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The nutritional selectivity of a siderophore-catabolizing bacterium*

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The ability of a siderophore-catabolizing bacterium to assimilate ferric ion was examined. While the bacterium utilizes the siderophore deferrioxamine B (DFB) as a carbon source, it was incapable of using the ferric ion analogue (ferrioxamine B) as an iron source. It did, however, assimilate the ferric ion of the chelator ferric nitrilotriacetic acid and of the siderophore ferrirhodotorulic acid (ferriRA). Neither ferriRA nor its deferrated analog (RA), however, were capable of functioning as carbon sources for the bacterium. The microbe thus employs a 'nutritional selectivity' with respect to these two siderophores. That is, it does not use the siderophore it employs as a carbon source (DFB) as an iron source nor does the siderophore utilized as an iron source, i.e. ferriRA, nor its deferrated analog (RA), serve as carbon sources for the organism.

Keywords: deferrioxamine B, nutritional selectivity, rhodotorulic acid, siderophore catabolism

Introduction

Siderophores are avid ferric ion chelating molecules synthesized by a wide variety of microbes, including many genera common to terrestrial environments (Powell *et al.* 1980, Emery 1982, Castignetti & Smarrelli 1986, Neilands & Leong 1986, Fekete *et al.* 1989, Mielczarek *et al.* 1989). Little attention, however, has been focused on how siderophores are recycled into the carbon cycle. Only two microbial isolates, one a soil pseudomonad which metabolized ferrichrome A and ferrichrome (Warren & Neilands 1964, Villavicencio & Neilands 1965, Warren & Neilands 1965), and the other a Gram-negative rod of uncertain taxonomic identity which catabolized deferrioxamine B (DFB) (Castignetti & Siddiqui 1990), have been noted to efficiently use siderophores as sole sources of carbon for growth.

The latter bacterium, which was given the trivial name DFBC 5 (deferrioxamine B catabolizer 5), is an obligate aerobe and grew in a medium where the concentration of the deferrated siderophore, DFB, greatly exceeded that of the ferrated analog, i.e. ferrioxamine B (FB). The molar ratio of DFB/FB in the growth medium was approximately 400/1 (Castignetti & Siddiqui 1990). While DFB functions as a carbon source for DFBC 5, FB cannot be similarly used by the bacterium nor can it be metabolized by either resting cells or cell-free extracts of the organism grown with DFB as the sole carbon source (Castignetti & Siddiqui 1990). A pertinent question, therefore, is how does DFBC 5 obtain the iron required for its growth?

Although not applicable to its growth in the DFB-containing medium, the organism may mimic other procaryotes in that it may use the ferrisiderophore of another microbe as an iron source (Neilands 1982, Neilands & Leong 1986). When growing in the DFB medium, the microbe may solve its nutritional iron requirements by utilizing FB or perhaps by synthesizing its own siderophore which would then compete for the available Fe^{3+} with DFB. The current study was conducted to determine if DFBC 5 utilizes FB as an iron source and thus to ascertain whether the same siderophore (or its deferrated analog) functions as both a source of iron and carbon to the bacterium.

*This paper is dedicated to the memory of Professor Thomas Emery. Professor Emery was instrumental in giving support and advice at a time when such mentorship greatly aided the corresponding author in developing a program concerning the catabolism of siderophores by microbes.

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Materials and methods

Bacterium and growth conditions

DFBC 5 is a soil isolate. Its ability to utilize DFB as a carbon source and the growth conditions of the bacterium when it was cultured as a DFB catabolizer have been previously described (Castignetti & Siddiqui 1990). Experiments to determine if DFBC could grow with either rhodotorulic acid (RA) or its ferrated analog (ferriRA) used cells grown in DFB–mineral salts–vitamins medium as inocula (1% v/v of a mid-logarithmic culture) and were conducted in the vitamins–mineral salts medium as described (Castignetti & Siddiqui 1990) except that the siderophore RA or ferriRA was substituted for DFB. In some experiments, 0.05% yeast extract (YE) (Difco, Detroit, MI) was added to the RA– or ferriRA–mineral salts–vitamin media to insure the growth of DFBC 5. RA was dissolved in water, filter sterilized and added to the mineral salts–vitamins or mineral salts–vitamins–YE media to a final concentration of 2.5–3.0 mM. For those experiments where ferriRA was the carbon source, ferriRA was prepared by adding an aliquot of 0.2 M $FeCl_3$–0.05 M HCl to a RA solution to yield a 95 mol% mixture. The ferriRA was vigorously stirred and then filtered through 0.8 μm and then 0.45 μm filters, and the sterilized ferriRA was then added to the mineral salts–vitamins medium to yield a final concentration of 2.5–3.0 mM ferriRA. As an indication of whether DFBC 5 could synthesize its own siderophore, the organism was inoculated from a DFB–mineral salts–vitamins plate onto a CAS agar plate (Schwyn & Neilands 1987).

Chemicals

DFB was a gift of the Ciba-Geigy Corporation (Suffern, NY) and was obtained as the mesylate salt. Rhodotorulic acid was isolated as described (Atkin & Neilands 1968, Atkin *et al.* 1970) from a culture of *Rhodotorula pilimanae* UCD 67–64 which was a gift from J. B. Neilands. Verification of the isolated compound as authentic RA was performed via melting point analysis and visible spectrum characteristics as described by Atkin & Neilands (1968). Sodium nitrilotriacetic acid (NTA), disodium salt, was obtained from Aldrich (Milwaukee, WI). ^{55}Fe was obtained as $^{55}FeCl_3$–5 N HCl from New England Nuclear (Boston, MA) and had a specific activity of 44 mCi mg^{-1}. The isotope was stored as a 200 μM solution of ^{55}Fe–0.1 N HCl as suggested by Rosenberg (1979). All other chemicals were reagent grade or better.

Assays

Cells were collected from mid-logarithmic cultures by centrifugation, washed twice in the uptake medium described by Rosenberg (1979) and resuspended in the uptake medium to yield final cell densities of 0.21–0.61 mg cell protein ml^{-1}. To insure that Fe^{3+} had been removed from the uptake medium, it had been passed through a Chelex 100 (Bio-Rad Laboratories, Richmond, CA) column which was prepared as described (Davey *et al.* 1970). Uptake assays were performed as described by Rosenberg (1979) except that the membrane filters were suction dried before being placed into scintillation vials containing the water miscible scintillation fluid Scinti-Verse Bio-HP (Fisher Scientific, Itaska, IL). At least two experiments were performed for each of the chelating agents studied, with two individual samples taken at each of the specific time intervals. Magnitude of uptake was based on the average of the replicates, with counts determined relative to an internal standard performed in each experimental run (Rosenberg 1979). Controls consisted of uptake experiments in which the organisms were maintained at 0 °C or where the cells had been previously incubated (30 min at room temperature with gentle agitation) with 5 mM sodium azide (NaN_3). This concentration of azide was chosen as it was sufficient to inhibit by 86% the metabolism of DFB by resting cells of the bacterium. These experiments were conducted as described by Castignetti *et al.* (1985) and Castignetti & Siddiqui (1990) except that cell suspensions without NaN_3 served as the control while cell suspensions with varying concentrations of NaN_3 were the experimental groups.

In either the isolation procedures for the RA or in the growth experiments which employed RA and ferriRA as sole carbon sources, determination of RA or ferriRA concentrations was performed according to the assay of Leong & Neilands (1982) and as described by Castignetti & Siddiqui (1990) except that RA and ferriRA were substituted for DFB and FB. Deferration of glassware was performed when necessary by use of the method of Lodge & Emery (1984) which employs EDTA as the iron chelator. The use of Chelex 100 to remove Fe^{3+} from solutions and buffers was as described by Davey *et al.* (1970).

Results

DFBC 5 grew very poorly on the CAS plates of Schwyn & Neilands, requiring an inordinate amount of time (about 3 weeks) to form colonies and displaying a very small orange halo around the colony. As DFBC 5 grows readily in the DFB–mineral salts medium described by Castignetti & Siddiqui (1990) and must thus satisfy its iron requirements within the few days of its growth, experiments were initiated to determine if the bacterium could use FB as a source of iron.

NTA is used as a stabilizing agent in the iron assimilation procedure of Rosenberg (1979). As part of the controls of the ^{55}Fe uptake experiments, DFBC 5 was given [^{55}Fe]ferriNTA as a Fe^{3+} source in the absence of any other chelator (Figure 1). [^{55}Fe]FB was also supplied the bacterium with NTA as the stabilizing agent (Figure 1). DFBC 5 assimilated the Fe^{3+} of ferriNTA displaying a rate of 2.2

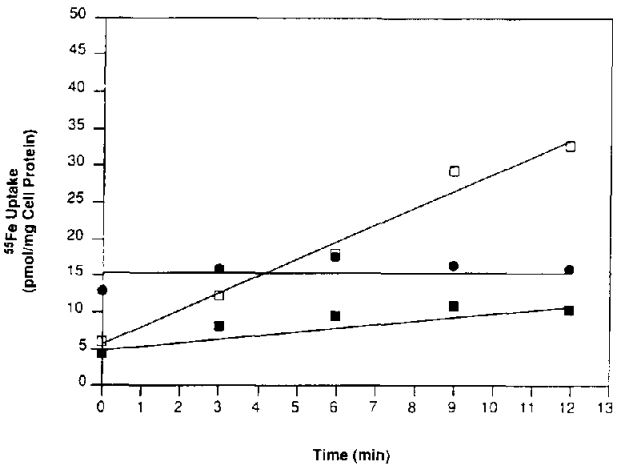

Figure 1. Rate of uptake of ^{55}Fe from [^{55}Fe]ferriNTA (□) and [^{55}Fe]FB (●) by DFBC 5. DFBC 5 cells used as controls for [^{55}Fe]ferriNTA assimilation (■) were at 0 C. All others were at 21°C. The bacterium was grown in DFB–vitamins–mineral salts medium described in Materials and methods and cells were present at a concentration of 0.3 mg cell protein ml^{-1}. [^{55}Fe]ferriNTA was present at a concentration of 1 μM, NTA was at a concentration of 499 μM. During the [^{55}Fe]FB assimilation experiment, [^{55}Fe]FB was at a concentration of 1 μM as was DFB.

pmol [^{55}Fe]ferriNTA min^{-1} mg cell protein^{-1} while displaying no inability to use the Fe^{3+} of FB.

While DFBC 5 demonstrated an ability to use FB as a Fe^{3+} source, the bacterium readily used rhodotorulic acid an iron source (Figure 2). With

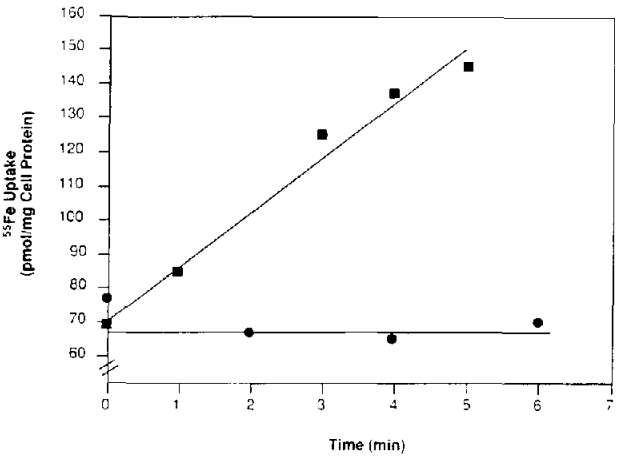

Figure 2. Rate of uptake of ^{55}Fe from [^{55}Fe]ferriRA (■). DFBC 5 was grown in DFB–vitamins–mineral salts medium described in Materials and methods and cells were present at a concentration of 0.26 mg cell protein ml^{-1}. DFBC 5 cells used as controls for [^{55}Fe]ferriRA assimilation (circles) had 5 mM sodium azide present. The assays were performed at 21 °C and the concentrations of [^{55}Fe]ferriRA and RA were each 5 μM.

5 μM [^{55}Fe]ferriRA as the substrate, DFBC 5 assimilated the Fe^{3+} of ferriRA at a rate of 15 pmol min^{-1} mg cell protein^{-1}. The apparent K_m and V_{max} values for ferriRA assimilation for DFBC 5 are 4.3 μM and 27.8 pmol min^{-1} mg cell protein^{-1} which were calculated from the Lineweaver–Burk plot of [^{55}Fe]ferriRA uptake (Figure 3).

The use of either ferriRA or RA as a carbon source by DFBC 5 was investigated in experiments where the compounds served as sole sources of carbon. Neither ferriRA nor RA supported the growth of DFBC 5 nor did the bacterium demonstrate any ability to metabolize the molecules as evidenced by consumption of the compounds from the growth medium. Attempts to supplement the ferriRA– or RA–mineral salts medium with a readily metabolizable substrate, 0.05% (w/v) YE, resulted in growth of the DFBC 5 but did not cause any of either the ferriRA or the RA to be metabolized by the microbe.

Discussion

While DFBC 5 grows well when the siderophore DFB is the sole carbon source, the bacterium demonstrated no ability to use FB as a carbon source (Castignetti & Siddiqui 1990). Not only was DFBC 5 incapable of using FB as a carbon source, cells or cell-free extracts of cells grown with DFB as the sole carbon source were unable to metabolize FB as the ferrisiderophore was not consumed nor was it degraded to a monohydroxamic acid as was DFB. The current study demonstrates that the lack of FB

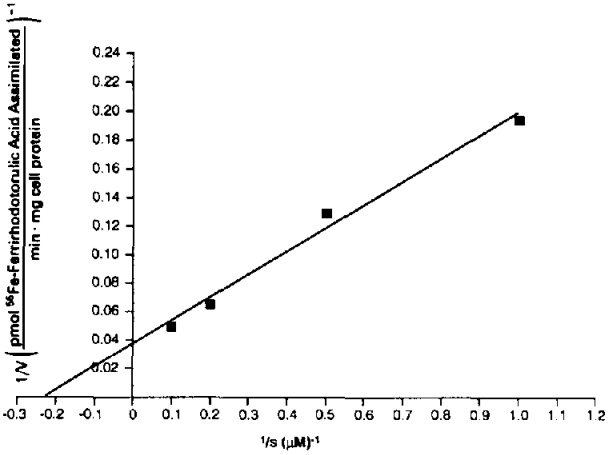

Figure 3. Kinetics of assimilation of ferriRA by DFBC 5. DFBC 5 was grown in the DFB medium described in Materials and methods section and cells were present at concentrations of 0.21–0.61 mg cell protein ml^{-1}. The concentrations of [^{55}Fe]ferriRA and RA were equimolar for every point noted, and were 1, 2, 5 and 10 μM.

use by DFBC 5 is complete in that FB also does not serve as a Fe^{3+} source for the bacterium.

While bacteria such as *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas cepacia* are capable of employing ferriNTA as an iron source (Meyer & Hohnadel 1992), whether DFBC 5 would use ferriNTA was unknown. Even though ferriNTA supplied Fe^{3+} to DFBC 5, it was unable to do so when the superior Fe^{3+} chelator DFB was present (Figure 1). No Fe^{3+} assimilation was observed under this circumstance as NTA is apparently unable to remove Fe^{3+} from FB. An examination of the formation constants (Sillen & Martell 1964) of both NTA and FB with Fe^{3+} (log K_f values of 15.87 and 30.6, respectively) notes that DFB is some 15 orders of magnitude greater in its ability to sequester the cation. It is therefore not unexpected that NTA is unable to gain Fe^{3+} from FB and the FB assimilation rate of 0 is consonant with the inability of DFBC 5 to use FB as an iron source even in the presence of NTA.

While DFBC 5 was unable to use FB as an iron source, it readily used ferriRA. The apparent K_m and V_{max} values of ferriRA assimilation are 4.3 μM and 27.8 pmol min^{-1} mg cell $protein^{-1}$, respectively. Other investigators (Lammers & Sanders-Loehr 1982, Ecker *et al.* 1986, Bachhawat and Ghosh 1987, DeWeger *et al.* 1988) have generally reported lower K_m values for bacteria while V_{max} estimations vary widely, e.g. from 10 (Lammers & Sanders-Loehr 1982) to 270 (Bachhawat & Ghosh 1987) pmol min^{-1} mg cell $protein^{-1}$. Fungi, as exemplified by *Neurospora crassa* (Ernst & Winkelmann 1974, Huschka *et al.* 1985, Bailey *et al.* 1986, Carrano *et al.* 1986), have K_m values for ferrisiderophore uptake of 0.05–29 μM and V_{max} estimations of 100 to 1000 pmol min^{-1} mg dry $weight^{-1}$ of cells. DFBC 5 thus displays a K_m value for ferriRA assimilation closer to those values noted of the fungus *N. crassa* while its V_{max} estimation is more characteristic of those of the bacteria. A possible explanation as to why ferriRA use by DFBC 5 is somewhat anomalous may be that RA is not the native siderophore of the bacterium. Further experimentation, to determine what siderophores, if any, the bacterium synthesizes is needed to resolve this point; such studies are planned to begin in our laboratory shortly.

Rhodotorula pilimanae, the yeast which synthesizes RA, is incapable of assimilating the Fe^{3+} of FB (Raymond *et al.* 1984) while DFBC 5 readily assimilates the iron of ferriRA but not that of FB. The use of DFB as a carbon source, while FB was unable to serve as either a carbon (Castignetti and Siddiqui 1990) or as a Fe^{3+} source, suggests that DFBC 5 may discriminate with respect to whether it employs a particular siderophore to gain carbon or iron. That the bacterium was able to utilize ferriRA as a Fe^{3+} source but unable to use either ferriRA or RA as carbon sources validates the idea of this 'nutritional selectivity'. A microbe employing such a strategy would thus be prevented from denying itself a source of Fe^{3+} due to the use of either the ferrisiderophore or the deferrisiderophore as a carbon source. While DFBC 5 displays the nutritional selectivity of not using the same siderophore as both a source of carbon and iron, the only other organism reported as being capable of siderophore catabolism, a bacterium termed *Pseudomonas* FC 1 (Warren & Neilands 1964), dissimilated both the ferri and deferrated forms of ferrichrome and ferrichrome A (Warren & Neilands 1964). While not displaying an ability to metabolize siderophores or hydroxamic acid compounds in general, *Pseudomonas* FC1 used ferrichrome, ferrichrome A, deferriferrichrome, deferriferrichrome A and, to a limited extent, ferric coprogen as carbon sources. Whether these siderophores served as Fe^{3+} sources for *Pseudomonas* FC 1 was not reported; such studies would be of interest. As reports of siderophore catabolizing microbes emerge, it will be noteworthy to determine whether such organisms also display a carbon and iron nutritional selectivity with respect to those siderophores which they dissimilate.

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References

Atkin CL, Neilands JB. 1968 Rhodotorulic acid, a diketopiperazine dihydroxamic acid with growth factor activity. I. Isolation and characterization. *Biochemistry* **7**, 3734–3739.

Atkin CL, Neilands JB, Phaff HJ. 1970 Rhodotorulic acid from species of *Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus,* and *Sporobolmyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. *J Bacteriol* **103**, 722–733.

Bachhawat AK, Ghosh S. 1987 Iron transport in *Azospirillum brasilense*: role of the siderophore spirilobactin. *J Gen Microbiol* **133**, 1759–1766.

Bailey CT, Kime-Hunt EM, Carrano CJ, Huschka H-G,

Winkelmann G. 1986 A photoaffinity label for the siderophore-mediated iron transport system in *Neurospora crassa*. *Biochim Biophys Acta* **883**, 299–305.

Carrano CJ, Bailey CT, Bonadies. 1986 Transport properties of *N*-acyl derivatives of the coprogen and ferrichrysin classes of siderophores in *Neurospora crassa*. *Arch Microbiol* **146**, 41–45.

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

Castignetti D, Smarrelli J. 1986 Siderophores, the iron nutrition of plants and nitrate reductase. *FEBS Lett* **209**, 147–151.

Castignetti D, Yanong R, Gramzinski R. 1985 Substrate diversity of an active heterotrophic nitrifier, an *Alcaligenes* sp. *Can J Microbiol* **31**, 441–445.

Davey EW, Gentile JH, Erickson SJ, Betzer P. 1970 Removal of trace metals from marine culture media. *Limnol Oceanogr.* **15**, 486–488.

DeWeger LA, van Arendonk JJ, Recourt K, van der Hofstad GA, Weisbeek PJ, Lugtenberg B. 1988 Siderophore-mediated uptake of Fe^{3+} by the plant growth-stimulating *Pseudomonas putida* strain WCS358 and by other rhizosphere microorganisms. *J Bacteriol* **170**, 4693–4698.

Ecker DJ, Matzanke BF, Raymond KN. 1986 Recognition and transport of ferric enterobactin in *Escherichia coli*. *J Bacteriol* **167**, 666–673.

Emery T. 1982 Iron metabolism in humans and plants. *Am Sci* **70**, 626–632.

Ernst J, Winkelmann G. 1974 Uptake of iron by *Neurospora crassa*. IV. Iron transport properties of semisynthetic coprogen derivatives. *Arch Microbiol* **100**, 271–282.

Fekete F, Chadhoke V, Jellison J. 1989 Iron-binding compounds produced by wood-decaying basidiomycetes. *Appl Environ Microbiol* **55**, 2720–2722.

Huschka H, Naegeli HU, Leuenberger-Ryf H, Keller-Schierlein W, Winkelmann G. 1985 Evidence for a common siderophore transport system in the presence of different siderophore receptors in *Neurospora crassa*. *J Bacteriol* **162**, 715–721.

Lammers PJ, Sanders-Loehr J. 1982 Active transport of ferric schizokinen in *Anabaena* sp. *J Bacteriol* **151**, 288–294.

Leong S, Neilands JB. 1982 Siderophore production by phytopathogenic microbial species. *Arch Biochem Biophys* **218**, 351–359.

Lodge JS, Emery T. 1984 Anaerobic iron uptake by *Escherichia coli*. *J Bacteriol* **160**, 801–804.

Meyer J-M, Hohnadel D. 1992 Use of nitrilotriacetic acid (NTA) by *Pseudomonas* species through iron metabolism. *Appl Microbiol Biotechnol* **37**, 114–118.

Mielczarek EV, Royt PW, Toth-Allen J. 1989 Microbial acquisition of iron. *Comments Mol Cell Biophys* **6**, 1–30.

Neilands JB. 1982 Microbial envelope proteins related to iron. *Annu Rev Microbiol* **36**, 285–309.

Neilands JB, Leong SA. 1986 Siderophores in relation to plant growth and disease. *Annu Rev Plant Physiol* **37**, 187–208.

Powell PE, Cline GR, Reid CP, Szaniszlo PJ. 1980 Occurrence of hydroxamate siderophore iron chelators in soils. *Nature* **287**, 833–834.

Raymond KN, Muller G, Matzanke BF. 1984 Complexation of iron by siderophores: a review of their solution and structural chemistry and biological function. *Topics Curr Chem* **123**, 49–102.

Rosenberg H. 1979 Transport of iron into bacterial cells. *Methods Enzymol* **56**, 388–394.

Schwyn B, Neilands JB. 1987 Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.

Sillen LG, Martell AE. 1964 *Stability Constants of Metal–Ion Complexes*. Special publication 17. London: The Chemical Society.

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 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.