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Isolation and identification of ferrioxamine G and E in *Hafnia alvei*

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Summary. Under conditions of iron-deprivation *Hafnia alvei* (Enterobacteriaceae) produces ferrioxamine G as the principal siderophore. Maximum hydroxamate siderophore production occurred at medium iron limitation. The ferrioxamines were extracted, purified by gel filtration and chromatography on silica gel yielding a major and a minor siderophore fraction. The minor siderophore fraction contained three siderophores, among which ferrioxamine E could be identified by HPLC and FAB mass spectrometry. Reductive hydrolysis of the ferrioxamine G fraction yielded succinic acid and a mixture of diaminopentane and diaminobutane, as determined by gas-liquid chromatography and GLC/MS. HPLC and FAB mass spectrometry confirmed that the ferrioxamine G fraction consisted of two different species, G_1 and G_2, possessing molecular masses of 671 Da and 658 Da respectively.

Key words: Siderophores — ferrioxamine G — *Hafnia alvei* — Enterobacteria

Introduction

Hafnia alvei is a facultatively anaerobic, rod-like bacterium of the family Enterobacteriaceae which occurs not only in man and animals but also in natural environments such as soil, sewage and water. With the exception of plasmid-encoded aerobactin, hydroxamate siderophores are untypical iron chelators within the family of Enterobacteriaceae which are known to biosynthesize the chromosomally encoded catecholate siderophore enterobactin, also named enterochelin, a cyclic trimer of 2,3-dihydroxybenzoylserine (Earhart 1987). *Hafnia alvei*, previously named *Enterobacter hafniae*, is closely related to the genus *Enterobacter*. However, it has been transferred to a separate genus *Hafnia* because it has few phenotypic or genetic similarities with other *Enterobacter* species (Sakazaki 1981; Bergey's Manual 1984). We have recently shown that the ability to produce and transport ferrioxamine E is a common chemotaxonomic trait of the genus *Enterobacter agglomerans* which is also called *Erwinia herbicola* by the phytopathologists (Berner et al. 1988; Berner and Winkelmann, 1990). As shown in the present investigation, *Hafnia alvei* strains, although very similar to *Enterobacter agglomerans* in their ability to produce ferrioxamines, may be distinguished from *Enterobacter* strains by their characteristic ferrioxamine G synthesis. Thus, while the cyclic ferrioxamines E and D_2 are the predominant ferrioxamines of *Enterobacter agglomerans*, the corresponding linear forms, ferrioxamine G_1 and G_2, are the main siderophores of *Hafnia alvei* (Fig. 1).

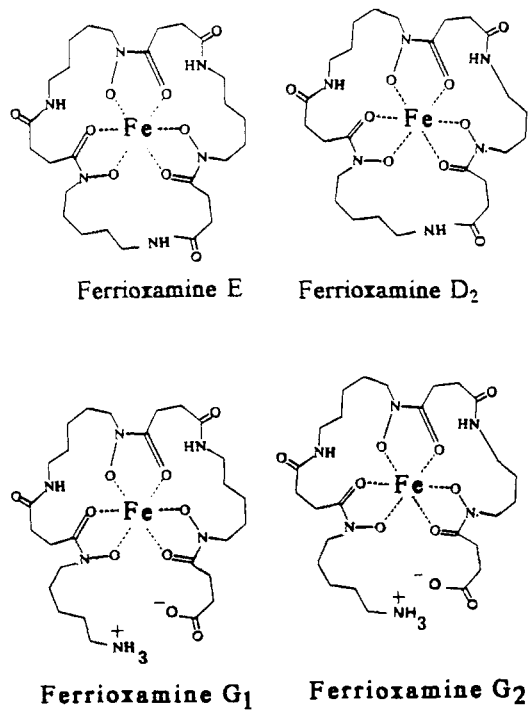

Fig. 1. Structures of the cyclic ferrioxamines E and D_2 and of the corresponding linear ferrioxamines G_1 and G_2

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Material and methods

Bacterial strains and growth conditions. *Hafnia alvei* strains IEE 1, IEE3, IEE6, IEE3338/84, IEE1081/80, IEE7473, IEE1075/80, IEE3/82, IEE1790/74, IEE1069/79, Biogroup 1 CDC 9013-81 were analyzed for siderophore production. The strain IEE7473 was selected for the chemical characterization of siderophores. This strain showed the typical physiological and biochemical characteristics of *H. alvei* as described in Bergey's Manual (1984). Bioassays were performed as described previously (Reissbrodt and Rabsch 1988). The ability of the isolated siderophores to stimulate bacterial growth was tested in iron-limited medium containing 200 μM 2,2'-dipyridyl (Dip) and nitrilotriacetic acid (NTA). The properties of the indicator strains *Escherichia coli* H1443, H854, and H1619 have been described by Hantke (1983). The first five indicator strains shown in Table 1 were described by Reissbrodt and Rabsch (1988). The indicator strain *Salmonella stanleyville* GRR 32 is a *fepA* mutant of *S. stanleyville* 207/81 as has been detected by outer-membrane protein analysis. This mutant was obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) mutagenesis and selected by the enterobactin-streptonigrin method (Braun et al. 1983). The indicator strain *S. thypimurium* SR 1001 is a *tonB* mutant of *S. thypimurium* enb-7. This mutant was obtained by NG mutagenesis and selected by the use of phage ES18 and by albomycin resistance. All glass ware was thorougly washed with ethylenediaminetetraacetic acid (2%), citric acid (2%), distilled and double-distilled water.

Chemicals. All chemicals used were of analytical grade. Rhodotorulic acid was a gift from J. B. Neilands (Department of Biochemistry, Berkeley, CA, USA). Schizokinen was kindly provided by J. Sanders-Loehr (Department of Chemical and Biological Sciences, Beaverton, OR, USA). The other siderophores used for comparison purposes were from the stock of the Institute at Tübingen or isolated and purified according to well known methods (Langman et al. 1972; Braun 1981). Desferal® was a gift from Ciba-Geigy AG (Basel, Switzerland).

Nutrient media. Cultivation of all strains was performed on nutrient broth for 18 h at 37° C. The strains were starved in Tris/succinate medium as described by Rabsch and Reissbrodt (1985). Iron-poor nutrient medium (basic medium) was supplemented by M9 Medium. The basic medium contained per litre: 7.5 g $Na_2HPO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 5.0 g protein hydrolyzate, 1.0 g Bacto-peptone, 4.0 g glucose, 0.25 g $MgSO_4 \cdot 7H_2O$, 2 mg $CaCl_2 \cdot 6H_2O$, 2.5 mg thiamine·HCl, 1 mg thiamine diphosphate, pH 7.2–7.4.

Cultivation. The cells were grown in supplemented M9 medium at 37° C. Iron-limited media contained increasing amounts of 2.2'-dipyridyl (Dip) and nitrilotriacetic acid (NTA). The production of siderophores was studied with 5-ml aliquots of culture filtrates according to Csaky (1948). The inoculum for each batch was pregrown in M9 medium (10^8 CFU/ml) as described above. The culture tubes (160 × 16 mm) were incubated for 18–48 h at 37° C with reciprocal shaking in the vertical position. Growth was controlled by means of the absorbance at 620 nm. Samples (1 ml) of each run were used for the estimation of hydroxylamine N.

Isolation and purification of the siderophores from Hafnia. Iron-limited media (50 ml) in 300-ml-conical flasks (containing 60, 75 or 100 μmol of both Dip and NTA) was inoculated with iron-starved *H. alvei* cell suspension (100 μl) and incubated for 22 h at 37° C on a shaker. Samples were centrifuged (5000 rpm) and the hydroxylamine content determined. After addition of $FeSO_4 \cdot 7H_2O$ (5 mg) the supernatants were collected and passed through a column of DEAE-cellulose (Renal, Budapest, Hungary; $d = 2.2$ cm, $l = 6$ cm) equilibrated with 0.1 M phosphate pH 7.0. After washing with the same buffer, eluent and washing buffer were evaporated to dryness. Traces of water were removed by further drying *in vacuo* over P_2O_5. The dry material was dissolved in methanol, filtered and concentrated. A further purification was achieved by gel filtration on a Bio-gel P2 column (Bio-Rad) and elution with distilled water. Ferrioxamine E was separated from ferrioxamine G on a silica gel column using chloroform/methanol (1:1) was an eluting solvent. Desferri-siderophores were prepared as described by Wiebe and Winkelmann (1975).

Gas-liquid chromatography and GLC/MS. The ferrioxamines were hydrolyzed with 57% HI or 6 M HCl in sealed vials and heated for 18 h at 110° C. After repeated evaporation to dryness, the residue was dissolved in water and extracted with chloroform. The chloroform extract was dried by evaporation, dissolved in pyridine and derivatized with an equal volume of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Serva). After a 1-h reaction time, the mixture was analyzed by gas-liquid chromatography on an SE30 capillary column using a temperature program of 80–150° C at 4° C/min. Silylated succinic acid served as a reference compound and the coinjection procedure was used for identification. The aqueous phase of the hydrolyzate was analyzed by GC/MS in order to identify the remaining diamines. The diamines were *N*-acylated with trifluoroacetanhydride yielding the *N,N'*-bis(fluoroacetyl) derivatives.

High-performance liquid chromatography. HPLC analysis was performed on C_{18} and C_8 reversed-phase material (Nucleosil, 3 μm, 250 × 4.5 mm, Grom, Ammerbuch, FRG) using acetonitrile (12%) and 10 mM ammonium acetate pH 4 or acetonitrile (8%) and acetic acid pH 4 as an eluting solvent and a flow rate of 1 ml/min. Samples were detected at a wavelength of 220 nm using a Shimadzu spectrometer.

FAB mass spectrometry. Fast-atom-bombardment (FAB) spectra were recorded with a Varian MAT 711 A instrument combined with an SS 200 data system with glycerol as a solvent and a xenon ionizing beam produced with a saddlefield primary atom gun (Ion Tech, England) at an ion source temperature of 25° C.

MS/MS spectra were recorded with an ion spray mass spectrometer (API III, Sciex, Toronto) at room temperature and atmospheric pressure ionization using argon as collision gas. Samples were solubilized in water/methanol (1:1) containing 1% formic acid and injected at a constant flow rate of 5 μl/min.

^{13}C-NMR spectra. The spectra of the desferri compounds were recorded in D_2O solution (c = 50 mg/ml, 303 K) with a Bruker WM 400 instrument (100.62 MHz) for ^{13}C. Resonances were assigned by chemical shift correlation with known siderophores of analogous structures (Keller-Schierlein et al. 1984; Borgias et al. 1989; Konetschny-Rapp 1990).

Results

The bioassays showed positive reactions with the following indicator strains: *Arthrobacter flavescens* JG9, *Salmonella stanleyville* GRR32, and *Salmonella typhimurium* enb-7 and negative reactions with *S. typhimurium* TA2700 and *E. coli* LG 1522 (Table 1). As the observed siderophore pattern differed markedly from the common pattern seen with other enterobacterial strains, the production of new siderophores was suspected. Moreover, since *S. typhimurium* SR1001 reacted positively with all siderophores from *H. alvei* strains, we suppose that 2,3-dihydroxybenzoic acid is also produced at 160 μM Dip and NTA. Isolation and purification of the siderophores for structure elucidation was performed with strain *H. alvei* 7473 (from the stock of the Institute of Experimental Epidemiology, Wernige-

Table 1. Bioassays of siderophores with different indicator strains

Indicator strains	2,3-Dihydroxybenzoic acid	Enterobactin	Ferrichrome	Rhodotorulic acid	Ferrioxamine B	Ferrioxamine G	Coprogen	Schizokinen	Aerobactin
S. typhimurium enb-7	+	+	+	+	+	+	+	+	−
S. typhimurium TA 2700	−	+	−	−	−	−	−	−	−
E. coli LG 1522	−	−	−	+	−	−	−	+	+
Arthrobacter flavescens JG-9	−	−	+	+	+	+	+	+	−
S. stanleyville 207/81	−	+	+	+	+	+	+	+	−
S. stanleyville GRR 32	−	−	+	+	+	+	+	+	−
S. typhimurium SR 1001	+	−	−	−	−	−	−	−	−
E. coli H 1443[a]	−	+	+	+	−[b]	−	+	−	−
E. coli H 854 (*fiu*)[a]	−	+	+	+	−[b]	−	+	−	−
E. coli H 1619 (*fhuE*)[a]	−	+	+	−	−	−	−	−	−

[a] The concentration of 2,2′-dipyridyl was increased twofold
[b] Positive at high concentrations

rode). Growth of *H. alvei* cells in iron-limited media was stimulated by 40 μM Dip and NTA. Above a concentration 60 μM of these chelators an increasing inhibitory effect was observed with respect to growth and hydroxamate production. At about 320 μM Dip and NTA, growth and hydroxamate production ceased. After 20 or 42 h of shaking at 37° C, the highest amounts of siderophores could be detected within the range of 40–120 μM Dip and NTA. Formation of hydroxylamino N and detection of microbiological activity were in accordance with the results from the bioassays. Differences in iron limitation (60, 75, 100 μM Dip and NTA) during cultivation had no influence on the amount of siderophores produced as the hydroxylamino N content was found to remain constant at about 11 μg/ml. More than 99% of hydroxylamino N was detected in the supernatant.

The crude siderophores of *H. alvei* were separated by thin-layer chromatography using chloroform/methanol/water (65:25:4) showing a major spot ($R_f = 0.07$; ferrioxamine G fraction) and three minor spots ($R_f = 0.16, 0.39, 0.43$). The crude extracts were dissolved in methanol and purified on an LH20 column. A comparison of the HPLC siderophore profiles on a C_{18} reversed-phase column using an isocratic system of 12% acetonitrile and 10 mM ammonium acetate pH 4 from *H. alvei* 7473 and *Erwinia herbicola* K4 (Fig. 2) revealed that in *H. alvei* the principal siderophore is ferrioxamine G while in *E. herbicola* it is ferrioxamine E. The total siderophore fraction from *H. alvei* was subsequently purified on Bio Gel P2. The less polar ferrioxamine fraction was separated on a silica gel column using chloroform/methanol (1:1) as an eluting solvent. HPLC analysis of this fraction confirmed the presence of ferrioxamine E which had been isolated earlier form *Erwinia herbicola* (*Enterobacter agglomerans*). The FAB mass spectrum provided evidence for the identity of the isolated compound with ferrioxamine E (Fig. 4a). Purification of the major siderophore fraction on Bio Gel P2 and subsequent HPLC separation on a C_8-reversed phase column (125 × 4.5 mm), using an isocratic system of acetonitrile (8%) and acetic acid pH 4, resulted in a main ferrioxamine G_1 peak preceded by a minor peak ferrioxamine G_2 (Fig. 3). Analysis of this ferrioxamine fraction ($G_1 + G_2$) by FAB mass spectrometry (Fig. 4b) yielded two MH^+ peaks (*m/z* 658 and 672) and their

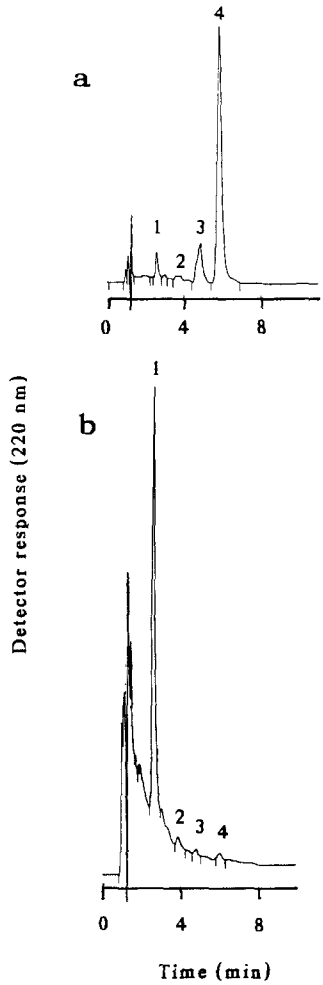


Fig. 2. HPLC separation of ferrioxamines isolated from (**a**) *Erwinia herbicola* K4 and (**b**) from *Hafnia alvei* 7473 on a C_{18} reversed-phase column (Nucleosil, 3 μm, 250 × 4.5 mm) using 12% acetonitrile and 10 mM ammonium acetate pH 4 and a flow rate of 1.5 ml/min. Peaks are assigned as follows: 1 = ferrioxamine G, 2 = unknown, 3 = ferrioxamine D_2, 4 = ferrioxamine E

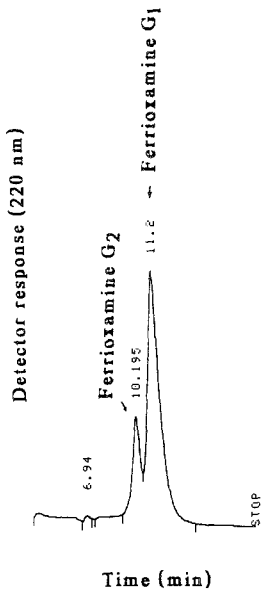


Fig. 3. HPLC separation of the ferrioxamine G fraction on a C_8-reversed phase column (125 mm) yielding ferrioxamine G_1 (10.195 min) and G_2 (11.2 min). conditions are: 8% acetonitrile and acetic acid, pH 4, flow rate 1 ml/min

corresponding ion fragments, $MH^+ - Fe + 3H$, $MH^+ - Fe + 3H - O$, and $MH^+ - Fe + 3H - 2O$. Thus, the FAB mass spectrum provided evidence for the existence of two different species of ferrioxamine G: ferrioxamine G_1 (M_1H^+, *m/z* 672) possessing three diaminopentane residues and ferrioxamine G_2 (M_2H^+, *m/z* 658), possessing two diaminopentanes and one diaminobutane. As the ferric forms were analyzed, the corresponding iron-free species could also be observed: *m/z* $619 = M_1H^+ - Fe + 3H$, *m/z* $658 = M_2H^+ - Fe + 3H$ as well as several mass species lacking one or two oxygens: *m/z* $603 = M_1H^+ - Fe + 3H - O$; *m/z* $587 = M_1H^+ - Fe + 3H - 2O$; *m/z* $589 = M_2H^+ - Fe + 3H - O$.

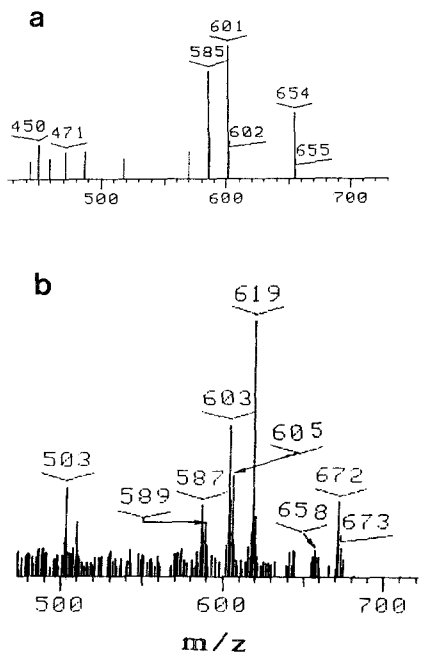


Fig. 4. Fast-atom-bombardment (FAB) mass spetrum of ferrioxamine E and G isolated from *H. alvei*. (**a**) Ferrioxamine E (*m/z* $654 = MH^+$; *m/z* $601 = MH^+ - Fe + 3H$). (**b**) Ferrioxamine G_1 *m/z* $672 = MH^+$; *m/z* $619 = MH^+ - Fe + 3H$; *m/z* $603 = MH^+ - Fe + 3H - O$; *m/z* $587 = MH^+ - Fe + 3H - 2O$. Ferrioxamine G_2 *m/z* $658 = (MH)^+$; *m/z* $605 = MH^+ - Fe + 3H$; *m/z* 589 $MH^+ - Fe + 3H - O$

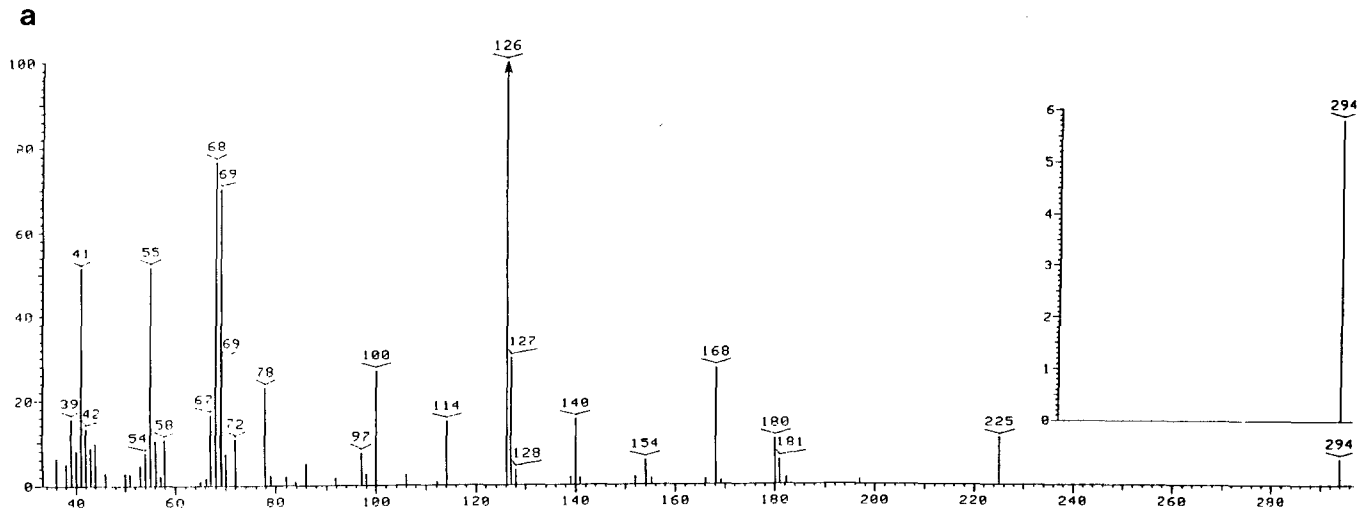
HI hydrolysis of the ferrioxamine G fraction, derivatization and gas chromatography on a Chirasil-Val column provided evidence that amino acids were absent. Silylation of the hydrolysis products and subsequent gas chromatography on SE30 indicated the presence of succinic acid and two diamines which were identified by GLC/MS analysis (Fig. 5). Thus the FAB mass spectrum was in accordance with the GLC/MS analysis of the diamine residues. Ferrioxamine G_1 containing solely diaminopentane residues seems to be a pecursor of ferrioxamine E, while ferrioxamine G_2 containing at least one residue of diaminobutane seems to be the corresponding precursor of ferrioxamine D_2 (Fig. 1). The total amount of ferrioxamine G_2 in the ferrioxamine G fraction was approximately 5–10%. The position of the diaminobutane residue within the molecule, however, remains to be determined. The mass spectrum of purified desferrioxamine G_1 (Fig. 6) showed a characteristic fragmentation pattern (daughter profile). The corresponding ion fragments (found) and (calculated) allowed a complete structural assignment as shown in Table 2.

^{13}C-NMR analysis of the isolated desferrioxamine G revealed besides the spectrum of the major component G_1 several minor peaks (less than 10% according to peak area integration) which may be assigned to the minor component G_2. Furthermore the sample contained an additional compound indicated by three resonances at 34.9, 34.4, and 30.9 ppm, each of them showing less than 50% of the mean peak area of all other methylene signals. Compared to the recently published ^{13}C-NMR data of desferrioxamine B (Borgias et al. 1989), the signals of most carbon atoms of the corresponding diaminopentane and succinic acid residues in desferrioxamine G_1 are shifted to lower field by 1–2 ppm. The spectrum of ferrioxamine G reported by Keller-Schierlein et al. (1984) has been recorded in dimethyl sulfoxide and, due to lower magnetic field strength, some signals overlap. Despite these problems and the lack of 2D-NMR experiments at 500 or 600 MHz, we could make a preliminary assignment of the ^{13}C-NMR signals as shown Table 3. The carbonyl region exhibits one weak carboxylate signal at 183.1 ppm followed by the two peptide carbonyl resonances at 177.4 and 177.3 ppm. The two peaks of equal intensity at 176.6 and 176.4 ppm are assigned to the three hydroxamate carbonyls. The three methylene carbons directly attached to the hydroxamate groups appear within the narrow range of 50.5, 50.4, and 50.3 ppm. The aminomethyl groups of the diaminopentane moieties are found at 42.0 ppm ($—CH_2—NH_3^+$) and 41.8 ppm (two $—CH_2—NH$ groups). In the region of 25–35 ppm the assignment follows that of Borgias et al. (1989).

Diaminopentane

$F_3C-CO-NH-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CF_3$

69 | 97 | 112 | 126 | 140 | 154 | 168 | 182 | 197 | 225 | 294



Diaminobutane

$F_3C-CO-NH-CH_2-CH_2-CH_2-CH_2-NH-CO-CF_3$

69 | 97 | 112 | 126 | 140 | 154 | 168 | 182 | 211 | 280

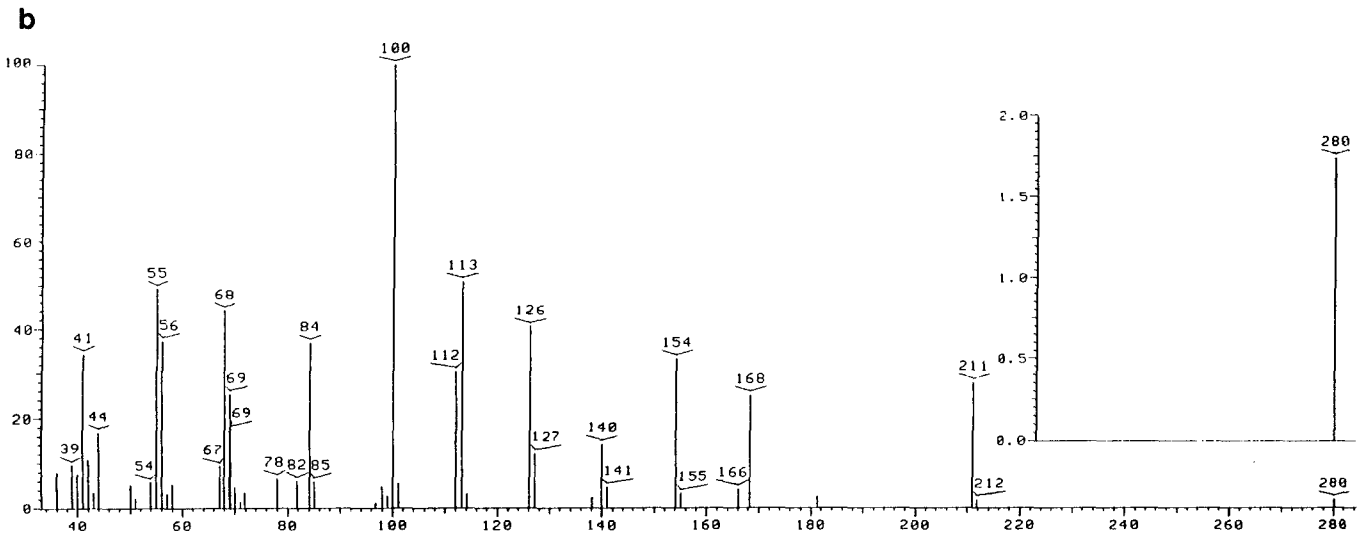


Fig. 5. Mass spectra of the diamines from the ferrioxamine G fraction after trifluoroacetylation and separation by gas-liquid chromatography (GLC/MS). (**a**) Trifluoroacetylated diaminopentane; (**b**) trifluoroacetylated diaminobutane

Discussion

Ferrioxamines D_2, E, and G were first isolated from *Streptomyces pilosus* and several other *Streptomyces* strains (Keller-Schierlein and Prelog 1961, Keller-Schierlein 1962, Keller-Schierlein et al. 1965). The chemical and structural features of the ferrioxamines and other siderophores have recently been compiled by van der Helm et al. (1987). While ferrioxamines are generally considered as typical bacterial siderophores of the Gram-positive actinomycetes, the present investigation shows that members of the Gram-negative ente-

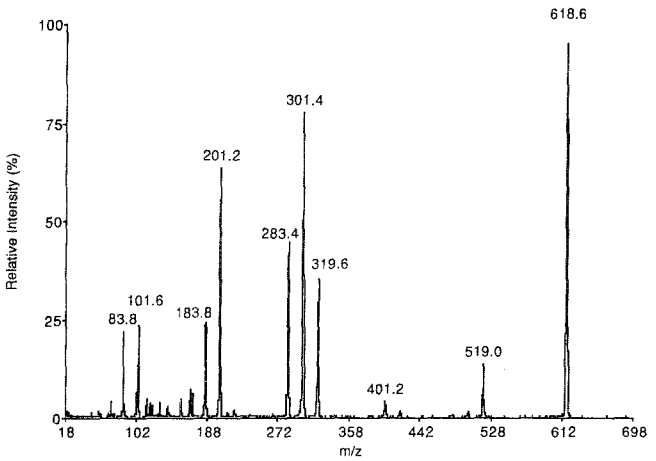


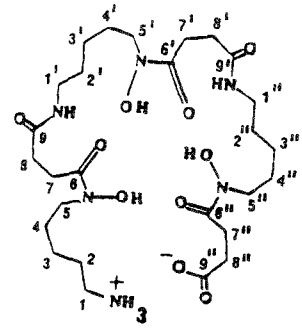
Fig. 6. Mass spectrum of purified desferrioxamine G_1. The spectrum was recorded with an ion spray mass spectrometer at room temperature and atmospheric pressure ionization (API III, Sciex, Toronto) using argon as collision gas. The sample was injected in formic acid (1%)/methanol (1:1) at a flow rate of 5 µl/min. The corresponding fragmentation pattern is given in Table 2

robacteria, such as *H. alvei* and *Enterobacter agglomerans* (*Erwinia herbicola*), also produce ferrioxamines as their principal siderophores.

Bioassays have been successfully used for the detection of siderophores as growth-promoting compounds. Recently Reissbrodt and Rabsch (1988) reported a siderophore pattern analysis for further differentiation of Enterobateriaceae. The siderophores of *H. alvei* were first detected by a siderophore pattern analysis which allowed a rapid preliminary assignment of the siderophores produced. Using this siderophore pattern analysis, the siderophores of *H. alvei* supported growth of *Arthrobacter flavescens* JG-9, *S. typhimurium* enb-7, *S. stanleyville* 207/81 and *S. stanleyville* GRR 32, but not *S. typhimurium* TA 2700 or *E. coli* LG 1522, indicating the presence of hydroxamate siderophores other than aerobactin and the absence of a catecholate siderophore. Moreover, the production of ferrichrome, coprogen, Fe-rhodotorulate, shizokinen and aerobactin could be excluded by the results obtained with *S. typhimurium* TA 2700, *S. typhimurium* SR 1001, *E. coli* LG 1522 and *E. coli* H1619 (*fhuE*).

Table 3. ^{13}C-NMR chemical shifts of desferrioxamine G_1 in D_2O (100. 6 MHz)

Shift (ppm)	Assignment of carbon atom
25. 3	3
25. 6	3′, 3″
27. 9	4
28. 1	4′, 4″
28. 9	2
30. 5	2′, 2″
30. 2	7, 7′, 7″
30. 3	
33. 0	8, 8′, 8″
33. 1	
41. 8	1′, 1″
42. 0	1
50. 3	5, 5′, 5″
50. 4	
50. 5	
176. 4	6, 6′, 6″
176. 6	
177. 3	9, 9′
177. 4	
183. 1	9″



Studies on the production of siderophores in relation to the prevailing iron content of the cultivation medium revealed that the ferrioxamines of *H. alvei* were produced in the presence of medium iron concentrations. This indicates that desferrioxamine biosynthesis in *Hafnia* is not as sensitive to iron repression as is enterobactin biosynthesis in *E. coli*.

Table 2. Assignments of fragments ions of the mass spectrum of desferrioxamine G_1 depicted in Fig. 6

m/z		Fragment
Found	Calculated	
618. 6	618. 7	M^+ (molecule ion)
519. 0	518. 7	$MH^+ - CO—(CH_2)_2—COOH$
401. 2	401. 4	$M^+ - NH—(CH_2)_5—N(OH)—CO—(CH_2)_2—COOH$
319. 6	319. 4	$M+2H^+ - CO—(CH_2)_2—CONH—(CH_2)_5—N(OH)—CO—(CH_2)_2—COOH$
301. 4	301. 4	$H_2N—(CH_2)_5—N(OH)—CO—(CH_2)_2—CONH—(CH_2)_5—NH^+$
283. 4	283. 4	$H_2N—(CH_2)_5—N(OH)—CO—(CH_2)_2—CONH—(CH_2)_5—NH^+ - H_2O$
201. 2	201. 4	$H_2N—(CH_2)_5—N(OH)—CO—(CH_2)_2—CO^+$
183. 8	184. 4	$H_2N—(CH_2)_5—N(OH)—CO—(CH_2)_2—CO^+ - OH$
101. 6	101. 1	$CO—(CH_2)_2—COOH^+$

The present report on the occurrence of ferrioxamines in *H. alvei* supports our earlier finding that ferrioxamines are characteristic siderophores in the family Enterobacteriaceae. *Enterobacter agglomerans* (*Erwinia herbicola*) has been shown earlier to produce several ferrioxamines among which ferrioxamine E was the principal siderophore (Berner et al. 1988). *Enterobacter* and *Hafnia* are closely related genera as discussed in detail in Bergey's Manual of Systematic Bacteriology. Moreover, *H. alvei* has been separated earlier from the genus *Enterobacter* because of several biochemical characteristics. The present investigation confirms this finding on a chemotaxonomical basis by showing that *E. agglomerans* produces predominantly the cyclic ferrioxamine E, whereas *H. alvei* produces mainly the corresponding linear ferrioxamine G. This suggests that *H. alvei* strains produce the linear ferrioxamine G as a result of an inefficient cyclization enzyme in the biosynthetic route to ferrioxamine E. The cyclic ferrioxamines, ferrioxamine E and D_2, were not completely absent in *H. alvei* strains. Approximately 5% of the ferrioxamines produced were cyclic. We have shown earlier that *Erwinia herbicola* (*Enterobacter agglomerans*) produces, besides ferrioxamine E, several other ferrioxamines. Among these we identified ferrioxamine D_2 according to its retention time in HPLC. Ferrioxamine D_2 is a cyclic ferrioxamine which contains two diaminopentane moieties and one butanediamine residue (Keller-Schierlein et al. 1965). In analogy to this finding we observed diaminobutane in the ferrioxamine G fraction, suggesting the presence of two distinct ferrioxamine G species, ferrioxamine G_1, containing exclusively diaminopentane and ferrioxamine G_2, containing diaminobutane and diaminopentane residues. Thus ferrioxamine G_2 represents the corresponding linear form of ferrioxamine D_2. Although for structure elucidation of the ferrioxamine G species only strain *H. alvei* 7473 was used in this investigation, thin-layer chromatography of 10 further strains showed a corresponding ferrioxamine pattern suggesting that ferrioxamine G is generally the principal siderophore of *H. alvei* strains. Thus the siderophore pattern may be taken as an additional characteristic trait for the differentiation of *H. alvei* strains from *Erwinia herbicola* (*Enterobacter agglomerans*) strains.

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