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A new approach to isolating siderophore producing actinomycetes.

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Running title: Siderophore producing actinomycetes

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ABSTRACT

Aims: The current study was conducted to investigate the application of 2, 2’-dipyridyl as a new approach to isolating siderophore producing actinomycetes.

Methods and Results: Isolation of actinomycetes from soil was conducted by a soil dilution plate technique using starch-casein agar. Iron starvation was fostered by the incorporation of the iron chelator 2, 2’-dipyridyl in the isolation medium. Pre-treatment of the samples at an elevated temperature (40°C) ensured that the majority of non-sporulating bacteria were excluded. The survivors of this treatment were largely actinomycetes. Of the viable cultures grown in the presence of 2, 2’-dipyridyl, more than 78-88 % (average of three separate studies) were reported to produce siderophore like compounds compared to 13- 18% (average of three separate studies) when grown on the basic media in the absence of the chelating agent. The most prolific producers as assessed by the Chromo Azurol Sulphate (CAS) assay were further characterised and found to belong to the genus Streptomyces.

Conclusions: Selective pressure using 2, 2’-dipyridyl as an iron chelating agent in starch-casein media increased the isolation of siderophore producing actinomycetes compared to the unamended medium.

Significance and Impact of the Study: The study described represents a new approach to the isolation of siderophore producing actinomycetes using a novel procedure that places a selection on cell population based upon the incorporation of a chelating agent in the medium.

KEYWORDS: Actinomycetes, 2, 2’-dipyridyl, iron chelation, siderophores, soil, Streptomyces.
INTRODUCTION

Iron is an essential element for microbial growth. However, due to the di-oxygen evolution of the atmosphere, iron, at physiological pH, forms insoluble polymeric oxyhydroxide complexes, bio-unavailable to microorganisms (Wandersman and Delepelaire, 2004). Bacterial iron metabolism involves the transcription of specific genes, which are responsible for siderophore production (Gunter et al. 1993). The latter (from the Greek word ‘iron carriers’) are low molecular weight compounds (600-1500 Daltons) that chelate iron from ferric complexes (Neilands, 1995). They are synthesised under iron-limited conditions and are responsible for the uptake and transport of iron and other metals to the cell (Kalinowski et al. 2000; John et al. 2001; Rodriguez and Smith, 2003).

Siderophores are divided into two main chemical groupings, hydroxamates and catechols, and they are classified on the basis of the chemical functional groups that they use to chelate iron (Hofte, 1993; Payne, 1994; Crosa and Walsh, 2002).

Members of streptomycetes are well acknowledged for their ability to produce multiple siderophores, which are independently regulated and act contingently in order to compete more efficiently in their environment (Challis and Hopwood, 2003). Streptomyces coelicolor, the model streptomycete, is known to have the capacity to produce desferrioxamine E and G1 as well as coelichelin and coelibactin (Challis and Ravel, 2000). The complex biosynthetic and uptake pathway for iron-siderophore sequestration has been previously investigated in this organism (Baroma-Gomez et al. 2006).

Siderophores are highly valuable molecules with environmental, agricultural and clinical applications (Arceneux et al. 1984; Kalinowski et al. 2000; John et al. 2001; Rodriguez and Smith, 2003; Kalinowski and Richardson, 2005).

Desferrioxamine B (trade name Desferal), a hydroxamate produced by Streptomyces pilosus, is marketed as the mesylate salt and it is administrated as a drug to patients
suffering iron intoxication (Cramer, 1984; Neilands, 1995). However the drug has to
be administered subcutaneously for a long period due to its hydrophilic nature leading
to poor patience compliance (Kalinowski and Richardson, 2005).
There is an increasing need for the application of siderophores in chelation therapies
and phlebotomy as part of new treatment or in a combination with ‘traditional’
approaches (Ratledge and Dover, 2000; Meyer, 2006).
In order to fully understand the potential value of siderophores, the search for new
candidates must continue. Here we report the incorporation of 2, 2’-dipyridyl in
starch-casein media in order to apply selective pressure for siderophore producing
actinomycetes.

**MATERIALS AND METHODS**

**Isolation of actinomycetes from soil:**

Isolation of actinomycetes from soil was conducted by a soil dilution plate technique
using starch-casein agar (0.4g casein, 1.0 starch, 0.5 g KNO₃, 0.2 g K₂HPO₄, 0.1 g
MgPO₄, 0.1 g CaCO₃, 15 g agar l⁻¹dH₂O). Supplementation of the culture media with
150 μmol l⁻¹ of 2, 2’-dipyridyl (DIP) was also investigated. One gram of each soil
sample was suspended in 100 ml of sterile distilled water and incubated overnight at
40° C. Serial aqueous dilutions (50 μl of 10⁻⁰ – 10⁻⁶) of the soil suspension were
applied onto the agar plates and incubated at 27° C for up to four weeks. Selective
colonies, chosen on the basis of their hairy appearance, self limited colonies and
penetration into the agar matrix, were further sub-cultured and pure actinomycetes
were isolated (Kieser et al. 2000).

**Siderophore production:**

Siderophore production was confirmed using the Chromo Azurol Sulphate (CAS)
assay (Renshaw et al. 2003). The presence of a catecholic compound was further
verified by Arnow’s assay (Arnow, 1937); Hydroxamate producers were identified
using the colorimetric assay of Atkin, 1970.
Ferrioxamine E was identified using an authentic standard by HPLC using a
SphereClone 5μ ODS (2) [150 x 4.60 mm] column. The mobile phase included 0.1 %
phosphoric acid (A) and 100 % acetonitrile (B) delivering the solvent A to the HPLC
system (Biorad, Model 2700) at a concentration of 95 % - 75 % at a flow rate of 1.5
ml/min. The sample volume was 20 μl of the filtered ferric culture supernatant (5 μl
of FeCl₃ ml⁻¹ of supernatant) and the effluent was monitored at 435 nm using a
Biorad-Bio-dimension™ UV/VIS detector

Strain characterisation:
Actinomycete colonies were characterised following directions given by Bergey’s
Manual of Systematic Bacteriology (Locci, 1989); cultural and morphological
characteristics were observed by light and scanning electron microscopy.

16S rRNA amplification:
Extraction of genomic DNA was performed using the ‘Kirby mix procedure’ (Kieser
et al. 2000). Amplification of the 16S sequences were performed with a Perkin-Elmer
Cetus GeneAmp Thermal Cycler, 9600, using 35 cycles of 95°C for 1 minute, 52°C
for 1 minute and 72°C for 2 minutes. Reaction mixtures contained 45 μl of 1.1 x PCR
MasterMix (1.5 mm l⁻¹ MgCl₂) [ABgene], 2 μl of DMSO, 1 μl of genomic DNA, 1 μl
of sterile dH₂O and 0.5 μl of each primer (forward and reverse). In order to achieve
maximum sequencing of the 16S rRNA genes two sets of streptomycete-specific
primers were used: Strep B- StrepE and StrepB- StrepF with the sizes of the PCR
amplification products expected to be of 519bp and 1074bp respectively (Weisburg et
al. 1991; Rintala et al. 2001). The amplified products were visualised on an ethidium-
bromide-stained 2 % agarose gel prior to purification with the QIAquick® PCR
purification kit (50) [Qiagen]. PCR products were sent to Lark™ Technologies, Inc
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RESULTS

Isolation of actinomycetes

In an attempt to investigate a new approach to isolating siderophore producing actinomycetes we have isolated and purified cultures from soil samples collected in Thailand (Tak province). Isolation of actinomycetes from soil was conducted by a soil dilution plate technique using starch-casein agar as regular medium and starch casein agar supplemented with 150 μmol l⁻¹ DIP (higher levels of DIP resulted in very few survivors) as the selective medium. DIP is a powerful chelating agent, which scavenges any ferric complexes from the environment and it is intended to apply selective pressure for organisms adapted to iron deficiency. The majority of colonies recovered from the soils were considered to belong to actinomycetes on the evidence of the specific media applied for their isolation with a further verification by phenotypic examination under the dissecting microscope (self-limited colonies, hairy appearance and substrate-aerial mycelia).

In total 196 actinomycetes were purified and 87 of these gave a positive result on the CAS assay plates indicating the presence of catecholic or hydroxamate moieties (Figure 1). Out of 87 siderophore producers 70 strains were isolated on the selective starch-casein media supplemented with DIP. Addition of the chelating agent reduced the number of surviving actinomycete colonies compared to the regular media and was thought to be likely to have physiological attributes that enabled them to survive iron limitation.

Three different soil samples were investigated. Each soil sample, following treatment at 40°C in sterile distilled water overnight, was plated onto both isolation medium
without DIP and also onto isolation medium containing DIP. The isolates were then
grown on media in the absence of DIP and assessed for the production of siderophores
by their ability to produce a decolourisation of the CAS containing agar Fig 1. In soil
sample 1 the number of siderophore producing isolates detected from isolation
medium unamended with DIP was 37 of which 4 screened positive for siderophore
production (representing 11% of the total). The same sample plated on isolation media
containing DIP resulted in 27 isolates of which 21 screened positive for siderophore
production (representing 78% of the total). The presence of DIP had a deleterious
effect on the total number of actinomycetes isolated however it did significantly
increase the percentage of those isolates that were screened as positive for siderophore
production. A similar pattern was seen with the other soil samples with sample 2 in
the absence of DIP during isolation yielding 12.5% siderophore producers while
isolation in the presence of DIP produced 83% siderophore producers and sample 3
giving 15% in the absence of DIP and 87.5% in the presence of DIP.

Of the isolates 5 strains exhibited prolific siderophore production and 16S rRNA
gene analysis confirmed that the isolates were streptomycetes (GenBank accession
number EF585403- EF585407). Liquid based assays were conducted on culture
supernatants in order to categorise the siderophore producers. The results classified
the strains EF585404- EF585407 as catechol producers, whereas EF585403 was
categorised as a hydroxamate synthesiser. Following HPLC analysis this chelating
agent produced by EF585403 was further classified as desferrioxamine E with
reference to an authentic standard.

**DISCUSSION**

It is anticipated that the isolation and characterisation of new actinomycetes might
lead to novel siderophores of significant commercial interest. The actinomycetes are
notorious environmental scavengers and have evolved well-developed processes to
capture ions in conditions depleted of elements; they are therefore an ideal target
group for exploitation in ion binding technology (Meiwe et al. 1990; Challis and
Ravel, 2000; Bentley et al. 2002). The method described significantly increased the
proportion of siderophore producers. Whether the nature of the selective chelating
agent dictates the type of siderophore produced remains to be determined. The
prohibitive cost of many of the natural siderophore currently available renders such a
systematic approach non-viable at present. Apart from the pharmaceutical values of
siderophores these compounds are recognised as important mediators of metal ion
remediation in the environment (John et al. 2001) and also plant growth promoters
(Katyar and Goel 2004). Exploitation of these compounds requires cost effective
production methods. It is hoped that the methodology described will move us towards
a greater range of siderophores and also realise the opportunity to use this technology
where bulk production is required.

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Figure 1: Total number of actinomycetes and siderophore producers isolated on starch 
and casein agar (S-C) and starch-casein agar supplemented with DIP (S-C-DIP).
Figure 1: Total number of actinomycetes and siderophore producers isolated on starch casein agar (S-C) and starch-casein agar supplemented with DIP (S-C-DIP).