strain level.

Keywords: 16S rDNA sequencing; Colour grouping; FT-IR; *Streptomyces*

1. Introduction

Actinomycetes, especially *Streptomyces*, are of particular industrial importance in antibiotics production and account for more than 70% of the world's naturally occurring antibiotics. In spite of this genus unification, the current classification systems have not yet solved the taxonomy within the genus, a pending issue that is being discussed by many taxonomic microbiologists [1]. As the biggest group of actinobacteria, the identification of *Streptomyces* species is still short of a universal standard even though the International *Streptomyces* Project had been carried out for decades.

Nowadays, physico-chemical whole-organism fingerprinting techniques such as Curie-point pyrolysis mass spectrometry [2], Fourier transform infrared (FT-IR) spectroscopy [3–5] and Raman microscopy [6–8] provide rapid and highly reproducible discrimination and have been applied to characterise bacteria including actinomycetes. They are automated and offer high throughput analyses. Recently, FT-IR has been used in combination with other methods for qualitative and quantitative studies on intraspecific diversity. A series of FT-IR spectral reference databases have been developed so far [5,9]. FT-IR is a rapid, non-destructive spectroscopic approach for whole-organism fingerprinting [3,4]. This technique is based on the absorption of IR light directed onto a sample. The amount of light absorbed depends on the molecules found within the sample. It measures dominantly vibrations of functional groups and highly polar bonds. Therefore it gives a lot of information about the total biochemical composition of a sample regarding the molecule composition, structure and interactions. FT-IR spectrometers record the interaction of IR radiation with samples, measuring the frequencies at which the sample absorbs the radiation and the intensities of these absorptions. Using our established reflection-based sampling protocol, FT-IR [4] has the major advantages that it is non-destructive, reproducible and is very rapid both for a single sample (1–10 s

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is typical) and with respect to the automated high throughput of samples in batches of 96 or 384. Thus FT-IR can be used as a rapid dereplication technique in the future so that only novel isolates are put through expensive natural product screening programs.

The aim of this study is to compare the characterisation of *Streptomyces* soil isolates using FT-IR with 16S rRNA gene sequences analysis and colour grouping and to confirm that FT-IR spectroscopy is a reliable, rapid and whole-organism fingerprint technique which can be applied to characterise *Streptomyces* at least at species level.

2. Materials and methods

2.1. Micro-organisms

All the isolates used in this study are listed in Table 1. They were maintained as suspension of spores and mycelia in 30% (v/v) glycerol at −20 °C and on oatmeal agar plates [10] at room temperature.

2.2. Soil sample preparation

Sandy soil samples were collected at Kefalloniá, Greece. Soil was air-dried at room temperature for a week and was sieved to get rid of the large particles. It was then treated with dispersion and differential centrifuge (DDC) method [11]. Mechanical blending of the soil was achieved using an Ultra-Turax T25 homogeniser at low speed using an 8 mm diameter probe. All pooled supernatants from the DDC techniques were centrifuged at 10,000 × *g* for 10 min at 4 °C separately and the pellets were suspended in 9 ml one-fourth strength Ringer's solution and 10-fold serially diluted.

2.3. Bacterial isolation

The above extracted and diluted soil suspensions were plated onto the isolation agar media: Starch-Casein agar and Raffinose-Histidine agar which were supplemented with cycloheximide and nystatin each at 50 μg ml^{-1} [12,13]. Plates were incubated at 25 °C. One to four weeks old colonies with obvious *Streptomyces* cultural characteristics were picked up and subcultured onto oatmeal agar plates for purification, morphology observation and further analysis.

Table 1
Streptomyces isolates used in this study

Isolates	Accession number	Colour group	Phylogenetic group
313	AY582728	3	C
312	AY582727	3	C
232	AY582724	3	C
287	AY582725	3	C
310	AY582726	1	B
204	AY582723	1	B
464	AY582735	1	B
432	AY582733	1	B
416	AY582730	4	D
173	AY582721	4	D
414	AY582729	4	D
86	AY582722	4	D
458	AY582734	2	A
420	AY582731	2	A
426	AY582732	2	A

2.4. Colour grouping

The selected isolates bearing typical *Streptomyces* morphology were inoculated onto oatmeal agar plates and incubated at 25 °C for 3 weeks. The plates were examined by naked eyes and a National Bureau of Standards [14] colour name chart was used to determine the colour of the substrate mycelia, aerial mycelia, spores mass and pigment production.

2.5. 16S rDNA PCR amplification and sequencing

Genomic DNA was isolated following the method of Marmur [15]. The 16S rDNA was amplified using universal primers 27f (5′-AGAGTTTGATCMTGCCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) [16]. Each PCR mixture (25 μl) contained 2.5 μl 10× PCR buffer (Promega), 0.2 mM dNTPs, 0.1 μM primers (MWG Biotechnology, Germany), 1 U Taq DNA polymerase (Promega). Amplification was carried out with an initial incubation of 5 min at 94 °C followed by 30 cycles of 1 min denaturing at 94 °C, 1 min annealing at 50 °C and 3 min elongation at 72 °C, followed by 10 min final extension at 72 °C. The PCR products were purified using a QIAquick PCR Extraction Kit (QIAGEN). Direct sequencing of PCR products was conducted using 109f and 1115r sequencing primers [16] on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using Big Dye fluorescent terminator chemistry (version 2.0) at the University of Cardiff sequencing facility.

2.6. Phylogenetic analysis

Proofreading of sequence data was conducted using AlignIR (Licor Biosciences Ltd., Lincoln, NE, USA) and the 16S rDNAs were compared with the sequences in RDP-II [24]. The sequences were aligned using RDP-II online analysis—RDP Phylip interface (http://35.8.164.52/cgis/phylip.cgi) alongside four *Streptomyces* sequences obtained from the public GenBank database [17] which are most related with those four colour groups. The distance matrix generated here uses Jukes–Cantor method in the Dnadist program and the phylogenetic tree is generated using the neighbour program included in Joe Felsenstein's Phylip 3.5c distribution (phylogeny inference package (PHYLIP), http://cmgm.stanford.edu/phylip/). The resulting trees were viewed using TreeView 1.5 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.7. Nucleotide sequences accession numbers

The nearly complete (∼1400 nt) 16S rRNA gene sequences of those 15 selected *Streptomyces* isolates have been deposited

in the GenBank database under accession numbers AY582721–AY582735 (Table 1).

2.8. FT-IR analysis

Isolates were inoculated onto the nylon membrane on top of the non-sporulating agar plates and incubated at 25 °C for a week [18]. Biomass was collected and suspended into 0.9% NaCl and subsequently homogenized 15 s to disrupt the lump. Ten microlitres of each sample was loaded into the 96-well aluminium IR plate and dried at 50 °C for 30 min. There are six biological replicates (i.e., each isolate was cultivate six times) and three machine replicates (i.e., each of the biological replicates was analysed three times by FT-IR) for each isolate.

A Bruker IFS28 FT-IR spectrometer (Bruker Spectrospin Ltd., Coventry, UK) equipped with an mercury–cadmium–telluride (MCT) detector cooled with liquid N_2 was used for the collection of FT-IR spectra. The loaded aluminium plate was analysed as detailed in [4]. OPUS version 2.1 was used to collect FT-IR spectra over the wavenumber range 4000–600 cm^{-1}. Spectra were acquired at a rate of 10 s^{-1}. The spectral resolution used was 4 cm^{-1}. To improve the signal-to-noise ratio, 256 scans were co-added and averaged. All spectra were collected in reflectance mode and were displayed in terms of absorbance as calculated from the reflectance–absorbance spectra using OPUS [19]. Fig. 1 shows typical spectra for the four different colour groups.

2.9. Cluster analysis of FT-IR spectra

As detailed in [4,9] the initial stage involved the reduction of the dimensionality of the FT-IR data by principal components analysis (PCA). PCA is a well-known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. Discriminant function analysis (DFA) was then used to discriminate between groups on the basis of the retained principal components (PCs) and the a priori knowledge of which spectra were replicates. Finally, the Euclidean distance between a priori group centres in DFA space was used to construct a similarity measure, with the Gower similarity coefficient S_G, and these distance measures were then processed by an agglomerative clustering algorithm to construct a dendrogram.

In order to validate the PC–DFA model, projection analysis of two of the biological replicates of each bacterial isolate into the PC–DFA space constructed from the other four biological replicates was performed. This procedure has previously been reported in [6,7]. The first four biological replicates of each isolate were used to construct a PC–DFA model as described above by performing PCA followed by DFA on only *the training set* (the first to fourth biological replicates). The test data from the fifth and sixth biological replicates were first projected into the PCA space and then the resultant PCs projected into the DFA space. All of the data from test and training sets are the average of three machine replicates. The training and test clusters were then plotted on the same PC–DFA ordination plots for comparison. Finally, the resultant training set DFs and the projected DFs from the test set were used to construct a dendrogram by HCA as detailed above.

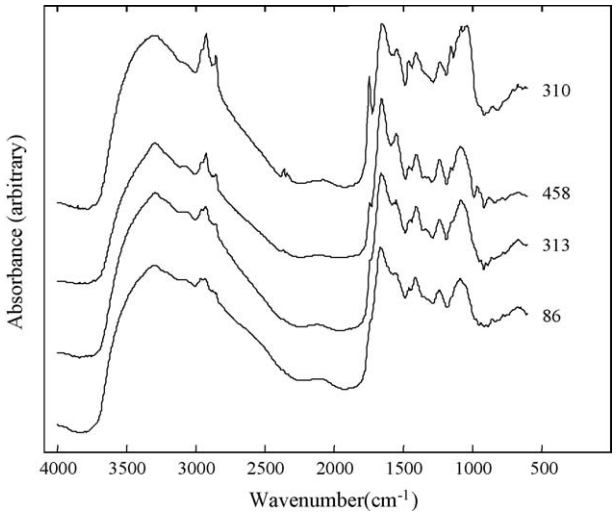

Fig. 1. Typical FT-IR spectra for the *Streptomyces* isolates. Representatives of the four colour groups (see text and Fig. 2 for details) are shown: 310 from group 1; 458 from group 2; 313 from group 3; and 86 from group 4. For clarity the spectra have been offset.

3. Results and discussion

3.1. Isolation of Streptomyces spp.

In total 337 putatively *Streptomyces* spp. were selected from Starch-Casein and Raffinose-Histidine agar plates. Most of these isolates show typical morphology of *Streptomyces*, they had branched and non-fragmented substrate mycelia, abundant aerial hyphae and short or long spore chains with or without pigmentation.

3.2. Colour grouping

Streptomyces isolates were grouped together based on macro-morphological characters, the production of distinct aerial spore mass, the colours of substrate mycelia and diffusible pigment colours on oatmeal agar plates. Out of 337 *Streptomyces*-like isolates recovered, there were 26 multi-member groups and 37 single-member colour groupings.

In order to validate FT-IR as a potential microorganism differentiation method 15 isolates were selected for further study using FT-IR and the relatively more expensive, time consuming 16S rDNA sequence analysis. These 15 isolates fell into four colour groups (1–4), and photographs of these groups are shown in Fig. 2. Group 1 contains isolates 464, 204, 432 and 310, they show greyish reverse colour, brownish-white aerial mycelia and spore mass and no diffusible pigment production. Group 2 includes the isolates 458, 420 and 426 that have very deep reddish-purple vegetative mycelium, light violet spores mass and vivid violet diffusible pigment which relates very well to the phenotypes of *S. coelicolor* AL939114. Isolates 313, 312, 232 and 287 were included in group 3 having dark orange-yellow reverse colour, light greenish-blue spores and light orange-yellow diffusible pigment. Whilst moderate yellow-

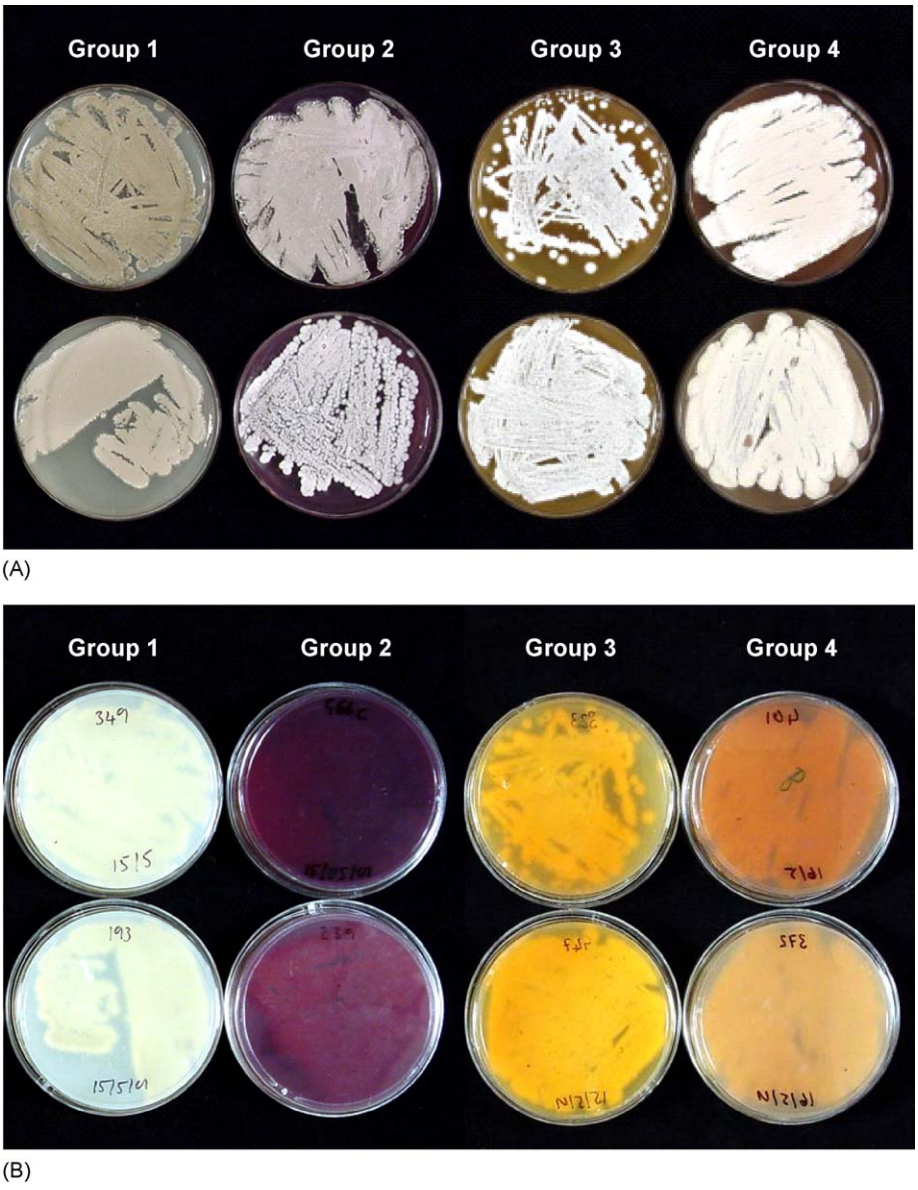

Fig. 2. Colour grouping of the *Streptomyces* isolates according to (A) their aerial mycelia and spore mass characteristics, and (B) the colour of the substrate mycelium and pigmentation. All isolates were grown on oatmeal agar plates at 25 °C for 3 weeks.

green nutritional mycelia, pale purplish-blue aerial hyphae and spores, light greenish-grey diffusible pigment was used to describe group 4 which contains isolates 173, 414, 416 and 86.

3.3. Phylogenetic analysis

All isolates were classified as belonging to the genus *Streptomyces* using the Sequence Match program in Ribosomal Database Project II Online Analysis (http://rdp.cme.msu.edu/html/analyses_preview.html). This is consistent with the assignment according to the morphological characteristics. All of the higher hits in the RDP-II database were *Streptomyces* species, and these were used to select the four most related, published and validated *Streptomyces* sequences for sequence comparison with our isolates and with the 16S rDNA sequence of the non-streptomycete *Nocardia carnea* as an outgroup. It was found that four groups (A–D) were obtained (Fig. 3). Groups A–D correspond to colour groups 2, 1, 3 and 4, respectively. Group A contains isolates 420, 426 and 458 that have same 16S rDNA sequence as *S. coelicolor* AL939114. Isolates 204, 310, 432 and 464 are clustered into Group B which has 100% 16S rDNA similarity with each other and 99.9% similarities with *S. bellus* AJ399476. Group C consists of isolates 287, 232, 312 and 313. The RDP-II Sequence Match analysis shows that whilst these four isolates are closely related to each other, they are not closely related to other characterised *Streptomyces* spp. in that database. This suggests that they could be new species which need to be further analysed. All the remaining isolates 86, 416, 414 and 173 were clustered in Group D and isolates 86, 416, 414 have the same 16S rDNA as *S. setonii* D63872.

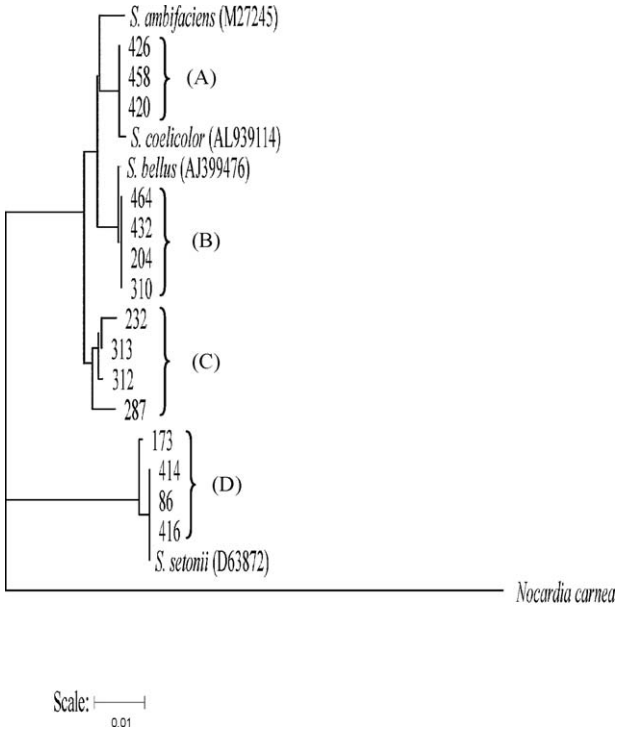

Fig. 3. A rooted neighbour-joining tree for the 15 *Streptomyces* spp. sequences obtained during this study alongside four published 16S rDNA sequences from *Streptomyces* using *Nocardia carnea* DSM 43397 as outgroup. GenBank accession numbers are shown alongside the published sequences used for these analyses. Bar is the inferred 0.01 substitution per nucleotide position. A–D indicates four phylogentic groups.

As the genus *Streptomyces* includes the largest number of species and strains in the *Actinobacteria*, their phenotypic characteristics are far more complicated than other actinomycetes. The taxonomy of *Streptomyces* was broadly based on phenotypic characters especially numerical classification [20] who assigned species-groups for nearly 400 *Streptomyces* type strains. However, it is very difficult to define them below the species level. Genotypic approaches such as DNA–DNA homology are currently thought as a gold standard for taxonomy [21], and sequences of 16S rDNA have also been successfully used to determine phylogenetic relationships in bacteria although there are exceptions [22,23]. However, the database of complete 16S rDNA sequences is insufficient for phylogenetic analysis within the genus *Streptomyces*. Thus it is still very difficult to classify *Streptomyces* reliably below the species level due to the complexity of this taxon, and alternative methods are needed.

3.4. FT-IR spectroscopy

Streptomycetes exhibit macro-morphological heterogeneity, they are mycelia-forming bacteria in nature and they undergo sporulation at late life cycle. Thus there may be some biological variation from the same isogenic sample grown several times. Therefore, in order to analyse these bacteria by FT-IR all of the isolates were collected from nylon membranes which had been placed on top of non-sporulating agar before incubation. A homogeniser was used to produce homogenous biomass before it was loaded onto an FT-IR sample plate. Note that this sample preparation process may also cause some changes in the chemical makeup of these samples. For each isolate 18 FT-IR spectra were produced, that is, each isolate was cultivated six times (so-called biological replicates), and each biological replicate was loaded three times on IR plate (machine replicates).

In order to test the robustness of streptomycetes growth and sample preparation, a supervised projection analysis was used where four of the biological replicates (chosen randomly) were used to construct PC–DFA and the remaining two were projected into this PC–DFA space. A dendrogram was then produced using agglomerative clustering. This was initially done on all 15 isolates and the dendrogram clearly recovered the projected test spectra into their respective colour groups (data not shown). However, within each of the clusters the assignment to isolate level was not evident. It is likely that this was due to the fact that the cluster analysis had found the greatest differences in the FT-IR spectra and these were at the level of colour groups. Any smaller differences were overshadowed. Therefore the projection analysis was performed on each of the colour group sub-clusters. As an example of this process the PC–DFA plot for *Streptomyces* isolates belonging to colour group 3 is shown in Fig. 4 where it is clear that each of the projected test set spectra were recovered very close to their correct clusters although there are overlapped area for isolates 312 and 313. It shows that may be the two isolates are too closely related to be analysed by this projection analysis. Similar results were found for each of the other three colour groups (data not shown).

The above suggests that our growth and sampling protocol was reproducible. A dendrogram was constructed from the FT-IR spectra which revealed four well-separated groups (Fig. 5).

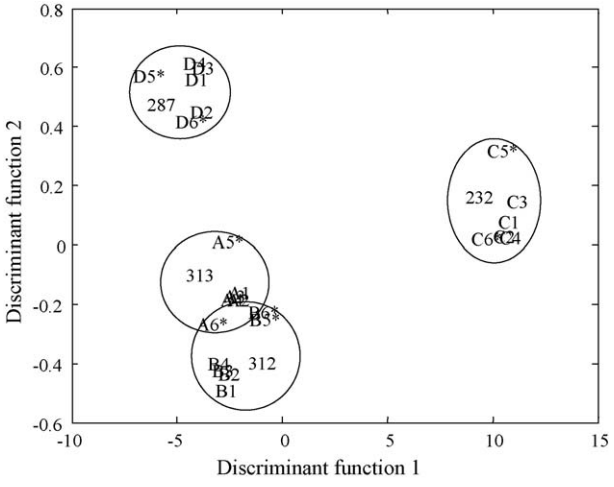

Fig. 4. PC–DFA projection analysis for colour group 3. The first four biological replicates X1–X4 were used to construct the model, and the last two replicates X5* and X6* were projected into this PC–DFA space. The circles are drawn as a guide and do not have any statistical meaning. The codes used were: A1–A6 = 313 (AY582728); B1–B6 = 312 (AY582727); C1–C6 = 232 (AY582724); D1–D6 = 287 (AY582725). The data used are the mean averages of three machine replicates.

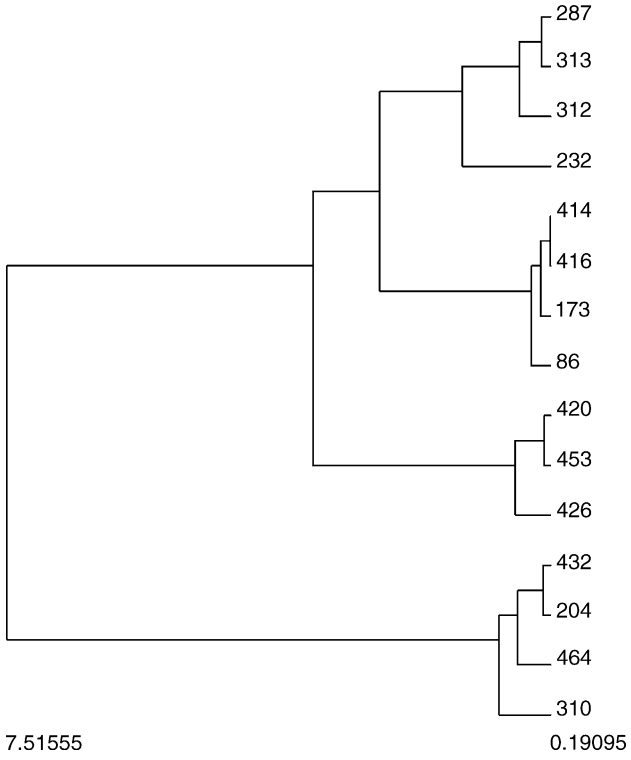

Fig. 5. Unsupervised hierarchical cluster analysis (HCA) on FT-IR data, revealing clusters according to the same four colour groups and 16S rDNA phylogenetic tree seen in Figs. 2 and 3. Data used are the mean averages of six biological replicates and three machine replicates, respectively.

In order to simplify the visualisation of this dendrogram the group means in the PC–DFA were used in the HCA. It is clear from Fig. 5 that all four groups were in excellent congruence with the colour groups (Fig. 2) and 16S rDNA phylogeny tree (Fig. 3). In particular, all those isolates that have 100% 16S rDNA similarities cannot be separated from each other in the phylogenetic analysis (Fig. 3). However, using FT-IR, most of them can be differentiated. For example, isolates 86, 414 and 416 have same 16S DNA sequence. But 86 was separated from 414 and 416 using FT-IR analysis (Fig. 5). Isolate 426 was also separated from 420 and 458 which have 100% 16S rDNA. 310, 464 were separated from 432 and 204 while these four isolates have same 16S rDNA as well. Thus it shows that FT-IR is likely to be more discriminative than the 16S rDNA-based phylogeny analysis at sub-species or strains level. Whilst we appreciate the small number of isolates studied we believe that it is worth performing further analyses in the future accommodating a large number of taxonomically validated *Streptomyces* isolates (including different species which are distantly related or closely related, and different isolates from the same species). This will allow for the validation of FT-IR as a rapid tool for the classification of *Streptomyces* spp. at sub-species or even strain level.

All the previous studies regarding the taxonomy of *Streptomyces* denote that a combination of phenotypic, genotypic and phylogenetic data should be used to define *Streptomyces* species. This emphasizes the importance of polyphasic taxonomic approaches and we also believe that FT-IR will have a role to play here as well.

The selected *Streptomyces* isolates that were studied were all clearly classified to the same four clusters using colour grouping, 16S rDNA sequences analysis and FT-IR spectroscopy. It only takes 1–10 s to collect the FT-IR fingerprints from each sample, and this approach can be performed in serial using automation. Thus FT-IR can be used as a rapid, high throughput screening technique for preliminary differentiation of isolates at least at species level. Indeed, the construction of a FT-IR spectral database with a large number of fully characterised *Streptomyces* strains would be highly attractive for screening novel isolates in the future.

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References

[1] A.S. Anderson, E.M.H. Wellinton, Int. J. Syst. Environ. Microbiol. 51 (2001) 797–814.
[2] J. Chun, et al. FEMS Microbiol. Lett. 114 (1993) 115–119.
[3] D. Helm, H. Labischinski, G. Schallehn, D. Naumann, J. Gen. Microbiol. 137 (1991) 69–79.
[4] R. Goodacre, E.M. Timmins, R. Burton, N. Karderbhai, A.M. Woodward, D.B. Kell, P.J. Rooney, Microbiology 144 (1998) 1157–1170.
[5] H. Oberrueuter, H. Seiler, S. Scherer, Int. J. Syst. Environ. Microbiol. 52 (2002) 91–100.
[6] E.C. López-Díez, R. Goodacre, Anal. Chem. 76 (2004) 585–591.
[7] R.M. Jarvis, R. Goodacre, Anal. Chem. 76 (2004) 40–47.
[8] K. Maquelin, L.-P. Choo-Smith, H.P. Endtz, H.A. Bruining, G.J. Peppels, J. Clin. Microbiol. 40 (2002) 594–600.
[9] E.M. Timmins, S.A. Howell, B.K. Alsberg, W.C. Noble, R. Goodacre, J. Clin. Microbiol. 36 (1998) 367–374.
[10] E. Küster, Int. Bull. Bacteriol. Nomencl. Taxon. 9 (1959) 97–104.
[11] D.W. Hopkins, S.J. Macnaughton, A.G. O'Donnell, Soil Biol. Biochem. 23 (1991) 217–225.
[12] E. Küster, S.T. Williams, Nature 22 (1964) 928–929.
[13] J.C. Vickers, S.T. Williams, G.W. Ross, in: L. Ortiz-Ortiz, L.F. Bojalil, V. Yakoleff (Eds.), Biological, Biochemical and Biomedical Aspects of Actinomycetes, Academic Press, London, 1984, pp. 553–561.
[14] K.L. Kelly, J. Res. Natl. Bureau Standards USA 61 (1958) 427.
[15] J. Marmur, J. Mol. Biol. 3 (1961) 208–218.
[16] D.J. Lane, in: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acid Techniques in Bacterail Systematics, 1991, 115–175.
[17] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, Nucl. Acids Res. 24 (1997) 4876–4882.
[18] J.J. Sanglier, D. Whitehead, G.S. Saddler, E.V. Ferguson, M. Goodfellow, Gene 115 (1992) 235–242.
[19] P.R. Griffiths, J.A. de Haseth, Fourier Transform Infrared Spectrometry, John Wiley, New York, 1986.
[20] S.T. Williams, M. Goodfellow, G. Alderson, E.M.H. Wellinton, P.H.A. Sneath, M.J. Sackin, J. Gen. Microbiol. 129 (1983) 1743–1813.
[21] L.G. Wayne, et al. Int. J. Syst. Bacteriol. 37 (1987) 463–464.
[22] G.E. Fox, J.D. Wisotzkey, P.J. Jurtshuk, Int. J. Syst. Bacteriol. 42 (1992) 166–170.
[23] W.H. Yap, Z. Zhang, Y. Wang, J. Bacteriol. 181 (1999) 5201–5209.
[24] J.R. Cole, B. Chai, T.L. Marsh, R.J. Farris, Q. Wang, S.A. Kulam, S. Chandra, D.M. McGarrell, T.M. Schmidt, G.M. Garrity, J.M. Tiedje, Nucleic Acids Res. 31 (2003) 442–443.