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Molecular recognition of synthetic siderophore analogues: A study with receptor-deficient and *fhu*(A-B) deletion mutants of *Escherichia coli*

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Abstract

The biological activity of six synthetic siderophore analogues (two dihydroxamates, two trihydroxamates, one tetrahydroxamate and one 3-hydroxy-4(1H)pyridinone) has been studied in *Escherichia coli*, *Morganella morganii* 13 and *Proteus mirabilis* 8993 strains by using growth promotion tests. Various transport-deficient mutants of *E. coli* were used to study the route of entry into gram-negative bacteria. The results indicated that the synthetic hydroxamate compounds are transported via Fhu-mediated transport systems, although receptor specificity was low. This could be proven by using a delta (*fhu*A-B) *E. coli* mutant as a control in which growth promotion by natural hydroxamates was completely abolished, suggesting that a periplasmic binding-protein-dependent transport system (FhuB, C, D) is required for the transport of all synthetic ferric hydroxamate complexes. Although utilization of the synthetic hydroxamates was generally lower than that of the natural siderophores, differences in growth promotion could be detected. Highest activity was observed with the dihydroxamate DOCYDHAMA ligand which supported growth at concentrations <1 mM. In comparison with other polyamino-polyhydroxamate ligands studied, this dihydroxamate ligand has an extra diamide backbone that could be important for the interaction with the receptors or with FhuD. The synthetic trihydroxamate and tetrahydroxamate ligands showed a relatively low siderophore activity. Studies with *Proteus* and *Morganella* in the presence of increasing bipyridyl concentrations showed a decreased growth promotion with the synthetic ferric hydroxamates, suggesting the involvement of a reduction step during iron mobilization or an increased toxicity of bipyridyl. This was not observed in the case of the 3-hydroxy-4(1H)pyridinone where bipyridyl had no effect.

Introduction

Iron is essential for the growth of all cells but its supply is restricted by the extreme insolubility of iron hydroxide in water at neutral pH. For this reason microorganisms produce a class of molecules, siderophores, which selectively bind and transport iron from the environment into the cell. Usually, siderophores contain catecholate or hydroxamate moieties for chelation to iron(III) (Drechsel & Winkelmann 1998). On the other hand, iron overload is a significant health problem. Thus, there has been a considerable interest in the development of new siderophore analogues that can be used as drugs in the treatment of iron overload diseases (Crichton 1991). Synthetic siderophore analogues mostly have catecholates or hydroxamates as functional groups. More recently, heterocycles containing hydroxy groups, such as 3-hydroxy-4(1H)-pyridinones, have received much attention (Feng *et al.* 1993; Ohkanda & Katoh 1995).

In Gram-negative bacteria uptake of siderophores and utilization of siderophore-bound iron has been shown to be dependent on outer membrane receptors (Braun 1995). Every siderophore utilized by *E. coli* has its corresponding outer membrane receptor: ferrichrome (FhuA), coprogen and Fe-rhodotorulate

(FhuE), aerobactin (Iut), ferric enterobactin (FepA), ferric citrate (FecA) and other catecholates (Cir and Fiu). *E. coli* does not utilize ferrioxamine E and only very little ferrioxamine B. A ferrioxamine receptor protein (FoxA) has been identified in other enterobacterial genera such as *Erwinia herbicola* (syn. *Pantoea agglomerans*) (Berner & Winkelmann 1988) and *Yersinia enterocolitica* (Bäumler & Hantke 1992).

We have previously synthesized six compounds: one 3-hydroxy-4(1H)-pyridinone (3-aminopropyl-2-methyl-3-hidroxy-4(1H)-pyridinone – APM-3,4-HOPY), two cyclic diamino-dihydroxamic acids (1,4,8,11-tetraazacyclotetradecane-12,14-dioxo-4,8-*bis*(*N*-methyl-acetohydroxamic acid) – DOCYDMAHA and 1,4-piperazine-*N*,*N*′-*bis*(*N*-methyl-acetohydroxamic acid) – PIPDMAHA), two macrocycles polyamino-trihydroxamic acids (1,4,8,11-tetraazacyclotetradecane, *N*,*N*′, *N*′′-*tris*(*N*-methyl-acetohydroxamic acid)-TETMAHA and 1,5,9-triazacyclododecane-*N*,*N*′,*N*′′-*tris*(*N*-methyl-acetohydroxamic acid) – DOTRMAHA) and one diamino-tetrahydroxamic acid (cyclohexane-1,2-dinitril-*N*,*N*,*N*′,*N*′′-*tetra*(*N*-methyl-acetohydroxamic acid) – CDTMAHA) (Figure 1).

The aim of this work was to probe if the synthesized compounds have any biological activity and whether or not outer membrane receptors or subsequent periplasmic and plasma membrane hydroxamate binding proteins are involved when enterobacteria utilize these compounds. For that purpose we used several transport-defective mutants of *E. coli*. We also included *Morganella morganii* 13 and *Proteus mirabilis* 8993, as these genera are known to utilize simple ferric complexes of keto-hydroxy bidentate ligands as siderophores and allow the discrimination between carboxylate and hydroxamate siderophore uptake (Thieken & Winkelmann 1993; Winkelmann & Drechsel 1997). Carboxylate siderophore receptors are not well studied in bacteria. However, two genes, *rum*A and *rum*B (**r**hizoferrin **u**ptake into **M***organella*), encoding an outer membrane protein and a periplasmic protein, respectively, have been identified in *M. morganii* (Kühn *et al.* 1996).

Materials and methods

Bacterial strains and growth conditions

All receptor mutant strains of *Escherichia coli* used in this study are *aro*B strains, which are unable to synthesize its own siderophore, enterobactin, unless a precursor is added. These strains allow a clear interpretation of exogenous siderophore-iron utilization, so that inter-ligand exchange of iron with its own siderophore (enterobactin) is excluded (Rabsch & Winkelmann 1991). The *E. coli* mutants were kindly provided by K. Hantke and were used to identify possible receptors involved in the transport of the synthetic compounds: the mutant MS 172 has a deletion in the coprogen receptor (FhuE); the mutant 235 has a deletion in the ferrichrome receptor (FhuA); the mutant HK97 is a strain that is defective in both ferrichrome and coprogen receptors and the strain HK97 pFU2 is a transformant in which a FoxA receptor from *Yersinia* was cloned via a plasmid (pFU2). The mutant H1880 was used as a control for ferric hydroxamate uptake. It has a deletion in the enterobactin biosynthetic genes (*ent*) like the aroB strains and in addition has a deletion in the genes for the FhuA receptor and subsequent transport proteins FhuD, FhuB and FhuC (Mademidis & Köster 1998). The mutant H1876 is deficient in the catecholate receptors (FepA, Fiu, Cir) and the mutant H1728 is deficient only in the Fiu and Cir receptors (Hantke 1990). *Morganella morganii* 13 and *Proteus mirabilis* 8993 were included for the discrimination of hydroxamate and carboxylate siderophores (Thieken & Winkelmann 1993). Strains and relevant genotypes are listed in Table 1.

All strains were from the Institute of Mikrobiologie, University of Tübingen. Strains were grown in LB medium containing (per liter) 25 g of LB (Luria Broth base from Gibco, BRL, Life Technologies). Erlenmeyer flasks containing 20 ml of LB medium were used. All material and media were autoclaved 15 min at 121 °C and 1 atm.

The bacteria were inoculated in the LB medium and incubated overnight at 37 °C and 200 rotations per minute in a rotary shaker (HT Infors AG). The strain HK97 pFU2, that has a plasmid, was grown in LB medium supplied with 200 μl of ampicillin (5 mg/ml) to a final concentration of 50 μg/ml. The stock solution of ampicillin (5 mg/ml) was sterilized with a membrane filter (0.2 μm, FP 03013 from Schleicher & Schuell).

Iron chelates and siderophores

The bioassays were performed with six synthetic compounds (APM-3,4-HOPY, DOCYDMAHA, PIPDMAHA, DOTRMAHA, TETMAHA and CDTMAHA), three natural trihydroxamates (ferrioxamine E, coprogen and ferrichrome) and enterobactin. The syn-

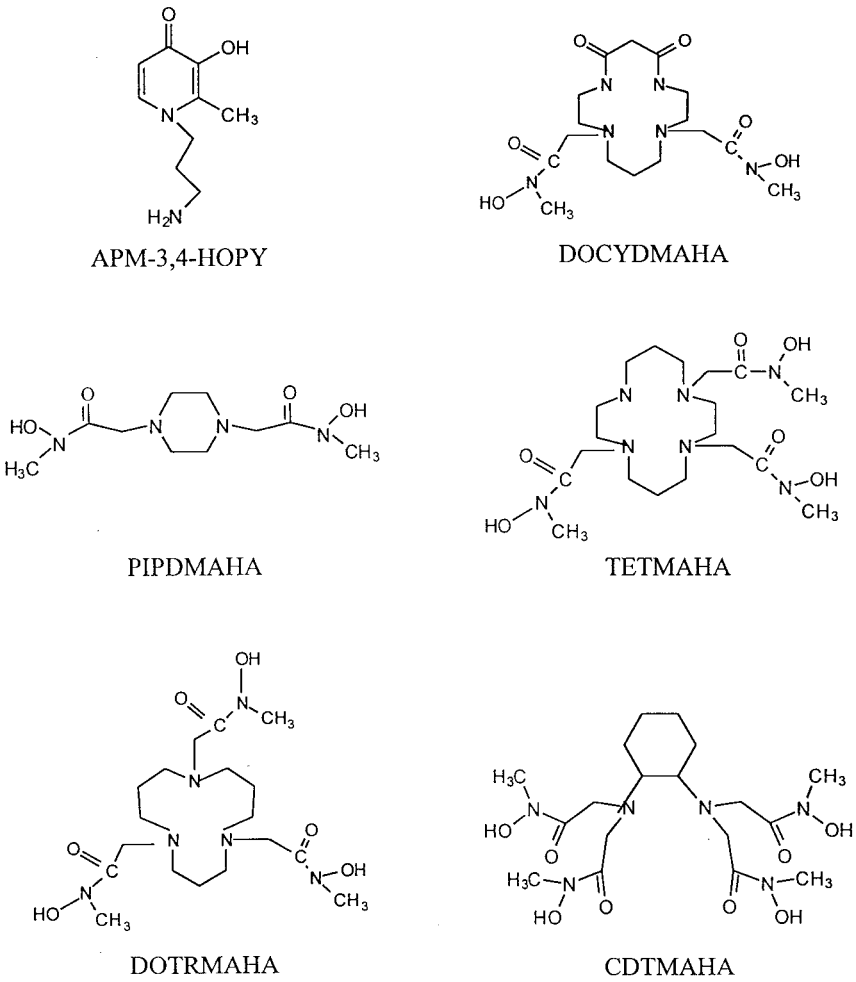

Figure 1. Structural formulas of the synthetic siderophore analogues.

Table 1. Characteristics and sources of strains used in the bioassays. All *E. coli* strains are *aroB* (or *ent*) indicating no enterobactin production

Strain	Relevant genotype	Origin
E. coli MS 172	*fhuE*$^-$	Microbiology Tübingen
E. coli 235	*fhuA*$^-$	Microbiology Tübingen
E. coli HK97	*fhuE*$^-$, *fhuA*$^-$	Microbiology Tübingen
E. coli HK97 pFU2	*fhuE*$^-$, *fhuA*$^-$, *foxA*$^+$	Microbiology Tübingen
E. coli H1880	*fepA*$^-$, *ent*$^-$, Δ (*fhu*A-B)	Microbiology Tübingen
E. coli H1876	*fepA*$^-$, *fiu*$^-$, *cir*$^-$	Microbiology Tübingen
E. coli H1728	*fiu* $^-$, *cir*$^-$	Microbiology Tübingen
Proteus mirabilis 8993	–	Robert Koch Institute
Morganella morganii 13	–	Robert Koch Institute

thetic compounds were synthesized in the Instituto Superior Técnico (Portugal), according to described procedures: APM-3,4-HOPY (Grazina *et al.* 1998), DOCYDMAHA (Santos *et al.* 1998), PIPDMAHA (Santos *et al.* 1993); DOTRMAHA (Esteves *et al.* 1995); TETMAHA (Gaspar *et al.* 1999); CDTMAHA (Santos *et al.* 1997). The natural siderophores were from the Laboratory stock of the Institute for Mikrobiologie and Biotechnologie, University of Tübingen, Germany. The natural compounds were isolated in Tübingen, ferrioxamine E was isolated from *Streptomyces pilosus* (Meiwes *et al.* 1990), coprogen from *Neurospora crassa* (Wong *et al.* 1983), ferrirubin from *Aspergillus ochraceus* (Jalal *et al.* 1984) and enterobactin from *E. coli AN311* (Berner *et al.* 1991). Aqueous solutions of different concentrations of each compound and with iron(III) (molar ratio 1:1) were prepared in a buffer solution pH = 8.0, tris/HCl 1M. Enterobactin was the only compound that was prepared without iron because ferric-enterobactin is unstable. The concentrations of the solutions were determined by their molar absorption coefficients. A buffer solution was used to maintain a neutral pH because the growth of bacteria could be affected by acidic medium and because inter-ligand iron exchange is a very slow event at neutral pH (Tuffano & Raymond 1981; Emery 1986), allowing the use of wild type strains when exogenously siderophores are supplied.

Performance of the bioassay

Bacterial over night cultures (10 μl) were added to 10 ml of TA medium (containing per liter 5 g NaCl, 8 g Tryptone, 5 g of Agar stored at 65 °C) containing 150 μM 2,2-bipyridyl (a ferrous ion chelator to reduce the bioavailable iron in the medium), 150 μM ethylenediamine-N,N′-bis(2-hidroxyphenylacetic acid) (EDDHA a ferric ion chelator) and 0.4% of glucose (deferrated on a chelex 100 column and autoclaved separately) and mixed thoroughly. The inoculated media were subsequently poured into Petri dishes. Sterile filter paper disks (6 mm diameter from Schleicher & Schuell) were loaded with 10 μl of the compounds to be tested, sterilized by heating for 1 min in a microwave oven (Moulinex 1100 W) and laid on the seeded agar surface. The resulting growth zones were examined after 24 h incubation at 37 °C upside down.

With *Proteus* and *Morganella* some competition studies were made using ferrirubin and coprogen, pipetting equal amounts of the synthetic compound and of ferrichrome or coprogen on the filter disks. In order to study the effect of bipyridyl on chelate iron uptake, agar plates were prepared containing bipyridyl (150, 300 and 450 μM).

Results and discussion

E. coli *mutants*

The ability of natural and synthetic siderophores to support the growth of *E. coli* mutants possessing different siderophore receptors was examined (Table 2). Although ferrichrome, ferrirubin and coprogen represent fungal products (Leong & Winkelmann 1998), enterobacteria possess receptor-mediated transport systems for their utilization (Braun & Hantke 1997). As shown in Table 2, the mutant MS172 utilized ferrichrome and little coprogen. This means that the corresponding FhuA receptor has a high specificity for ferrichrome but small amounts of coprogen are also taken up. The mutant 235 utilized mainly coprogen as the ferrichrome receptor was deleted. The mutant HK97 pFU2 has a cloned FoxA receptor and utilizes ferrioxamine E and coprogen. As the original mutant HK97 does not transport any natural trihydroxamate (Killmann & Braun 1992), this means that the introduced FoxA receptor can recognize both trihydroxamates. In fact, it has been reported that the FoxA receptor from *Yersinia* recognizes both ferrioxamine and coprogen (Deiss *et al.* 1998; Winkelmann 1997). Due to the complete deletion of all *fhu* genes, the mutant H1880 cannot utilize any hydroxamate siderophores. In contrast, although defective in the transport of enterobactin and its derivatives the mutants H1876 and H1728 can still take up all trihydroxamates including some ferrioxamine E. This is attributed to the fact that ferrioxamines can also be recognized by the FhuE receptor (Bäumler & Hantke 1992) which is not deleted in these mutants.

It is well known that siderophore receptors loose their specificity when they are supplied with high concentrations. So, to find specific interactions between a receptor and a siderophore one should use the lowest possible concentration. Therefore, we tested the synthetic compounds at different concentrations.

APM-3,4-HOPY is taken up by the mutants HK97pFU2, H1880, H1876 and H1728, in a 1.0 μmol/ml concentration and 1.5 μmol/ml by MS172, thus suggesting some specific interactions with receptors. In fact, APM-3,4-HOPY should be

Table 2. Growth promotion activity of ferric complexes with mutants of *Escherichia coli*

Iron chelator μmoles/ml		MS172 (− FhuE)	235 (− FhuA)	HK97 pFU2 (+FoxA) (− FhuE) (− FhuA)	HK97 (− FhuE) (− FhuA)	H1880 (− Fep A) (− FhuA) (− FhuD) (−FhuB, C)	H1876 (− Fep A) (− Fiu) (−Cir)	H1728 (− Fiu) (− Cir)
Ferrichrome	1	++++	−	−	−	−	++++	++++
Coprogen	1	+	++++	++	−	−	++++	++++
Ferrioxamine E	1	−	−	++++	−	−	+	+
APM-3,4-HOPY								
	1	−	−	+	−	+	++	++
	1.5	++	+	++	+			
	2.0	++	++	+++	++	++	++	++
	3,3	+++	++	+++	++			
DOCYDMAHA								
	0.5	−	−	−	+			
	0.67	++	++	++	++	−	++	++
	0.8	++	+	++	++			
	1	++	++	++	++	+	++	++
PIPDMAHA								
	0.5	−	−	−	−			
	1	++	++	++	+	−	++	++
	1.25	++	+	+	−	−	++	++
	2.5	+++	++	++	++			
TETMAHA								
	1	−	−	−	−			
	2	++	+	++	+	−	++	++
	3.8	++	−	++	−			
	10	+++	++	+	++	+	+++	+++
DOTRMAHA								
	1	−		−	−			
	2.5	−	−	−	−			
	5	++	+	+	+	−	+	+
	10	++	−	++	++	+	++	++
CDTMAHA								
	1	−	−	−	−			
	2	−	−	−	−	−	−	−
	8	+	−	+	+	−	++	++

−, indicates no zone of growth around the disk; +, indicates a <10 mm halo of growth; ++ = 10–15 mm halo; +++ = 15–20 mm halo; ++++ = > 20 mm halo.

preferentially transported by FoxA and FhuA (ferrioxamine and ferrichrome receptors) taking into account the growth of HK97pFU2 and MS172. The growth of H1876 and H1728 mutants also supports the existence of a transport performed by the FhuA and the FhuE receptors (these mutants do not have a FoxA receptor). On the other hand, APM-3,4-HOPY might also use catecholate receptors (perhaps Fiu or/and Cir) taking into account the growth of HK97 and H1880, where no hydroxamate transport is possible. Another hypothesis for the growth of HK97 is the existence of an unspecific iron transport. APM-3,4-HOPY might also enter via the FoxA and FhuA receptors because HK97pFU2 and MS172 showed a better growth than HK97.

DOCYDMAHA is taken up by all mutants of *E. coli* except H1880 at 0.7 μmol/ml concentration, thus suggesting easy entry via the hydroxamate transport system. In fact DOCYDMAHA could use the FoxA, FhuA and FhuE receptors due to the growth of HK97pFU2, MS172 and 235 strains. The growth

of H1876 and H1728 mutants also supports the existence of a transport performed by the FhuA and FhuE receptors.

PIPDMAHA is taken up by all mutants of *E. coli*, except H1880, in a 1.0 μmol/ml concentration. This indicates that the synthetic compound might enter via different receptors. The growth of the HK97pFU2, MS172 and 235 mutants suggests that PIPDMAHA could be transported by FhuA, FoxA and FhuE. However, the highest growth observed with MS172 and HK97pFU2 suggests a slight preference for the FhuA and FoxA receptors. The fact that H1876 and H1728 grow with PIPDMAHA confirms that this compound use the trihydroxamate receptors. On the other hand the little growth of HK97 indicates low receptor specificity.

TETMAHA could support growth of all strains of *E. coli* only at a very high concentration (10.0 μmol/ml). However for a 2.0 μmol/ml concentration which can still indicates a specific interaction, TETMAHA can feed MS172 and HK97pFU2, with FhuA and FoxA receptors, respectively. The fact that H1876 and H1728 grow with TETMAHA confirms that this compound is recognized as a trihydroxamate.

DOTRMAHA is only taken by the *E. coli* mutants if supplied in a 5.0 μmol/ml concentration. This means that the bacteria do not recognize the compound when low concentrations are used and/or that the observed transport is unspecific. However, we can see that the mutant 235 shows a reduced growth, suggesting a possible interaction with FoxA or FhuA.

CDTMAHA is only taken by the *E. coli* strains if supplied at a 8.0 μmol/ml concentration, indicating that the bacteria cannot easily utilize this compound. This indicates that the bacteria either do not recognize the compound when low concentrations are supplied or that the transport is unspecific. However, 235 and H1880 revealed no growth even with so high concentration, identifying FoxA or FhuA as the most probable receptor for entry.

Table 3 shows a set of thermodynamic and electrochemical characteristics of the synthetic ferric hydroxamates, studied at physiological conditions, namely the global stability constants, the p[M] values and the reduction potential ($E_{1/2}$). p[M] values give an idea of the strength with which a compound can complex iron(III) and allow a direct comparison of the stability at defined pH and concentration. All the synthetic compounds have lower p[M] values than the natural trihydroxamate siderophores and enterobactin. The lower affinity of these synthetic compounds towards iron (III) may explain why these compounds, even DOCYDMAHA (the most active compound), showed less growth promotion than the natural siderophores. On the other hand, the synthetic ferric hydroxamates are easier reduced than the natural siderophores, as suggested by the lower reduction potentials: $E_{1/2} = -0.690$ V for ferrichrome, $E_{1/2} = -0.698$ V for ferrioxamine B (Brockway *et al.* 1980), $E_{1/2} = -0.603$ V for DOCYDMAHA (Santos *et al.* 1998), $E_{1/2} = -0.560$ V for PIPDMAHA (Santos *et al.* 1993), $E_{1/2} = -0.609$ V for TETMAHA (Gaspar *et al.*), $E_{1/2} = -0.585$ V for DOTRMAHA (Esteves *et al.* 1995) and $E_{1/2} = -0.586$ V for CDTMAHA (Santos *et al.* 1997), (SCE). Finally, these synthetic ferric hydroxamates reveal an electron transfer mechanism that is slightly different from that of the natural siderophores. All of them show a reversible or quasi-reversible electrochemistry reaction followed by a more or less rapid chemical dissociation of the reduced species. This last step has not been described in the literature for the natural siderophores, which suggests that probably it is not detectable at the scale of the electron transfer measurements (cyclic voltammetry). Thus, easy reduction of the iron(III) complex and the following chemical dissociation could explain the low biological activity of all these com-

Table 3. Global stability constants, values of p[M] and $E_{1/2}$ of some siderophores or related analogous ferric complexes

Ligand	Log $\beta_{Fe_pH_qL_r}$ *,#	p[M]	$E_{1/2}$ (mV)	Ref.
Rhodotorulic acid	(2,0,3) = 62.3	21.9	−601	a, b
DOCYDMAHA	(2,0,3) = 60.5	21.1	−603	c
PIPDMAHA	(2,0,3) = 61.7	21.5	−560	d
Ferrioxamine E	(1,0,1) = 32.5	27.7	–	a
Ferrioxamine B	(1,0,1) = 30.99	26.3	−698	e, f
Ferrichrome	(1,0,1) = 29.1	25.2	−690	a, e
TETMAHA	(1,1,1) = 37.8	22.8	−609	g
DOTRMAHA	(1,1,1) = 24.2	21.7	−585	h
CDTMAHA	(2,3,2) = 78.4	20.2	−586	i
Enterobactin	(1,0,1) = 52.0	37.6	−996	a, f
APM-3,4-HOPY	(1,1,3) = 51.25	21.34	−786	j

p[M] = −log [Fe^{3+}] at pH = 7.4, [Fe^{3+}] = 1×10^{-6} M, [L] = 1×10^{-5} M; # PSEQUAD program; * The (p,q,r) symbolism means a species with stoichiometry ($Fe_pH_qL_r$). [a] from ref. Raymond & Carrano 1979; [b] from ref. Carrano *et al.* 1979; [c] from ref. Santos *et al.* 1998; [d] from ref. Santos *et al.* 1993; [e] from ref. Brockway *et al.* 1980; [f] from ref. Motekaitis & Martell 1991; [g] from ref. Gaspar *et al.* 1999; [h] from ref. Esteves *et al.* 1995; [i] from ref. Santos *et al.* 1997; [j] from ref. Grazina *et al.* 1998.

Table 4. Growth promotion activity and competition studies with ferric complexes in *Morganella morganii* 13 and *Proteus mirabilis* 8993

Iron chelator (μmol/ml)		Proteus Mirabilis 8993	Proteus mirabilis 8993 + Ferrirubin	Proteus mirabilis 8993 + Coprogen	Proteus mirabilis 8993 2B	Proteus mirabilis 8993 3B	Morg. morganii 13	Morg. morganii 13 + Ferrirubin	Morg. morganii 13 + Coprogen	Morg. morganii 13 2B	Morg. morganii 13 3B
Ferrirubin											
	1	−					−				
	5	−					−				
	10	−					−				
Coprogen											
	1	−					−				
	5	−					++				
	10	−					++				
Fox E											
	1	−					−				
Enterobactin											
	1	++++					++++				
APM-3,4-HOPY											
	1	++	++	++	++	++	+++	+++	++	++	++
	2	+++									
DOCYDMAHA											
	0.5	++					++				
	1	+++	+++	+++	+++	−	+++	+++	++	++	+++
PIPDMAHA											
	0.5	−					++				
	1	++	++	++	++	−	++	++	++	++	++
TETMAHA											
	1	−					++				
	2	+					++				
	3.8	++	++	++	+	−	++	+++	++	++	++
DOTRMAHA											
	2.5	−					+				
	5	++	+	+	−	−	++	++	++	++	+
CDTMAHA											
	1	−					−				
	2	++					+				
	4	++	++	++	−	−	++	++	++	+	−

For the competition equal amounts of the synthetic compound and of ferrirubin or coprogen were placed on the filter disks. 2B = 300 μM of bipyridyl; 3B = 450 μM of bipyridyl. − indicates no growth halo around the disc; + = < 10 mm halo of growth; ++ = 10–15 mm, +++ = 15–20 mm; ++++, = > 20 mm halo.

pounds. In the case of APM-3,4-HOPY the reduction potential is much higher than any of the hydroxamate siderophores ($E_{1/2} = -0.786$ V), although it is lower than the reduction potential of the catecholate siderophores (enterobactin $E_{1/2} = -0.750$ V vs NHE, $E_{1/2} = -0.996$ V vs SCE, pH = 7) which is out of the range of any physiological reductants (Raymond & Carrano 1979). This shows that this compound has intermediate properties between the catecholate and the hydroxamate siderophores enabling recognition by both receptors.

Among all the synthetic hydroxamates studied in the present work, the best compound was DOCYDMAHA, a *bis*(amide,amine)hydroxamate ligand that was utilized by all hydroxamate receptors. This compound has $E_{1/2}$ and p[M] values similar to those of

the Fe-rhodotorulic acid, a natural di-hydroxamate that is transported in the producing fungi by a taxi cab mechanism (Winkelmann & Drechsel 1997). This natural siderophore has a cyclic backbone with two amide groups. The fact that DOCYDHAMA is the only siderophore analogue with a diamide macrocycle backbone may contribute to its good biological activity. The hydroxypyridonate APM-3,4-HOPY complex was transported by all strains including the delta (FhuA-B), *fep*A$^-$, *ent*$^-$ strain, confirming independence of a FepA receptor or a fhu system for iron utilization. The lowest biological activity was revealed by the tetrahydroxamate ligand, CDTMAHA. Since it has more hydroxamate groups than the three necessary ones for the coordination with iron(III), it cannot be excluded that it forms polymers that are unable to transverse the cytoplasmatic membranes or may precipitate. Compared to the synthetic dihydroxamate compounds the trihydroxamates TETMAHA and DOTRMAHA are not so well utilized perhaps because of the rigid macrocycle backbone which prevents entry of the complex via the receptors.

Proteus and morganella

As shown in Table 4, *Proteus* and *Morganella* utilized APM-3,4-HOPY, DOCYDMAHA, PIPDMAHA, TETMAHA and CDTMAHA in the range of concentrations of the natural siderophores (1 or 2 μmol/ml), thus indicating that these bacteria can use the iron bonded to these compounds. DOTRMAHA is also taken but in higher concentrations. These genera are known to use ferric complexes of keto hydroxy bidentate ligands as siderophores (Winkelmann & Drechsel 1997). However, the receptors used are not yet identified and little is known about ferric transport systems in these genera. Only two genes, *rum*A and *rum*B have been identified in *M. morganii* (Kühn *et al.* 1996). So, we do not know the system used in the transport of the studied compounds. As a control, enterobactin is well taken up, indicating the existence of a FepA receptor in both strains.

We have performed some inhibition studies with ferrirubin, a ferrichrome-type siderophore, and coprogen and also studies with increasing concentrations of bipyridyl, a chelator of ferrous ions.

Proteus does not take ferrirubin or coprogen, even at higher concentrations but *Morganella* can take coprogen at a concentration of 5 μmol/ml. In the two bacteria there is no inhibition for all compounds tested with the two siderophores, even in *Morganella* with coprogen. This probably means that the system used to transport iron with our compounds do not have any interaction with these two trihydroxamates.

The studies with bipyridyl in *Proteus* reveal that a double concentration of bipyridyl (300 μM) is enough to completely inhibit the grow promotion by DOTRMAHA and CDTMAHA and that a triple concentration (450 μM) is enough to completely inhibit the grow promotion by TETMAHA, PIPDMAHA and DOCYDMAHA. APM-3,4-HOPY can promote growth, even at a 450 μM concentration. This could mean that *Proteus* could reduce Fe(III) bound to the compounds to Fe(II) and then transport Fe(II) into the cell. Besides the ferric enterobactin uptake route, *Proteus* may also reduce simple ferric chelates and then transport the ferrous ion into the cell, which is still a hypothesis because in this genera we do not know the real mechanism of iron uptake from synthetic complexes. In fact, it is easier to reduce the synthetic hydroxamate compounds than the natural siderophores as suggested by their lower reduction potential (see Table 3). This agrees with the fact that our compounds can promote growth in *Proteus* but not the natural trihydroxamates. So it is possible that *Proteus* first reduces Fe(III) and then transports Fe(II) into the cell. APM-3,4-HOPY may be transported by another mechanism, because the reduction potential is much higher than any of the hydroxamate siderophores ($E_{1/2} = -0.786$ V for APM-3,4-HOPY), although it is lower than the reduction potential of the catecholate siderophores (enterobactin $E_{1/2} = -0.996$ V vs SCE, pH = 7) which is out of the range of any physiological reductants (Raymond & Carrano 1979). The studies with bipyridyl in *Morganella* revealed that only high concentrations (450 μM) inhibit growth promotion by CDTMAHA.

Conclusions

In the present investigation we have shown that some of the synthetic compounds studied, APM-3,4-HOPY, DOCYDMAHA and PIPDMAHA have good biological activity in the tested organisms *Escherichia coli*, *Proteus* and *Morganella* at relatively high concentrations (1–8 mM) compared to the natural siderophores (0.01 mM) (Deiss *et al.* 1998). DOCYDMAHA supports growth of *E. coli* strains at a concentration <1 mM. TETMAHA can be utilized by *Proteus*, *Morganella* and some mutants of *E. coli* at a concentration of 2 mM. DOTRMAHA can be utilized at 5 mM

and CDTMAHA can be utilized by *Proteus* and *Morganella* at 2 mM and by some *E. coli* mutants at a concentration of 8 mM.

Although it was not possible to identify only one specific receptor for any of the compounds studied we observed different transport activities with different receptor mutants. The best compound was DOCYDMAHA, a *bis*(amide, amine)hydroxamate ligand that entered the cells of all receptor mutants. The hydroxypyridonate APM-3,4-HOPY seems to preferentially use the FoxA and the FhuA receptors. The diaminodihydroxamate (PIPDMAHA) and tetraaminotrihydroxamate (TETMAHA) ligands were transported by all the receptors of the hydroxamate transport system, but preferentially by FhuA and FoxA. The tris- and tetra-hydroxamate ligands (DOTRMAHA and CDTMAHA) do not seem to be recognized, unless when high concentrations are used. All compounds seem to be transported, at different degrees, in a more or less unspecific manner with regard to the hydroxamate outer membrane receptors. However, the control strain H1880 lacking the periplasmic binding protein FhuD and the plasma membrane transport proteins (FhuB, C) showed that all synthetic ferric hydroxamates require a functional hydroxamate transport system. Also the results with the FepA, Fiu and Cir mutants confirm that the synthetic ferric hydroxamates can enter these mutants via the Fhu transport system, which is still active in these mutants.

It is generally accepted that the specificity for natural siderophores resides in the outer membrane receptors of *E. coli* and other gram-negative bacteria. However, this was not the case with the synthetic siderophore analogues of the present investigation. The low receptor specificity might be attributed to the fact that these synthetic siderophores are small molecules without specific contacts to the receptor proteins. They are also easier reduced than the natural ones and their global reduction mechanism may lead to a kinetic chemical dissociation. Another hypothesis is that these compounds can fit into a variety of receptors because of their partial structural identity. It would be interesting to know if the amide groups of the DOCYDMAHA and the keto-hydroxy group of APM-3,4-HOPY are important for the interactions with the receptor proteins.

With respect to *Proteus* and *Morganella* (Table 4) the receptors involved in the ferric transport systems are not well known and therefore it is difficult to obtain conclusions about the transport routes. Inhibition studies showed that the uptake routes for the synthetic compounds do not interfere with added ferrirubin and coprogen, thus excluding competition for receptors. From the studies with bipyridyl it was concluded that *Proteus* may reduce the Fe(III) compounds to Fe(II) and then transport Fe(II) into the cell interior. APM-3,4-HOPY could probably transport iron by another mechanism, which is independent of the Fhu system. In *Morganella* a reducing mechanism might be involved in the case of CDTMAHA but not in the case of the other compounds.

Thus, summarizing the results with the synthetic hydroxamates we were unable to identify receptor specificity but we have proven that the growth promotion requires the function of the Fhu transport system.

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