CHARACTERISATION OF FIVE SIDEROPHORE PRODUCING ACTINOMYCES FROM SOIL SAMPLES AND THE USE OF ANTIBIOTIC RESISTANCE TO DIFFERENTIATE THE ISOLATES

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Abstract- Organisms were isolated on their basis of survival in an iron-limited environment. The survivors of this treatment were largely actinomycetes. Of the viable cultures, most were found to produce siderophore like compounds. The most prolific producers as assessed by the Chromo Azuerol Sulphate assay were further characterised and found to belong to the genus Streptomyces. Attempts to taxonomically characterise these organisms illustrated the conserved nature of the 16S rRNA gene in this group of organisms. Physiological characterisation was undertaken and it was found that the resistance of these organisms to a range of antibiotics proved to be a useful discriminating factor.

Key words- Actinomycetes, iron chelation, siderophores, soil, streptomycetes, classification, resistance

Introduction
Iron is a fundamental element to microbial nature. Due to the evolution of the di-oxygen atmosphere in the earth’s terrestrial environment, iron forms insoluble, bio unavailable ferric moieties at physiological pH [1]. In order to capture iron microorganisms have developed specific mechanisms involving the formation of siderophores [2]. The latter are low molecular weight compounds synthesised under iron starvation; they are produced extracellularly to bind iron and other ions forming a meta-siderophore complex. These complexes are then bound by the cells and transported intracellularly [3-6].

The actinomycetes are notorious environmental scavengers and have well-developed processes to capture ions in conditions depleted of elements; they are therefore an ideal target group for exploitation in iron binding technology [7-9]. Here we report the characterisation of four siderophore producing actinomycetes. The taxonomy of streptomycetes has been extensively studied by many groups and comprehensive reviews are available in the literature [10-12]. Modern molecular taxonomical approaches involve the sequence analysis of the 16S rRNA gene, a particularly powerful tool in the classification of streptomycetes. Although 16S rRNA is a highly conserved area of the genome, it contains enough variations to allow us to investigate relationships at the genus, species and strain levels. Nevertheless it can be misleading due to intragenic variation, which highlights the need of genotypic and phenotypic observations [12]. One possible discriminatory factor is the resistance profile to antibiotics. This article describes data that uses antibiotic resistance to differentiate the siderophore producing isolates.

Materials and Methods
Purification of actinomycetes from soil:
A soil sample (1g) was suspended in 100ml of sterile distilled water and incubated 40°C for 24 hours. Actinomycetes were isolated from the soil suspension using a dilution plate technique on starch-casein agar (1.0 of starch, 0.4g of casein, 0.5 g of KNO₃, 0.1 g of MgPO₄, 0.2 g of K₂HPO₄, 0.1 g of CaCO₃ and 15g of agar per litre of distilled water [dH₂O]) supplemented with 150

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mM 2,2’-dipyridyl (DIP). The agar plates were incubated at 27°C for up to four weeks. Selective colonies were further sub-cultured and pure actinomycetes were isolated [13].

Siderophore production

Siderophore production was confirmed using the Chromo Azurol Sulphate (CAS) assay (Renshaw et al. 2003). Arrow’s [14] and Atkin’s [15] assays confirmed the presence of a catecholic and hydroxamate compounds respectively.

Actinomycete characterisation

Actinomycete colonies were characterised according to Bergey’s Manual of Systematic Bacteriology [16]: specific characteristics were observed by light and scanning electron microscopy. The ability of the organisms to utilise different nutrient sources was tested using the SF-N2 (OXOID) and GP2 (OXOID) Biolog Microplates. Antibiotic resistance of the strains was examined using MASTRINGS (Mast Diagnostics) and susceptibility discs (OXOID) performing the disc diffusion test. The model streptomycete, Streptomyces coelicolor (S. coelicolor) was also included in this study for comparison. The strains were also tested for their ability to grow and sporulate on a range of liquid or solid media, including yeast extract-malt extract (YEME), Mannitol-Soya bean flower (MS), Muller-Hinton (MH), MH glucose, MH fructose and starch-casein agar [13].

16S rRNA amplification and taxonomic analysis

Genomic DNA was extracted using the ‘Kirby mix procedure’ [13]. Amplification of the 16S sequences were performed according to Rintaia et al. 2001 [17]. The amplified products were purified using the QIAquick® PCR purification kit (50) (Qiagen) and sent to Lark® Technologies, Inc (United Kingdom) according to their requirements. Pairwise sequence comparisons and retrieval of homologous sequences were conducted by the NCBI BLAST database (available online- http://www.ncbi.nih.gov/). Similar sequences, including an outgroup (ancestral sequence), were multiple aligned by BioEdit sequence alignment Editor 7.0.4.1 (Copyright 1997-2005, Hall, 1999). Taxonomic analysis was performed by the ClustalX software, version 1.83 and Bootstrap confidence values (1000) were provided by the Neighbour- Joining (NJ) algorithm. Finally the phylogenetic tree was presented using TreeView (Win 32) 1.6.6 (Copyright 2001) by Roderic. D. M. Page, available online: http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Results

Strain isolation

In an attempt to identify novel siderophore compounds we have isolated and purified 154 cultures from soil samples collected in Thailand (Tak province). The majority of strains are considered to be members of actinomycetes on the evidence of phenotypic examination. All the isolates were grown on starch-casein agar containing 150 mM DIP. This concentration of DIP was chosen as it reduced the number of surviving actinomycete colonies by approximately 99%. The remaining 1% was thought to be likely to have physiological attributes that enabled them to survive iron limitation. Twenty-two strains were confirmed CAS-assay positive with five strains (23F, 31B, 31C, 33D and 29C- GenBank accession numbers EF585403 - EF585407) exhibiting prolific siderophore production. Liquid based assays were conducted on culture supernatants in order to categorise the siderophore producers. The results classified the strains 31B, 31C, 33D and 29C as catechol producers, whereas 23F was categorised as a hydroxamate synthesiser.

16S rRNA gene sequence analysis and phylogenetic studies

16S rRNA gene sequence analysis was carried out to elucidate the taxonomic position of the isolates. Almost complete 16S rRNA sequences of the representatives were determined following the isolation of genomic DNA and sequencing of the amplified genes. The strains’ phylogenetic positions are reported in figure 1. The levels of 16S rRNA sequence similarities between the isolates and actinomycetes species ranged from 97-99 %, confirming that the strains are members of the family, mainly streptomycetes.

The high percentage similarity values amongst species of the same genus are very common due to the highly conserved nature of the gene. For example although Streptomyces macrosporus and Streptomyces megasperos share 98 % similarities, they are clearly different on their phenotypic characterisation. A second paradigm includes Streptomyces thermocarboxydyovorans (S. thermocarboxydyovorans) and Streptomyces thermodiastaticus (99 %) or S. thermocarboxydyovorans and S. thermoviolaceus (98 %) [18]. Moreover Amycolatopsis albidoflavus share 97.4 % similarities with Amycolatopsis azurea and Amycolatopsis orientalis [19].

Characterisation of the strains

All the streptomycete stains were further investigated in order to determine their morphological, physiological, growth and biochemical characteristics. Their ability to use a variety of nutrient compounds is described in Table 1.

| Nutrient utilisation of: (Measured using the Biolog system) | Isolates |
|--------------------------------------------------------------|---------|
|                                                               | 23F    | 29C | 31B | 31C | 33D |
| a-Cyclodextrin                                               | +      | -   | +   | +   | +   |
| b-Cyclodextrin                                               | +      | -   | +   | +   | +   |
| Dextrin                                                      | -      | +   | -   | +   | +   |
| Glycogen                                                    | +      | -   | +   | +   | +   |
| Inulin                                                       | -      | +   | +   | -   | +   |
| Mannan                                                      | +      | +   | -   | +   | +   |
| Tween 40                                                    | +      | +   | +   | +   | +   |
| Tween 80                                                     | +      | +   | +   | +   | +   |
| N-Acetyl-D-Glucosamine                                       | -      | +   | -   | +   | +   |
| N-Acetyl-b-D-Mannosamine                                     | +      | -   | -   | +   | +   |
| Amygdalin                                                   | -      | +   | +   | +   | +   |
| L-Arabinose                                                  | +      | +   | -   | -   | +   |
| D-Arabinose                                                 | -      | +   | -   | +   | +   |
| Arbutin                                                     | -      | +   | +   | +   | +   |
| D-Cellobiose                                                 | -      | +   | +   | +   | +   |
| D-Fructose                                                  | +      | +   | +   | +   | +   |
| L-Fucose                                                    | +      | +   | +   | +   | +   |
| D-Galactose                                                 | -      | -   | +   | +   | +   |
| D-Galacturonic Acid                                          | -      | +   | -   | +   | +   |
| Gentibiose                                                  | -      | -   | +   | +   | +   |
| D-Glucosic Acid                                              | -      | +   | -   | +   | +   |
| a-D-Glucose                                                  | +      | +   | -   | +   | +   |
| m-Inositol                                                  | -      | -   | +   | +   | +   |
| a-D-Lactose                                                 | -      | -   | +   | +   | +   |
| Lactulose                                                   | -      | -   | +   | +   | +   |
| Maltose                                                      | -      | -   | +   | +   | +   |
| Maltoluctose                                                 | -      | -   | +   | +   | +   |

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Bioinfo Publications

203
Table 1 Continue

| Table 1 - Nutrient utilisation of strains 29C (GenBank accession number EF585407), 33D (GenBank accession number EF585406), 31B (GenBank accession number EF585404), 31C (GenBank accession number EF585405) and 23F (GenBank accession number EF585403). |
|---------------------------------------------------------------|
| D-Mannitol | - | - | + | - |
| D-Mannose | - | - | - | - |
| D-Melezitose | - | - | + | - |
| D-Melibiose | + | - | + | - |
| a-Methyl-D-Galactoside | - | - | - | - |
| b-Methyl-D-Galactoside | - | - | - | - |
| 3-Methyl Glucose | + | - | + | - |
| a-Methyl-D-Glucoside | + | - | + | - |
| b-Methyl-D-Glucoside | - | - | + | - |
| a-Methyl-D-Mannoside | - | - | - | - |
| Palatinose | + | - | + | - |
| D-Psicose | - | - | - | - |
| D-Raffinose | - | - | - | - |
| L-Rhamnose | - | - | + | - |
| D-Ribose | + | - | + | - |
| Salicin | + | - | - | - |
| Sedoheptulanos | + | - | - | - |
| D-Sorbitol | - | - | + | - |
| Stachyose | + | - | + | - |
| Sucrose | + | - | + | - |
| D-Tartarose | + | - | - | - |
| D-Trehalose | - | - | + | - |
| Turanose | + | - | + | - |
| Xylose | - | - | + | - |
| D-Xylose | - | - | - | - |
| Acetic Acid | + | - | + | - |
| a-Hydroxybutyric Acid | - | - | + | - |
| b-Hydroxybutyric Acid | - | - | + | - |
| g-Hydroxybutyric Acid | - | - | + | - |
| p-Hydroxy-Phenylacetic Acid | - | - | + | - |
| a-Ketoglutaric Acid | + | - | + | - |
| a-Ketovaleric Acid | + | - | + | - |
| Lactamide | + | - | + | - |
| D-Lactic Acid Methyl Ester | + | - | - | + |
| L-Lactic Acid | + | - | - | - |
| D-Malic Acid | + | - | + | - |
| L-Malic Acid | + | - | + | - |
| Pyruvic Acid Methyl Ester | + | - | + | - |
| Succinic Acid Mono-methyl Ester | + | - | + | - |
| Propionic Acid | + | - | + | - |
| Pyruvic Acid | + | - | + | - |
| Succinic Acid | + | - | + | - |
| Succinic Acid | + | - | + | - |
| N-AcetyL-D-Glumatic Acid | - | - | + | - |
| L-Alaminamide | + | - | + | - |
| D-Alanine | - | - | + | - |
| L-Alanine | + | - | + | - |
| L-Alanyl-Glycine | + | - | + | - |
| L-Lysine | + | - | + | - |
| L-Asparagine | + | - | + | - |
| L-Glutamic Acid | + | - | + | - |
| Glycyl-L-Glutamic Acid | - | - | + | - |
| L-Prolylglutaric Acid | - | - | + | - |
| L-Serine | + | - | + | - |
| Putrescine | + | - | + | - |
| 2,3-Butanediol | + | - | + | - |
| Glycolaldehyde | + | - | + | - |
| Adenosine | + | - | + | - |
| 2-Deoxy Adenosine | + | - | + | - |
| Inosine | + | - | + | - |
| Thymidine | + | - | + | - |
| Uridine | + | - | + | - |
| Adenosine-5’-Monophosphate | - | - | + | - |
| Thymidine-5’-Monophosphate | - | - | + | - |
| Uridine-5’-Monophosphate | - | - | + | - |
| D-Fructose-6-Phosphate | + | - | + | - |
| a-D-Glucose-1-Phosphate | + | - | + | - |
| D-Glucose-6-Phosphate | + | - | + | - |
| D-L-Glycerol Phosphate | + | - | + | - |

Ismini Nakouti and Glyn Hobbs

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(28°C - 37°C) conditions. It grew quickly on MS (3 days) agar, nevertheless much slower in starch-casein medium (approximately 10 days). It did not sporulate on YEME agar and on MHG and its optimum growth conditions involved cultivation on MS or starch-casein, pH 7, at 37°C.

**Antibiograms**

Antibiotic resistance data offer valuable information in terms of strain characterisation and identification (Table 2). In the specific small-scale experiment 14 out of 24 antibiotics demonstrated different patterns amongst the streptomycetes. Careful examination of the data revealed specific profiles for *Streptomyces coelicolor* (S. coelicolor) and strains 23F, 29C and 31B. For example sensitivity to amoxicillin, cefuroxim or oxaxillin was a unique characteristic of isolate 29C. Stain 31B was the only candidate that was resistant to vancomycin. A combination of growth in the presence of erythromycin and levofloxacin differentiated strain 23F from *S. coelicolor*. Culture 33D could be identifiable by its sensitivity against ampicillin and vancomycin. Finally *S. coelicolor* demonstrated a distinctive pattern of tetracycline (10 μg) and erythromycin resistance.

**Table 2-Antibiograms of the siderophore producing strains. *S. coelicolor* is included as a reference**

| Antibiotics | Isolates | 23F | 29C | 31B | 31C | 33D | S. coelicolor |
|-------------|----------|-----|-----|-----|-----|-----|---------------|
| Amoxicillin 2mg | -+ | + | + | + | + | + | + |
| Amoxicillin/clavulanic acid 3mg | + | + | + | + | + | + | + |
| Ampicillin 25 mg | + | + | - | - | - | - | - |
| Cefotaxime sodium 5 mg | + | + | + | + | + | + | + |
| Cefuroxime 5 mg | + | + | + | + | + | + | + |
| Cephalxin 30 mg | + | + | + | + | + | + | + |
| Chloramphenicol 50 mg | - | + | + | - | + | + | + |
| Ciprofloxacin 1 mg | + | + | + | + | + | + | + |
| Colistin sulphate 100 mg | + | + | + | + | + | + | + |
| Erythromycin 5 mg | + | + | + | + | + | + | + |
| Gentamicin 10 mg | - | - | - | - | - | - | - |
| Kanamycin 30 mg | - | - | - | - | - | - | - |
| Levofloxacin 1 mg | + | + | + | + | + | + | + |
| Nalidixic Acid 30 mg | + | + | + | + | + | + | + |
| Neomycin 10 mg | - | - | - | - | - | - | - |
| Nitrofurantoin 50 mg | + | + | + | + | + | + | + |
| Nitrofurantoin 200 mg | + | + | + | + | + | + | + |
| Oxaillin 1 mg | + | + | + | + | + | + | + |
| Penicillin G 1 unit | + | + | + | + | + | + | + |
| Piperacillin/ tazobactam 85 mg | + | + | + | + | + | + | + |
| Spectinomycin 25 mg | - | + | + | + | + | + | + |
| Streptomycin 25 mg | - | - | - | - | - | - | - |
| Tetracycline 10 mg | - | - | - | + | + | + | + |
| Tetracycline 100 mg | - | - | - | - | - | - | - |
| Trimethoprim 2.5mg | + | + | + | + | + | + | + |
| Vancomycin 5 mg | - | - | - | - | - | - | - |

Table 2- Antibiograms of the siderophore producing isolates 29C (GenBank accession number EF585407), 33D (GenBank accession number EF585406), 31B (GenBank accession number EF585404), 31C (GenBank accession number EF585405) and 23F (GenBank accession number EF585403). *S.coelicolor*, the model streptomycetes, is included as a reference organism.

**Discussion**

It is anticipated that the isolation and characterisation of new actinomycetes might lead to novel siderophores of significant pharmaceutical interest. Although 16S RNA analysis is a very powerful tool in phylogenetic relationships, it does not provide enough evidence for classification of closely related species. Therefore a ‘universal’ approach, which will include the combination of modern molecular approaches with a number of morphological, physiological and biochemical characteristics, will facilitate the understanding of the genotypic and phenotypic behaviour of these precocious siderophore producers. Antibiotic resistance data provide useful information on the nature of the cells. A report by Hopwood reaffirms the importance of actinomycetes as a major reservoir of antibiotic resistance genes [20]. Clearly these characteristics can be a useful way of classifying strains. This could be a valuable evolutionary route to differentiate strains within a population. Given the fact that *Streptomyces* species have been found to have the capacity to produce multiple “antimicrobial” compounds [9, 21] and hence the ability to be resistant to these compounds, it will be interesting to develop a resistance fingerprint system for strains and to revisit phylogenetic analysis in the light of this information.

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**Fig. 1**- Phylogenetic relationships of the isolates and related Actinomycetes based on the 16S rRNA sequence analysis. The tree is bootstrapped by 1000 times by Neighbour-Joining (N-J). M. tuberculosis was employed as an outgroup.