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# Siderophore production by actinomycetes isolates from two soil sites in Western Australia

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**Abstract** The actinomycetes are metabolically flexible soil micro-organisms capable of producing a range of compounds of interest, including siderophores. Siderophore production by actinomycetes sampled from two distinct and separate geographical sites in Western Australia were investigated and found to be generally similar in the total percentage of siderophore producers found. The only notable difference was the proportion of isolates producing catechol siderophores with only 3% found in site 1 (from the north-west of Western Australia and reportedly containing 40% magnetite) and 17% in site 2 (a commercial stone fruit orchard in the hills east of Perth with a soil base ranging from sandy loam to laterite). Further detailed characterization of isolates of interest identified a *Streptomyces* that produced extracellularly excreted enterobactin, the characteristic Enterobacteriaceae siderophore, and also revealed some of the conditions required for enterobactin production. Carriage of the *entF* gene, which codes for the synthetase responsible for the final assembly of the tri-cyclic structure of enterobactin, was confirmed by PCR in this isolate. Another separate *Streptomyces* produced a compound that matched the UV/VIS spectra of heterobactin, a siderophore previously only described in *Rhodococcus* and *Nocardia*.

**Keywords** Siderophores · Actinomycetes · *Streptomyces* · Enterobactin · Heterobactin

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## Introduction

Iron is an essential trace element required by bacteria for physiological processes necessary for survival. However, iron in the environment is present as various forms of insoluble iron oxides and not easily accessible by bacteria. The production of siderophores is one strategy employed by bacteria to overcome low iron availability. Siderophores are low molecular weight chelators of ferric iron (Fe(III)) that are synthesized under conditions of iron starvation by non-ribosomal peptide synthetases (NRPS) or NRPS-independent pathways (Oves-Costales et al. 2009; Walsh and Marshall 2004). Most siderophores are then excreted extracellularly to bind Fe(III) and the ferric complexes are recovered by bacteria via specialized receptors. Fe(III) is dissociated from the siderophore for use by the bacterium (Crosa 1989).

Siderophores are a structurally and architecturally diverse group of molecules and can be classified on the basis of the chemical structure of the functional group that interacts with Fe(III). The two most common classifications of siderophores are catechol and hydroxamate and some siderophores have a mix of groups, i.e. mixed carboxylate–hydroxamate (Hider and Kong 2010; Raymond and Dertz 2004). Siderophores can also incorporate other, less common, functional groups that will complex with iron.

The actinomycetes are Gram-positive bacteria found in soil and include a range of genera shown to produce siderophores. *Streptomyces* are actinomycetes and most commonly produce some type of trihydroxamate siderophore known as desferrioxamine (Imbert et al. 1995; Meiwes et al. 1990; Yamanaka et al. 2005) but reports have described isolates capable of producing enterobactin (Fiedler et al. 2001) the characteristic siderophore of Enterobacteriaceae, that was not excreted into the external environment but remained in the bacterial biomass. Others have described the *Streptomyces* siderophores coelichelin (Challis and Ravel 2000; Lautru et al. 2005) and griseobactin (Patzer and Braun 2010), while the novel heterobactin siderophores have been found in *Rhodococcus* (Carrano et al. 2001) and *Nocardia* (Mukai et al. 2009).

This study examined siderophore production by environmental isolates of actinomycetes collected from soil in Western Australia. The general ability of isolates to produce siderophore was investigated and siderophores classified as catechol or hydroxamate. More detailed assessment of a subset of isolates using HPLC analysis identified the known siderophores produced. The kinetics of siderophore production in response to iron concentration and over an extended period of time was also monitored to determine the optimum conditions for maximum siderophore production. Two novel isolates were found among the isolates examined: a *Streptomyces* producing excreted enterobactin and a *Streptomyces* putatively producing heterobactin.

## Materials and methods

### Collection and isolation of actinomycetes

Soil was collected and serially diluted from $10^{-1}$ to $10^{-4}$ in sterile distilled water. Aliquots of 100 µl from each dilution were plated in duplicate on modified ISP4 agar (4 g/l soluble starch, 0.3 g/l casein, 2 g/l $KNO_3$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l $CaCO_3$, 0.01 g/l $FeSO_4 \cdot 7H_2O$ and 25 g/l agar) and starch nitrate agar plates containing 0.01% pimafucin, 10 µg/ml acridione, 5 µg/ml oxytetracycline and 0.5 µg/ml polymyxin B (Al-Zarban et al. 2002; Shirling and Gottlieb 1966). After incubation at 26°C for 1 week actinomycete colonies were picked off the plates, inoculated onto ISP4 agar slopes and incubated at 26°C for 1 month.

### Growth of actinomycetes

Each actinomycete isolate was inoculated into 5 ml of modified ISP4 broth, an iron-limiting and chemically defined growth medium. The composition of the ISP4 broth was modified by the removal of casein and $FeSO_4 \cdot 7H_2O$, and the inclusion of 1 g/l of yeast extract. In experiments where isolates were monitored for the ability to produce siderophore in the presence of iron, the modified ISP4 broth was supplemented with 1 mM $FeCl_3$ in 1 mM HCl to the required iron concentration. Isolates were grown in glass bottles or flasks at 26°C, with shaking, until sporulation occurred or after at least 1 week of growth.

### Screening for siderophore production by CAS assay

The liquid chrome azurol S (CAS) assay was used to assess siderophore production by isolates during growth in modified ISP4 broth (Schwyn and Neilands 1987). Culture supernatant was sterilized by filtration through a 0.2 µm membrane and 200 µl was removed to a clear plastic tube. An equal volume of CAS assay solution was added and the mixture was then observed for any change in color. A color change from blue to pink or orange was indicative of the presence of siderophores. Un-inoculated ISP4 broth was used as a negative control and remained blue in the assay.

On the basis of fast (<15 min) or immediate positive reactions in the CAS assay, isolates were classified as potentially strong siderophore producers. Isolates were classified as borderline siderophore producers if the color change was incomplete and took longer than 12 h to occur.

### Classification of siderophores produced as catechol or hydroxamate

Isolates that returned a positive or negative reaction for siderophore production in the CAS assay were screened further to determine if the siderophore produced was a catechol or a hydroxamate. The Arnow test and $FeCl_3$ test were used to classify siderophores as, respectively, either catechol or hydroxamate (Arnow 1937; Meyer et al. 1995). In the Arnow test, 200 µl of sterilized supernatant was mixed with equal volumes of 0.5 M HCl, nitrite/molybdate reagent and 1 M NaOH in a clear microcentrifuge tube, mixing vigorously between the addition of each reagent. The presence of catechol siderophores was indicated by a pink color change; the mixture remained clear if catechol siderophores were absent. In the $FeCl_3$ test, 1 ml of sterilized supernatant was mixed with 200 µl of 100 mM $FeCl_3$ in 0.1 M HCl. The presence of trihydroxamate siderophores was indicated by orange and dihydroxamate siderophores by pink.

### Solvent extraction of siderophores

The protocol for solvent extraction of siderophores was modified according to whether siderophores were catechol or hydroxamate. For catechol siderophores, 8 ml of supernatant was adjusted to pH 2–3 by the dropwise addition of 0.5 M HCl. Siderophore was then extracted with 1:5 volume of ethyl acetate, repeated three times to ensure maximum recovery of siderophore. Ethyl acetate was evaporated at room temperature and extracted siderophore was resuspended in 300 µl of methanol.

For hydroxamate siderophores, NaCl was added to 8 ml of supernatant to a concentration of 300 g/l and mixed thoroughly. Siderophore was then extracted with a 1:5 volume of benzyl alcohol and three volumes of diethyl ether and driven into a 1:20 volume of sterile distilled water. Diethyl ether was removed by evaporation at room temperature. Water was evaporated by incubation at 50°C and extracted siderophore was resuspended in methanol.

### HPLC of extracted siderophore

HPLC was conducted on an Agilent 1200 series DAD-HPLC system using a Zorbax Eclipse Plus C-18 Solvent Saver Plus column (3 mm diameter × 150 mm length, 3.5 µm particle size) with a guard column (4.6 mm length × 12.5 mm diameter, 5 µm particle size). The mobile phase employed consisted of 0.1% orthophosphoric acid in Milli-Q water (solvent A) and HPLC grade acetonitrile (solvent B). A 10 µl volume of each sample was injected into the column and run using a linear gradient from 0% solvent B to 100% solvent B in 20 min with a 5 min hold at 100% solvent B and 5 min post-time at initial conditions. A constant flow rate of 0.4 ml/min was used. Peaks were detected at 204 nm with a 1 nm step using a slit width of 2 nm and a data sampling rate of 500 ms.

The identity of analytes was established by comparison of UV spectra against a library of standards purchased from Professor H. P. Fiedler (Universität Tübingen) (Fiedler 1993). Analytes were considered a match if a score of 990 or greater was returned.

### Quantification of siderophore production by CAS dilution assay

Serial 1:2 dilutions of sterilized culture supernatant were prepared in 96 well plates to a final volume of 100 µl in each well; dilutions ranged from neat to 1/128. Chelex-treated iron-free ISP4 broth was processed in the same way and used as a control. A 100 µl aliquot of CAS reagent was added to and mixed in each well and 96 well plates were then incubated at room temperature for an hour to allow for any color change to develop. The absorbance at 630 nm was read in an xMark Microplate Absorbance Spectrophotometer (Biorad) for each dilution of each sample of supernatant. Zeroed dilution data, where average control absorbance had been subtracted, was used to plot a curve with dilution factor on the X-axis and absorbance at 630 nm on the Y axis. Lower absorbance values indicate greater siderophore activity.

### Calculation of % siderophore units

The curve generated by the CAS dilution assay was used to calculate the % siderophore units present in neat sample of sterilized supernatant. Linear regression analysis was conducted on the data points within the linear portion of the curve, using at least three data points and excluding data points that were obvious outliers. This produced an equation describing the distribution of those data points in the form of $y = ax + b$. This equation was used to calculate:

- $x$ when $y = 0$
- $y$ when $x = 1$

The value of $x$ when $y = 0$ is the dilution factor when supernatant is so dilute that the presence of siderophore can no longer be detected by CAS assay. The value of $y$ when $x = 1$ is the absorbance in the CAS assay when neat supernatant is used (i.e. when dilution factor is 1). This value was then used to calculate % siderophore units in the neat supernatant of an isolate using the equation:

$$\%\ \text{siderophore units} = \left[\frac{|y\ \text{when}\ x = 1|}{\text{reference absorbance}}\right] \times 100$$

where $|y\ \text{when}\ x = 1|$ is the absolute value of $y$ when $x = 1$ and reference absorbance is the average control absorbances.

Due to the zeroing of the raw data prior to analysis, where the average control readings was subtracted from isolate absorbances, the above equation for calculation of % siderophore units is actually a form of the function used to calculate % siderophore units (Payne 1994), traditionally expressed as:

$$\%\ \text{siderophore units} = \left[\frac{|\text{sample absorbance} - \text{reference absorbance}|}{\text{reference absorbance}}\right] \times 100$$

PCR amplification of *entF* gene

A 203 bp fragment of the *entF* gene that encodes the final NRPS in the enterobactin biosynthetic pathway was amplified with the *entF* forward (5′-CGCAACT TTCAGGTGTGCAGCG-3′) and *entF* reverse (5′-TT CCGGCAGCGTTTCTTGCG-3′) primers. Template DNA for use in PCR was prepared by boiling pelleted cells from 1 ml of culture. Amplification was done in a 25 µl PCR reaction mixture containing 2 µl of boiled lysate template DNA, 1.5 mM $MgCl_2$, 0.2 mM each of four dNTPs, 0.6 µM of each primer, 1.25 U of AmpliTaq Gold DNA polymerase and 1 × PCR buffer.

Reactions were heated to 95°C in a thermal cycler for 10 min to activate the DNA polymerase. This was followed by 35 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 1 min), followed by a final extension cycle (72°C for 10 min). Samples were electrophoresed in 2.5% agarose gels containing ethidium bromide, and then visualized under UV light. A positive control reaction with template DNA from *Escherichia coli* MG1655 was also done.

Structure determination by mass spectrometry

Siderophore purified by HPLC underwent structural analysis by negative ion-electro spray ionization (ESI) on an Agilent 6540 UHD Q-TQF mass spectrometer. Analysis was conducted using a Phenomomex Luna C18 column (3 µm – 2 × 50 mm) at a flow of 0.4 ml/min. The mobile phases were 5 mmol ammonium formate (pH 3) and methanol, programmed from 5% methanol to 100% methanol over 8 min.

Ion spectra was obtained on the Agilent 1100 MSD-Trap mass spectrometer using an Agilent Extend C18 column (5 µm – 2 × 150 mm) at a flow of 0.5 ml/min. The mobile phases were 10 mmol ammonium formate (pH 3) and methanol, programmed from 20% methanol to 100% methanol over 9 min.

Taxonomic identification by 16S rRNA sequencing

Taxonomic identification of isolates by 16S rRNA sequencing was conducted by PathWest Laboratory Medicine WA. Sequences were matched to known isolates by BLAST and aligned with BioEdit version 7.0.5.3 using default CLUSTAL W settings (Altschul et al. 1990; Thompson et al. 1994). MEGA5 was used to generate a phylogenetic tree using 1,339 bp of aligned sequence data from the 10 closest matches to each isolate that were identified to the species level (Tamura et al. 2011). The phylogenetic tree was generated using the Neighbour-Joining method and the bootstrap test (1,000 replicates) test of phylogeny (Felsenstein 1985; Saitou and Nei 1987). Evolutionary distances were calculated using the p-distance method (Nei and Kumar 2000).

## Results

### Siderophore production by actinomycetes from geographically distinct sites

A total of 112 actinomycete isolates were recovered from soil at two geographically distinct sites in Western Australia; 64 isolates from (1) a site in the north-west of WA reportedly containing 40% magnetite in soil, and 48 isolates from (2) a commercial stone fruit orchard in the hills east of Perth with a soil base ranging from sandy loam to laterite. The isolates were assessed for their ability to produce siderophore during growth in iron-free ISP4 broth. It was found that the populations of actinomycetes from the two sites had very similar siderophore production characteristics (Table 1). Both populations had similar overall rates of siderophore production; the rates of strong and borderline siderophore production were also very similar though there tended to be more borderline siderophore producers from site 2. All isolates excreted hydroxamate siderophores, while only 10 (9%) co-produced a catechol-type siderophore (Table 1). The main point of difference between the two sites was that the rate of catechol production was much higher among the isolates of site 2, which was 17%, compared to the 3% detected among isolates of site 1 (Table 1).

### HPLC analysis of extracted siderophore

HPLC analysis conducted on extracted siderophore from each of 22 strong siderophore-producing isolates found extensive variation in the repertoire of analytes produced by each isolate. Previous screening with the $FeCl_3$ and Arnow tests had found all 22 isolates were positive for trihydroxamate production and nine isolates were positive for catechol production. The number and size of peaks produced varied between isolates, ranging from a single large peak to multiple large peaks accompanied by many smaller peaks.

Comparison of the UV spectra associated with each analyte peak to a library of known analytes identified the presence of known siderophores or their precursors and breakdown products. Only isolate 1481 produced intact enterobactin with a retention time of 9.450 min, which returned a matching score of 995 to enterobactin in the HPLC library (Fig. 1). Isolate 1481 also produced dihydroxybenzoylserine monomers, dimers and linear trimers (retention time 7.726 min) that matched the library with scores of 994. Dihydroxybenzoylserine monomers and dimers were also produced by isolates 2342 and 2349, which were positive for catechol production by the Arnow test, along with isolate 1481.

Isolate 2350 produced a peak at retention time 8.644 min, which returned a matching score of 993 to heterobactin, a mixed catechol-hydroxamate siderophore in the library of analytes (Fig. 2). Similar heterobactin-matching peaks were also detected in HPLC of isolates 2332 (retention time 8.820 min), 2342 (retention time 8.631 min), 2344 (retention time 8.666 min), 2351 (retention time 8.648 min) and 2366 (retention time 8.629 min). Isolate 2332 returned a match specifically to heterobactin A. Isolates 2342, 2344 and 2351 were positive for catechol production using the Arnow test.

Many large, distinct peaks present in the isolate chromatograms found no definitive match in the library. The most significant of these unidentified analytes produced a peak at approximately 2 min in all 22 isolates screened by HPLC. Analysis of its associated UV spectra returned a match to a range of hydroxamate siderophores.

**Table 1** Siderophore production in actinomycetes isolates from two geographically distinct sites

| | Site 1 | Site 2 |
|---|---|---|
| Total CAS positive | 55/64 (86%) | 37/48 (77%) |
| Borderline CAS positive | 9/64 (14%) | 11/48 (23%) |
| Strong CAS positive | 13/64 (20%) | 9/48 (19%) |
| Hydroxamate production | 64/64 (100%) | 48/48 (100%) |
| Catechol production | 2/64 (3%) | 8/48 (17%) |

*Site 1* North-west of WA, *site 2* orchard east of Perth

### Determination of enterobactin structure

Examination of the HPLC peak by negative ESI gave a molecular weight of 669.1455 Da, represented as a negative ion of 668.1377. That was consistent with the calculated molecular weight for enterobactin ($C_{30}H_{27}N_3O_{15}$) of 669.1442. The compounds had isotope ratios in agreement with that expected for enterobactin (Fig. 3a).

Fragmentation analysis of the compound gave product ion spectra characterized by major fragments

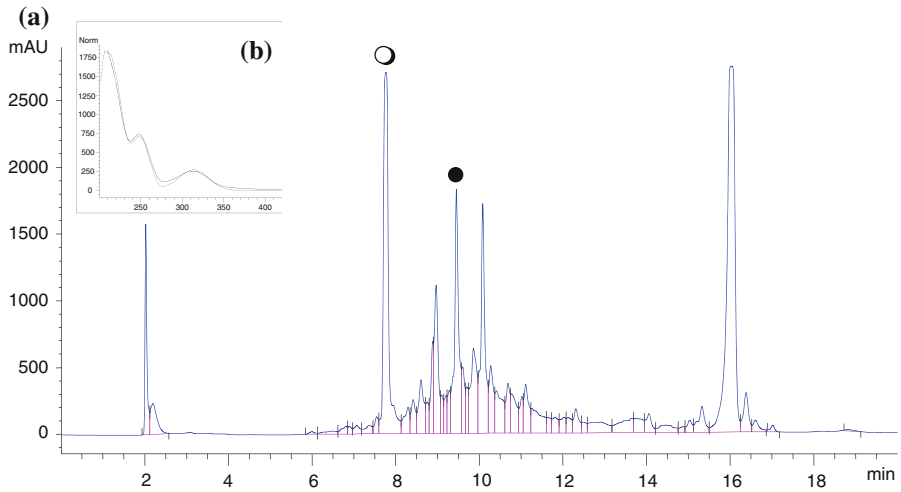


**Fig. 1** HPLC chromatogram of analyte production by isolate 1481 under iron starvation (**a**). Each peak represents an analyte present in the supernatant. *Black circle* Enterobactin, *open circle* dihydroxybenzoylserine monomer, dimer or linear trimer. Peaks without an associated symbol represent unidentified analytes. UV spectrum of enterobactin (**b**). *Solid line* UV spectrum of enterobactin produced by isolate 1481, *dotted line* UV spectrum of enterobactin in HPLC library

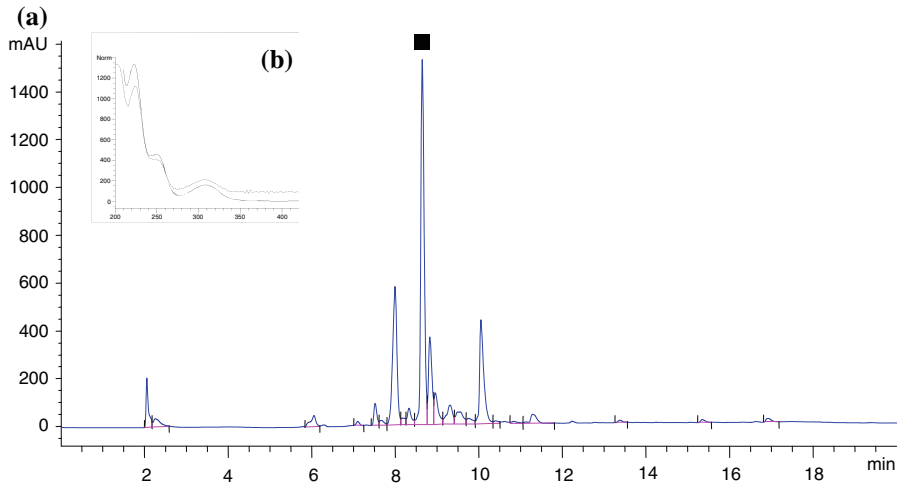


**Fig. 2** HPLC chromatogram of analyte production by isolate 2350 under iron starvation (**a**). Each peak represents an analyte present in the supernatant. *Black square* Heterobactin. Peaks without an associated symbol represent unidentified analytes. UV spectrum of heterobactin (**b**). *Solid line* UV spectrum of heterobactin produced by isolate 2350, *dotted line* UV spectrum of heterobactin in HPLC library

at 222 and 445 Da, with minor fragments at 240 and 463 (Fig. 3b). The fragmentation results were consistent with cleavage of the lactone bonds of the cyclic trimer structure of enterobactin.

Isolate 1481 was screened for carriage of the *entF* gene, which encodes the final NRPS in the enterobactin biosynthetic pathway. This was confirmed by PCR amplification of a 203 bp internal fragment of the gene (Fig. 4).

## Evaluation of siderophore production in response to external iron concentration

The effect of external iron concentration on siderophore production by isolates 1481, 1528, 1548, 2342 and 2366, which are isolates capable of high levels of siderophore production as determined by calculated % siderophore units, was examined at iron concentrations ranging from 0 to 20 μM. Earlier screening had

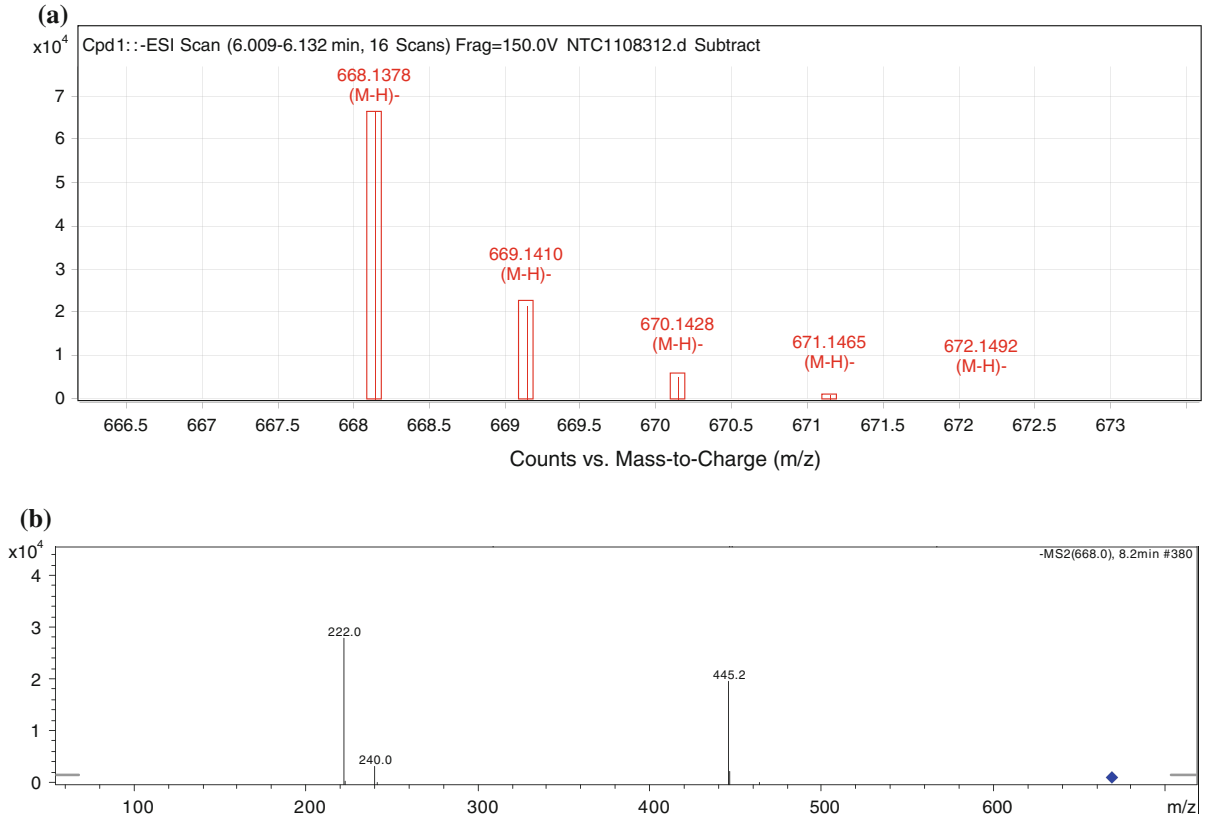


**Fig. 3** Isotope ratios (**a**) and fragmentation ion spectra (**b**) of enterobactin produced by isolate 1481

revealed that all isolates were positive for trihydroxamate production and isolates 1481, 2342 and 2366 were also positive for catechol production. For these isolates, the greatest siderophore production occurred during growth in media that did not contain iron. The presence of even small amounts of supplementary iron in the ISP4 broth resulted in reduction in siderophore production to levels comparable with the negative control containing no siderophore.

## Evaluation of siderophore production by isolate 1481 over time

The siderophore production by isolate 1481 was assessed weekly over the course of 6 weeks with the 20 ml volume of the culture maintained by periodic addition of sterile distilled water to counter the loss due to evaporation. Siderophore activity increased weekly from weeks 1 to 3, reaching a maximum after 3 weeks of incubation (Fig. 5a). Thereafter, siderophore activity fell and remained steady at levels similar to week 2. HPLC analysis of the culture at weeks 1 and 6 found that HPLC profiles differed between the two time points and although isolate 1481 is known to be capable of enterobactin production, it was not detected.

When the culture was allowed to deplete naturally by evaporation over 6 week incubation period, enterobactin was detected in the culture at week 6. Siderophore activity also increased from weeks 1 to 5 then fell in week 6 to levels similar to week 4 (Fig. 5b).

## Taxonomic identification of isolates 1481 and 2350

16S rRNA sequencing of isolates 1481 and 2350 generated 1,488 and 1,367 bp of quality sequence, respectively. All matches found by BLAST belonged to genus *Streptomyces* with identity of at least 98%. The phylogenetic tree generated using 1,339 bp of aligned sequence from isolates 1481, 2350 and the 10 closest matches to each isolate that were identified to

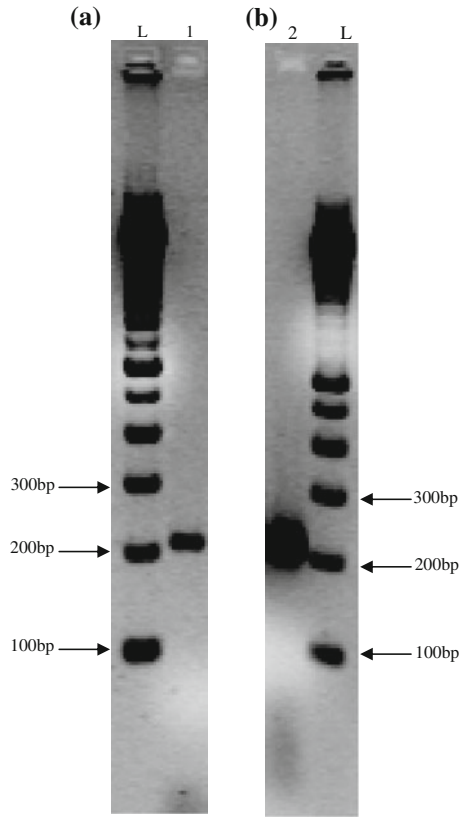


**Fig. 4** PCR amplification of 203 bp fragment of *entF* gene in *Streptomyces* isolate 1481 (**a**) and *E. coli* MG1655 (**b**). *Lane L* 100 bp ladder, *lane 1 entF* PCR from isolate 1481 DNA template, *lane 2 entF* PCR from *E. coli* MG1655 DNA template

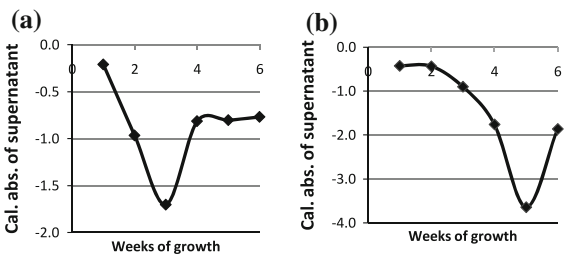


**Fig. 5** Calculated absorbance of neat supernatant from isolate 1481 over 6 weeks of growth under conditions of iron starvation when, **a** culture volume was maintained at 20 ml over 6 weeks, and **b** culture volume reduced by evaporation over 6 weeks. Lower absorbance indicates greater siderophore activity

the species level found that isolates 1481 and 2350 were located in distinct and separate clades (Fig. 6). Isolate 1481 was most closely related to *Streptomyces achromogenes* subsp. *rubradiris* strain NBRC 14000. Isolate 2350 was most closely related to *S. showdoensis* strains NBRC 13417 and ISP 5504 and *S. viridobrunneus* strain NBRC 15902.

### Identification of isolates capable of siderophore production in the presence of iron

Less well regulated siderophore production in actinomycetes was sought by growing isolates in ISP4 broth supplemented with 5 μM of iron, then applying the CAS assay to detect siderophore production. A total of 273 new isolates were screened and seven were positive for siderophore production; a further six isolates returned borderline positive results in the CAS assay. All 13 isolates were negative for catechol production by the Arnow test and nine isolates were positive for trihydroxamate siderophore production by $FeCl_3$ test.

HPLC analysis of sterilized supernatant detected the presence of a large analyte peak at 2 min, also found in all previous HPLC chromatograms generated. A small peak representing dihydroxybenzoylserine monomer and dimer was also detected in the sterilized supernatant of isolate 2813. However, it returned a negative result for catechol production in the Arnow test, which may indicate that dihydroxybenzoylserine monomer and dimer production occurs at low levels below the detection threshold of the Arnow test. HPLC analysis conducted on extracted siderophore from the sterile supernatant of each isolate found generated very similar profiles for each isolate. The large peak at 2 min, matching assorted hydroxamates, was present along with large peaks representing unknown analyte at 9.8, 10.6 and 11.7 min.

## Discussion

In this study, actinomycetes isolates were collected from two geographically distinct sites: (1) a site in north-west Western Australia reportedly containing 40% magnetite, and (2) a commercial orchard with a base ranging from sandy loam to laterite. Screening of these actinomycetes isolates found that the siderophore production characteristics were highly similar, despite the fact that the isolates originated from separate bacterial populations. All isolates screened were positive for some degree of siderophore production and this is consistent with the necessity of bacterial iron acquisition systems to cope with the

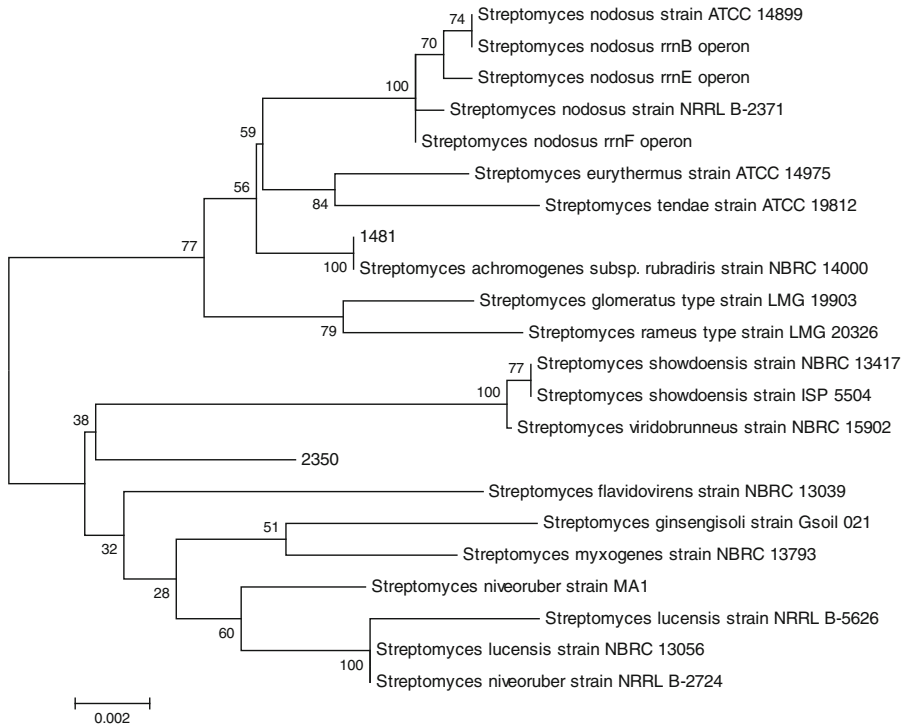


**Fig. 6** Evolutionary relationships of *Streptomyces* species. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,325 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

inaccessibility of iron in the environment where it is present as various insoluble iron oxides. Both populations had comparable proportions of strong and weak siderophore producing isolates, which may suggest that strong siderophore production is not essential for survival at either site. Weak siderophore production alone may still recruit sufficient levels of iron for bacterial survival. Alternatively, weak siderophore production could be supplemented by the utilization of siderophores produced by other environmental organisms.

The most notable difference in siderophore production characteristics among the isolates from the two populations was that a significantly higher proportion of isolates from site 2 produced catechol siderophores (17%) compared to site 1 (3%). The reason for this difference is not known. However, the siderophore with the highest known iron binding constant is enterobactin, a catechol siderophore. It could be speculated that the differences in iron content between the soils at the two sites selected for the production of catechol siderophores, with potentially greater capacity to bind iron, at site 2. Population differences in the environmental bacteria may also have exerted an influence in the selection of catechol siderophore production in this population. Although non-actinomycete bacteria were beyond the scope of this study, greater catechol production by actinomycetes isolates at site 2 could be a strategy to outcompete other bacteria for a scare resource, as shown by one experiment the catechol siderophore may only be synthesized at times of extreme iron stress and not before. Thus some of the actinomycetes in this study may have a hierarchical induction of their siderophores with the hydroxamates being induced first. If the iron stress becomes greater, catechols with higher

iron-binding constants, or other siderophores that may be more competitive, such as the heterobactins, are induced.

Further investigation by HPLC revealed that isolate 1481 from site 1 excreted into the supernatant a compound with a UV spectrum matching intact enterobactin. Subsequent mass spectrometry confirmed that the excreted compound was enterobactin. Carriage of the *entF* gene, which encodes the final NRPS in the enterobactin biosynthetic pathway, by isolate 1481 was also confirmed by PCR. The production of enterobactin, the characteristic siderophore of the Enterobacteriaceae, by actinomycetes has only been reported once previously. Fiedler et al. (2001) described two isolates of *Streptomyces* that produced membrane-bound enterobactin, as confirmed by HPLC analysis and mass spectrometry. 16S rRNA sequencing and morphological characterization identified this isolate as a member of genus *Streptomyces*. Our study is the first report describing enterobactin production by *Streptomyces* in which the siderophore is excreted extracellularly. HPLC detected the presence of dihydroxybenzoylserine monomer and dimer in the culture supernatants of this isolate and also in isolates 2342, 2349 and 2813. This raises the possibility that these isolates may also be capable of enterobactin production under the right conditions.

Monitoring of siderophore production by isolate 1481 over 6 weeks of growth provided insight into changes in siderophore production by this isolate over an extended period of time. Siderophore production occurred in late log or early stationary phase cultures as siderophores are secondary metabolites. The effect of culture volume on enterobactin production was also revealed. The inability to detect enterobactin when the volume was maintained at 20 ml contrasted with strong enterobactin production when the culture volume was allowed to decline due to evaporation. Clearly enterobactin was only produced when the organism was under extreme stress and it is not known if other factors such as variation in aeration and agitation and the concentration of accumulated waste products in the broth played a role in its induction. It should be noted that examination of siderophore production over time was conducted only for isolate 1481 and it is not known whether all isolates follow the same pattern and optimum timing of siderophore production in an extended growth experiment.

Enterobactin is a cyclic trimer of dihydroxybenzoylserine. Both the dimer and the linear trimer of dihydroxybenzoylserine are preliminary synthesis products and are capable of siderophore activity. The EntF protein is responsible for creating the monomer, adding subsequent subunits for the formation of first the dimer, then the linear trimer and, finally, cyclization of the linear trimer into the mature enterobactin cyclic trimer (Walsh and Marshall 2004). The presence of the monomer and dimer forms in culture supernatant of isolates 2342 and 2349 suggests strongly that these isolates possess functional EntF. The inability to detect enterobactin production by these isolates may indicate that enterobactin biosynthesis was not complete at the time of screening or that any enterobactin produced had degraded into monomer and dimer breakdown products. It should be noted that HPLC detected dihydroxybenzoylserine monomer, dimer and linear trimer in the culture supernatant of isolate 1481, as well as mature cyclic enterobactin.

HPLC analysis also detected a peak with a UV spectrum matching the siderophore heterobactin in extract from isolate 2350, as well as isolates 2332, 2342, 2344, 2351 and 2366. This may suggest that these isolates produce and excrete intact heterobactin. Heterobactin siderophores are mixed catechol-hydroxamate siderophores and were first discovered in *Rhodococcus erythropolis* IGTS8, a member of the actinomycetes (Carrano et al. 2001). The production of a heterobactin-like siderophore by *Nocardia tenerifensis* NBRC 101015, also an actinomycete, was later discovered (Mukai et al. 2009). Isolate 2350 was identified as a *Streptomyces* species on the basis of its morphological characteristics and 16S rRNA sequencing results and this is the first report describing putative heterobactin production by *Streptomyces* species. Although HPLC UV spectrum data indicated that heterobactin is produced, further structural determination is required for confirmation. The reactivity of heterobactins in the Arnow test for detection of catechol groups is not known. Heterobactins contain at least one catechol group but in this study not all heterobactin-positive isolates returned a positive Arnow test and this may suggest that reactivity in the Arnow test is a concentration dependent phenomenon.

All BLAST matches to 16S sequences from isolates 1481 and 2350 belonged to organisms from genus *Streptomyces* and had sequence identity of 98% or greater. Despite the similarity in 16S sequences, the

percentages generated by the bootstrap test of the phylogenetic tree suggested that the topology of particular branches and nodes is uncertain. These ambiguities are consistent with the challenging nature of *Streptomyces* taxonomy, which is well documented. Identification of isolates 1481 and 2350 beyond the genus level requires further investigation.

Our results indicate that in the majority of actinomycetes, siderophore production is subject to strict regulation in response to external iron concentration. Detailed examination of five isolates known to be capable of high siderophore production in the absence of iron in the growth medium found that siderophore production fell dramatically once even small concentrations of iron were introduced. Screening of a further 273 isolates identified only 13 that were positive or borderline for siderophore production when grown in liquid medium with 5 μM added iron present, which supported the earlier findings that siderophore production is strictly regulated in actinomycetes. Tight regulation of siderophore production in response to the presence of iron avoids the metabolically wasteful situation of unnecessary siderophore production and processing when iron is readily available in small but sufficient quantities. The much smaller number of isolates capable of siderophore production, even in the presence of iron, represents isolates where siderophore production is less tightly regulated. The similarity in HPLC profiles for these isolates may suggest that common analytes, particularly that found repeatedly at 2 min in all isolates tested and which matched with assorted hydroxamates, may be potential siderophores produced by this subset of organisms.

In conclusion this paper has the first report of a *Streptomyces* species synthesizing and excreting enterobactin as a siderophore. It also contains the first reports of apparent heterobactin production by a streptomycete, the siderophore only reported previously in *Rhodococcus* and *Nocardia*.

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