A new approach to isolating siderophore producing actinomycetes.

Journal:	*Applied Microbiology*
Manuscript ID:	Draft
Journal Name:	2 Letters in Applied Microbiology - LAM
Manuscript Type:	LAM - Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Nakouti, Ismini; Liverpool John Moore's University, Pharmacy and Biomolecular Sciences Hobbs, Glyn; John Moores University, School of Pharmacy and Biomolecular Sciences Sihanonth, Prakitsin; Chulalongkorn University, Department of Microbiology
Key Words:	Actinomycetes, Streptomycetes, Soil

A new approach to isolating siderophore producing actinomycetes.

Ismini Nakouti[1], Prakitsin Sihanonth[2], and Glyn Hobbs[1]*

[1]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK

[2] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, 10300, Thailand

Running title: Siderophore producing actinomycetes

* Author for correspondence: School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool. Tel: 0151 2312198. Fax: 0151 2312170. E-mail: g.hobbs@ ljmu.ac.uk

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 (40^0 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 Azuerol 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 KNO_3, 0.2 g K_2HPO_4, 0.1 g $MgPO_4$, 0.1 g $CaCO_3$, 15 g agar $l^{-1}dH_2O$). 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 40^0 C. Serial aqueous dilutions (50 µl of $10^0 - 10^{-9}$) of the soil suspension were applied onto the agar plates and incubated at 27^0 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 Azuerol 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 $FeCl_3$ ml^{-1} of supernatant) and the effluent was monitored at 435 nm using a Biorad-Bio-dimension^{TM} 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 95^0 C for 1 minute, 52^0 C for 1 minute and 72^0 C for 2 minutes. Reaction mixtures contained 45 µl of 1.1 x PCR MasterMix (1.5 mm l^{-1} $MgCl_2$) [ABgene], 2 µl of DMSO, 1 µl of genomic DNA, 1 µl of sterile dH_2O 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^{R} PCR purification kit (50) [Qiagen]. PCR products were sent to Lark$_{TM}$ Technologies, Inc

(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/).

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 40^0 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 (Meiwes 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.

REFERENCES

Arceneaux, J. E. L., Boutwell, M. E. and Byers, B. R. (1984) Enhancement of copper toxicity by siderophores in *Bacillus megaterium*. *Antimicrob agents Chemother* **25**, 650-652.

Arnow, L. E. (1937) Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine-tyrosine mixtures. *J Biol Chem* **118**, 531-537.

Atkin, C. L., Neilands, J. B. and Phaff, H. J. (1970) Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. *J Bacteriol* **103**, 722-733.

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G, Chen, C. W., Colllins, M., Cronin, A., Fraser, A., Goble, A., Hilalgo, J., Hornsby, T., Howarth. S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Robbinowitsch, E., Rajandream, M. A., Rutherford, B. G., Rutter, S.,

Seeger, K., Saunders, D., Sharp, S., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill. J. and Hopwood, D. A (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). *Nature* **417**, 141-147.

Baroma-Gomez, F., Lautru, S., Francou, F-X., Leblond, P., Pernodet, J-L. and Challis, G. L. (2006) Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3 (2) and *Streptomyces ambofaciens* ATCC23877. *Microbiology* **152**, 3355-3366.

Challis, G. L. and Hopwood, D. A (2003) Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc Natl Acad Sci* **100**, 14555-14561.

Challis, G. L. and Ravel, J. (2000) Coelichelin, a new peptide siderophore encoded by the *Streptomyces coleicolor* genome: structure prediction from the sequence of its non-ribosomal peptide synthetase. *FEMS Microbiol lett* **187**, 111-114.

Cramer, S. M., Nathanael, B. and Horvath, C. (1984) High-performance liquid chromatography of desferrioxamine and ferrioxamine: interference by iron present in the chromatographic system. *J Chromatog* **295**, 405-411.

Crosa, J. H. and Walsh, C. T. (2002). Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol Mol Biol Rev* **66**, 223-249.

Gunter, K., Toupet, C. and Schupp, T (1993). Characterization of an iron-regulated promoter involved in desferrioxamine B synthesis in *Streptomces pilosus*: Repressor-binding site and homology to the dipheria toxin gene promoter. *J Bacteriol* **175**, 3295-3302.

Höfte, M. (1993) Classes of microbial siderophores. In *Iron chelation in plants and soil Microorganisms* ed Barton, L. L. and Hemming, B. C. pp 3-26. Academic Press, San Diego, Calif.

Hopwood, D. A. (2007) How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? *Mol Microbiol* **63**, 937–940.

John, S. G., Christy, E., Ruggiero, L. E., Tung, S. and Neu, M. P (2001) Siderophore production mediated plutonium accumulation by *Microbacterium flavescens* (JG-9). *Environ Sci Techol* **35**, 2942-2948.

Kalinowski, D. S. and Richardson, D. R. (2005) The evolution of iron chelators for the treatment of iron overload disease and cáncer. *Pharmacol Rev* **57**, 547-583.

Kalinowski, B. E., Liermann, L. J., Givens, S. and Brantley, S. L. (2000) Rates of bacteria-promoted solubilization of Fe from minerals: a review of problems and approaches. *Chem Geol* **169**, 357-370.

Katiyar, V. and Goel, R. (2004) Siderophore plant growth promotion at low temperature by mutant of fluorescent pseudomonad. *Plant Growth Regul* **42** 239-244

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. and Hopwood, D. A. (2000) In *Practical Streptomyces genetics*, pp 8, 168-169 & 342-343. The John Innes Foundation, Norwich.

Kim, C., Walter Lorenz, W., Hoopes. J. T. and Dean, F. F. D. (2001) Oxidation of phenolate siderophores by the multicoppers oxidase encoded by the *Escherichia coli yacK* gene. *J Bacteriol* **183**, 4866-4875.

Lee, S. D. and Hah, Y. C. (2001). *Amycolatopsis albidoflavus* sp. Nov. *Int J Syst Evol Microbiol* **51**, 645-650.

Locci, R. (1989) *Streptomyces* and related Genera. In *Bergey's Manual of Systematic Bacteriology* ed. Williams Wikins Company, Baltimore. pp 2451-1508.

Meiwes, J., Fiedler, H. P., zahner, H., Konetschny-Rapp, S. and Jung, G. (1990) Production of desferrioxamine E and new analoques by directed fermentation and feeding fermentation. *Appl Microbiol Biotechnol* **32**, 505-510.

Meyer, D. (2006) Iron chelation therapy for HIV and *Mycobacterium tuberculosis* co-infection under conditions of iron overload. *Curr Pharm Des* **12**, 194-1947.

Neilands, J. B. (1995) Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**, 26723-26726.

Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y. and Hattori, M. (2001) Genome sequence of an industrial microorganism *Streptomyces avermitilis***:** Deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci USA* **98**, 12215-12220

Payne, S. M. (1994) Detection, isolation and characterisation of siderophores. *Methods Enzymol* **235**, 329-344.

Ratledge, C. and Dover, L. G. (2000) Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**, 881–941.

Renshaw, J. C., Halliday, V., Robson, G. D., Trinci, A. P. J., Wiebe, M. G., Livens, F. R., Collison, D. and Taylor, R. J. (2003) Development and application of an assay for uracyl complexation by fungal metabolites, including siderophores. *Appl Environl Microbiol* **69**, 3600-3606.

Rodriquez, M. G. and Smith, I. (2003) Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. *Molecul Microbiol* **47**, 1485-1495.

Wandersman, C. and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* **58**, 611-647.

List of figures:

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).

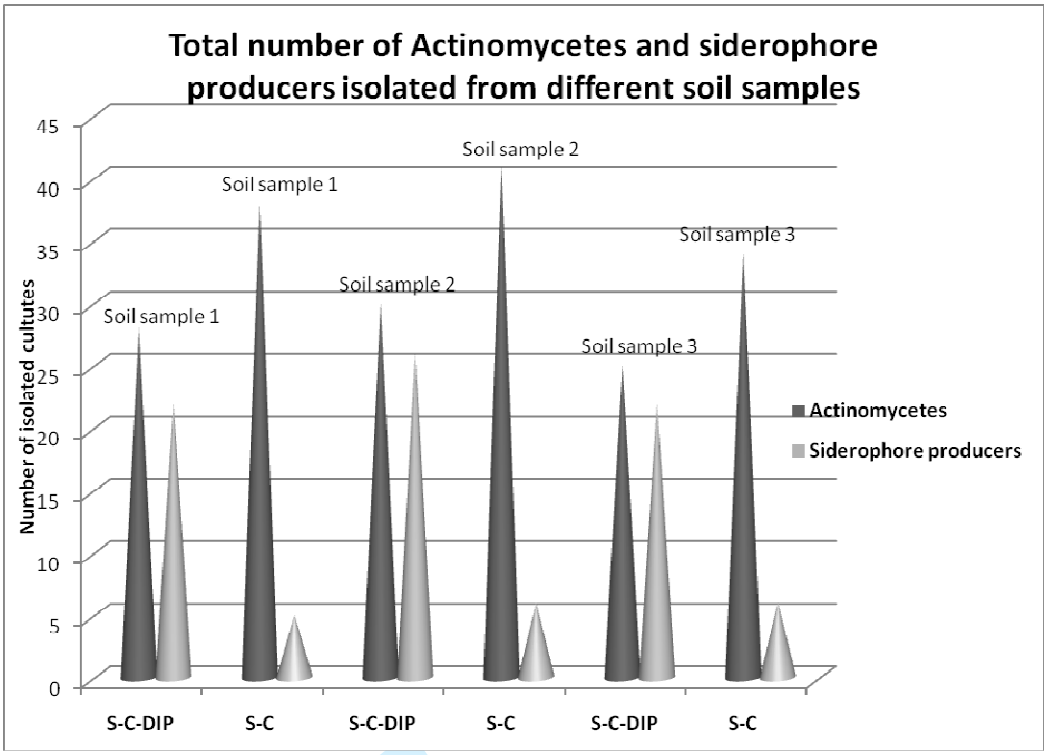

Figure 1: Total number of actinomycetes and siderophore producers isolated on starch casein agar (S-C) and starch-casein agar supplemented with DIP (S-C-DIP).