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**A new approach to isolating siderophore producing actinomycetes.**

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Manuscripts

Review

1 **A new approach to isolating siderophore producing actinomycetes.**

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9 **Running title:** Siderophore producing actinomycetes

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27 **ABSTRACT**

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

30 **Methods and Results:** Isolation of actinomycetes from soil was conducted by a soil  
31 dilution plate technique using starch-casein agar. Iron starvation was fostered by the  
32 incorporation of the iron chelator 2, 2'-dipyridyl in the isolation medium. Pre-  
33 treatment of the samples at an elevated temperature (40<sup>0</sup> C) ensured that the majority  
34 of non-sporulating bacteria were excluded. The survivors of this treatment were  
35 largely actinomycetes. Of the viable cultures grown in the presence of 2, 2'-dipyridyl,  
36 more than 78-88 % (average of three separate studies) were reported to produce  
37 siderophore like compounds compared to 13- 18% (average of three separate  
38 studies)when grown on the basic media in the absence of the chelating agent. The  
39 most prolific producers as assessed by the Chromo Azuerol Sulphate (CAS) assay  
40 were further characterised and found to belong to the genus *Streptomyces*.

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

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

48 **KEYWORDS:** Actinomycetes, 2, 2'-dipyridyl, iron chelation, siderophores, soil,  
49 *Streptomyces*.

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54 **INTRODUCTION**

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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).

65 Siderophores are divided into two main chemical groupings, hydroxamates and  
66 catechols, and they are classified on the basis of the chemical functional groups that  
67 they use to chelate iron (Hofte, 1993; Payne, 1994; Crosa and Walsh, 2002).  
68 Members of streptomycetes are well acknowledged for their ability to produce  
69 multiple siderophores, which are independently regulated and act contingently in  
70 order to compete more efficiently in their environment (Challis and Hopwood, 2003).  
71 *Streptomyces coelicolor*, the model streptomycete, is known to have the capacity to  
72 produce desferrioxamine E and G1 as well as coelichelin and coelibactin (Challis and  
73 Ravel, 2000). The complex biosynthetic and uptake pathway for iron-siderophore  
74 sequestration has been previously investigated in this organism (Baroma-Gomez et al.  
75 2006).

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

79 Desferrioxamine B (trade name Desferal), a hydroxamate produced by *Streptomyces*  
80 *pilosus*, is marketed as the mesylate salt and it is administrated as a drug to patients

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81 suffering iron intoxication (Cramer, 1984; Neilands, 1995). However the drug has to  
82 be administered subcutaneously for a long period due to its hydrophilic nature leading  
83 to poor patient compliance (Kalinowski and Richardson, 2005).

84 There is an increasing need for the application of siderophores in chelation therapies  
85 and phlebotomy as part of new treatment or in a combination with 'traditional'  
86 approaches (Ratledge and Dover, 2000; Meyer, 2006).

87 In order to fully understand the potential value of siderophores, the search for new  
88 candidates must continue. Here we report the incorporation of 2, 2'-dipyridyl in  
89 starch-casein media in order to apply selective pressure for siderophore producing  
90 actinomycetes.

## 91 **MATERIALS AND METHODS**

### 92 **Isolation of actinomycetes from soil:**

93 Isolation of actinomycetes from soil was conducted by a soil dilution plate technique  
94 using starch-casein agar (0.4g casein, 1.0 starch, 0.5 g KNO<sub>3</sub>, 0.2 g K<sub>2</sub>HPO<sub>4</sub>, 0.1 g  
95 MgPO<sub>4</sub>, 0.1 g CaCO<sub>3</sub>, 15 g agar l<sup>-1</sup>dH<sub>2</sub>O). Supplementation of the culture media with  
96 150 μmol l<sup>-1</sup> of 2, 2'-dipyridyl (DIP) was also investigated. One gram of each soil  
97 sample was suspended in 100 ml of sterile distilled water and incubated overnight at  
98 40<sup>0</sup> C. Serial aqueous dilutions (50 μl of 10<sup>0</sup> – 10<sup>-9</sup>) of the soil suspension were  
99 applied onto the agar plates and incubated at 27<sup>0</sup> C for up to four weeks. Selective  
100 colonies, chosen on the basis of their hairy appearance, self limited colonies and  
101 penetration into the agar matrix, were further sub-cultured and pure actinomycetes  
102 were isolated (Kieser et al. 2000).

### 103 **Siderophore production:**

104 Siderophore production was confirmed using the Chromo Azuerol Sulphate (CAS)  
105 assay (Renshaw et al. 2003). The presence of a catecholic compound was further

106 verified by Arnow's assay (Arnow, 1937); Hydroxamate producers were identified  
107 using the colorimetric assay of Atkin, 1970.  
108 Ferrioxamine E was identified using an authentic standard by HPLC using a  
109 SphereClone 5 $\mu$  ODS (2) [150 x 4.60 mm] column. The mobile phase included 0.1 %  
110 phosphoric acid (A) and 100 % acetonitrile (B) delivering the solvent A to the HPLC  
111 system (Biorad, Model 2700) at a concentration of 95 % - 75 % at a flow rate of 1.5  
112 ml/min. The sample volume was 20  $\mu$ l of the filtered ferric culture supernatant (5  $\mu$ l  
113 of FeCl<sub>3</sub> ml<sup>-1</sup> of supernatant) and the effluent was monitored at 435 nm using a  
114 Biorad-Bio-dimension™ UV/VIS detector

115 **Strain characterisation:**

116 Actinomycete colonies were characterised following directions given by Bergey's  
117 Manual of Systematic Bacteriology (Locci, 1989); cultural and morphological  
118 characteristics were observed by light and scanning electron microscopy.

119 **16S rRNA amplification:**

120 Extraction of genomic DNA was performed using the 'Kirby mix procedure' (Kieser  
121 et al. 2000). Amplification of the 16S sequences were performed with a Perkin-Elmer  
122 Cetus GeneAmp Thermal Cycler, 9600, using 35 cycles of 95<sup>0</sup> C for 1 minute, 52<sup>0</sup> C  
123 for 1 minute and 72<sup>0</sup> C for 2 minutes. Reaction mixtures contained 45  $\mu$ l of 1.1 x PCR  
124 MasterMix (1.5 mm l<sup>-1</sup> MgCl<sub>2</sub>) [ABgene], 2  $\mu$ l of DMSO, 1  $\mu$ l of genomic DNA, 1  $\mu$ l  
125 of sterile dH<sub>2</sub>O and 0.5  $\mu$ l of each primer (forward and reverse). In order to achieve  
126 maximum sequencing of the 16S rRNA genes two sets of streptomycete-specific  
127 primers were used: Strep B- StrepE and StrepB- StrepF with the sizes of the PCR  
128 amplification products expected to be of 519bp and 1074bp respectively (Weisburg et  
129 al. 1991; Rintala et al. 2001). The amplified products were visualised on an ethidium-  
130 bromide-stained 2 % agarose gel prior to purification with the QIAquick<sup>R</sup> PCR  
131 purification kit (50) [Qiagen]. PCR products were sent to Lark™ Technologies, Inc

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(United Kingdom) according to their requirements. The sequences obtained were individually checked for errors and manually aligned. Retrieval of homologous sequences and pairwise sequence comparisons and were conducted by the NCBI BLAST database (available online- <http://www.ncbi.nih.gov/>).

## 136 **RESULTS**

### 137 **Isolation of actinomycetes**

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

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

157 Three different soil samples were investigated. Each soil sample, following treatment  
158 at  $40^{\circ}\text{C}$  in sterile distilled water overnight, was plated onto both isolation medium



159 without DIP and also onto isolation medium containing DIP. The isolates were then  
160 grown on media in the absence of DIP and assessed for the production of siderophores  
161 by their ability to produce a decolourisation of the CAS containing agar Fig 1. In soil  
162 sample 1 the number of siderophore producing isolates detected from isolation  
163 medium unamended with DIP was 37 of which 4 screened positive for siderophore  
164 production (representing 11% of the total). The same sample plated on isolation media  
165 containing DIP resulted in 27 isolates of which 21 screened positive for siderophore  
166 production (representing 78% of the total). The presence of DIP had a deleterious  
167 effect on the total number of actinomycetes isolated however it did significantly  
168 increase the percentage of those isolates that were screened as positive for siderophore  
169 production. A similar pattern was seen with the other soil samples with sample 2 in  
170 the absence of DIP during isolation yielding 12.5% siderophore producers while  
171 isolation in the presence of DIP produced 83% siderophore producers and sample 3  
172 giving 15% in the absence of DIP and 87.5% in the presence of DIP.

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

## 181 **DISCUSSION**

182 It is anticipated that the isolation and characterisation of new actinomycetes might  
183 lead to novel siderophores of significant commercial interest. The actinomycetes are  
184 notorious environmental scavengers and have evolved well-developed processes to  
185 capture ions in conditions depleted of elements; they are therefore an ideal target

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186 group for exploitation in ion binding technology (Meiwes et al. 1990; Challis and  
187 Ravel, 2000; Bentley et al. 2002). The method described significantly increased the  
188 proportion of siderophore producers. Whether the nature of the selective chelating  
189 agent dictates the type of siderophore produced remains to be determined. The  
190 prohibitive cost of many of the natural siderophore currently available renders such a  
191 systematic approach non-viable at present. Apart from the pharmaceutical values of  
192 siderophores these compounds are recognised as important mediators of metal ion  
193 remediation in the environment (John et al. 2001) and also plant growth promoters  
194 (Katyar and Goel 2004). Exploitation of these compounds requires cost effective  
195 production methods. It is hoped that the methodology described will move us towards  
196 a greater range of siderophores and also realise the opportunity to use this technology  
197 where bulk production is required.

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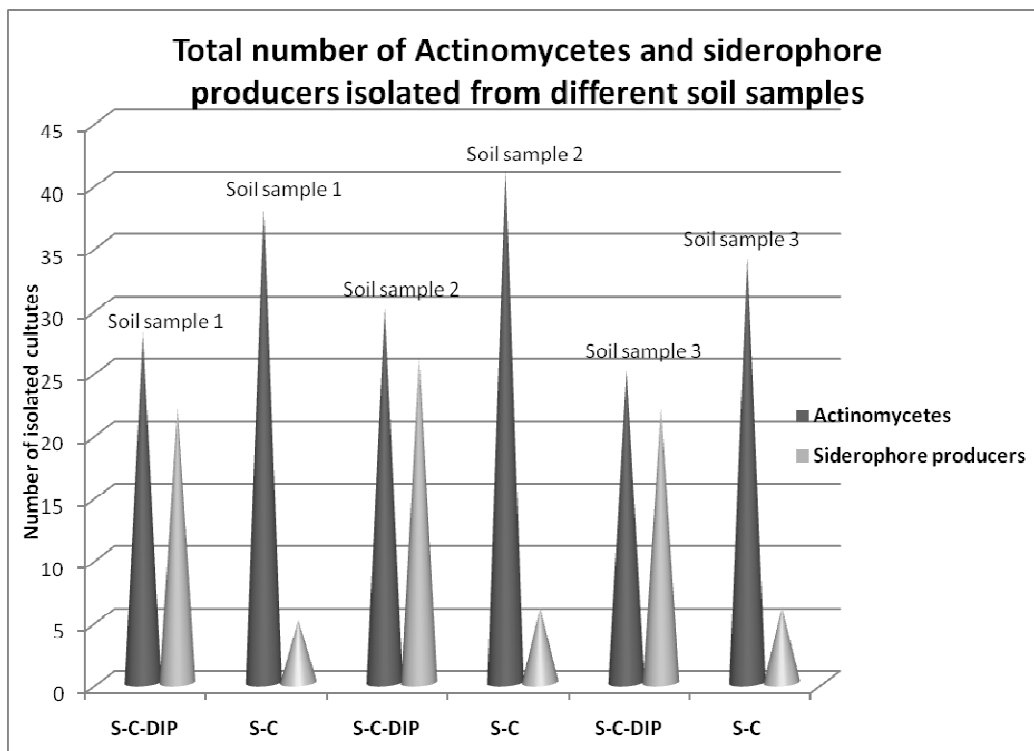
288 **List of figures:**

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

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