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Heterobactins: A new class of siderophores from *Rhodococcus erythropolis* IGTS8 containing both hydroxamate and catecholate donor groups

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Abstract

We report here on a new class of siderophores isolated from *Rhodococcus erythropolis* IGTS8, the first structurally characterized from any species of *Rhodococcus* and for which we suggest the name heterobactins. These siderophores consist of a tripeptide of sequence (N-OH)-L-Orn-Gly-D-Orn-(δ-N-dihydroyxbenzoate). The alpha amino group of the D-Orn is derivatized either as a 2-hydroxybenzoxazolate in heterobactin A or remains free in heterobactin B. The structures were determined by a combination of amino acid analysis, mass spectrometry and NMR methods. The two new compounds are true siderophores in that they relieve iron limited growth in the producing strain. The heterobactins are also transported by other non-producing bacteria. Growth promotion tests using various transport mutants revealed that in *E. coli* heterobactin A is only recognized by the catecholate receptor Cir while heterobactin B is taken up in both *E. coli* and *A. flavescens* JG9 via a hydroxamate transport system.

Introduction

It is well known that the growth of many microorganisms is controlled by the availablity of the essential micronutrient, iron. Other aspects of microbial behavior such as the virulence of some pathogens is also linked to their need for iron. Bacteria and fungi have evolved highly sophisticated systems based on siderophores to acquire, transport and process this essential, but biologically unavailable, metal ion. Some hundreds of siderophores (low molecular weight iron chelating compounds secreted by microorganisms under iron stress) are known and extensive studies of their isolation, structure, transport and molecular genetics have been undertaken in the last two decades (Winkelmann & Carrano 1997). It is therefore surprising that there have been no definitive reports of siderophores from any species of *Rhodococcus*. The rhodococci are related to the mycobacteria and Nocardia, which differ from most bacteria in the possession of a thick lipoidal cell envelope (Komagata & Suzuki 1987). This thick envelope acts as a barrier for the uptake of various materials and hence it could be expected that rhodococci and mycobacteria would elaborate a more complex system for iron acquisition than other bacteria. Indeed in mycobacteria hydrophilic, peptidic, extracellular siderophores known as exochelins and carboxymycobactins scavenge iron from the environment but then pass it on to other more lipophilic siderophores (mycobactins) permanently lodged in the cell membrane (Ratledge & Dover 2000). Some species formerly classified as rhodococccci, now assigned to the "Gordona" group, are also reported to contain mycobactins (Ratledge & Patel 1976; Rainey et *al*. 1995). While the general approach adopted by the two groups of organisms might be expected to be similar, clearly the details of iron transport are different as no true

Rhodococcus expresses any known mycobactin. We have therefore undertaken to isolate and characterize the siderophores and iron transport mechanisms from a strain of *Rhodococcus erythropolis* designated IGTS8. This organism is one of some commercial interest as it is being considered for use in the biodesulfurization of fuel oil (Kayser *et al*. 1993). The result of the studies reported herein is the identification and structural determination of a previously unknown class of siderophores, the first documented for any species of *Rhodococcus*.

Materials and methods

Bacterial growth

R. erythropolis type strain DSM 43066^T (ATCC 25544) and *R. rhodochrous* DSM 43241 (ATCC 13808) were from institute stocks. A further *R. erythropolis* strain (DSM 43188) was kindly supplied by Dr. Kroppenstedt of the DSM. *R. erythropolis* IGTS8 was obtained from Prof. Linette Watkins, Department of Chemistry and Biochemistry, Southwest Texas State University and was grown in one of two defined salt media containing the following per liter. Medium A: Na_2HPO_4, 26 g, KH_2PO_4, 15.9 g, NH_4Cl, 6 g, $MgCl_2$, 3.84 g, Na_2SO_4, 1.28 g, $CaCl_2$, 0.2 g, $ZnSO_4$, 15.6 mg, $MnCl_2$, 6 mg, $CuSO_4$, 1 mg, $CoSO_4$, 1 mg, EDTA, 7.5 mg. Glucose, treated with CHELEX resin to remove adventitious iron, was autoclaved separately and added to the medium at 20 g/l prior to innoculation. Medium B: Tris, 12.1 g, succinic acid, 10 g, KH_2PO_4, 2.72 g, Na_2SO_4, 1.42 g, NH_4NO_3, 3.0 g, $MgCl_2$, 0.64 g, $CaCl_2$, 33 mg, $ZnCl_2$, 2.6 mg, $MnCl_2$, 1.0 mg, $CuCl_2$, 0.15 mg, and $Co(NO_3)_2$, 0.125 mg, adjusted to pH 7.2 as needed. In order to maintain iron limited growth and thereby induce siderophore production, all glassware was stored for several hours with a dilute solution of HCl to remove trace iron from the surface prior to use.

Siderophore isolation and purification

For siderophore production 6 l cultures (3 × 2 l) were shaken at 100 rpm and 30 °C for one week. The cells were removed by centrifugation and the supernatant passed down a column of XAD-2 resin to absorb the desferri form of the siderophore. The column was then washed extensively with water and the siderophores eluted with MeOH. Further purification was achieved by fractionation of the crude methanol extract on a P2 (BioRad) column, eluting with water and monitoring fractions for iron binding capacity with Chrom-Azurol-S (CAS). After all activity had passed, a second CAS positive fraction could be eluted with 2% formic acid. All CAS positive fractions were concentrated by rotary evaporation and lyophilized.

HPLC and TLC analysis

Thin layer chromatography (TLC) was performed on cellulose plates (Merck) using either n-BuOH/HOAc/H_2O (4:2:2), or EtOAc/HCOOH/H_2O, (4:2:2) as eluting solvents. Iron binding compounds were identified by spraying with CAS reagent. HPLC employed a Shimadzu LC-10 instrument with a reversed phase 250 × 4.6 mm Nucleosil C18 column (Grom, Herrenberg, Germany), and a gradient program starting with a mixture of 94% H_2O, 6% ACN, 0.1% TFA increasing to 40% ACN over 20 min, remaining at 40% for 5 min, and finally increasing to 100% for an additional 5 min with detection at 220 nm.

Mass Spectrometry

FAB mass spectra were recorded on a Varian MAT 711 instrument coupled with a SS 200 data system. The FAB spectra were measured from a matrix of glycerol at an ion source temperature of 323 K. High resolution mass spectrometry was carried out with a passively shielded 4.7 Tesla APEX II-ESI/MALDI-Fourier Transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonik, Bremen, Germany). XMASS software (version 5.0.10), supplied by the manufacturer and running on a Silicon Graphics O2 Workstation, was used for mass claculation, data aquisition, and processing. For direct infusion determinations via syringe pump, 512 k data points were typically acquired within the mass range of 200–2000 Da giving a resolution of up to 80 000 (full width half height) for the compounds of interest. Positive ion electrospray (ESI) was performed without nebulizer gas using a grounded capillary sprayer needle (Analytica, Branford, USA) mounted 60° off-axis and samples were injected at a flow rate of 1 μl/min using a carrier solvent of 0.5% acetic acid/acetonitrile (1:1). Internal calibration of the spectrometer was achieved via a four point approach using mixtures of known composition. For fragmentation studies, high resolution FTICR-MS/MS were aquired by sustained off-resonance irradiation collision induced dissociation (SORI-CID) using argon as the collision gas. Before adding collision gas, correlated sweep isolation

was used to isolate precursor ions and reject all others from the analyzer cell.

NMR spectrometry

High resolution two-dimensional NMR spectra were recorded on a Bruker AMX 400 spectrometer (Bruker Physics, Karlsruhe, Germany) at 400 MHz (^{1}H) in DMSO-d6 at 305 K. One dimensional ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 instrument at 250 MHz (^{1}H) also in DMSO-d6 at 305 K. Chemical shifts of both ^{1}H and ^{13}C are internally referenced to residual protio solvent.

Amino acid analysis

Purified samples were hydrolyzed with either 57% HI (reductive) or 8M HCl (normal) at 110 °C for 18–24 h. The hydrolysate was derivatized as the TMS ethers and analyzed on a Chirasil Val glass capillary with FID detector. GC-MS of these derivatives was performed with a Varian MAT 112 S mass spectrometer with electron impact ionization.

Biotests

Filter disks (6 mm) containing 10 μl of a solution of either heterobactin A and B (both desferri and ferri forms) were sterilized by microwave heating and placed on freshly prepared LB-soft agar (0.6%) containing 150 μM each of the iron chelators, 2,2-bipyridyl and ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA). Growth zones were read after 48 h incubation at 27 °C. Growth promotion assays with the ferric-hydroxamate auxotroph *A. flavescens* JG9 or strains of *E. coli* deficient in various outer membrane receptors were performed as previously described (Rabsch & Winkelmann 1991).

Results

Cultures of IGTS8 grown on low iron glucose containing medium A showed a very prolonged lag phase. However, after 1 week of growth, a TLC of the crude mixture obtained from XAD extraction of the supernatant of such cultures revealed the presence of three iron binding substances. The most abundant and most lipophilic of these (Rf in solvent system 1, 0.63) was designated compound **1**, the next most abundant, (Rf, 0.46) which was ninhydrin positive, was designated compound **2**. Compound **3** (Rf, 0.19) was only present in small amounts and its characterization was not pursued further. The two major siderophores were separated and purified by fractionation on a P2 column. HPLC analysis confirmed that both compound **1** and **2** were pure (retention time 19.8 min for **1** and

Table 1. ^{1}H NMR Assignments (ppm) for heterobactin A and B in d6-DMSO.

Assignment	Heterobactin A	Heterobactin B
α-L-orn	4.32(m)	4.32(m)
β-L-orn	ca.1.63(m)	ca 1.82(m)
γ-L-orn	ca.1.87(m)	ca.1.87(m)
δ-L-orn	3.45(m)	3.45(m)
α-D-orn	4.57(m)	4.52(m)
β-D-orn	ca 1.63(m)	ca.1.82(m)
γ-D-orn	ca.1.87(m)	ca.1.87(m)
δ-D-orn	3.30(m)	2.77(m)
gly	3.74(d)	3.74(d)
DHB meta	6.67(t)	6.43(t)
DHB para	6.90(d)	6.77(d)
DHB ortho	7.40(d)	7.27(d)
α-L-orn amide	8.08(d)	8.16(d)
α-gly amide	8.26(t)	8.41(t)
α-D-orn amide	8.61(t)	n/a
δ-D-orn amide	8.90(d)	ca. 8.4
meta benzoxazole	7.28(m)	n/a
para benzoxazole	7.10(m)	n/a
oxazole	7.11(m)	n/a

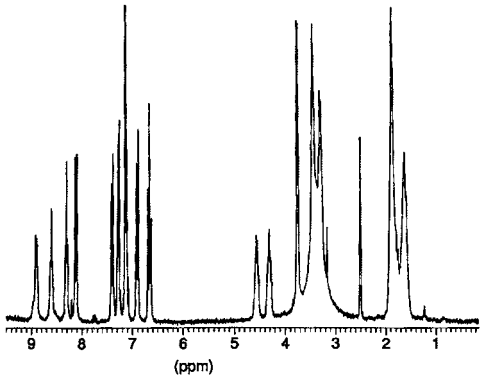

Fig. 1. 400 MHz ^{1}H NMR spectrum of heterobactin A in d6-DMSO.

13.1 min for **2**). On occasion it was found necessary to repeat the P2 column fractionation in order to obtain sufficiently pure material. Amino acid analysis obtained after reductive hydrolysis gave one equivalent of D-ornithine, one equivalent of L-ornithine and one equivalent of glycine for both **1** and **2**. Normal HCl hydrolysis however gave for both only one equivalent of D-ornithine and one of glycine, suggesting that the L-ornithine might be N-hydroxylated and that both species had the same amino acid backbone. Initial positive ion mode mass spectral analysis employing either ESI-FT-ICR, or FAB gave a strong $[M+H]^+$ ion at 599 for **1** and 438 for **2**. In the presence of p-toluene sulfonic acid the FAB-MS of **1** also showed a major fragment ion at 438 suggesting that **2** could be derived from **1** by the loss of a 161 mass unit fragment. High resolution FTICR-MS on **1** gave an exact mass of 599.20959 with relative error of 0.05 ppm and only one formula consistent with the known amino acid composition arises from this, $C_{27}H_{31}N_6O_{10}$ (599.20962). Similar data obtained for **2** gives an exact mass of 438.19820 and a molecular formula of $C_{19}H_{28}N_5O_{16}$ (438.19832). ^{1}H NMR on **1** clearly revealed the presence of four amide, a set of dihydroxobenzoate (DHB), two ornithine alpha C-H and the glycine CH_2 protons (Figure 1). Another, not obviously assigned, set of three aromatic protons was also evident. The ^{1}H spectra of **2** was very similar to that of **1** minus the extra set of aromatic peaks and the fact that the amide region was partially obscured by the presence of a formate counter ion (protonation of the free amine known to be present in **2** occurs during elution from P2 with formic acid). The connectivities defining the complete structures of **1** and **2** were finally determined by a combination of 2D NMR techniques including TOCSY, HMBC and HSQC.

TOCSY of both **1** and **2** showed the presence of three non aromatic spin systems. One consisted only of the glycine, another contained two amides and corresponded to a dhb derivatized ornithine and finally another ornithine containing only a single amide proton as expected for a cyclized N-hydroxyornithine. HMBC established that the cyclic N-hydroxy ornithine was in a terminal position attached via the α-N to the glycine. The glycine in turn was connected to the second ornithine whose α-N was derivatized with the unknown aromatic moiety and whose δ-N was derivatized to the dihydroxybenzoate. This sequence was confirmed by SORI-CID FTICR MS/MS (Figure 2). The identification of the final aromatic metal binding fragment in **1** proved to be somewhat more

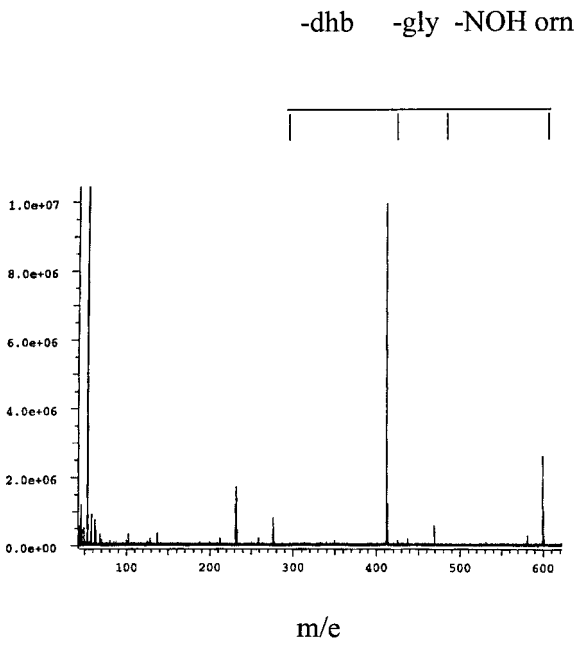

Fig. 2. SORI-CID FTICR MS/MS of heterobactin A. Loss of the characteristic fragment ions is shown above (NOH orn = N-hydroxyornithine, gly = glycine, dhb = dihydroxybenzoate).

problematic. From mass spectrometry the formula of the unkown piece corresponded to $C_8H_4NO_3$. Based on the fact that the three observable protons were all aromatic and a third iron binding moiety was in all probability needed, we formulate the final fragment as the salicylate derived benzoxazole. Detailed analysis of the observed coupling constants and chemical shifts are entirely in keeping with the proposed structure but alternate isomeric possibilities cannot be unequivocally ruled out at this time. Final ^{1}H and ^{13}C NMR assignments are given in Tables 1 and 2, respectively. Given the unusual presence of both hydroxamate and catecholate donor groups in the same siderophore we propose the names heterobactin A and B for compounds **1** and **2**, respectively (Figure 3).

Interestingly, when cultures were grown on succinate medium B, distinctly different results were obtained. Growth of IGTS8 under these conditions was both more rapid and reached much higher cell densities than with medium A. In addition only very small amounts of the peptidic siderophores **1** and **2** were detected. However extraction of the media with the less hydrophobic resin, XAD-4, elution with MeOH and fractionation of the extract on Sephadex G-10 revealed the presence of two low molecular mass iron binding compounds. One of these was unequivically identified

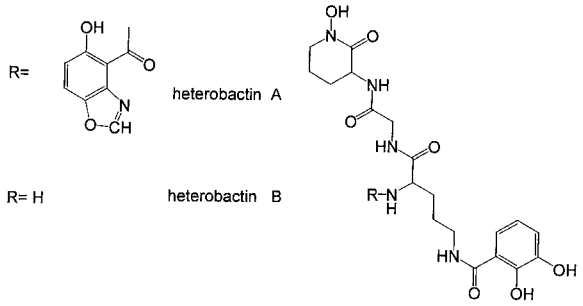

Fig. 3. Structures of heterobactin A and B as determined by amino acid analysis, NMR and mass spectrometry.

Table 2. Major ^{13}C NMR Assignments (ppm) for heterobactin A and B in d6-DMSO.

Assignment	Heterobactin A	Heterobactin B
L-orn carbonyl	164.6	165.0
α-L-orn	49.6	49.6
D-orn carbonyl	171.5	171.8
α-D-orn	52.7	52.3
δ-D-orn	51.1	51.2
gly carbonyl	168.3	165.7
gly methylene	41.9	42.1
DHB carbonyl	168.9	168.5
Benzoxazole carbonyl	165.9	n/a

by NMR and ESI-MS as dihydroxybenzoic acid. The other compound was also aromatic, and believed to be 1-carboxy-2-hydroxybenzoxazole but we were unable to obtain enough of it in a sufficiently pure state for unequivocal identification.

Given the reported lack of siderophores from any species of *Rhodococcus* we have screened another strain of *R. erythropolis,* (DSM 43066$^{\mathrm{T}}$) as well as the common species, *R. rhodochrous* (DSM 43241) and were surprised to find no trace of either heterobactin A or B in either organism tested. Rather we found that two different iron binding compounds seemed to be expressed by both the type strain of *R. erythropolis* and *R. rhodochrous* when grown under conditions that produced heterobactin A and B in IGTS8. The first of these has been determined to be salicylic acid while the other is of a yet unknown structure but apparently unrelated to heterobactin A or B. The formation of salicylic acid by *R. erythropolis* and *R. rhodochrous* and DHB by IGTS8 is similar to the situation seen with the mycobacteria which when grown under iron deficient conditions produce not only peptidic sideophores, but large quantities of salicylic acid, a precursor of mycobactin and the carboxymycobactins (Lane *et al.* 1998). Salicylic acid was once thought to function as a siderophore in its own right, but it now appears that its binding constant for iron is too low for it to compete with other media components and solubilize the element except under acidic conditions (Chipperfield & Ratledge 2000). The same has been suggested to be true for dihydroxybenzoate.

Bioassays in media rendered iron limiting by the presence of chelators, 2,2′-bipyridyl and EDDHA, revealed that both **1** and **2** are able to relieve iron induced growth inhibition in the producing organism as expected of true siderophores and do so with approximately equal efficiency. Growth promotion tests on the related organisms *R. erythropolis*^{T} (type strain) and *R. rhodochrous*, using either heterobactin A or B, confirm the surprising fact that they neither express nor recognize these new siderophores. It is noteworthy that the hydroxamate reporter organism, *A. flavescens* JG9, recognizes only heterobactin B as being a hydroxamate containing siderophore (heterobactin A gives a weak but observable responce) although it is utilized relatively poorly as compared to simple trishydroxamate siderophores such as the ferrioxamines and ferrichromes. We also examined heterobactin uptake in various mutants of *E. coli*, which contains both catecholate and hydroxamate transport systems. The results (Table 3) show that heterobactin A was transported only via the cir (catecholate) receptor while heterobactin B was transported via the fhuE (coprogen) system.

Discussion

Since iron is often the growth limiting nutrient in microorganisms we have searched for and found a novel siderophore from IGTS8, a strain of *Rhodococcus* being considered for industrial scale biodesulfurization of fuel oils. The current methodology for removing the 0.025 to 11% sulfur content of petroleum and coal fuels that on combustion generate sulfur dioxide, a major component in acid rain, is catalytic hydrodesulfurization. As fossil fuel consumption increases, governments have implemented stricter controls on sulfur dioxide emissions and hydrodesulfurization is ineffective for the desulfurization of many classes of

Table 3. Growth promotion tests on ferri-heterobactin A and B using *E. coli* strains deficient in various outer membrane receptors involved with catecholate and hydroxamate uptake.

Strain	relevant genotype	heterobactin A	heterobactin B
H1876	*fiu, cir, fepA*	−	+
H1875	*cir, fepA*	−	+
H1877	*fiu, fepA*	+	+
H1728	*fiu, cir*	−	+
AB2847	*wild type*	(+)	+
MS172	*fhuE*	(+)	−
AN311	*fhuA (fep B)*	+	+

Fiu catecholate receptor.
Cir catecholate receptor.
Fep A enterobactin receptor.
Fhu E coprogen receptor.
Fep B enterobactin periplasmic binding protein.

organosulfur compounds and thus there is an urgent need for new methods. This has led to a substantial interest in alternative technologies including microbial biodesulfurization. The many advantages that bacterial systems offer over current chemical desulfurization processes, particularly their ability to remove sulfur from a broader range of organosulfur compounds without the formation of toxic by-products, have been well established (Kayser *et al.* 1993). However, to achieve a viable industrial microbial process, attention will have to be paid to obtaining optimal growth of the organism chosen in a difficult environmental millieu (an oil-water suspension). Indeed we attribute the long lag phase in growth of IGTS8, even in simple iron limited media, to the need to produce and secrete the siderophores heterobactin A and B, suggesting that iron limitation will be an important consideration in the culturing of this organism.

The strain IGTS8 was assigned to *Rhodococcus erythropolis* based on morphological characteristsics, fatty acids, mycolic acids (C_{32-42}) and 16S rDNA similarity (100% to *R. erythropolis* DSM 43188 reference strain). However, 16S rDNA similarity to *R. erythropolis* type strain DSM 43066^T was only 98.4%, suggesting that the species *R. erythropolis* is not a homogenous group. The strain 43188 was previously named *Nocardia calcarea*, but was transferred to *R. erythropolis* due to significant homology in DNA/DNA hybridization experiments. The observation that heterobactins could be detected and supported growth in the producing strain IGTS8, and in the reference strain *R. erythropolis* (DSM 43188), but not in the type strain (DSM 43066) or *R. rhodochrous* (DSM 43241) underlines this inhomogeneity. An examination of the various iron binding compounds produced within the Rhodococci suggests that structurally different siderophores are produced within the species and even within the erythropolis group (unpublished results).

A particularly noteworthy aspect of the heterobactins is their dual nature in receptor recognition. Like the two faced Roman god Janus, one part of the molecule is recognized by a hydroxamate transport system as evidenced by JG9 activity while the other part is recognized by a catecholate receptor (*E. coli*) providing further justification for the name, heterobactins. A more detailed analysis of the different catecholate (Fiu, Cir and FepA) and hydroxamate receptors (Fhu A, FhuE) revealed that heterobactin A enters the *E. coli* cells only via the Cir receptor, while heterobactin B enters the E. coli cells via the FhuE receptor. Previous studies in *E. coli* have demonstrated that the Fiu and Cir receptors recognize enterobactin breakdown products such dihydroxybenzoylserine (DHBS) or the linear dimer and trimer of DHBS (Hantke 1990) as well as other natural catechol-containing siderophores such as the amonabactins (Rabsch & Winkelmann 1991). Fiu and Cir have also been reported to enable uptake of catechol-siderophore-drug conjugates which then function as trojan horses and deliver the conjugate to the target by entering via the iron uptake route (Nikaido & Rosenberg 1990; Diarra *et al*. 1996; Moellmann *et al*. 1998). While these previous studies showed that there was little or no preference for uptake of substrates between Fiu and Cir, the present work clearly shows that Cir is the only receptor that recognizes heterobactin A.

It is perhaps significant that we have isolated heterobactin A and B in their desferri forms rather than as is more usual, their corresponding iron complexes (Drechsel & Winkelmann 1997). Given the very low aqueous solubility we have observed for the iron complexes at neutral to slightly acidic pH, this has important consequences and may explain why no siderophore has been reported previously from this group. As anticipated from the similarities between the rhodococci and mycobacteria, heterobactin A is quite non-polar, eluting late in our standard HPLC assay and is only poorly soluble in water. Notable however, is the lack of long chain fatty acid groups found in other lipophilic bacterial siderophores (Ratledge & Dover 2000; Martinez *et al*. 2000).

The iron binding centers in heterobactin A and B are not known for certain at present, but both the dihy-

droxybenzoate and the hydroxamate group from cyclic N-OH ornithine are well known in other siderophores and are almost certainly involved. The hydroxybenzoxazole moiety of heterobactin A, expected to be the third iron binding domain, is previously unknown in siderophore chemistry. This latter group has several potential coordination sites but we favor the bidentate, so called, 'salicylate' mode of binding to iron via the phenyl hydroxyl and alpha amide carbonyl. This type of binding has been crystallographically characterized in model complexes and is implicated in the iron binding of several catecholate siderophores at low pH (Cohen *et al*. 1998). Molecular modeling using the program CAChe shows that all three donor groups can wrap around the Fe^{3+} ion in a relatively strain free way to give an octahedral, 1:1, mononuclear iron complex. Heterobactin B, which does not contain sufficient donor groups to satisfy the six coordinate octahederal geometry required by Fe^{3+} in a 1:1 complex, nevertheless functions as a true siderophore for IGTS8. Structurally it has distinct similarities to the amonabactins (Telford & Raymond 1997) which have been shown to produce both 1:1 and coordinatively saturated, dinuclear iron complexes of stoichiometry Fe_2L_3 (Telford & Raymond 1998). A similar situation is expected to occur here. Indeed preliminary ESI-MS studies reveal that both heterobactin A and B produce mainly 1:1 complexes with Fe(III) and Ga(III) at neutral pH although there is evidence for higher molecular weight species as well. Further studies on the iron complexation chemistry and transport studies of these new siderophores will be reported later.

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