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Further characterization and proposed pathway of deferrioxamine B catabolism

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Most siderophores are catabolism resistant because of their non-peptidic or cyclic peptide chemical structures. Siderophore degradation is thus a rare event, and one which has received little attention. Based on literature precedence and the generation of monohydroxamic acid(s) by cell-free extracts of a deferrioxamine B degrading bacterium, a catabolic scheme of the siderophore is proposed. Data are also presented concerning parameters such as the pH profile, inducible nature of the siderophore-degrading metabolism and the ability of the deferrioxamine B degrading bacterium to metabolize some of the hypothesized products of deferrioxamine B dissimilation.

Keywords: catabolism, deferrioxamine B, nutrient recycling, siderophore

Introduction

Siderophores are avid ferric ion chelators produced by a wide array of microorganisms (Neilands 1981, 1982, Emery 1982, Guerinot 1994). During the last three decades, research has defined the role of siderophores in microbial nutrition, agriculture and pathogenesis. In a similar manner, the application of molecular biology procedures has elucidated some of the mechanisms of siderophore genetic regulation. An area which has received little attention, however, is that of siderophore catabolism and recycling. As siderophores are often either cyclic peptides or non-peptidic, they tend to be resistant to degradation, with such activity being a rare event (Winkelmann *et al.* 1996).

Neilands and colleagues (Warren & Neilands 1964, 1965, Villavicencio & Neilands 1965) were the first to note and characterize siderophore degradation. These investigators isolated a soil pseudomonad capable of ferrichrome, ferrichrome A and coprogen catabolism. Studies concerning a deferrioxamine B (DFB) catabolizing bacterium named DFBC#5, also isolated from soil and resembling a pseudomonad (Castignetti *et al.* 1988, Castignetti & Siddiqui 1990, DeAngelis *et al.* 1993), indicated that the siderophore was used as a source of carbon but not of iron. Winkelmann *et al.* (1996) successfully recovered from lake water a *Spirillum*-like bacterium that catabolizes DFB and deferrioxamine E (DFE), and generates two unique dihydroxamates from the trihydroxamate DFB. While the study of siderophore catabolism and recycling is still in its genesis, these studies have presented data that indicate both similarities and differences by which the respective microbes degrade siderophores. Of interest is that the latter two bacteria showed limited or no capacity to degrade the ferric analogs of DFB (ferrioxamine B-FB) or DFE (ferrioxamine E-FE), while the first organism demonstrated a much greater activity when degrading the ferric analogs of the siderophores with which it was active. A noteworthy difference between the two DFB degraders is that the pseudomonad-like bacterium did not use FB as either an iron or carbon source while the

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Spirillum-like bacterium could utilize both FE and FB as iron sources.

The assay used by Castignetti *et al.* (1988) to monitor DFB degradation by a cell-free extract (Castignetti & Siddiqui 1990) measured the relative quantities of monohydroxamates and trihydroxamates present when the enzyme trivially named 'siderophore hydrolase' was presented with DFB. We have currently adopted the name 'DFB hydrolase' to represent the enzymatic activity required to initiate DFB catabolism, as this term more precisely describes the enzyme's function. As DFB hydrolase has not been purified to homogeneity, it is currently unknown whether the activity is due to the catalysis of one or more enzymes. Similarly, whether DFB hydrolase is capable of degrading its substrate from either end (the molecule is not symmetrical), thereby completely dissimilating DFB by generating dihydroxamate and subsequently monohydroxamate fragments, or whether a subsequent dihydroxamate hydrolyzing enzyme is also required, is also unknown. While the data of Winkelmann *et al.* (1996) noted that two different dihydroxamates were generated by cell-free extracts of their organism, whether these molecules become hydrolyzed to form their corresponding monohydroxamates, or the number of enzymes required to perform this catabolism, is currently unknown. In the current paper we present data to support a hypothesized scheme of DFB degradation and provide additional information concerning the DFB hydrolyzing enzymatic activities of our pseudomonad-like bacterium.

Materials and methods

Growth of the microbe and characterization of DFB hydrolase

DFBC#5 was grown in a mineral salts medium supplemented with 5 ml of vitamin solution and 0.1–0.3% (w/v) of DFB as described previously (Castignetti & Siddiqui 1990). DFB was generously supplied as the mesylate salt by the Ciba-Geigy Corporation (Summit, NJ, USA). For those experiments where acetate, succinate or cadaverine were assayed as carbon sources for the bacterium, sodium acetate trihydrate was the acetate source (Baker Chemical Co., Phillipsburg, NJ, USA), succinic acid was the succinate source (Eastman Organic Chemicals, Rochester, NY, USA) while 1,5-diaminopentane dihydrochloride, 99% (cadaverine) was the cadaverine source (Aldrich Chemical Co., Milwaukee, WI, USA). Control and experimental cultures were each conducted in triplicate and the values presented are the means of these replicates. Control and experimental cultures were the same except that the experimental cultures were given a 1% inoculum of cells previously grown in either the acetate– or cadaverine–mineral salts medium.

To obtain cell-free extracts, and subsequently the soluble and particulate fractions of the cell, DFBC#5 was harvested during mid- to late-logarithmic growth, washed and resuspended in either 50 or 100 mM, pH 7.0 phosphate buffer, ruptured via sonication and separated from unbroken cells and cell debris by centrifugation at 3000 × *g* at 4ºC as described (Castignetti & Siddiqui 1990). The only exception to this was when preparing cells for determination of the pH activity profile in which the washed DFBC#5 cells were suspended in 0.8% NaCl, and an equal volume of the buffer required to adjust the pH as desired (i.e. pH 4.2, 5.0, 6.0, 7.0 and 8.0) was added prior to sonication. For pH 4.2 and 5.0, a phthalate–sodium hydroxide buffer was used, while pH values of 6.0, 7.0 and 8.0 were obtained using a phosphate buffer (Gomori 1955). Extract boiled for 5 min served as the controls while unboiled extracts served as the experimental samples. In all cases of pH profile determinations, control samples were without enzymatic activity; this was measured, as were the experimental samples, via the spectral assay whereby the absorption maxima shifts noted of tri-, di- and monohydroxamates at pH 1.8–2.0 were determined and used to calculate the rate of conversion of DFB to monohydroxamate(s) (Castignetti *et al.* 1988). Protein concentrations of cell-free extracts, soluble and particulate cellular fractions, and ammonium sulfate precipitated enzymatic fractions were determined using the bichinchonic acid procedure (Pierce, Rockford, IL, USA).

To obtain particulate and soluble fractions of the crude extract, the preparation was centrifuged at 140 000 × *g* at 4ºC for 90 min (Brodie 1962). The precipitated particulate fraction was resuspended in an appropriate volume (usually 1–5 ml) of either 50 or 100 mM, pH 7.0 phosphate buffer while the soluble fraction was used as such. To obtain ammonium sulfate precipitated preparations of the cell-free soluble fraction, saturated ammonium sulfate solution aliquots were added to the soluble fraction according to the data listed in the ammonium sulfate precipitation tables of Scopes (1994). Precipitated fractions were resolubilized using appropriate volumes of 50 mM, pH 7.0 phosphate buffer while those fractions which remained soluble after such treatments were used as such. Storage of DFB hydrolase crude extract or ammonium sulfate precipitated fractions was in either 50 or 100 mM phosphate, pH 7.0 buffer at 4ºC. On occasion, crude extract was stored frozen in the above buffer at a temperature of –20ºC.

Chromatographic resins employed in this study were Amberlite XAD-16, Sephadex G-75, Sephadex G-100, DEAE Sephadex A-25, DEAE Sephadex A-50, Sephadex C-25 and Sephadex C-50 (Sigma, St. Louis, MO, USA). The resins were prepared and used as described by their manufacturer (Pharmacia LKB, Piscataway, NJ, USA). Nominal molecular weight cutoff (NMWC) filters were used as directed by their manufacturers (Micron Separations Inc., Westboro, MA–UltraFuge 100 000

NMWC filters) and Spectrum-Microgon (Centri/Por Centrifuge Concentrator, 50 000 NMWC, Laguna Hills, CA). An SDS–PAGE discontinuous system of a 4%T, 2.7%C stacking gel and a resolving gel of 10%T, 2.7%C was prepared and used with the Sturdier Slab Gel Electrophoresis unit as described by the Hoefer Scientific Co. (San Francisco, CA, USA). Protein samples were dissolved in the Tris–HCl, SDS, glycerol, bromophenol blue, 2-mercaptoethanol buffer of Lugtenberg *et al.* (1975) while the silver stain was conducted as described by Blum *et al.* (1987).

DFBC#5 induction experiments and usage of proposed DFB catabolism products

To determine if DFBC#5 was constitutive or inducible for the catabolism of DFB, the bacterium was grown in 1/2 strength King B medium (King *et al.* 1954) overnight and a 0.75% inoculum of these cells was used to inoculate fresh 1/2 strength King B medium. The culture was harvested during mid-logarithmic growth (14–18 h), collected by centrifugation and washed once with 50 mM phosphate, pH 7.0 buffer and placed on ice. Cells were suspended to an optical density at 660 nm (1/100) of 0.36 with the 50 mM phosphate buffer. This was followed by the removal of a 3 ml volume of the suspension which was then placed into a beaker and allowed to stir at room temperature for 5 min. 3 ml of a DFB solution dissolved in 50 mM phosphate, pH 7.0 buffer were added to the suspension at this time and the conversion of DFB to monohydroxamate(s) was monitored as described above by removing aliquots which were analyzed in triplicate. The induction experiment was repeated on two separate occasions and representative data are presented below. Controls consisted of cells grown in a DFB-containing mineral salts medium, harvested and prepared as resting cells as previously described (Castignetti & Siddiqui 1990). These cells were resuspended to an optical density at 660 nm (1/100) of 0.25 and used as above to begin the determination of DFB catabolism. DFB-grown cells actively consumed DFB (data not shown) confirming that the experimental conditions were appropriate to monitor such activity.

The catabolism of acetate, succinate and cadaverine, and the resultant growth of DFBC#5, was assayed by obtaining growth curves of the bacterium when these molecules served as sole carbon sources. The designated carbon source, 0.1–0.3%, was added aseptically to the mineral salts medium previously described and experiments were conducted as were the original growth experiments which established DFB as a carbon source for the microbe (Castignetti & Siddiqui 1990). Experiments were repeated on at least two separate occasions and triplicates of two different sets of control flasks (with a 0.1% inoculum but containing no carbon source; with no inoculum but containing carbon sources) and experimental flasks (with a 1% inoculum) were assayed for growth as measured by cell protein increases. The latter was determined by correlating the amounts of cell protein present in suspensions of the medium where absorbencies at 660 nm were taken and the protein content determined using the bichinchonic acid system of Pierce. Aliquots of these suspensions were removed, solubilized by boiling for 5 min in 0.2 N NaOH, and curves relating the amount of cell protein present versus a particular optical density at 660 nm were established. The amount of protein present in a sample was in turn determined by comparing the absorbance values obtained and correlating them with bovine serum albumin standards.

Acetate concentrations were assayed using a modified method to determine organic acids (Clesceri *et al.* 1989). The total amount of the acetate was determined in an aliquot without prior binding to a silica gel resin, i.e. by extracting it from the medium using 15 ml of chloroform–butanol reagent, and by using thymol blue as the sole indicator of pH in the ranges of 1.0–1.2 and 8.7–8.9. When pure sodium acetate standards were prepared and analyzed via the modified method, good correlation between the amounts of acetate present and the NaOH required to raise the pH to 8.7–8.9 was obtained and thus verified the applicability of the modified method. We suspect that since acetate was the sole organic acid in the growth medium, the need to bind and then elute it from the silica gel was obviated.

Results and discussion

Monohydroxamates, when ferrated, have absorption maxima of about 420–450 nm when at neutral pH; these shift to maxima of approximately 510 nm as the pH becomes acidic, i.e. about pH 1.0 (Neilands 1966, 1967). Conversely, trihydroxamic acids (for example ferrichrome and FB) have absorption spectra much less affected, with shifts from about 420–450 nm being observed as the pH is decreased from neutrality to 1.0–2.0 (Warren & Neilands 1964, Neilands 1966, 1967, Monzyk & Crumbliss 1982, 1983). Dihydroxamic acids, as exemplified by rhodotorulic acid, display intermediate absorption maxima shifts. Rhodotorulic acid demonstrates a shift from 425–480 nm as the pH decreases from 7 to 1 (Atkin & Neilands 1968).

Utilizing a final pH of about 1.8, the principle of absorption maxima differences between tri-, di- and monohydroxamates was exploited (Castignetti *et al.* 1988, Castignetti & Siddiqui 1990) to note the catabolism of DFB by cell-free extracts of DFBC#5. As the assay measures the ratio of monohydroxamate(s) to trihydroxamate (DFB), it was impossible to determine if only monohydroxamates were being formed, or indeed dihydroxamates were also being formed, when absorption maxima of about 460–480 nm were realized during and after enzymatic degradation of DFB. Maxima of 490 nm were observed,

however, when DFB hydrolase recovered from a 45% ammonium sulfate precipitated preparation was given sufficient time to catabolize DFB (Table 1). In separate experiments, absorbance maxima as large as 500–505 nm have been noted when the resolubilized 45% ammonium sulfate precipitated preparation was given DFB and sufficient time. The generation of monohydroxamate(s) by the enzyme thus appears likely.

By contrast, Winkelmann *et al.* (1996), studying DFB dissimilation by their *Spirillum*-like bacterium, were unable to detect the generation of monohydroxamates even though they noted the synthesis of dihydroxamates from DFB. As suggested by these authors, any monohydroxamates generated by their cell-free system may be quickly metabolized to other products or may be undetectable using the system (HPLC) employed to monitor DFB degradation. It will be of interest to determine if monohydroxamates are made from either DFB or its degradation dihydroxamates by the *Spirillum*-like bacterium of Winkelmann *et al.* and thus to determine if the two bacteria use similar or different schemes of DFB catabolism.

In comparison to the bacteria, differences with respect to DFB metabolism exist between these microbes and humans, as represented by a thalassemia major patient undergoing DFB infusion (Singh *et al.* 1990). DFB metabolites, resultant from oxidation, transamination, decarboxylation, and oxygenation, with the metabolites, however, still remaining trihydroxamates, were noted in the urine of the patient (Singh *et al.* 1990). The ability of the bacteria to dissimilate DFB to common intermediary metabolites, as proposed below, is most likely the reason why these organisms produce di- and monohydroxamates and can also use DFB as a sole source of carbon for growth.

Table 1. Formation of monohydroxamate(s) from DFB by a partially purified preparation of DFB hydrolase[a]

Time (min)	Absorption maximum (nm)
0	450
30	464
60	481
90	490

[a]The preparation of DFB hydrolase was that obtained after resolubilizing the 45% ammonium sulfate precipitate of the soluble fraction. The initial concentration of DFB was 2 mM and the rate of DFB degradation observed during this experiment was 23.3 nmol monohydroxamate formed per min per mg protein. The absorption maximum of 490 nm represents a greater than 81/1 ratio of monohydroxamate(s) to trihydroxamates.

Table 2. Metabolism of DFB by DFBC#5 cells grown as chemoheterotrophs and prepared as resting cells[a]

Time (min)	DFB concentration (mM)	Absorption maxima (nm)
0	2.2	450
30	2.2	451
60	2.2	452
120	2.2	451

[a]For the experimental conditions refer to the Materials and methods section.

The ability of DFBC#5 to catabolize DFB was not constitutive. DFBC#5, grown as a chemoheterotroph utilizing the King B medium as a nutrient source and prepared as resting cells, demonstrated no ability to consume or convert DFB (Table 2). This conclusion was reached as these cells demonstrated an inability to alter the absorption maxima of the DFB-containing medium, indicating that the trihydroxamate had not been metabolized (Table 2) . Conversely, DFBC#5 cells that had been grown as DFB catabolizers and prepared as resting cells, consumed approximately 75% of the DFB presented to them under these experimental conditions. DFBC#5 is not unique in its inducible ability to use DFB as a carbon source. Winkelmann *et al.* (1996) also noted that their DFB-consuming *Spirillum*-like bacterium has an inducible ability to consume DFB.

The ability of DFBC#5 to generate monohydroxamate(s) from DFB led us to consider possible products of DFB dissimilation. Scheme 1 is proposed as a pathway of DFB catabolism by DFBC#5 and its enzyme(s). Precedence for this scheme is derived from the ability of DFB hydrolase to generate monohydroxamates. Similarly, the overall scheme of generating dihydroxamates, monohydroxamates, amides and amines from a siderophore agrees with the earlier work concerning the degradation of ferrichrome and ferrichrome A by a soil pseudomonad (Warren & Neilands 1964, 1965, Villavicencio & Neilands 1965). These authors noted the production of monohydroxamates, the corresponding amides and their deacylation to yield amines. Further, they presented evidence that the enzyme initially responsible for ferrichrome A dissimilation was an intracellularly located alkaline peptidase. Recently, Winkelmann *et al.* (1996) noted the generation of two different dihydroxamates, termed metabolites I and II, by the cell-free extract of their *Spirillum*-like bacterium. The dihydroxamates were most likely generated by an enzyme(s) which cleaved DFB at its peptidic bonds, as proposed in the current

scheme. Such a generation of dihydroxamates would appear inevitable with 'DFB hydrolase' or similar enzymes and suggests that a likely fate of the dihydroxamates would be their conversion into their respective, constituent monohydroxamates as noted in the scheme. Whether this conversion is performed by DFB hydrolase or another enzyme remains to be determined.

As to the nature of DFB hydrolase, since DFB is a dipeptide, the enzyme may be classified as a dipeptidase (Enzyme Nomenclature 1984, Barrett 1992). Winkelmann *et al.* (1996), studying their *Spirillum*-like bacterium, reached a similar conclusion with the enzyme(s) responsible for DFB degradation, suggesting that an amidase-like activity was responsible for the generation of dihydroxamates from DFB. They also observed that L-alanine aminopeptidase, L-leucine aminopeptidase and dipeptidyl peptidase activities increased during the partial purification of the enzyme responsible for DFB hydrolysis.

Once generated, the reduction of hydroxamates to their corresponding amides is favored due to the electrophilic nature of the N of the N–O bond; such electrophiles are prone to reduction (Spain 1995). Indeed, guinea pig and rabbit liver aldehyde oxidases perform such reductions with salicylhydroxamic acid (Sugihara *et al.* 1983).

Neilands (1966), citing the work of Prelog & Walser (1962), noted that the complete hydrolysis of DFB yielded 1-amino-5-hydroxyaminopentane, succinic acid and acetic acid. Should the 1-amino-5-hydroxyaminopentane undergo reduction to the corresponding amine, cadaverine (1,5-diaminopentane) would result. The ultimate generation of cadaverine, acetic and succinic acids from DFB is thus a plausible pathway by the enzymes of DFBC#5. As these compounds are common intracellular metabolites, the gain to the microbe of performing DFB dissimilation would be to generate presumably readily metabolizable products suitable for supporting growth. To that end, we investigated whether DFBC#5 could use these molecules as carbon sources.

Figure 1 depicts the growth of DFBC#5 when either acetate or cadaverine served as sole sources of carbon. That the organism was able to use these two carbon sources gives credence to the postulated degradation products noted, suggesting that cell growth would be a logical outcome of DFB catabolism.

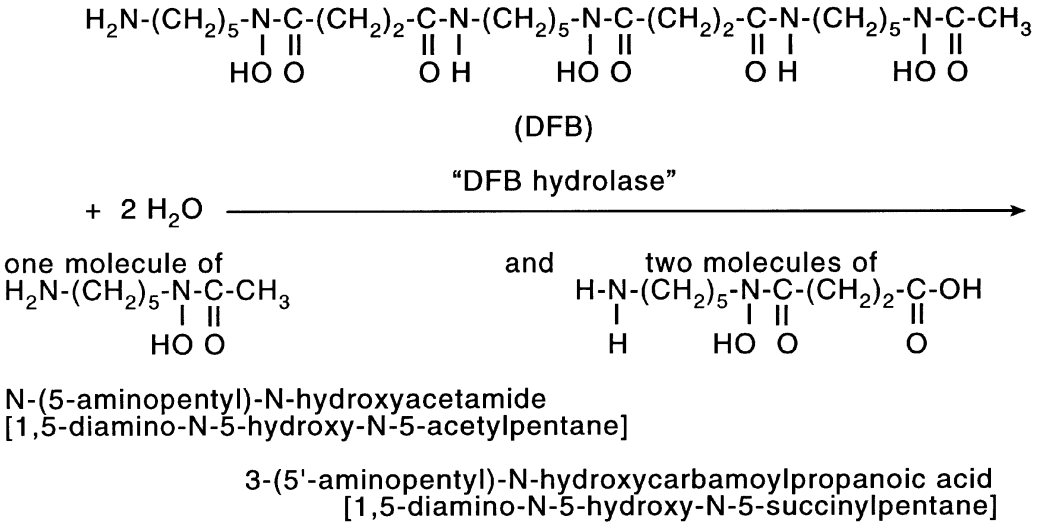
Growth with acetate was typical of that of a bacterium with a readily metabolized substrate; stationary phase was reached by the third day of culture. Cadaverine growth was much more prolonged with DFBC#5, requiring about 25 days to reach stationary phase. While the precise reason for this extensive period of time is unknown, two possibilities exist. The first would be that cadaverine itself was not metabolized, but rather that a dissimilation product of the molecule was. This product was not immediately available to DFBC#5 but arose with time as the culture was incubated. The second possibility is that a subset of the inoculated DFBC#5 cells, and not the entire culture, possessed the ability to metabolize cadaverine. If the subset was sufficiently small, an amount of time, perhaps as long as two to three weeks, would be necessary to allow sufficient numbers of these cells to accumulate to the point where an increase in the culture density could be observed.

DFBC#5 was unable to use succinic acid as a carbon source. This result is somewhat surprising as the bacterium is an obligate respirer (Castignetti & Siddiqui 1990) and is thus expected to contain a functional Krebs cycle. The inability to utilize exogenous succinic acid is perhaps due to a lack of transport of this molecule across DFBC#5's cytoplasmic membrane.

DFB hydrolase was noted as being substantially (> 85%) inhibited when the peptidase inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A and EDTA were present. Surprisingly, DFB hydrolase exhibited no proteolytic activity when commercial peptide substrates were presented to it (Castignetti & Siddiqui 1990). To further characterize the enzyme, experiments were conducted to discern additional parameters pertinent to its activity. DFB hydrolase activity was found only in the soluble fraction of the cell ($140\,000 \times g$ for 90 min); no activity was present in the particulate fraction. This result substantiates those previously noted by both Castignetti & Siddiqui (1990) and Winkelmann *et al.* (1996), namely that the DFB degrading activity is cell-associated and not found in the extracellular medium.

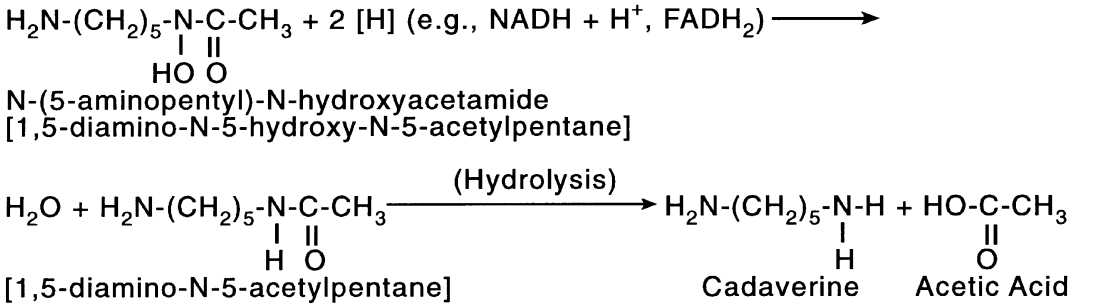
DFB hydrolase activity was sensitive to pH, with an optimum pH of 7.0 being noted (Figure 2). The enzyme is also remarkably stable, with either crude extract or ammonium sulfate precipitated preparations remaining active for at least two weeks when present in 50–100 mM, pH 7.0 phosphate buffer stored at 4°C. Indeed, we have noted that preparations refrigerated for periods of up to nine months retained their DFB hydrolytic activity. Frozen samples of enzyme, prepared similarly, were also with activity after at least one week although about 25% of the activity was lost when compared with the refrigerated enzyme.

Proposed Initial Hydrolysis of DFB

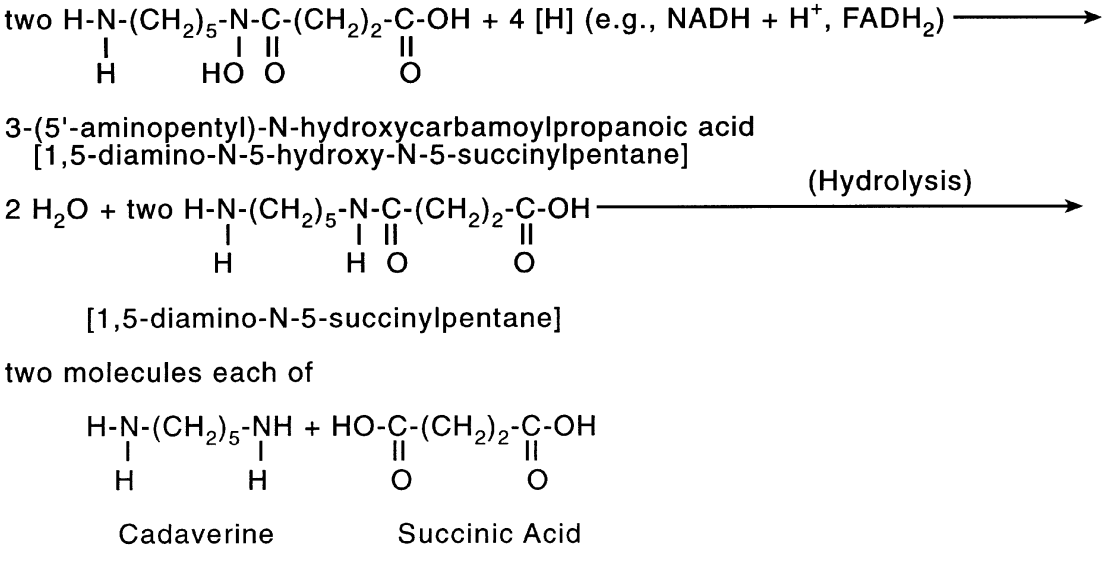


Proposed Pathway of Further DFB Catabolism

[First Hydroxamate]



[Second Hydroxamate]



Scheme 1. Pathway of DFB catabolism by DFBC#5 and its enzyme(s).

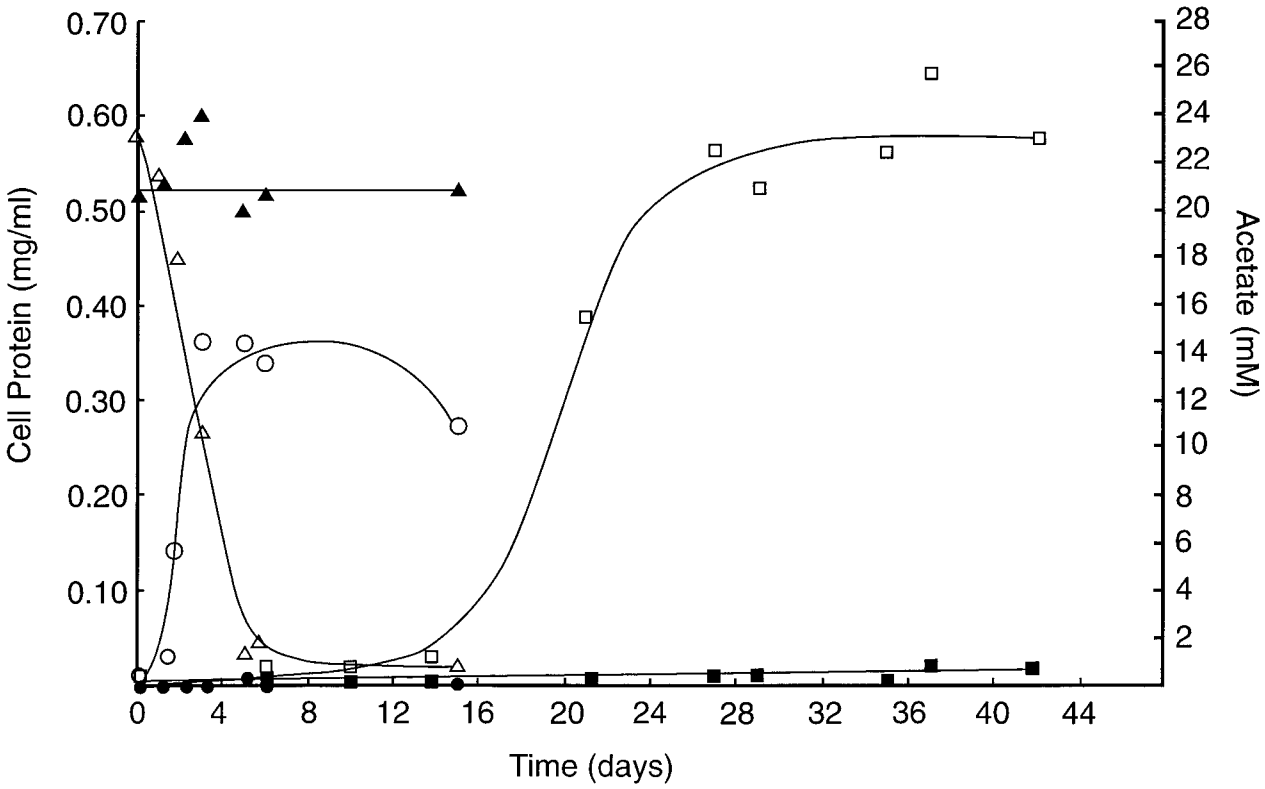


Figure 1. Growth of DFBC#5 with either acetate (0.3%) or cadaverine (0.1%) as sole sources of carbon. Open symbols represent parameters measured in the inoculated samples; filled (black) symbols represent parameters measured in uninoculated controls. Triangles denote acetate concentrations; circles denote growth of the cultures or controls given acetate as the sole carbon source; squares denote growth of the cultures or controls given cadaverine as a sole source of carbon. Growth was monitored by measuring the mg of cell protein per ml of medium.

When the crude extract of the enzyme was subjected to centrifugation to yield the soluble fraction and then treated with ammonium sulfate, all DFB hydrolase activity was precipitated between 25 and 45% of the salt, with the majority of the enzymatic activity precipitating between 25 and 35% ammonium sulfate saturation. Partial purification of DFB hydrolase after resolubilization from the ammonium sulfate precipitation resulted only with Sephadex A-50, indicating that the other resins used (see the Materials and methods section) displayed no affinity for the enzyme.

DFB hydrolase is thus most likely a weak anion at pH 7.0; it did not demonstrate either hydrophobic or cationic qualities. Binding to Sephadex A-50 was, however, rather weak as only 0.4–0.5 mg of protein bound per ml of resin (Table 3). SDS–PAGE revealed the presence of nine bands after passing the ammonium sulfate precipitated enzyme through a Sephadex A-50 column. At this point, however, so little material remained in the active fraction collected that it was not possible accurately to measure the sample's protein content or to purify the enzyme further.

DFB hydrolase activity eluted in the void volume from Sephadex G-75 columns, but was retained by Sephadex G-100 columns. As the useful Mr fractionation ranges of the two resins are 3000–80 000 and 4000–150 000, respectively, DFB hydrolase should have an Mr $\geq$ 80 000 but $\leq$ 150 000. Use of NMWC filters in two separate experiments, resulted in DFB hydrolase activity being retained by a 50 000 NMWC filter but passing through a 100 000 NMWC filter. These data suggest that DFB hydrolase has an Mr between 80 000 and 100 000.

We are currently continuing with our attempts to purify and isolate DFB hydrolase. The aim of these studies will be to characterize both the enzyme and the products it makes from DFB. Whether the enzymatic activity noted to date is due to a single (or multiple) enzyme, as well as whether dihydroxamates and monohydroxamates are produced due to the actions of the enzyme, will be of interest to discern. Finally, whether the postulated non-hydroxamate

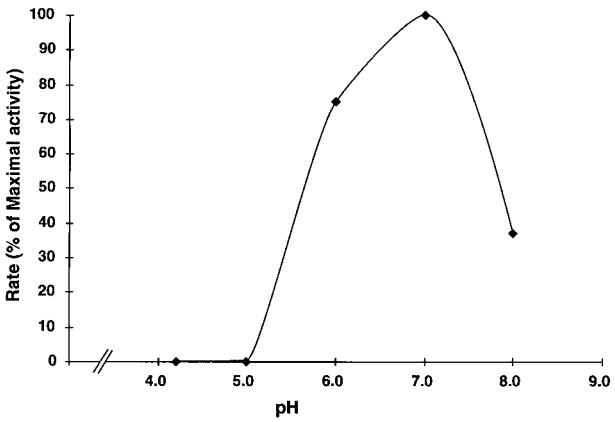


Figure 2. pH activity of DFB hydrolase. 100% of activity corresponds to 80 nmol monohydroxamate synthesized per min per mg protein.

products of DFB catabolism can be identified from either crude extracts, or reconstituted active fractions, supplied with DFB will be the subject of future studies.

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Table 3. Partial purification data of DFB hydrolase

Fraction	Protein (mg ml^{-1})	Specific activity (nmol per min per mg protein)
Crude	9.05	39.1
Soluble	6.25	48.2
20–45% ammonium sulfate	6.00	94.2

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