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Characterization of a novel *Spirillum*-like bacterium that degrades ferrioxamine-type siderophores

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A novel Gram-negative *Spirillum*-like bacterium (ASP-1) was isolated from lake water by enrichment culture on desferrioxamine B as sole source of carbon and energy. ASP-1 was able to degrade the siderophores desferrioxamine B and E. The property of siderophore degradation was inducible in the presence of desferrioxamine B. The ferric complexes, however, were not measurably degraded but served as an iron source. Degradation of desferrioxamines in culture was followed by measuring the residual ferrioxamines colorimetrically at 430 nm after addition of iron. Degradation in cell-free assays was followed quantitatively by HPLC on a reversed-phase column measuring the time-dependent disappearance of the desferrioxamines B and E. Cell-free assays also revealed that degradation of the cyclic desferrioxamine E was rapid and complete, whereas degradation of the linear desferrioxamine B yielded two intermediate iron-binding metabolites of shorter chain length. Preparative isolation by HPLC and mass spectrometric analysis of the metabolites revealed masses at 361 and 419 a.m.u., respectively, suggesting a splitting at the two amide bonds. ASP-1 is a nitrogen fixing *Spirillum* bacterium which could also use ammonium and glucose or several organic acids as a carbon source but grew poorly with amino acids. Physiological comparisons with *Aquaspirillum* and *Azospirillum* failed to assign ASP-1 to any of the presently known *Spirillum* species. Based on 16S rDNA sequence analysis the strain could be placed within the radiation of the *Azospirillum/Rhodocista* group. The closest relative was *Azospirillum irakense*, showing 98.8% similarity.

Keywords: degradation, ferrioxamines, siderophores

Introduction

Earlier reports on the occurrence of siderophore degrading bacteria have shown that strains of *Pseudomonas* were able to split hexapeptide siderophores of the ferrichrome family (Warren & Neilands 1964, 1965). *Pseudomonas* FC-1 was isolated from soil by enrichment culture on ferrichrome A as sole source of carbon and nitrogen. Moreover, it was shown that an inducible peptidase was able to cleave the hexapeptide ring in ferrichrome A at the acyl serine bond. The enzyme was isolated and partially characterized (Villavicencio & Neilands 1964). Recently Castignetti and coworkers resumed work on microbial degradation of siderophores by isolating a novel Gram-negative soil isolate, DFBC5, capable of growing on desferrioxamine B as the sole carbon source (Castignetti & Siddiqui 1990). Cell-free extracts from DFBC5 converted desferrioxamine B into monohydroxamates, a reaction that could be inhibited by peptidase inhibitors. Several microbial proteases were inactive, suggesting that desferrioxamine degrading enzymes may differ from known proteases. Further studies on the nutritional selectivity of this siderophore catabolizing bacterium revealed that the ferric form, ferrioxamine B, did not serve as an iron source, while ferric rhodotorulate was capable of delivering iron to the cells but the ligand was not degradable (DeAngelis *et al.* 1993). In the present investigation we report on a novel *Spirillum*-like bacterium, ASP-1, which was able to grow using the iron-free forms of the linear ferrioxamine B and the cyclic ferrioxamine E. ASP-1 was isolated from lake water by enrichment culture on desferrioxamine B and turned out to induce a novel cell-bound desferrioxamine degrading enzyme. Physiological, chemotaxonomic and genetic studies have so far failed to identify the isolated *Spirillum*-like bacterium. According to the currently known procaryotic phylogenetic tree (Olsen *et al.* 1994), the organism can be placed in the α-1 subdivision within the *Azospirillum/Rhodocista* group.

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Materials and methods

Siderophores

Desferrioxamine B was used as the methane sulphonate salt (Desferal®) obtained from Ciba Geigy (Basel, Switzerland). Ferrioxamine E was kindly provided by H. Zähner (Microbiology, Universität Tübingen).

Enrichment, isolation and identification of the bacterium

ASP-1 was isolated from lake water by enrichment culture on desferrioxamine B as sole source of carbon in minimal medium. The minimal medium for enrichment contained per litre: $K_2HPO_4 \cdot 3H_2O$ (10.6 g), $NaH_2PO_4 \cdot 1H_2O$ (6.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $(NH_4)SO_4$ (2.0 g), $Ca(NO_3) \cdot 4H_2O$ (10 mg), pH 7. Lake water (200 µl) was added to minimal medium (20 ml) containing 150–220 µM desferrioxamine B and incubated on a rotary shaker at 27°C for several days. Degradation was then followed photometrically at 430 nm and 200 µl of the culture was transferred into fresh medium prior to complete degradation. Transfer into fresh medium was repeated five times and the residual culture was finally plated on agar medium to select single colonies. From these colonies an isolate named ASP-1 was selected which showed inducible degradation of desferrioxamines. Identification was performed using the API 20 NE test system (Bio-Merieux, Nürtingen, Germany) in addition to various other carbon and nitrogen sources. Gram-reaction, catalase, oxidase and OF test were determined according to common microbiological methods. Isolation of genomic DNA, amplification of 16S rDNA and direct sequencing of PCR products were performed at the Deutsche Sammlung für Mikroorganismen according to procedures described previously (Rainey *et al.* 1992). The 16S rDNA sequence of ASP-1 was aligned with sequences of members of the genus *Azospirillum*, *Rhodospeudomonas*, *Rhodospirillum* and related taxa. The phylogenetic tree was reconstructed from the distance matrix by the neighbour-joining method (Saitou & Nei, 1987). The EMBL accession number of the 16S rDNA sequence of the strain ASP-1 is ×92464.

Measurement of degradation from culture samples

Degradation was followed in minimal medium (20 ml) containing 150–500 µM desferrioxamine B as sole carbon source on a rotary shaker at 27°C. Samples (1.2 ml) were taken at intervals and the cells were sedimented by centrifugation. Ferric chloride (10 µl, 100 mM) was added to the supernatant (1 ml), mixed and the decrease of absorbance was measured at 430 nm after incubation for 30 min. Degradation was calculated from an uninoculated control and a ferrioxamine B standard.

Preparation of cell-free extracts

Cell-free extracts were prepared from cells grown in 2 l minimal medium containing 0.4% glucose on a rotary shaker at 27°C until an OD_{546} of 0.4 was reached. Then desferrioxamine B (1 ml, 20 mM) was added for induction and incubated further until an OD of 0.8. Cells were harvested by centrifucation, resuspended in MOPS buffer (100 mM, pH 7), and washed two times in the same buffer and stored at −20°C. Cell-free extracts were obtained with an X-Press (HAWE) and the cell debris was removed by repeated centrifugation. The supernatant served as a cell-free extract for degradation studies.

Bioassays and enzyme assays

Bioassays to determine the preferred carbon source were performed on agar plates containing minimal medium without glucose plus 0.4% agar. Substrates (10 µl, 3% solutions) used as carbon sources were pipetted on filter discs (6 mm diameter), sterilized and dried in a microwave oven for 1 min, and subsequently laid on the agar surface and incubated for 3 days at 27°C. Bioassays to determine the growth promotion activity of siderophores were performed according to Thieken & Winkelmann (1993) with the modification that *Spirillum* medium plus 0.4% agar and 300 µM bipyridyl was used. The *Spirillum* medium contained per litre: peptone 5.0 g, Na-succinate 1.0 g, $(NH_4)_2SO_2$ (1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $MnSO_4 \cdot H_2O$ 2 mg, pH 7. Siderophores (10 µl, 0.1%, 0.0%, 0.001%), pipetted on filter discs, dried and sterilized in a microwave oven for 1 min, were used for growth promotion tests and the growth halos were read after several days of incubation at 27°C.

HPLC separation of ferrioxamines

Samples of standard solutions of ferrioxamine B and E as well as samples taken from the degradation assays were separated by HPLC on a reversed-phase column (C18 nucleosil, 5 µm, 250 × 4.6 mm; Grom, Herrenberg, Germany) using a flow rate of 1.0 ml min^{-1} and a gradient of 6–40% acetonitrile/water in 19.2 min (LC-9A pumps, equipped with an integrator C-R4AX, gradient controller SCL-6B and automatic sampler SIL-6B; Shimadzu, Duisburg, Germany). Detector wavelength was set at 430 nm. Semipreparative isolation of metabolites was performed on a column (C18 nucleosil, 5 µm, 250 × 8 mm; Grom) using a flow rate of 1.6 ml. The separated peaks were collected in a fraction collector FRCA-10A with system controller SCL-10A.

Enzyme isolation and purification

Cell-free extracts diluted with MOPS buffer (100 mM, pH 7) were separated by gel filtration on a Sephadex G100 column and also by FPLC (Pharmacia, Freiburg, Germany) on a hightrap Q column using a gradient of 1–100% NaCl (1 M). Fractions with the highest desferrioxamine B degrading activity were analysed further by SDS-PAGE (12%) according to the Laemmli procedure by silver staining to check the purity of the isolated proteins.

Mass spectrometry

Samples were analyzed by electrospray mass spectrometry on a Sciex API triple-quadrupole mass spectrometer with a

2400 Da mass range equipped with an ion spray source (Sciex, Toronto, Canada). Aqueous solutions containing 0.1% formic acid were introduced with a microlitre syringe (infusion pump) at a flow rate of 5 μl min^{-1}.

Electron micrographs

Cells were grown for 24 h in Luria-Bertini broth, sedimented and washed two times with phosphate buffered saline. Fixation of cells was performed by resuspending in PBS containing 2.5% glutaraldehyde.

Results and discussion

The isolate, ASP-1, is a Gram-negative, *Spirillum*-like bacterium with one polar flagellum (Figure 1). Older cultures show several intracellular coccoid bodies. Cells grow under aerobic and microaerophilic conditions, and are catalase and oxidase positive. Although ammonium ions can serve as nitrogen source, ASP-1 is able to fix nitrogen in a nitrogen-deficient medium under microaerophilic conditions. Growth factors are not required. Starch was hydrolysed; however, gelatine was not. Amino acids were generally not well utilized, whereas most sugars and several organic acids, like succinate, malate, pyruvate and ketoglutarate, were good substrates. A comparison with the substrate profiles of *Aquaspirillum* and *Azospirillum* species revealed no accordance. Therefore ASP-1 could physiologically not be assigned to the known genera of *Aquaspirillum* and *Azospirillum*. Analysis of the quinones revealed Q-9, indicative of membership of either the α- or γ-subclasses of the Proteobacteria. Polar lipids and fatty acid composition is typical of a certain group within the α-subclass of the Proteobacteria. Due to the fact that insufficient data is available on the chemical composition of the rest of this group, it is not possible to place ASP-1 in any known taxa. Although 16S rRNA sequencing showed 98.8% homology with a recently described strain of *Azospirillum irakense* (Fani *et al.* 1995), the sequencing data would suggest that the present isolate is probably distinct, both on the basis of 16S rRNA and chemical composition. A phylogenetic dendrogram based on 16S rDNA sequence comparison showing the position of ASP-1 is shown in Figure 2.

Ferrioxamine degradation is regarded as a rare event among common bacterial groups and there is so far only one report in the literature where a ferrioxamine degrading bacterium has been analysed in detail (Castignetti & Siddiqui 1990). A culture of ASP-1 (20 ml) was able to rapidly degrade desferrioxamine B (250 μM) within 30 h as shown in Figure 3. The disappearance of desferroxamine B from the culture medium was measured at 430 nm from samples taken at intervals after complex formation by addition of ferric chloride. A similar degradation was observed with desferrioxamine E (not shown). Concomitant with the degradation of desferrioxamines the OD_{546} of the culture increased to about 0.15. Thus desferrioxamines are degraded to serve as a carbon source for this organism. Utilization of desferrioxamines could also be visualized by using growth promotion assays on Petri dishes (not shown), similar to the growth promotion assays used for iron nutrition (Thieken & Winkelmann 1993). Filter discs soaked with desferrioxamine B and E were placed on inoculated agar medium where glucose was omitted as a carbon source.

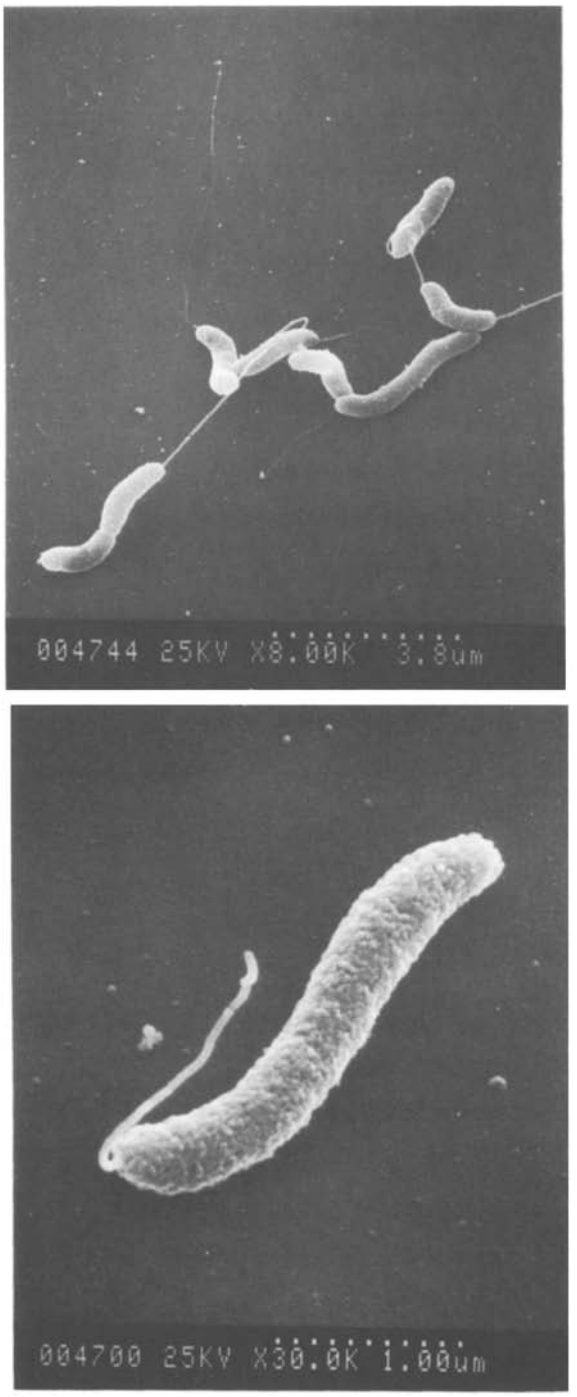

Figure 1. Electron micrographs of ASP-1 cells, showing the Spirillum-like shape and the polar flagella. The scale bar is 3.5 μm (upper) and 1 μm (lower).

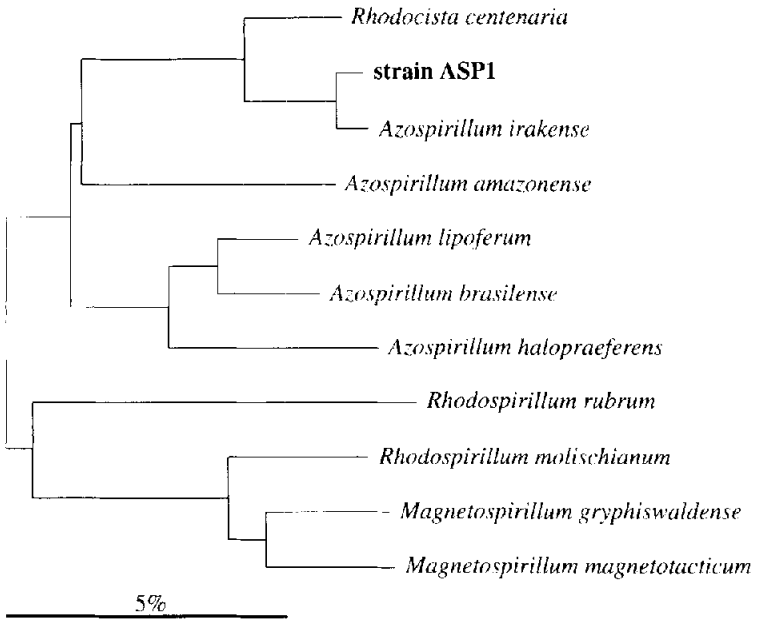

Figure 2. Phylogenetic dendrogram based on 16S rDNA sequence comparison indicating the phylogenetic position of strain ASP-1. The scale bar represents five nucleotide substitutions per 100 nucleotides.

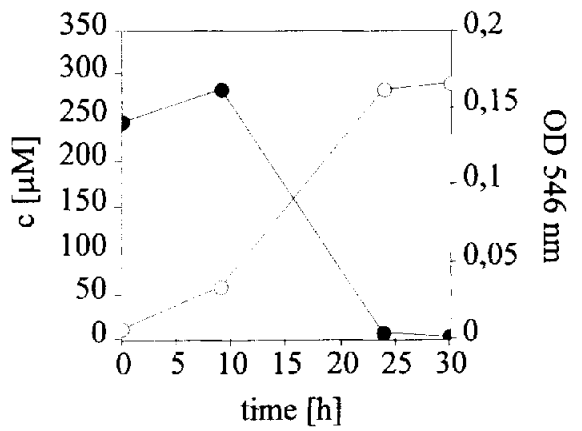

Figure 3. Growth (○) and time-dependent degradation (●) of desferrioxamine B by a culture of ASP-1 in minimal medium. Degradation of desferrioxamine B was measured colorimetrically at 430 nm from samples after addition of iron as described in Materials and methods.

Faint growth halos indicated utilization of desferrioxamines as a carbon source. Glucose and malate served as a control. To demonstrate cell-free degradation of desferrioxamines, cells grown in desferrioxamine containing media were crushed with an X-press and the cell-free supernatant was mixed with desferrioxamine (0.5 μmol ml^{-1}) and incubated at 27°C. Samples were taken at intervals and separated by HPLC at 430 nm detector wavelength after addition of iron to form the ferrioxamine complex. As shown in Figure 4, the ferrioxamine B peak at 12.3 min was reduced to nearly zero after 2 h of incubation and the intermediate appearance of two peaks (I and II) was observed. Degradation of desferrioxamine E under the same conditions was even more rapid and was complete after 1 h of incubation. Unlike to desferrioxamine B, no metabolites could be detected during desferrioxamine E degradation. The two major peaks appearing during desferrioxamine B degradation were collected by semipreparative HPLC and analysed by electrospray mass spectroscopy, yielding ion peaks at $m/z = 361.5$ for metabolite I and $m/z = 419.5$ plus $m/z = 441.5$ for metabolite II, corresponding to the MH^+ and MNa^+ ions, respectively (Figure 5). These two metabolites were consistent with a hydrolytic cleavage of the two amide bonds in desferrioxamine B as shown in the structural formula of Figure 6. Thus an amidase-like enzyme is acting yielding two different dihydroxamates which can be detected in their iron complex forms during HPLC. Smaller monohydroxamate fragments, where both amide bonds are cleaved, could not be detected. Monohydroxamates may be more difficult to isolate and separate on HPLC or may even be metabolized faster. The isolation of metabolites of desferrioxamine B from serum has been previously shown in desferrioxamine B treated patients, and suggested the involvement of transamination and oxidation reactions (Singh *et al.* 1990). The present investigation shows that only hydrolytic cleavage products could be observed during microbial degradation. Why no intermediary metabolites could be found during degradation of desferrioxamine E is unclear, but may be due to a more rapid metabolization. Gel filtration and FPLC separation of cell-free extracts yielded protein fractions which still contained several protein bands when analysed by SDS gel electrophoresis (data not shown). L-alanine aminopeptidase, L-leucine aminopeptidase and dipeptidyl peptidase activity increased, concomitant with the purification of the ferrioxamine degrading enzyme activities, suggesting that the partially purified enzyme has

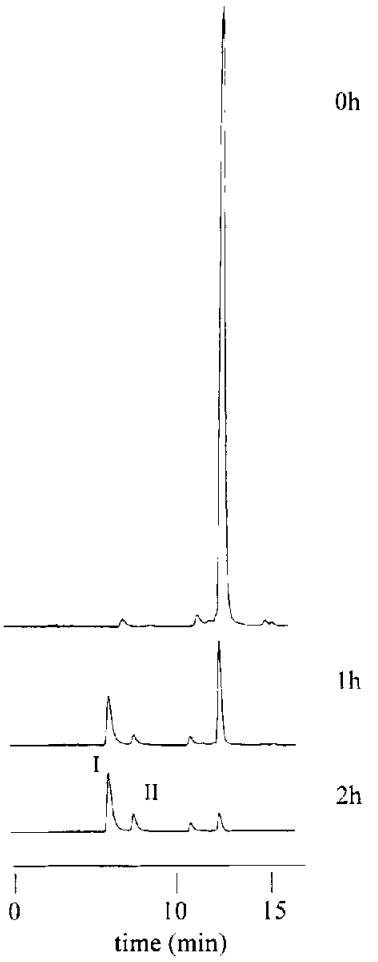

Figure 4. Degradation of desferrioxamine B by cell-free extracts of ASP-1 followed by HPLC. Samples (1 ml) were taken at 0, 1 and 2 h, heated (10 min, 100°C), centrifuged and iron (10 μl $FeCl_3$, 100 mM) was added to allow complex formation. The clear supernatant was then separated by HPLC on a C18 reversed-phase column using a gradient of acetonitrile/water and a detector wavelength at 430 nm as described in Materials and methods. Retention times of HPLC peaks were as follows: metabolite I (8.34 min), metabolite II (9.63 min) and ferrioxamine B (12.4 min).

similar activities or might still be contaminated by other enzymes.

Most natural siderophores are resistant to degradation by virtue of being either cyclic peptides like the ferrichromes or by being non-peptidic like the ferrioxamines. As the function of siderophores is primarily to scavenge iron in an extracellular environment, degradation seems to be a rare event. However, as shown in the present investigation, enrichment cultures allowed the isolation of a bacterium with high degradation activities towards ferrioxamine-type siderophores. The ASP-1 isolate degrades both, linear and cyclic desferrioxamines, by using them as sole carbon source. Although the ferric forms seem not to be degradable by ASP-1, they also served as an iron source. It may be assumed that ASP-1 produces its own siderophore. The fact that the iron-free forms of siderophores are degraded after uptake is not a unusual feature. For example, after uptake of ferric enterobactin via the fepA receptor in *Escherichia coli* and subsequent iron removal, enterobactin is degraded into mono-, di- and linear trimers (Winkelmann *et al.* 1994). However, in this case the degradation products are finally excreted again and are not metabolized. Contrary to the iron-containing siderophores, the iron-free

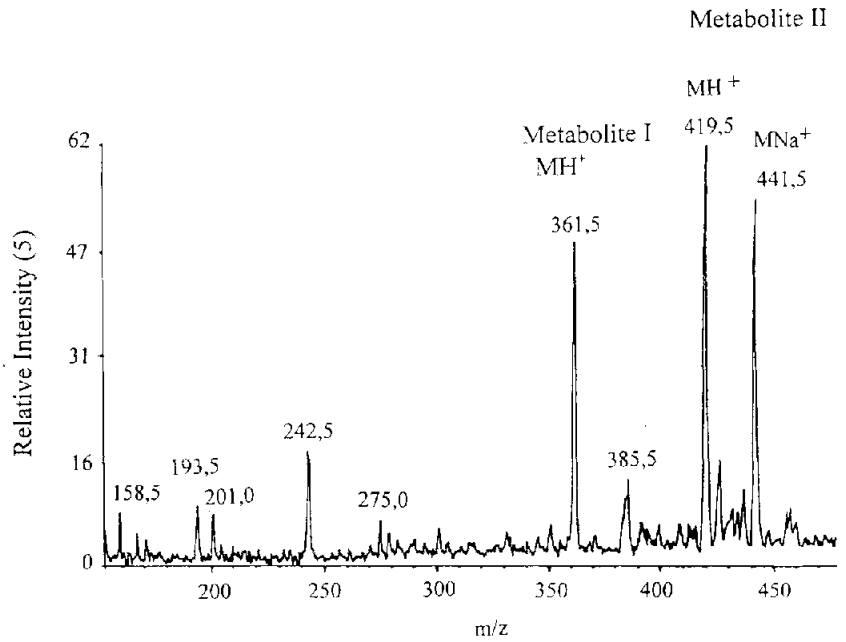

Figure 5. Electrospray mass spectrum of the metabolites (mixture of I and II) isolated by preparative HPLC.

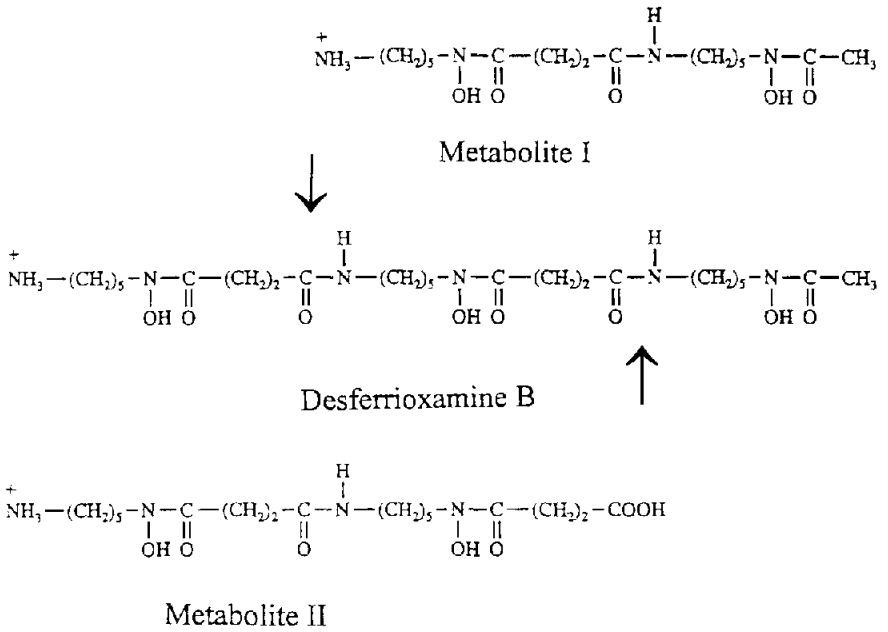

Figure 6. Structural formula of desferrioxamine B showing the positions of hydrolytic attack (arrows) at the amide bonds resulting in two different dihydroxamate metabolites I and II.

desferrisiderophores have never been shown to be taken up in large amounts. Although a simultaneous function as an iron donor and as a carbon source cannot be excluded in the case of the ASP-1, degradation of exogenously supplied desferrioxamines seems to be an independent route developed for utilization of certain carbon sources of which the desferrioxamines are only one possible candidate. Moreover, increased degradation of exogenously added desferrioxamines as a carbon source requires prior induction of degrading enzymes in the presence of desferrioxamines. Degradation activities could not be detected in the culture fluids but were always associated with the cells. The desferrioxamine degrading enzyme(s) could be released by crushing the cells with an X-press, enabling degradation studies in cell-free extracts. In conclusion, the present paper gives a physiological and taxonomical description of a novel desferrioxamine degrading bacterium ASP-1 and reports on the cell-free activity of siderophore degradation. Further studies are under way to characterize the novel desferrioxamine degrading enzyme.

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