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ORIGINAL ARTICLE

Identification of erythrobactin, a hydroxamate-type siderophore produced by *Saccharopolyspora erythraea*

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Abstract

Aims: To investigate the production of siderophores by *Saccharopolyspora erythraea* SGT2 and how this production is affected by the inoculum.
Methods and Results: When grown in a low-iron, chemically defined medium (CDM), the soil dwelling actinomycete *S. erythraea* secretes a substance that is reactive in the nonspecific chrome azurol S (CAS) assay. Importantly, the production of CAS-reactive substance is highly reduced upon the addition of 0·925 μmol l^{-1} iron to the cultures and has a peak of production in the late-log to early stationary growth phase. In addition, the culture supernatants tested were negative in the Arnow and Rioux assays but positive in the Csáky procedure. Interestingly, we also found evidence that the production of this CAS-reactive substance in CDM was highly reduced, when inoculated with cells that had been previously grown to late-stationary phase. Conversely, inocula derived from late-log to early stationary cultures presented high levels of CAS activity.
Conclusions: These results indicate that *S. erythraea* produces a hydroxamate-type siderophore that we have generically designated as erythrobactin. Additionally, the inocula growth stage plays a key role in siderophore production in *S. erythraea*.
Significance and Impact of the Study: It is the first evidence for siderophore synthesis in *S. erythraea* and one of the first examples of nonpolyketide secondary metabolite production by this organism.

Introduction

Iron is an essential trace element for most micro-organisms. It is used as cofactor for several electron-transport proteins, reduction of ribotide precursors of DNA, formation of haeme and other essential purposes (Neilands 1995). Despite being one of the most abundant elements on earth, the aerobic environment has decreased its bioavailability due to the oxidation of ferrous to ferric ion. To overcome these growth limiting conditions, many micro-organisms secrete high-affinity low-molecular-mass iron-chelating compounds termed siderophores. It is known that many soil bacteria (e.g. *Streptomyces* spp.) produce siderophores (Neilands 1981). Although desferrioxamine (DFX) is the typical siderophore produced by *Streptomyces* (Ōmura *et al.* 2001; Bentley *et al.* 2002; Challis and Hopwood 2003; Barona-Gómez *et al.* 2004), structurally different siderophores have recently been reported to be coproduced in some species [like coelichelin and coelibactin in *Streptomyces coelicolor* (Challis and Ravel 2000; Bentley *et al.* 2002) or enterobactin in *Streptomyces tendae* (Fiedler *et al.* 2001)]. The related *Saccharopolyspora erythraea* is a Gram-positive mycelium-forming actinomycete, known to be the main producer of the important macrolide antibiotic erythromycin A. In fact, only erythromycin and a red pigment (Cortés *et al.* 2002), have been reported as main products of *S. erythraea*'s secondary metabolism.

To investigate the eventual production of siderophores by *S. erythraea*, the nonspecific chrome azurol S (CAS) assay was used (Schwyn and Neilands 1987). This assay consists in a competition for Fe(III) ions between the CAS-Fe(III) complex and the excreted siderophore. The higher affinity constant of the siderophore for iron will

lead to a colour change of CAS (usually from blue to orange). This work reports not only the detection of iron-binding compounds from solid and liquid media cultures of *S. erythraea* using the CAS assay, but also presents evidence on the nature of the compound and how this production is strongly affected by the inoculum growth stage.

Materials and methods

Bacterial strains and growth conditions

A triple deletion mutant in the erythromycin gene cluster, *S. erythraea* SGT2 (Δ*eryA* Δ*eryBV* Δ*eryCIII*), was used in the experiments (Gaisser *et al.* 2000). Spore stocks were stored in a 50% (v/v) glycerol solution at −70°C. Nutrient agar (for the solid media experiments) and nutrient broth (NB) were purchased from Oxoid (Basingstoke, UK). Chemically defined medium (CDM) for siderophore production had the following composition (given in g l^{-1}): glucose anhydrous 30; K_2HPO_4 0·1; $NaNO_3$ 11·1 and morpholinepropanesulfonic acid buffer 21. Trace elements were prepared as a filter-sterilized stock and added to the medium as follows (given in g l^{-1}); $MgSO_4{\cdot}7H_2O$ $2{\cdot}50 \times 10^{-1}$, $CuCl_2{\cdot}2H_2O$ $5{\cdot}30 \times 10^{-4}$, $CoCl_2{\cdot}6H_2O$ $5{\cdot}30 \times 10^{-4}$, $CaCl_2{\cdot}2H_2O$ $1{\cdot}32 \times 10^{-2}$, $ZnCl_2$ $1{\cdot}04 \times 10^{-2}$, $MnCl_2{\cdot}4H_2O$ $6{\cdot}20 \times 10^{-3}$, $NaMoO_4{\cdot}2H_2O$ $3{\cdot}00 \times 10^{-4}$. Iron was also added to the trace element solution in the form of $FeSO_4{\cdot}7H_2O$, in concentrations ranging from 0 to $2{\cdot}50 \times 10^{-2}$ g l^{-1} (0–92·5 μmol l^{-1}).

Detection of siderophore production in solid media

For the detection of siderophore production in solid media, the modified ferric hexadecyltrimethylammonium bromide CAS agar plate assay was used as previously described (Machuca and Milagres 2003). Nutrient agar was used as solid media to grow *S. erythraea*. The plates were incubated at 28°C for 2 weeks in the dark. Uninoculated control plates were incubated in the same conditions stated above for a period of 3 weeks.

Detection of siderophore production in liquid media

To evaluate siderophore production in liquid media, frozen spore stocks of *S. erythraea* SGT2 were used to inoculate CDM under several iron limitation conditions. *Saccharopolyspora erythraea* was grown in baffled shake flasks at 28°C and 200 rev min^{-1} for 120 h. Samples were taken every 24 h and centrifuged in a Beckman CS-6R centrifuge (Beckman, Buckshire, UK) at 1606 ***g*** and 5°C for 15 min. One millilitre of supernatant was then carefully removed, filtered through a 0·20 μm 25-mm diameter filter (Millipore, Watford, UK) and mixed with an equal volume of CAS assay solution (Schwyn and Neilands 1987). A reference solution was prepared with uninoculated media and CAS assay solution. The sample and reference absorbances were measured at 630 nm after 1 h of incubation at room temperature. The Csáky, Arnow and Rioux (Arnow 1937; Csáky 1948; Gilliam *et al.* 1981; Rioux *et al.* 1983) assays were also performed to distinguish between hydromaxate and catechol type siderophores. DFX and hydroxylamine hydrochloride were used as standards for the CAS and Csáky assays, respectively, while 2,3-dihydroxybenzoic acid was used as a standard for the Arnow and Rioux procedures. All glasswares used were previously treated with concentrated HCl to remove iron and was rinsed with ultrapure water.

To determine an approximate value for the siderophore molecular weight, a dialysis step was performed with 1 kDa molecular size cutoff Spectra/Por cellulose ester tubes (Medicell Int., London, UK). The citrate and phosphate levels in the supernatant were determined by high performance liquid chromatography (HPLC) analysis using a Dionex Summit HPLC System (Dionex, Oakville, Canada, US) with an Aminex HPX-87H 30 cm column (Bio-Rad, Richmond, CA, USA). The mobile phase was 5 mmol l^{-1} of H_2SO_4 pumped at 0·6 ml min^{-1}. The injection volume was 20 μl, detection was performed at 200 nm and the analysis time was 20 min.

Influence of inoculum on *S. erythraea* CAS activity

To study the influence of the inoculum growth stage on CAS activity, frozen spore stocks of *S. erythraea* were used to inoculate NB. These cultures were grown in baffled shake flasks at 200 rev min^{-1} and 28°C, until late-log to early stationary phase (48 h) or late-stationary phase (96 h). Samples were then taken, centrifuged, resuspended in 10 ml of CDM and then centrifuged as before. The cells were finally introduced into 50 ml of iron-depleted CDM at a dry cell weight (DCW) of *c.* 1 g l^{-1}. To analyse the influence of the inoculum iron content on CAS activity, frozen spore stocks were used to inoculate CDM with iron concentrations ranging from 0 to 92·5 μmol l^{-1}. These cultures were allowed to grow until late-log to early stationary phase in the same conditions stated before and then re-introduced into 50 ml iron-depleted CDM at a DCW of *c.* 1 g l^{-1}.

Dry cell weight

In the shake-flask experiments performed, DCW was determined using predried Whatman GF/F filters (Millipore, Watford, UK). Five millilitres of samples were filtered through previously weighed filters, and these were consequently dried at 95°C until a constant weight was

achieved. The DCW was calculated by the difference between the weight of the dried filter plus biomass and the weight of the dried filter alone.

Results

Siderophore production in solid media

The modified CAS agar plate assay was used for the detection of siderophores in solid medium. The colour of the CAS agar changed from blue to dark orange (Fig. 1), which is indicative of a hydroxymate type of siderophore (Milagres *et al.* 1999). As expected, no colour change was observed in the uninoculated plates after incubation for 3 weeks (data not shown). Similarly to what was obtained with other siderophore producing organisms (Machuca and Milagres 2003), the distance of the colour front was also a linear function of the incubation time (data not shown).

Siderophore production in liquid media

Siderophore production was also detected in liquid media using the universal CAS assay. The relative concentration of iron-binding compounds produced by *S. erythraea* was evaluated in iron-deprived CDM cultures directly inoculated with frozen spore stocks at a DCW of 1 g l^{-1} (Fig. 2). The inclusion of micromolar amounts of iron in the medium drastically diminished the CAS activity observed in *S. erythraea* supernatants. In fact, cultures supplemented with 0·925 μmol l^{-1} of iron produced about half of the CAS activity observed in culture supernatants with 0 μmol l^{-1} of iron content. The inclusion of 9·25 and 92·5 μmol l^{-1} iron further reduced the CAS activity to 264 and 203 μmol l^{-1} DFX equivalents respectively. The peak of CAS activity occurred during the late-log to early stationary growth phase in cultures with iron concentrations of 0, 0·925 and 9·25 μmol l^{-1} and CAS activity was inversely correlated with biomass yield.

It has been shown that the presence of low-affinity iron chelators (like phosphate and citrate), is able to interfere with the CAS reaction (Schwyn and Neilands 1987; Guerinot *et al.* 1990). Therefore, we also evaluated if any of these compounds was interfering with the CAS activity obtained. Possible citrate synthesis by *S. erythraea* was evaluated by HPLC, and it was found to be much lower than the detection limit in the CAS assay (*c.* 500 μmol l^{-1}) (Guerinot *et al.* 1990). Regarding phosphate, as CDM only contains 0·57 mmol l^{-1} of

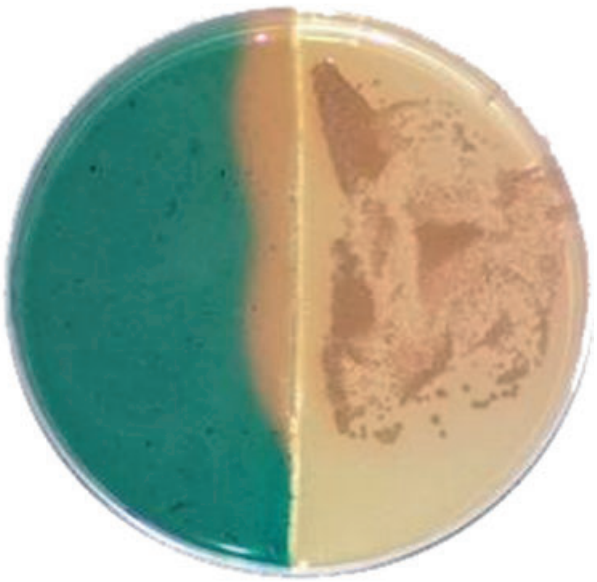

Figure 1 Modified chrome azurol S (CAS) agar plate assay showing the dark orange halo in the half containing CAS blue agar after 2 weeks of incubation at 28°C in the dark.

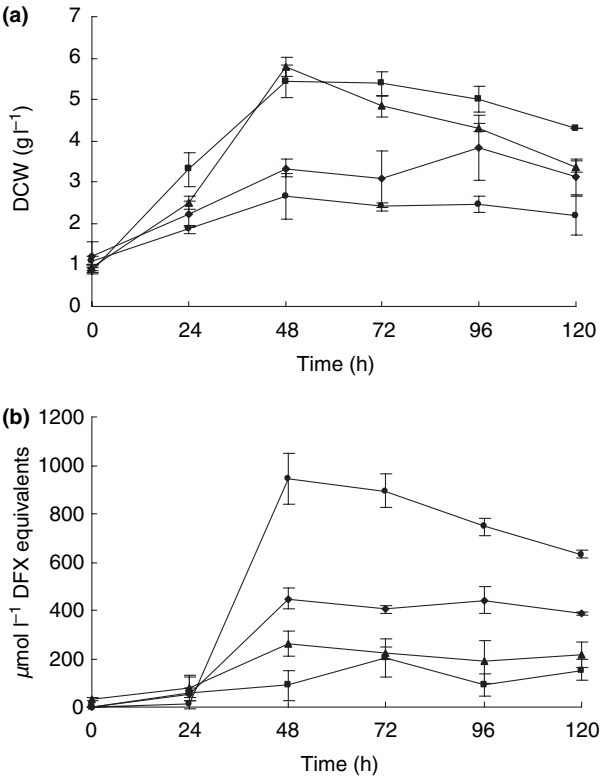

Figure 2 Change in dry cell weight (DCW) (g l^{-1}) (a) and siderophore production [expressed as μmol l^{-1} of desferrioxamine (DFX) equivalents] (b) in chemically defined medium cultures containing different concentrations of iron and inoculated at 1 g l^{-1} with frozen spore stocks of *Saccharopolyspora erythraea* SGT2. All data shown represent the mean values and SD from duplicate cultures. ■, 92·5 μmol l^{-1}; ▲, 9·25 μmol l^{-1}; ◆, 0·925 μmol l^{-1}; ●, 0 μmol l^{-1}.

phosphate, 99·43 mmol l^{-1} more phosphate would have to be present in the medium to account for any CAS activity (Schwyn and Neilands 1987). The phosphate levels in all cultures were determined by HPLC and never exceeded 0·27 mmol l^{-1}. To demonstrate that the CAS activity obtained was due to a low-molecular weight compound, *S. erythraea* supernatant was dialysed against a 1 kDa exclusion size membrane. Subsequent analysis of the retentate clearly indicated loss of CAS activity. In a separate experiment, the CAS activity was retained after the supernatant was boiled for periods of time between 5 and 20 min, which rules out the possibility of a CAS-reactive protein (Raaska and Mattila-Sandholm 1995).

Initial investigation into the structural nature of the CAS reactive substance

Saccharopolyspora erythraea supernatants with as much as 945 μmol l^{-1} DFX equivalents were also used in specific assays to search for hydroxamate or catechol type siderophores. The Arnow and Rioux assays yielded negative results, showing that the iron-binding compound was not a catecholate. On the other hand, a positive result using the Csáky assay indicated that the substance was of a hydroxamate type. To confirm the results obtained in the specific assays, *S. erythraea* supernatants were assayed for CAS activity after extraction with several organic solvents. CAS-negative results were obtained after extraction with ethyl-acetate, demonstrating that the substance was not a typical catecholate (Neilands 1981; Payne 1994). However, positive results were obtained after extraction with butanol and to a lesser extent with chloroform, providing further evidence for the hydroxamate nature of the substance (Neilands 1981; Payne 1994).

Effect of the inoculum on *S. erythraea* CAS activity

To determine if and how the inoculum growth stage influences the CAS activity in *S. erythraea*, iron-depleted cultures were inoculated with cells previously grown in NB for different lengths of time. Samples were then taken at regular intervals and used to determine DCW and CAS activity. Cells previously grown in NB until late-log to early stationary phase, presented biomass levels and CAS activity similar to the ones obtained in cultures derived from frozen spore stocks (Fig. 3). Like before, the maximum of CAS activity appeared to be coincident with the peak of biomass concentration at 48 h. On the other hand, inocula obtained from late-stationary phase CDM cultures, grew sluggishly and presented extremely low-levels of CAS activity.

We also analysed how different iron concentrations in the inoculum could influence growth and CAS activity in CDM. This was performed by inoculating iron-depleted CDM with *S. erythraea* cells previously grown in CDM with iron concentrations ranging from 0 to 92·5 μmol l^{-1} until late-log phase. It appears that cells previously grown in CDM with higher iron content, grew slightly better in iron-depleted CDM compared with inocula derived from CDM cultures containing 9·25, 0·925 and 0 μmol l^{-1} (Fig. 4). The latter, however, presented higher levels of CAS activity. These values were similar to the ones obtained in *S. erythraea* CDM cultures directly inoculated with frozen mycelia stocks.

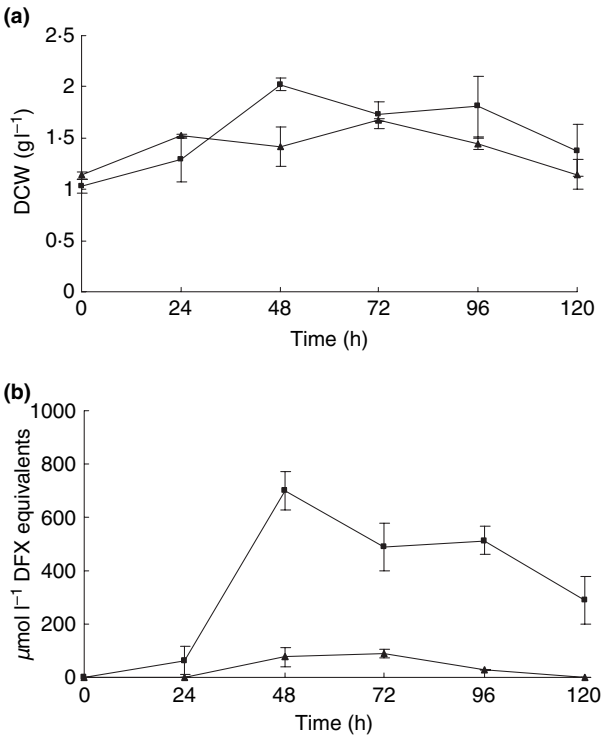

Figure 3 Change in dry cell weight (DCW) (g l^{-1}) (a) and siderophore production [expressed as μmol l^{-1} of desferrioxamine (DFX) equivalents] (b) in iron-deprived (0 μmol l^{-1}) chemically defined medium cultures, inoculated at *c.* 1 g l^{-1} with *Saccharopolyspora erythraea* cells previously grown in nutrient broth to late-log to early stationary phase (■) or late-stationary phase (▲). All data shown represent the mean values and SD from duplicate cultures.

Discussion

In this report, we present data that strongly suggest that the Gram-positive actinomycete *S. erythraea* produces a bonafide hydroxamate type siderophore. The first evidence obtained was the presence of a CAS-reactive substance in iron-deprived CDM. This CAS activity, besides being consistently observed, presented a maximum around late-log to early stationary growth phase and was

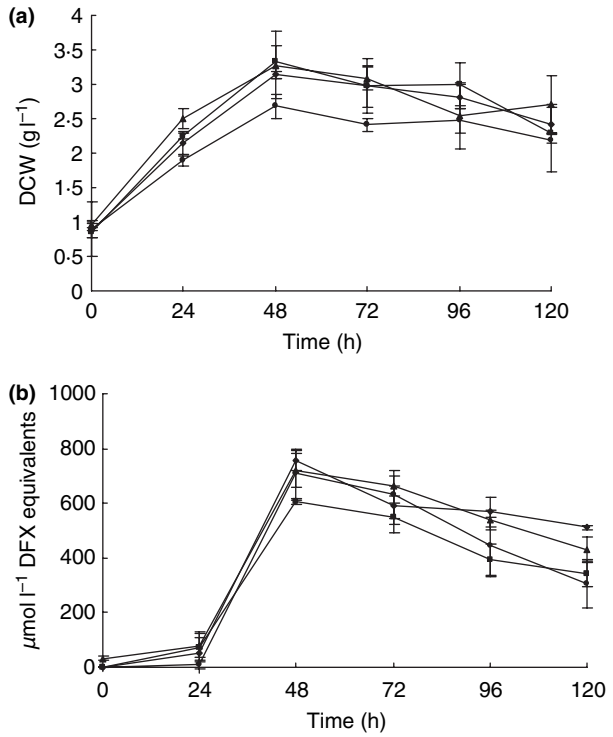

Figure 4 Change in dry cell weight (DCW) (g l^{-1}) (a) and siderophore production [μmol l^{-1} of desferrioxamine (DFX) equivalents] (b) in iron-deprived (0 μmol l^{-1}) chemically defined medium (CDM) cultures, inoculated at *c.* 1 g l^{-1} with *Saccharopolyspora erythraea* cells previously grown in CDM cultures to late-log to early stationary phase in different concentrations of iron. All data shown represent the mean values and SD from duplicate cultures. ■, 92·5 μmol l^{-1}; ▲, 9·25 μmol l^{-1}; ◆, 0·925 μmol l^{-1}; ●, 0 μmol l^{-1}.

significantly reduced by micromolar amounts of iron. Positive results were also obtained in the modified CAS agar plate assay, with formation of dark-orange halos in the CAS blue agar. Further analysis of *S. erythraea* iron-depleted supernatants, showed that the CAS-reactive substance has a molecular weight <1 kDa and is resistant to heat. Also, the presence of low-affinity iron chelators in the supernatant like phosphate and citrate, as determined by HPLC, was found to be below the detection limits of CAS. Initial structural evidence about this CAS-reactive substance was given by the specific Csáky, Arnow and Rioux assays. The positive results obtained only in the Csáky assay, strongly suggest the presence of a hydroxamate-type siderophore. This evidence was corroborated by the fact that butanol and chloroform (typically used for the extraction of hydroxamates), were the most effective organic solvents in the extraction of this substance. Preliminary purification using reverse-phase HPLC indicates that a single peak corresponds to the CAS activity. All these results, taken together, indicate that *S. erythraea* produces a hydroxamate-type siderophore that we have designated as erythrobactin. The production of this siderophore is not a particular attribute of *S. erythraea* SGT2, as similar results were obtained with the *S. erythraea* wild type strain (data not shown).

The influence of the inoculum's growth phase on *S. erythraea* CAS activity was also analysed, and the results obtained indicate that it plays an essential role in CAS activity. Inocula previously grown in NB until late stationary phase, consistently failed to produce siderophores in high titres. However, inocula derived from late-log to early stationary cultures, presented high levels of CAS activity. Apart from the lower cellular viability, it seems that other intrinsic mechanisms are preventing *S. erythraea* to produce siderophores from late-stationary phase cultures. A similar dependence between CAS activity and inoculum growth phase has been previously documented for the nonclassical siderophore legiobactin, produced by strains of *Legionella pneumophila* (Liles *et al.* 2000).

These results clearly show that for purposes of siderophore production optimization, it is necessary to take into account not only culture medium parameters, but also the inocula itself. Still remains to be explained what kind of metabolic or physiological modifications are behind the low siderophore production in late-stationary phase cells. Work is also in progress to structurally characterize this siderophore in more detail.

References

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

Barona-Gómez, F., Wong, U., Giannakopulos, A.E., Derrick, P.J. and Challis, G.L. (2004) Identification of a cluster of genes that directs desferrioxamine biosynthesis in *Streptomyces coelicolor* M145. *J Am Chem Soc* **126**, 16282–16283.

Bentley, S.D., Chater, K.F., Cerdeño-Tárraga, A.-M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A. *et al.* (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141–147.

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 USA* **252**, 675–679.

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

Cortés, J., Velasco, J., Foster, G., Blackbaby, A., Rudd, B. and Wilkinson, B. (2002) Identification and cloning of a type

III polyketide synthase required for diffusible pigment biosynthesis in *Saccharopolyspora erythraea*. *Mol Microbiol* **44**, 1213–1224.

Csáky, T.Z. (1948) On the estimation of bound hydroxylamines in biological materials. *Acta Chem Scand* **2**, 450–454.

Fiedler, H.-P., Krastel, P., Muller, J., Gebhardt, K. and Zeeck, A. (2001) Enterobactin: the characteristic catecholate siderophore of Enterobacteriaceae is produced by *Streptomyces* species. *FEMS Microbiol Lett* **196**, 147–151.

Gaisser, S., Reather, J., Wirtz, G., Kallenberger, L., Staunton, J. and Leadlay, P.F. (2000) A defined system for hybrid macrolide biosynthesis in *Saccharopolyspora erythraea*. *Mol Microbiol* **36**, 391–401.

Gilliam, A.H., Lewis, A.G. and Anderson, R.J. (1981) Quantitative determination of hydroxamic acids. *Anal Chem* **53**, 841–844.

Guerinot, M.L., Meidl, E.J. and Plessner, O. (1990) Citrate as a siderophore in *Bradyrhizobium japonicum*. *J Bacteriol* **172**, 3298–3303.

Liles, M.R., Scheel, T.A. and Cianciotto, N.P. (2000) Discovery of a nonclassical siderophore, legiobactin, produced by strains of *Legionella pneumophila*. *J Bacteriol* **182**, 749–757.

Machuca, A. and Milagres, A.M.F. (2003) Use of CAS-agar plate modified to study the effect of different variables on the siderophore production by *Aspergillus*. *Lett Appl Microbiol* **36**, 177–181.

Milagres, A.M.F., Machuca, A. and Napoleão, D. (1999) Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *J Microbiol Methods* **37**, 1–6.

Neilands, J.B. (1981) Microbial iron compounds. *Annu Rev Biochem* **50**, 715–731.

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

Ōmura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H. *et al.* (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 characterization of siderophores. *Methods Enzymol* **235**, 329–344.

Raaska, L. and Mattila-Sandholm, T. (1995) Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp. *J Ind Microbiol* **15**, 480–485.

Rioux, C., Jordan, D.C. and Rattray, J.B.M. (1983) Colorimetric determination of catechol siderophores in microbial cultures. *Anal Biochem* **133**, 163–169.

Schwyn, B. and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.