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Degradation of desferrioxamines by *Azospirillum irakense*: Assignment of metabolites by HPLC/electrospray mass spectrometry

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Abstract

Based on a recent finding that an Azos*pirillum* isolate ASP-1 possessing high 16S rDNA similarity to *Azospirillum irakense* was able to degrade desferrioxamine type siderophores (Winkelmann et al. BioMetals 9, 78-83, 1996), various members of the genus *Azospirillum* were analyzed for their ability to degrade desferrioxamines. While the desferrioxamine-degrading activity was absent or scarcely detectable in strains of *A. lipoferum*, *A. brasilense*, *A. amazonense*, degradation activity seemed to be confined to the species *A. irakense* (KBC-1, KA3). Also the identity of strain ASP-1 as *A. irakense* could be confirmed by species-specific oligonucleotide hybridization, Inter-LINE PCR fingerprinting and carbon source utilization pattern (BIOLOG) analysis. Products of desferrioxamine B degradation were analyzed by analytical HPLC and HPLC/electrospray mass spectrometry. Using whole cells and purified enzyme it was shown that the trihydroxamate desferrioxamine B (561 amu) is split at the N-terminal amide bond yielding a monohydroxamate (MH_1, 219 amu) and a dihydroxamate (DH_1, 361 amu) metabolite. A second monohydroxamate (MH_2, 319 amu) resulted from DH_1 after splitting the acetylhydroxamate bond. Minor amounts of a further dihydroxamate (DH_2, 419 amu) originated from splitting the second amide bond in desferrioxamine B. In addition to desferrioxamine B, several other linear and cyclic desferrioxamines and derivatives were degraded, whereas desferricoprogen and desferri-ferrichrome were not degraded, indicating high substrate specificity of the desferrioxamine hydrolase in *A. irakense* species. A simple microtiter plate assay was developed which can be used to phenotypically discriminate and identify species of *A. irakense* from other *Azospirillum* species by their characteristic feature of desferrioxamine degradation.

Introduction

We previously reported on the isolation of a novel *Spirillum*-like bacterium that degrades ferrioxamine-type siderophores (Winkelmann *et al.* 1996). Due to 16S rDNA sequence similarity to *Azospirillum irakense* we suspected desferrioxamine degradation in further members of the genus Azospirillum, e.g. *A. lipoferum*, *A. brasilense*, *A. amazonense.* The genus *Azospirillum* represents non-nodulating microaerophilic diazotrophic bacteria that frequently occur associated with roots of many plants (Döbereiner 1992). *Azospirillum* species could be isolated from root and soil samples all over the world and their favored carbon substrates are organic acids such as L-malate, succinate, pyruvate and – in some species – a variety of sugars and derivatives. Although N_2-fixation and NH_4^+ salts are commonly utilized as N source, the use of N-containing carbon sources is not well studied. As shown in the present investigation degradation of desferrioxamines results in the pro-

duction of various products that serve as carbon and nitrogen sources.

A. irakense has been first isolated by Khammas *et al.* (1989) from rhizosphere soil and roots collected from rice fields in Iraq. They are Gram-negative, alpha-2 protoebacteria with a typically pleomorphic shape (curved or vibroid in semisolid N-free medium and spirillum-like in nutrient broth). Due to a polar flagellum the movement is winding. Similar to *A. amazonense* it readily grows on sugars. *A. irakense* together with *A. amazonense*, *Rhodospirillum centenum* and two not further classified *Azospirillum* sp. (DSM 4834 and DSM 4835) forms a phylogenetic cluster distinct from the other four *Azospirillum* species (*A. brasilense*, *A. lipoferum*, *A. halopraeferens* and *A. largomobilis*) (Xia *et al.* 1994; Ben Dekhil *et al.* 1997). 16S- and 23S-rRNA-directed phylogenetic oligonucleotide probes are available to identify *A. irakense* and the other *Azospirillum* spp. by DNA-hybridization (Kirchhof *et al.* 1997).

Siderophores in their iron-free form represent a very uncommon C- and N-source. Moreover, most siderophores are rapidly converted to the iron complex form in the presence of iron-rich soils. Ferric siderophores possess a remarkable chemical stability and accumulate in the soil (Powell *et al.* 1983) probably due to the inability of hydrolytic enzymes to attack the chelate backbone after complexing with iron. However, ester-containing siderophores of the fusarinine group have been reported to be split by fungal esterases (Emery 1976) and peptidic ferrichrome-type siderophores have been shown to be degraded by bacteria of the Pseudomonas group (Warren & Neilands, 1964, 1965; Villavicencio & Neilands 1965). Degradation of desferrioxamines possessing amide bonds (succinic acid-diaminopentane bonds) instead of peptide bonds have been first described by Castignetti and coworkers (Castignetti & Siddiqui 1990; DeAngelis *et al.* 1993; Harwani *et al.* 1997). The isolate (DFB#5) analyzed by this group has been characterized as a *Rhizobium loti*-like organism and the enzyme involved has been described as a serine protease (Zaya *et al.* 1998). According to Castignetti and coworkers, the proposed pathway of the 'DFB hydrolase' showed complete degradation to monohydroxamates with cadaverine, succinic and acetic acid as hypothesized endproducts. Contrary to desferrioxamine B degradation by the *R. loti* strain (DFB#5), *Azospirillum irakense* strains revealed a different pattern of metabolites (Winkelmann *et al.* 1996). Thus, in the current paper a detailed analysis of the degradation products by HPLC/electrospray mass spectrometry and a final identification of the ASP-1 isolate as *A. irakense* was performed by comparing its characteristic metabolic and degradation properties with authentic strains of *A. irakense* and other *Azospirillum* species.

Materials and methods

Strains and growth conditions

Cultures of *A. lipoferum* 59b, *A. brasilense* Sp7, *A. amazonense* Y1 (DSM 2789), *A. amazonense* Y6 (DSM 2787), and *A. irakense* KBC1 were from the stock of the GSF, (Neuherberg, Germany). Strains DSM 4834 and DSM 4835 were from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). *A. irakense* KA3 was kindly provided by Dr. Paul Kaiser (Inst. Natl. Agronomique, Paris Gringnon, France). *Azospirillum* ASP-1 was from the stock of the Dept. of Micobiology & Biotechnology, Tuebingen, Germany and was isolated as previously described (Winkelmann et al. 1996). All strains were maintained aerobically on potato-glucose-agar. Shake cultures for desferrioxamine degradation were grown in minimal medium containing per litre: $K_2HPO_4 \cdot 3H_2O$ (13.5 g), $NaH_2PO_4 \cdot H_2O$ (6.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (2.37 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.1 g). Glucose (12 ml of a 50% w/v-stock solution, autoclaved separately), vitamins (1 ml of a stock-solution containing 10 mg biotin and 20 mg pyridoxol HCl per 100 ml, sterile filtered) and trace elements (2 ml of a stock solution containing per litre: H_3BO_3 (0.56 g), $MnSO_4$ (0.47 g), $NaMO_4 \cdot 2H_2O$ (0.4 g), $ZnSO_4 \cdot 7H_2O$ (48 mg), $CuSO_4 \cdot 5H_2O$ (1.6 mg), sterile filtered), were added to the minimal medium after autoclaving.

HPLC separation

Desferrioxamines were separated on a reversed phase column (Nucleosil C18, 5 μm, 4 $\times$ 250 mm, Grom, Herrenberg, Germany) using an acetonitrile/water gradient (6–40%) with 0.1% trifluoroacetic acid (TFA) added to both solvents. HPLC separation was run on a HPLC (LC-10AT pumps, equipped with gradient controller and automatic sampler (Shimadzu, Duisburg, Germany). Detector wavelength was set at 220 nm which allowed to detect peaks of desferrioxamines and its degradation products. Iron-containing ferrioxamines were detected at 435 nm.

Siderophores

Desferrioxamine B (Desferal^R, methanesulfonate salt of desferrioxamine B) was kindly provided by Dr. Schnebli, Novartis, Switzerland. Desferrioxamine E, desferrioxamine Et_2, desferrioxamine Et_3 were from the stock of the institute and were produced as described earlier from *Streptomyces olivaceus* Tü 2718 (Meiwes *et al.* 1990). For comparison purposes also the non-degradable siderophores desferricoprogen and desferri-ferrichrysin were used. These fungal siderophores are biosynthesized by *Neurospora crassa* and *Aspergillus melleus* respectively (Leong & Winkelmann 1998).

Degradation assays

Minimal medium (20 ml) supplemented with glucose, vitamines and trace elements was inoculated with one colony of an 24 h-grown agar slant culture. After growth overnight on a rotary shaker (120 rpm) at 25 °C, desferrioxamine B (0.15 mM) was added to induce the desferrioxamine hydrolyzing activity. Cells were grown further for 6 h, sedimented (6000 g), and resuspended in fresh medium without glucose but containing the desferrioxamines to be degraded.

Degradation of desferrioxamines was monitored form culture supernatants by taking samples (1 ml) at intervals (2, 4, 6, 24 h) and subsequent sedimentation at 13 000 rpm in a microfuge. The supernatant (0.5 ml) was analyzed either directly by HPLC separation of the desferrioxamines and their degradation products, or was photometrically determined as the residual ferric complex at 435 nm in a UV/vis spectrophotometer after addition of $FeCl_3$. Assays of desferrioxamine degradation in microtiter plates was as follows: 10 μl of a desferrioxamine solution (10 mM) were pipetted into wells containing 100 μl cell suspension taken from an induced overnight culture which had been sedimented, washed with fresh medium and suspended in minimal medium. After 24 h of incubation at 25 °C degradation was monitored by measuring the residual amounts of desferrioxamines by adding 10 μl of a $FeCl_3$ solution (20 mM). A red-brown color due to iron complex formation was seen when desferrioxamine was still present, while uncolored wells indicated complete degradation.

Mass spectrometry

Electrospray mass spectra (ES-MS) and tandem mass spectra (MS/MS) were recorded on a triple-quadrupole mass spectrometer API III equipped with a pneumatically assisted electospray (ion spray) ion source (Sciex, Thornhill, Canada). Aqueous samples (1 mg ml^{-1}) were injected at a continous flow of 5 μl min^{-1} using a medical syringe infusion pump (model 22, Harward Apparatus, USA) and measured in positive mode (orifice voltage 80 V). Tandem mass spectra (MS/MS) were obtained by collision induced dissociation (CID) of the molecular ions with argon as collision gas (50 keV). For online HPLC/electrospray mass spectrometry (LC-MS) an HPLC system consisting of a solvent delivery system 140 A (Applied Biosystem, Weiterstadt, Germany), an injection valve (Latek Eppelheim, Germany) and a Nucleosil C-18 column (100 × 2 mm, 5 μm) (Grom, Herrenberg, Germany) was connected to the electrospray interface. A gradient of acetonitrile in water (6–40%) within 20 min containing 0.1% trifluoroacetic acid was used at a flow rate of 200 μl min^{-1}.

Phenotypic tests

Carbon source utilisation pattern analysis was performed using the BIOLOG-GN^R-system (Stager & Davis 1992).

PCR-fingerprinting

The genetic fingerprints of the bacteria were obtained using Inter LINE PCR according to the method of Smida *et al.* (1996) modified by Kirchhof *et al.* (1997a).

Filter hybridization

PCR was performed using primers 317 and 939, which flank the 23S rDNA segment complementary to the 23S rDNA directed probes (317: 5′-GTG TCG GTT TGG GGT A-3′; 939: 5′-AGT AGY GGC GAG CGA A-3′) following the conditions developed by Ludwig *et al.* (1994). With another PCR the whole 16S rDNA was amplified (primers according to Marchesi *et al.* 1998). Both amplification products were dot-blotted on a positively charged nylon membrane under vacuum using a 96-well manifold apparatus (Schleicher & Schüll, Germany). The hybridization reaction was carried out using ^{32}P-labelled oligonucleotides following the procedure of Kirchhof and Hartmann (1992). The temperature conditions were adjusted as shown in Kirchof *et al.* (1997a). Autoradiography was used for detection.

% G+C-content determination

The mean% G+C content of bacterial DNA was determined by the thermal denaturation method (Johnson 1998) and the melting profiles were recorded photometrically. The% G+C content was calculated by the equation of Owen & Lapage (1976).

Purification of desferrioxamine B-hydrolase

Cells from overnight cultures of *A. irakense* were collected by centrifugation (6000 g), broken by passing through a French Press and centrifugated again (38 000 g). The supernatant (cytoplasma) was concentrated by ammonium sulfate precipitation between 45 and 80% saturation. The precipitated proteins were resuspended in Na-phosphate (50 mM, pH 6.0) containing 1 M ammonium sulfate and exposed to hydrophobic interaction chromatography on Butyl Sepharose® 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden). The active fractions were concentrated by ultracentrifugation (Macrosep, 10 000 Da, Pall Filtron, Dreieich, Germany) and transferred to Tris buffer (20 mM, pH 9.5) by gelfiltration with Column PD-10 (Pharmacia Biotech, Uppsala, Sweden). Anion exchange chromatography on HiTrap® Q (Pharmacia Biotech, Uppsala, Sweden) yielded highly active fractions of 'purified enzyme'.

Results

Time dependent desferrioxamine degradation studies with whole cells of the various *Azospirillum* species revealed that *A. irakense* KBC-1, *A. irakense* KA3, the ASP-1 isolate and an unidentified strain, *A.* sp. DSM 4834, exhibited significant degradation activity of desferrioxamines, whereas *A. lipoferum* Sp59b, *A. brasilense* Sp7, *A. amazonense* Y1 and A. sp. DSM 4835 failed to shown degradation activity. In the present investigation several degradation assays were used. The first method was to follow degradation of desferrioxamine B using different *Azospirillum* species during 24 h of incubation by measuring the residual desferrioxamine B by adding ferric iron and measuring complex formation at 435 nm (Figure 1). While *A. irakense* KBC 1 and strain ASP-1 showed rapid degradation with time, *A. lipoferum* and *A. brasilense* were inactive, as revealed by a constant level of non-utilized ferrioxamine B. A further screening method for the detection of desferrioxamine (B and E) degradation using microtiter plates (data not shown) revealed that of the two unidentified strains DSM 4834 and DSM 4835 only DSM 4834 showed desferrioxamine degradation, suggesting that DSM 4834 might be a close relative to the *A. irakense*. Attempts to establish a similar degradation assay based on chrome azuroleS (CAS) discoloration failed due to interference with medium constituents. Therefore, the assay based on iron complex formation and UV detection was superior when microtiter assays were performed.

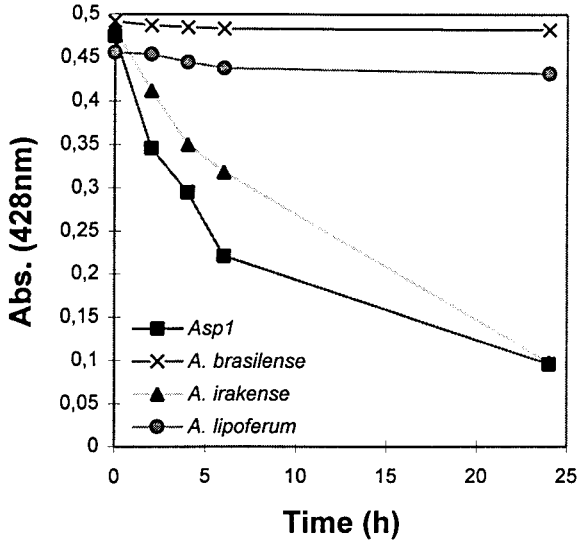

Figure 1. Degradation of desferrioxamine B by different *Azospirillum* species followed by spectrophotometric determination of residual ferrioxamine B in the culture medium after adding ferric iron.

In order to analyze the resulting degradation products of desferrioxamine B, time-dependent HPLC separation of culture supernatants was performed. Figure 2 shows a typical HPLC pattern of desferrioxamine B degradation after 0 h, 4 h and 24 h of incubation with whole cells of *A. irakense* KBC-1. Results were identical with strains ASP-1 and KA3.

The upper HPLC chromatogram (Figure 2a) shows undegraded desferrioxamine B at zero time (0 h). Degradation after 4 h (Figure 2b) and 24 h (Figure 2c) of incubation yielded several peaks which could be identified as dihydroxamates (DH_1 and DH_2), monohydroxamates (MH_1 and MH_2) and iron containing ferrioxamine (FO). The latter increased with time due to residual amounts of iron scavenged in the presence of added desferrioxamine B. The iron containing ferrioxamine B is not degraded at all. The dihydroxamates have already been identified as metabolites I and II (Winkelmann *et al.* 1996). The dihydroxamates rep-

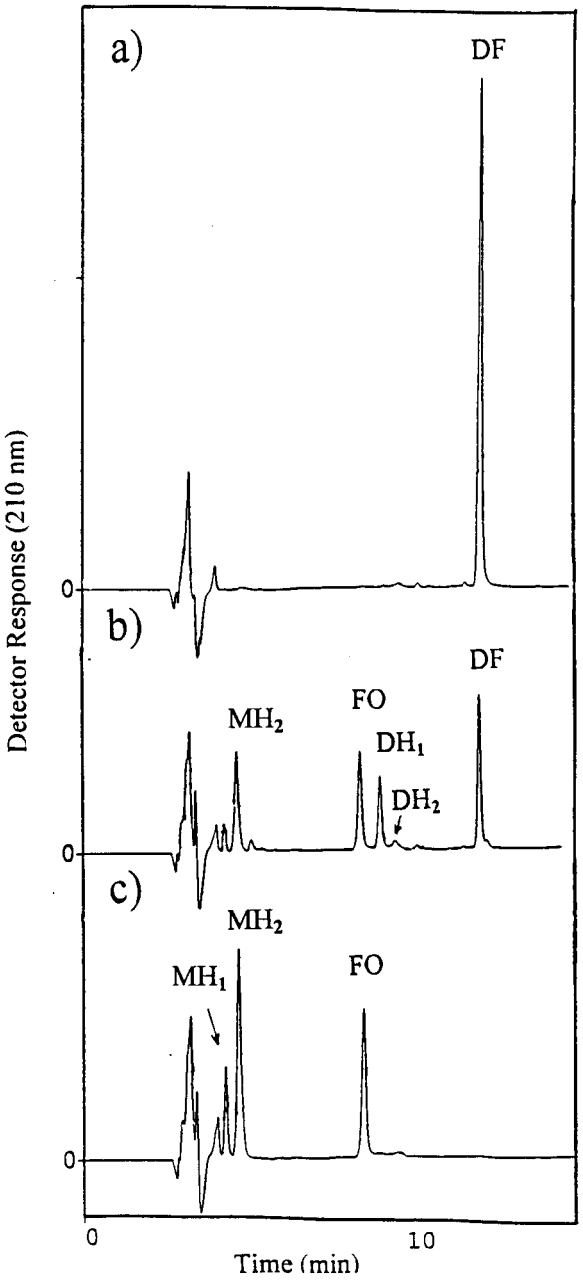

Figure 2. Degradation of desferrioxamine B by *A. irakense* KBC-1 followed by HPLC. Cells were sedimented and samples of culture supernatants were taken at intervals and measured directly by HPLC at 220 nm. (a) desferrioxamine B (DF) at sero time, (b) and (c) metabolites after 4 h and 24 h of incubation (DH_1, DH_2, MH_2 see text). FO = ferrioxamine B (ferric form).

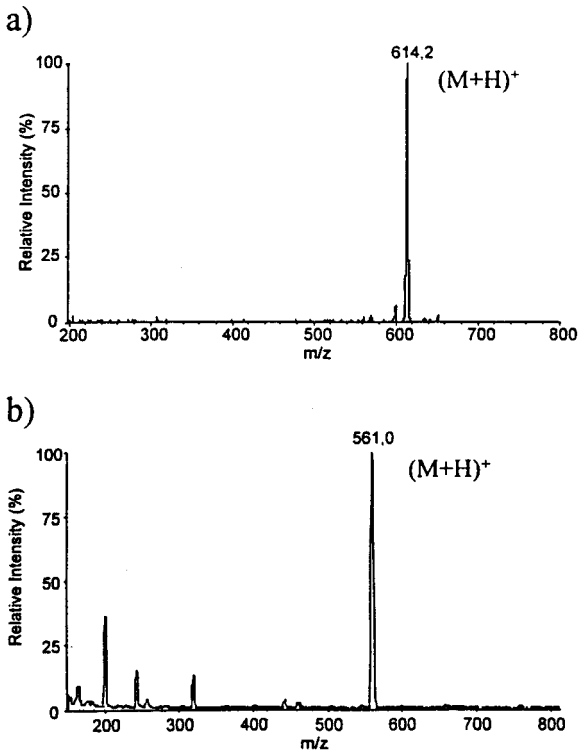

Figure 3. Electrospray mass spectra (ES-MS, positive mode) of (a) ferrioxamine B and (b) desferrioxamine B.

resent intermediate forms that are metabolized during further degradation and are no more detectable after 24 h of incubation with whole cells (Figure 2c).

Figure 3a and 3b represent electrospray mass spectra of desferrioxamine B (DF) and iron containing ferrioxamine B (FO), while Figure 4a-c show tandem mass spectra (MS/MS) of the the main metabolites during desferrioxamine B degradation from scans of the HPLC/electrospray on-line measurements. Thus, MS/MS of the parent dihydroxamate ions (MH^+ m/z 361 and m/z 419) and the monohydroxamate ion (MH^+ m/z 319) confirmed their identity. Fragmentation of the parent dihydroxamate ion (MH^+, m/z 361) yielded a daughter peak at m/z 243 which is obtained after splitting off a fragment (NH_2-$(CH_2)_5$-NOH, m/z 118) at the N-terminal hydroxamate bond. Further splitting at the C-terminal acetylhydroxamate bond (acetyl, m/z 43) yielded a daughter peak at m/z 200. A similar fragmentation (Figure 4b) was seen when the second dihydroxamate (DH_2) was fragmented into two daughter ions (m/z 300, m/z 200), resulting from splitting of the first (−118) and the second (−100) hydroxamate bond yielding fragment (CO-$(CH_2)_2$-COO^-, m/z 100). Thus, fragmentation

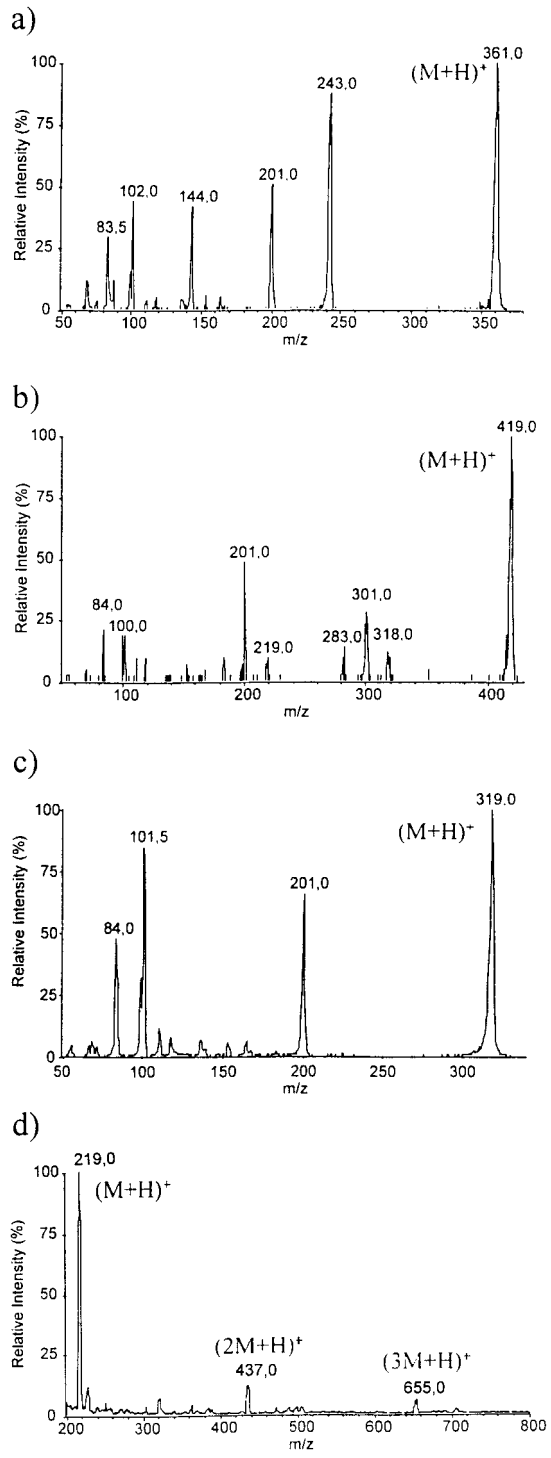

Figure 4. Electrospray mass spectra of metabolites from desferrioxamine B degradation. (a) ES-MS/MS of dihydroxamate DH_1, (b) ES-MS/MS of dihydroxamate DH_2, (c) ES-MS/MS of monohydroxamate MH_1, d) ES-MS of monohydroxamate MH_2 . Mass spectra were recorded by online HPLC/electrospray MS as described in material and methods.

of the two dihydroxamate metabolites (DH_1, DH_2) by tandem electrospray mass spectrometry gives evidence for the existence of intermediate degradation products.

We also detected two monohydroxamates (MH_1 and MH_2) which besides the ferric form (FO) accumulated after complete degradation of desferrioxamine B during 24 h of incubation (Figure 2c). These monohydroxamates are consistent with quasi molecular ions of m/z 218 and m/z 319 respectively, as shown by ion spray mass spectra in Figure 4c and 4d. While MH_1 represents a characteristic monohydroxamate metabolite obtained after splitting off the N-terminal half of DH_1, MH_2 represents a monohydroxamate metabolite resulting form splitting of the acetylhydroxamate bond of the dihydroxamate DH_1. As seen in the HPLC chromatogram after 24 h of incubation, MH_2 was the major monohydroxamate product which accumulated during further incubation (Figure 2c). A flow diagram of desferrioxamine B degradation (Figure 5) illustrates the results obtained by HPLC/electrospray mass spectrometry. Several low molecular weight products have been included in this scheme which have not been experimentally determined but which are regarded as end products that do not accumulate in the medium and are metabolized by the cells.

Figure 6 shows desferrioxamine B degradation using a purified enzyme preparation which was obtained after breaking off the cells and separating active fractions from hydrophobic chromatography and ion exchange columns (Sepharose Q). While the upper chromatogram shows complete degradation after 30 min of incubation at room temperature, the lower chromatogram shows intact desferrioxamine B in buffer solution in the absence of the enzyme preparation. Thus, the results with enzyme preparations resemble the degradation pattern obtained with whole cells after 4 h of incubation, where the dihydroxamates are still present. Besides desferrioxamine B also desferrioxamine E was utilized as a substrate by *A. irakense* strains (data not shown). However, contrary to the results obtained with linear desferrioxamine B, the cyclic desferrioxamine E did not show any accumulation of degradation products, suggesting that either rapid entrance of degradation products occured or that degradation took place after internalization. Also utilization of some ether-containing desferrioxamine derivatives (Et_2 and Et_3) was observed (data not shown) which had been isolated earlier by directed fermentation of *Streptomyces olivaceus* (Meiwes *et al.* 1990). Degradation of the two ether-containing desfer-

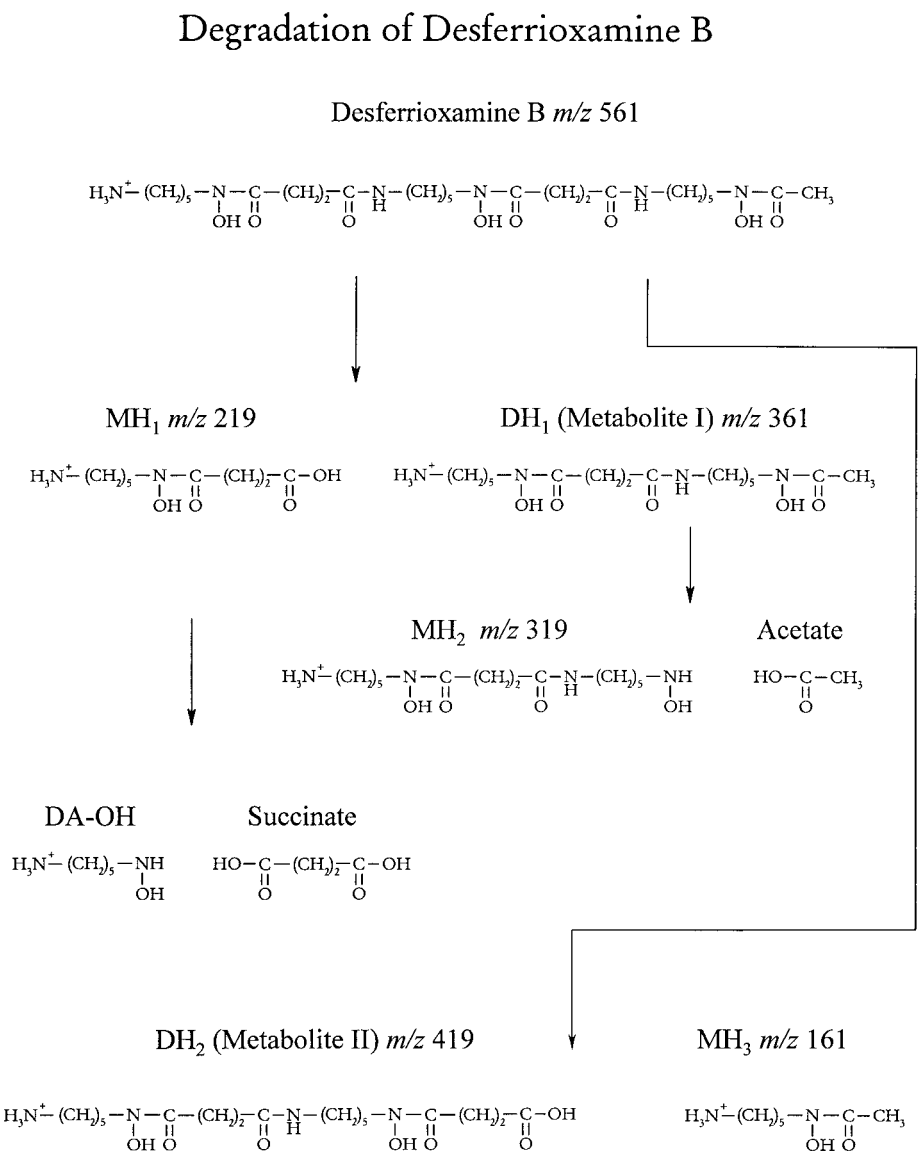

Figure 5. Scheme of desferrioxamine B degradation as revealed by HPLC and electrospray mass spectrometry.

rioxamine Et_2 and Et_3 indicated that the bulky ether groups did not prevent attack by the desferrioxamine hydrolase enzyme. This is an interesting finding which supports the idea that the desferrioxamine hydrolyzing enzymes tolerate some structural variations of the desferrioxamine backbone. Interestingly, the two fungal siderophores, desferricoprogen and desferrichrysin, were resistant to degradation (data not shown), indicating a high substrate specificity for desferrioxamines in *A. irakense*. While strains KBC-1 and KA3 were authentic *A. irakense* strains, the relationship of the isolate ASP-1 to *A. irakense* was suggested previously due to 16S rDNA homology (Winkelmann *et al.* 1996). In order to further prove its affiliation to the species *A. irakense*, we compared growth caracteristics and substrate utilization pattern using the BIOLOG GN® system (data not shown). In addition, various hybridization probes derived from 23S and 16S rDNA from other *Azospirillum* species, e.g. *A. amazonense*, *A. brasilense*, *A. lipoferum* and *A. irakense* proved the affiliation of the strain ASP-1 with *A. irakense* and also Interline PCR fingerprinting (Figure 7) was performed to corroborate the identification of ASP-1. The results (Table 1) showed that ASP-

Table 1. Characterization of *Azospirillum* sp. isolates

Analysis	KBC1	ASP-1	DSM 4834	DSM 4835
Growth in 1/2 DYGS	+	+	+	+
Growth in JNFB semisolid (no alkalization)	++	++	+/−	+/−
Growth in NFB semisolid	+++	+++	+/−	+/−
Growth in LGI semisolid	−	−	−	−
Growth on 1/2 DYGS plates	+	+	+	+
Growth on Congored medium	beige	beige	beige	beige
BIOLOG-GN (from1/2DYGS)		100% *Azospirillum irakense* KBC1	41% *Sphingomonas paucimobilis*	35% *Xanthomonas campestris*
%G+C-content	64.0	64.0	65.1	65.1
Hybridization: alfa-Proteobacteria (alf 1b, 16S)	+	+	+	+
Hybridization: *Azospirilla* (AZO 23S)	+	+	+	+
Hybridization: *A. amazonense* (AA 23S)	−	−	−	−
Hybridization: *A. brasilense* (AB 23S)	−	−	−	−
Hybridization: *A. irakense* (AI, 23S)	+	+	+/−	+/−
Hybridization: *A. irakense* (AI, 16S)	+	+	−	−
Hybridization: *A. lipopferum* (AL, 23S)	−	−	−	−
Hybridization: *Eubacteria* (EUB, 16S)	+	+	+	+
InterLINE-PCR fingerprint (see Fig. 7)		identical with KBC 1	different compared to KBC 1	different compared to KBC 1

Nitrogen-free NFB and JNFB media were use as described by Doebereiner (1995). The DYGS growth medium was prepared according to Rodrigues *et al.* (1986)

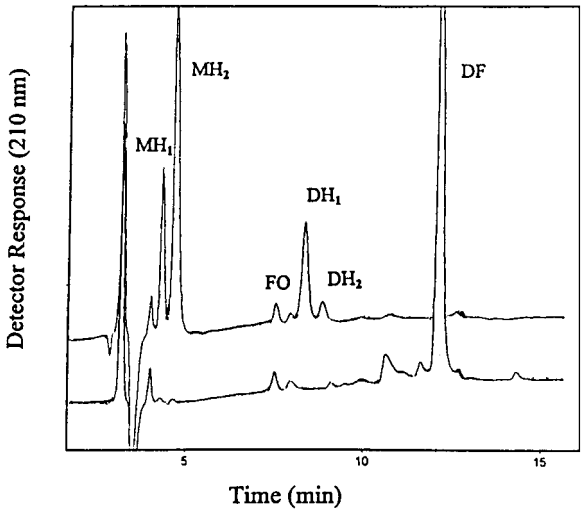

Figure 6. Desferrioxamine B degradation after 30 min using purified desferrioxamine hydrolase enzyme (upper chromatogram) and in the absence of enzyme (lower chromatogram).

1 is 100% identical to *A. irakense* (KBC-1). Although *Azospirillum* sp. DSM 4834 was different from ASP-1 and *A. irakense* in Biolog® -tests, oligonucleotide hybridization and InterLINE PCR fingerprinting, it also has the property of desferrioxamine degradation.

Discussion

The desferrioxamine hydrolyzing activity is a characteristic trait of *A. irakense* species. *Azospirillum* sp. DSM 4834, the only bacterium able to hydrolyze desferrioxamines outside the *A.irakense* species, was phylogenetically closely related (Ben Dekhil *et al.* 1997). Although *A. irakense* has similar diazotrophic properties like all other members of the genus *Azospirillum*, its degradative enzymes may be of advantage in habitats where larger amounts of desferrioxamines are produced. Desferrioxamine enrichment may occur in the vicinity of soil-inhabiting Streptomycetes which are well known producers of desferrioxamines. Also members of *Erwinia* and *Pseudomonas* are known to produce desferrioxamines (Berner *et al.* 1988; Meyer & Abdallah 1980). However, as revealed from degradation experiments, iron containing ferrioxamines are not substrates for degradation, suggesting that either the transport systems or the degradative enzymes are not able to recognize desferrioxamines after conformational change by complexation with iron. Thus, soils rich in iron may rapidly convert the desferri form to the ferric form leaving little if any desferrioxamines as substrates for *A. irakense*. On the other hand,

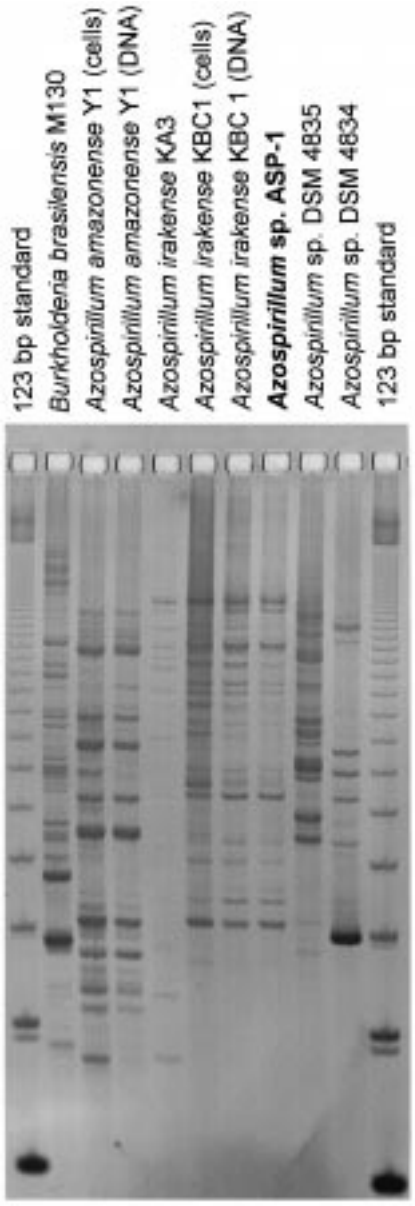

Figure 7. Inter LINE PCR of various Azospirillum strains proving the identity of *Azospirillum* ASP-1 with *A. irakense* strains.

low-iron environments may represent a rich source of desferrioxamines as substrates for *A. irakense* when iron is limiting and producing Streptomycetes are present. Possibly, a tight association of both organisms is required. Whether or not the desferrioxamines are natural or artificial substrates for *A. irakense* remains still an open question. The siderophore-mediated iron uptake in *A. irakense* has not been studied in detail. However, it has been shown for *A. brasilense*, that foreign hydroxamate-like siderophores can readily be utilized for iron uptake (Hartmann 1989; Hartmann *et al.* 1992) in addition to their own iron scavanging system (Bachhawat & Gosh 1987). Enzymatic degradation of desferrioxamines starts by splitting the N-terminal amide bond in desferrioxamines, resulting in a monohydroxamate metabolite (MH_1) and a dihydroxamate metabolite (DH_1). The existence of the former has now been confirmed by mass spectrometry. The second amide bond is also split resulting into a further dihydroxamate metabolite (DH_2), as suggested earlier (Winkelmann *et al.* 1996). Interestingly, an additional monohydroxamate metabolite (MH_2) accumulated which originated from splitting of the acetylhydroxamate bond of the accumulating dihydroxamate (DH_1). Splitting of the acetylhydroxamic bond seems to occur preferentially with the dihydroxamate metabolite (DH_1) while the same bond in the original trihydroxamate desferrioxamine B remained intact during incubation.

The fact that cell-free extracts and purified enzyme preparation yielded similar dihydroxamates as found with whole cells in short time experiments is evidence for the existence of a single desferrioxamine hydrolase that intracellularly attacks the amide bonds in desferrioxamine B. However, when cyclic desferrioxamine E or its ether-containing (3-oxa- 1,5-diaminopentane) derivatives Et_2 and Et_3were used as substrates no degradation products could be detected in the culture medium, although rapid and complete disappearance of desferrioxamines from the incubation medium was observed. This suggests that degradation of cyclic ferrioxamines occurs intracellularly without release of breakdown products. It is interesting to note that the bulky ether groups did not inhibit ferrioxamine hydrolases from attack on the amide bonds. Since desferrioxamines represent achiral nonpeptidic structures, the desferrioxamine hydrolases do not require the recognition of amino acid side chains as found in peptidases. Further studies are in progress to characterize the isolated desferrioxamine hydrolase enzymes.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Berner I, Konetschny-Rapp S, Jung G, Winkelmann G. 1988 Characterization of ferrioxamine E as the principal siderophore of *Erwinia herbicola* (*Enterobacter agglomerans*). *BioMetals* **1**, 51–56.

Bally R, Kabir M, Rahman M, Haurat J, Normand P 1996 *Azospirillum* phylogeny based on 16S rRNA sequences. In: Rahman M, ed. *Biological Nitrogen Fixation Associated with Rice Production*. Dordrecht: Kluwer Academic Publishers; 225–229.

Bachhawat SL, Gosh S. 1987 Iron transport in *Azospirillum brasilense*: Role of the siderophore spirilobactin. *J Gen Microbiol* **133**, 1759–1765.

Ben Dekhil S, Cahill M, Stackebrandt E, Sly LI. 1997 Transfer of *Conglomeromonas largomobilis* subsp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* comb. nov., and elevation of *Conglomeromonas largomobilis* subsp. *paroonensis* to the New Type Species of *Conglomeromonas*, *Conglomeromonas parooensis* sp. nov. *System Appl Microbiol* **20**, 72–77.

Castignetti D, Siddiqui AS. 1990 The catabolism and heterotrophic nitrification of the siderophore deferrioxamine B. *BioMetals* **3**, 197–203.

DeAngelis RM, Forsyth M, Castignetti D. 1993 The nutritional selectivity of a siderophore-catabolizing bacterium. *BioMetals* **6**, 234–238.

Deiss K, Hantke K, Winkelmann G. 1998 Molecular recognition of siderophores: A study wioth cloned ferrioxamine receptors (FoxA) from *Erwinia herbicola* and *Yersinia enterocolitica*. *BioMetals* **11**, 131–137.

Döbereiner J. 1992 The genera *Azospirillum* and *Herbaspirillum*. In: Ballows A, Trüper HG, Dworkin M, Harder W, Schleifer KH, eds. *The Prokaryotes*, 2nd edition, Vol. III, New York: Springer, 2236–2253.

Doebereiner J. 1996 Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. In: Alef K, Nannipieri P, eds. *Methods in Applied Soil Microbiology and Biochemistry* London: Academic Press; 134–141.

Emery T. 1976 Fungal ornithine esterases: Relationship to iron transport. *Biochemistry* **15**, 2723–2728.

Hartmann A. 1989 Ecophysiological aspects of growth and nitrogen fixation in *Azospirillum* spp. *Plant Soil* **110**, 225–238.

Hartmann A, Gündisch C, Bode W. 1992 *Azospirillum* mutants improved in iron aquisition and osmotolerance as tools for the investigation of environmental fitness traits. *Symbiosis* **13**, 271–279.

Harwani SC, Roginsky A, Vallejo Y, Castignetti D. 1997. Further characterization and proposed pathway of desferrioxamine B catabolism. *BioMetals* **10**, 205–213.

Khammas KM, Ageron E, Grimont PAD, Kaiser P 1989 *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. *Res Microbiol* **140**, 679–693.

Kirchhof G, Hartmann A. 1992 Development of gene-probes for *Azospirillum* based on 23S-rRNA sequences. *Symbiosis* **13**, 27–35.

Kirchhof G, Reis VM, Baldani JI, Eckert B, Döbereiner J, Hartmann A. 1997a Occurrence, phyiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil* **194**, 45–55.

Kirchhof G, Schloter M, Aßmus B, Hartmann A. 1997b Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. *Soil Biol Biochem* **29**, 853–862.

Leong SA, Winkelmann G 1998 Molecular biology of iron transport in fungi. In: Sigel A, Sigel H, eds, *Metal Ions in Biological Systems*, Vol. 35, *Iron Transport and Storage in Microorganisms, Plants, and Animals*. New York: Marcel Dekker, Inc.; 147–186.

Ludwig W, Dorn S, Springer N, Kirchhof G, Schleifer K-H. 1994 PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. *Appl Environ Microbiol* **60**, 3236–3244.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG 1998 Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**, 795–799.

Meyer JM, Abdallah MA. 1980 The siderochromes of non-fluorescent pseudomonads: production of nocardamine by *Pseudomonas stutzeri*. *J Gen Microbiol* **118**, 125–129.

Owen RJ, Lapage SP 1976 The thermal denaturation of partly purified bacterial desoxyribonucleic acid and ist taxonomic applications. *J Appl Bacteriol* **41**, 335–340.

Powell PE, Szaniszlo PJ, Reid CP. 1993 Confirmation of occurence of hydroxamate siderophores in soil by a novel *Escherichia coli bioassay*. *Appl Environ Microbiol* **46**, 1080–1083.

Rodrigues NJ, Malavolta Va, Victot O. 1986 Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. citri tipo B. *Suma Phytopathologica*, São Paulo, Vol. 12, 1986.

Smida J, Leibhard S, Nickel AM, Eckardt-Schupp F, Hieber L. 1996. Application of repetitive sequence-based PCR (Inter LINE PCR) for the analysis of genomic rearrangements and for the genome characterization on different taxonomic levels. *Gen Anal Biomol Eng* **13**, 95–98.

Stager CE, Davis JR 1992 Automated system for identification of microorganisms. *Clin Microbiol Rev* **5**, 302–327.

Villavicencio M, Neilands JB. 1965 An inducible ferrichrome A-degrading peptidase from pseudomonas FC-1. *Biochemistry* **4**, 1092–1097.

Warren RAJ, Neilands JB. 1964 Microbial degradation of the ferrichrome compounds. *J Gen Microbiol* **35**, 459–470.

Warren RAJ, Neilands JB. 1965 Mechanism of microbial catabolism of ferrichrome A. *J Biol Chem* **240**, 2055–2058.

Winkelmann G, Schmidtkunz K, Rainey F. 1996 Characterization of a novel *Spirillum*-like bacterium that degrades ferrioxamine-type siderophores. *BioMetals* **9**, 78–83

Xia Y, Embley TM, O'Donnell AG. 1994 Phylogenetic analysis of *Azospirillum* by direct sequencing of PCR amplified 16SrDNA. *System Appl Microbiol* **17**, 197–201

Zaya N, Roginsky A, Williams J, Castignetti D. 1998 Evidence that a desferrioxamine B degrading enzyme is a serine protease. *Can J Microbiol* **44**, 1–7.