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Isolation and Preliminary Characterization of Hydroxamic Acids Formed by Nitrogen-Fixing *Azotobacter chroococcum* B-8

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The free-living diazotroph *Azotobacter chroococcum* B-8 responded to iron-limited growth conditions by forming hydroxamic acids and an 85,000-dalton outer membrane protein. The Fe(III)-binding hydroxamate compounds stimulated the growth of *Arthrobacter flavescens* JG-9 and gave a positive Csaky reaction for bound hydroxylamines. The hydroxamates were isolated from liquid cultures by benzyl alcohol extraction and purified by size exclusion chromatography and high-performance liquid chromatography. Four high-performance liquid chromatography fractions, designated A, B, C, and D, had the characteristic hydroxamate absorption maximum at 420 to 423 nm, which did not shift over a pH range from 3.0 to 9.0. Cyclic voltammograms of the iron-hydroxamate complexes exhibited reduction potentials of −0.426 to −0.442 V for fractions A, B, and D and of −0.56 V for fraction C versus the normal hydrogen electrode at pH 8.0. Based on mass spectra, nominal molecular weights of 800 and 844 were assigned to ferrated compounds A and B, respectively. Reductive hydrolysis of compounds A and B in 57% hydriodic acid yielded ornithine as detected by gas chromatography-mass spectrometry. All of these physiological and chemical data strongly support the hypothesis that the high-affinity iron-binding compounds isolated from *A. chroococcum* B-8 are hydroxamic acids and probably function as siderophores for this diazotroph.

Members of the family *Azotobacteraceae* are a coherent group of aerobic, gram-negative, free-living diazotrophs. Some representatives of this family can produce associative growth, but not symbiotic growth, with higher plants (48). Members of the *Azotobacteraceae* are considered to be important agriculturally, since estimates of the contribution of nonsymbiotic nitrogen fixers to soil nitrogen range from 10 to 15 kg of N ha^{-1} $year^{-1}$ (4).

Nitrogenase enzyme and other proteins involved in nitrogen fixation, and those respiratory systems responsible for protecting nitrogenase against oxygen inactivation, require a high complement of iron (6, 43). Therefore, members of the *Azotobacteraceae* have evolved efficient iron acquisition systems. However, the mechanisms of iron assimilation have been examined in only a few members of this family (42).

Iron exists in aerobic soil and water environments predominantly in the insoluble Fe(III) state, which is not readily available for microbial assimilation. To solubilize and sequester Fe(III), many microorganisms have evolved efficient high-affinity iron acquisition systems (37, 38). Components of a high-affinity system include the synthesis and release of siderophores into the extracellular environment to chelate and solubilize Fe(III), the synthesis and deployment of specific cytoplasmic membrane receptor proteins, and, in gram-negative bacteria, outer membrane (OM) receptor proteins for the ferrisiderophore complex (37, 38).

Within the family *Azotobacteraceae*, high-affinity iron acquisition systems exhibit the following characteristics. When grown under iron-deficient conditions, *Azotobacter vinelandii* produces the phenolic compounds *N*,*N*-di(2,3-dihydroxybenzoyl)-L-lysine(azotochelin),2,3-dihydroxybenzoic acid (11), and a yellow-green fluorescent peptide (azotobactin) (7). Knosp et al. (33) have shown that the catechol azotochelin and the fluorescent compound azotobactin function as siderophores for iron-limited *A. vinelandii*. Recently, Demange et al. reported the structure of azotobactin D, the siderophore from strain D of *A. vinelandii* (15, 16). *A. vinelandii* produces three major proteins, of 93,000, 85,000, and 81,000 daltons, and a minor, 77,000-dalton protein in the OM of iron-limited cells (41). In another nonsymbiotic diazotroph, *Azomonas macrocytogenes*, we have found that iron-sufficient and iron-deficient cells produce the phenolate 3,4-dihydroxybenzoic acid (protocatechuic acid) and an uncharacterized yellow-green fluorescent compound (which has the same electrophoretic behavior as the yellow-green fluorescent peptide from *A. vinelandii*) only under conditions of iron deficiency (49). Iron-deficient *Azomonas macrocytogenes* expresses an 83,000-dalton OM protein coincident with the formation of the yellow-green fluorescent compound (49). Other workers have reported that both *Azomonas macrocytogenes* and *Azotobacter paspali* produce fluorescent compounds only under iron-limited growth conditions and form 3,4-dihydroxybenzoic acid when grown under both iron-sufficient and iron-deficient conditions (10). Page (40) has reported that the capsule-deficient, sodium-dependent strain 184 of *Azotobacter chroococcum* produces a hydroxamate compound only when grown in media supplemented with a small amount of soluble iron and not in the absence of iron sources in the culture medium. Production of this compound is repressed when strain 184 is grown in the presence of adequate iron. Iron limitation also causes strain 184 to produce four OM proteins with molecular weights ranging from 70,000 to 76,000 (40).

This investigation concerns the further elucidation of the iron assimilation systems in the B-8 strain of *A. chroococ-*

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cum. Members of the genus *Azotobacter* are ubiquitous in soil, water, rhizosphere, and phyllosphere environments (4). *A. chroococcum* appears to be the most widespread species, occurring mainly in neutral and alkaline soils (4). Many azotobacters produce copious amounts of capsular extracellular polysaccharide (4). We have shown previously that in response to both iron and molybdenum deprivation, *A. chroococcum* B-8 produces extensive extracellular polysaccharide exclusively under nitrogen-fixing conditions (21, 24). Iron deficiency elicits the formation of hydroxamate-type iron-binding compounds when strain B-8 is grown under both fixed-nitrogen (NH_4^+ and NO_3^-) and nitrogen-fixing conditions (21; P. Lapp, L. A. Profenno, M. Toder, G. A. Mabbott, and F. A. Fekete, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K134, p. 215). We report here the isolation, purification, and preliminary characterization of the hydroxamic acids formed by nitrogen-fixing *A. chroococcum* B-8. Also, we observed the coincident production of an OM protein by this diazotroph at an iron concentration which elicited hydroxamate formation.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. chroococcum* B-8 (ATCC 7491) was grown under nitrogen-fixing conditions at 28°C in medium B6 liquid or on solid-plating medium (agar purified; Difco Laboratories, Detroit, Mich.) as described by Dalton and Postgate (13), with 2% sucrose (grade I; Sigma Chemical Co., St. Louis, Mo.) used in place of mannitol. $FeSO_4 \cdot 7H_2O$ was omitted from the stock trace element solution and added as appropriate. To reduce iron contamination, deionized water was used in all medium components, and glassware was cleaned and deionized as previously described (23). Growth was monitored turbidimetrically in iron-limited and control cultures by reading the A_{550} in a Spectronic 20 spectrometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Isolation and purification of hydroxamate compounds. An *A. chroococcum* B-8 stock slant culture was used to inoculate 200 ml of iron-deficient medium B6 in a 500-ml baffled Erlenmeyer flask. Inoculation density of the culture was approximately 10^6 cells ml^{-1}. This culture was incubated at 28°C and 200 rpm on a Gyrotory water bath shaker (model G76; New Brunswick Scientific Co., Inc., Edison, N.J.) for 72 h. The chrome azurol S universal chemical assay for siderophores (46) and high-voltage paper electrophoresis of culture supernatant fluids were used to follow hydroxamate production in iron-deficient cultures of *A. chroococcum* B-8 as described previously (21, 22). Spent culture fluids were separated from cells by centrifugation at 7,000 × *g* for 15 min. The supernatant fluids were concentrated to one-fifth the original volume by rotary evaporation at 45°C. $FeSO_4 \cdot 7H_2O$ at 1 mg ml of original culture volume^{-1} was then added to the concentrated supernatant fluid. The solution was acidified with concentrated HCl to pH 3, saturated with ammonium sulfate, and extracted with benzyl alcohol as described by Garibaldi and Neilands (26). The extracts were combined, and the hydroxamates were partitioned into water by addition of 3 volumes of diethyl ether and 1/10 volume of water. The aqueous extracts were combined and then washed with diethyl ether to remove any benzyl alcohol present.

The aqueous extracts were concentrated to about 10 ml with a rotary evaporator. Portions of 5 ml were eluted on a Sephadex G-10-120 size exclusion column (2.5 cm [inside diameter] by 35 cm), using a 50% methanol-water mobile phase. The orange fractions were pooled and evaporated under vacuum to about 1 ml before injection of 250-μl portions onto a reverse-phase high-performance liquid chromatography (HPLC) column (Altex ODS, 5-μm packing, 10.0 mm [inside diameter] by 25 cm), using a 30% methanol-water mobile phase. The first three hydroxamate compounds, designated A, B, and C, were collected as separate peaks under these conditions. The fourth compound, D, eluted after the mobile phase was stepped to 100% methanol.

Siderophore assays. Aqueous samples of hydroxamates isolated from iron-deficient cultures of *A. chroococcum* B-8 by the benzyl alcohol extraction procedure were assayed as follows. The presence of *o*-dihydric phenols was tested by the Arnow assay (2). The positive control was 2,3-dihydroxybenzoic acid (Sigma). A modified Csaky reaction was used to detect secondary hydroxamic acids (28). The assay standard was prepared with 0.1 to 1.0 mM hydroxylamine hydrochloride. Benzyl alcohol-extracted iron-binding compounds were subjected to the *Arthrobacter flavescens* JG-9 bioassay for hydroxamate siderophores (8). Positive controls were 0.1 μM ferrichrome and rhodotorulic acid (Porphyrin Products, Logan, Utah).

UV-visible absorption spectrophotometry. The electronic absorption spectra of the hydroxamate compounds were recorded with a DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). To measure the effect of pH on the absorbance maxima of compounds A through D, the corresponding HPLC fractions were evaporated to dryness and suspended in the following aqueous buffers: 10 mM sodium formate (pH 3.0), 10 mM 1,4-piperazinediethanesulfonic acid (PIPES [pH 6.5]; Sigma), and 10 mM Tris hydrochloride (pH 9.0).

After initial absorption readings were taken, the hydroxamate complexes in 0.2 to 0.5 ml of the buffered solution were deferrated by addition of 2 to 5 mg of 8-hydroxyquinoline (99+% pure; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 0.1 ml of methanol. The iron-hydroxyquinoline complex and excess reagent were removed by six washes with equal volumes of chloroform. The deferrated hydroxamates were referrated by addition of 25 μM $FeSO_4 \cdot 7H_2O$.

FAB MS. Fast-atom bombardment (FAB) mass spectrometry (MS) spectra were obtained with a Kratos MS-50 triple analyzer tandem mass spectrometer, which was previously described (29). This instrument consists of a high-resolution MS-I of Nier-Johnson geometry followed by an electrostatic analyzer used as MS-II. Samples were dissolved in methanol (100 μl), and a 1-μl portion was added to the matrix (3-nitrobenzyl alcohol) for acquisition of positive-ion mass spectra. FAB by 7-keV argon atoms was used to desorb the preformed ions from the matrix, which was supported on a gold probe held at +8 kV (positive-ion mode).

Ligand hydrolysis and detection of ornithine. Reductive hydrolysis was performed by heating about 1 mg of deferrated hydroxamate in 57% hydriodic acid (Aldrich) for 12 to 16 h at 100 to 110°C in a vacuum-sealed ampoule (3). The solution was cooled and evaporated to dryness with a stream of dry N_2. The I_2 by-product was removed by washing the residue repeatedly with diethyl ether (reagent grade; VWR Scientific Co.) until only a light-yellow color remained in the solid. Traces of ether were removed by placing the vial in a vacuum desiccator. Volatile derivatives of free amino acids were formed by making the *N*-trifluoroacetamide methyl esters (14). These derivatives were prepared by first dissolving the residue in 0.5 to 1.0 ml of freshly prepared 3% methanolic hydrochloride (25) and heating the solution in a sealed vial for 30 min at 70°C. The sample was dried by

vacuum evaporation. The residue was redissolved in methanol (HPLC grade; Fisher Scientific Co., Pittsburgh, Pa.) twice more and evaporated to dryness under vacuum to remove traces of reagent. About 0.3 ml of trifluoroacetic anhydride (Aldrich) was used to take up the residue and transfer the solution to an ampoule, which was then sealed under vacuum and placed in an oven at 140°C for 10 min. After cooling, the solution was evaporated with a stream of dry N_2. The products were taken up in about 100 μl of methylene chloride (HPLC grade; Eastman Kodak Co., Rochester, N.Y.). About 2 μl of residual vapors from a sample-wetted syringe was injected onto a gas chromatograph-mass spectrometer (model 5890A/5970B; Hewlett-Packard Co., Palo Alto, Calif.), using a splitless injector. The vapors were cold trapped at the beginning of the column by cooling the initial 6 in. [ca. 15 cm] of column in an ice bath during the splitless injection period of 0.5 min. The 15-meter cyanopropylphenylmethyl silicone capillary column (0.25-mm inside diameter, DB-225; J & W Scientific) was programmed from 30 to 200°C at 15°C min^{-1}. A sample blank and authentic deferrated rhodotorulic acid were used as controls for the hydrolysis and derivatization procedures. A derivative was also prepared from authentic L-ornithine hydrochloride for comparison of retention times and mass spectra.

Electrochemical measurements. Cyclic voltammograms were recorded in either 0.1 M KCl–0.1 M Tris (primary standard grade; Fisher) buffer adjusted to pH 8.0 or 0.1 M KCl–0.01 M K_2HPO_4 solution adjusted to pH 6 to 8. The purified complexes recovered from HPLC were evaporated to dryness under vacuum, and then each was taken up in 100 μl of buffer. To test the stability of the complexes, voltammograms were also taken with these same solutions after addition of an excess (1 to 2 mg) of Na_2H_2 EDTA · $2H_2O$ (99+% pure; Aldrich) directly to the electrochemical cell. Buffers were prepared by using deionized (18 MΩ) water; other chemicals were reagent grade and were used without further purification.

Experiments were performed at a hanging mercury drop electrode, using a potentiostat (model 173; Princeton Applied Research). To enable work at a high concentration with only milligram quantities of complex, a special microvolume insert for the standard glass cell was machined out of Lucite (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). A tapered well of 6-mm outer diameter and about 2-mm depth was cut into a 1-cm length of 1.2-cm-diameter Lucite dowel. This well accommodated the sample solution, the mercury drop working electrode, a fine-Pt-wire (0.127-mm diameter) counter electrode, and the agar-sealed salt bridge made from a 1.5-mm glass tube that also housed a fine-wire Ag-AgCl reference electrode in 0.100 M KCl. Sample solutions of 80 μl were used to record voltammograms. The solutions were deaerated by passing wetted nitrogen through the cell for 20 to 25 min before recording a scan. The performance of this small-volume apparatus was tested by recording cyclic voltammograms of 12 mM Fe(III)-EDTA prepared from reagent-grade $Fe(NO_3)_3 \cdot 9H_2O$ and Na_2H_2 EDTA · $2H_2O$ (45).

OM isolation and analysis. Cells of nitrogen-fixing *A. chroococcum* B-8 were harvested by centrifugation at 8,000 × *g* for 10 min. The pellets were suspended in medium B6 salts (no sucrose), vortexed, and centrifuged at 28,000 × *g* for 10 min as previously described (21) to remove the copious capsular material. The cells were then lysed according to the osmotic shock procedure of Shah et al. (47). The lysate was pelleted by centrifugation at 28,000 × *g* at 4°C for 30 min and suspended in 8 mM Tris hydrochloride buffer (pH 7.4). The protein concentration of the lysate was determined by the Lowry assay (35), and the lysate was then shaken with sarcosyl (free acid; Sigma) in a 6:1 weight-to-protein ratio for 60 min at 25°C. The insoluble OM fraction was separated from the cytoplasmic membrane fraction by centrifugation at 28,000 × *g* for 60 min at 4°C. The pellet was washed with Tris buffer, assayed for protein concentration, and recentrifuged. The purity of the OM fraction was checked by the succinic dehydrogenase assay as described by Dobrogosz (17). The pellet was finally suspended in Laemmli (34) solubilization buffer to a protein concentration of 12 mg ml^{-1}.

OM proteins and molecular weight markers were solubilized and separated by the electrophoretic methods of Laemmli (34). Volumes (10 to 20 μl) of the OM samples from iron-sufficient and iron-deficient cultures were loaded onto 1.5-mm sodium dodecyl sulfate-containing vertical slab gels consisting of a 4.5% stacking gel and a 7.5% resolving gel. Gels were run at 24 mA for 4 to 6 h and stained with Coomassie brilliant blue R. Molecular weights of the OM protein bands were determined from a standard curve based on the following authentic molecular weight markers: carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), and phosphorylase *b* (97,400) (Sigma).

RESULTS

Hydroxamate formation. Iron-binding compounds produced by nitrogen-fixing *A. chroococcum* B-8 were initially detected by the siderophore paper electrophoresis assay (21, 22). Supernatant fluids from 6-day-old iron-deficient cultures produced two bands on high-voltage paper electrophoretograms. These two nonfluorescent bands were designated slow or fast, depending on their anodal migration in the electrophoretic field. High-voltage paper electrophoresis of supernatant fluids from iron-limited 72-h-old cultures produced iron-binding compounds that migrated only as the slow band. All work described here was done on cultures of *A. chroococcum* B-8 that were deprived of iron for 72 h (when only the slow band was present).

Isolation and confirmation of hydroxamates. The ferrated red-orange complexes from the supernatant fluids of cultures grown for 72 h were easily extractable into benzyl alcohol solvent, as is the case for many hydroxamate siderophores (38). Aqueous samples of the iron complexes, derived from the benzyl alcohol extracts of culture supernatants, were positive for the Csaky assay (28) for bound hydroxylamines and gave a positive reaction in the universal chrome azurol S siderophore assay (46) but were nonreactive in the Arnow (2) assay for *o*-dihydric phenols. The hydroxamate nature of the iron-binding compounds was further substantiated by the strongly positive growth response (relative to that of the ferrichrome positive control at 0.1 μM) of the hydroxamate auxotroph *Arthrobacter flavescens* JG-9 (data not shown).

Hydroxamate purification. None of the peaks in the cluster that eluted from HPLC in the first 4 min after the time of injection appeared to correspond to an iron-hydroxamate complex (based on the visible spectra of the eluates) (Fig. 1). Each of the eluates collected at approximately 6.5 min (fraction A), 11 min (fraction B), 26 min (fraction C), and 5 min after stepping to a mobile phase of 100% methanol (fraction D) exhibited an absorbance maximum near 420 nm (Fig. 1). Purity of the fractions was checked by FAB MS.

UV-visible spectrophotometry. UV-visible electronic absorption spectra of the Fe(III) complexes of the HPLC

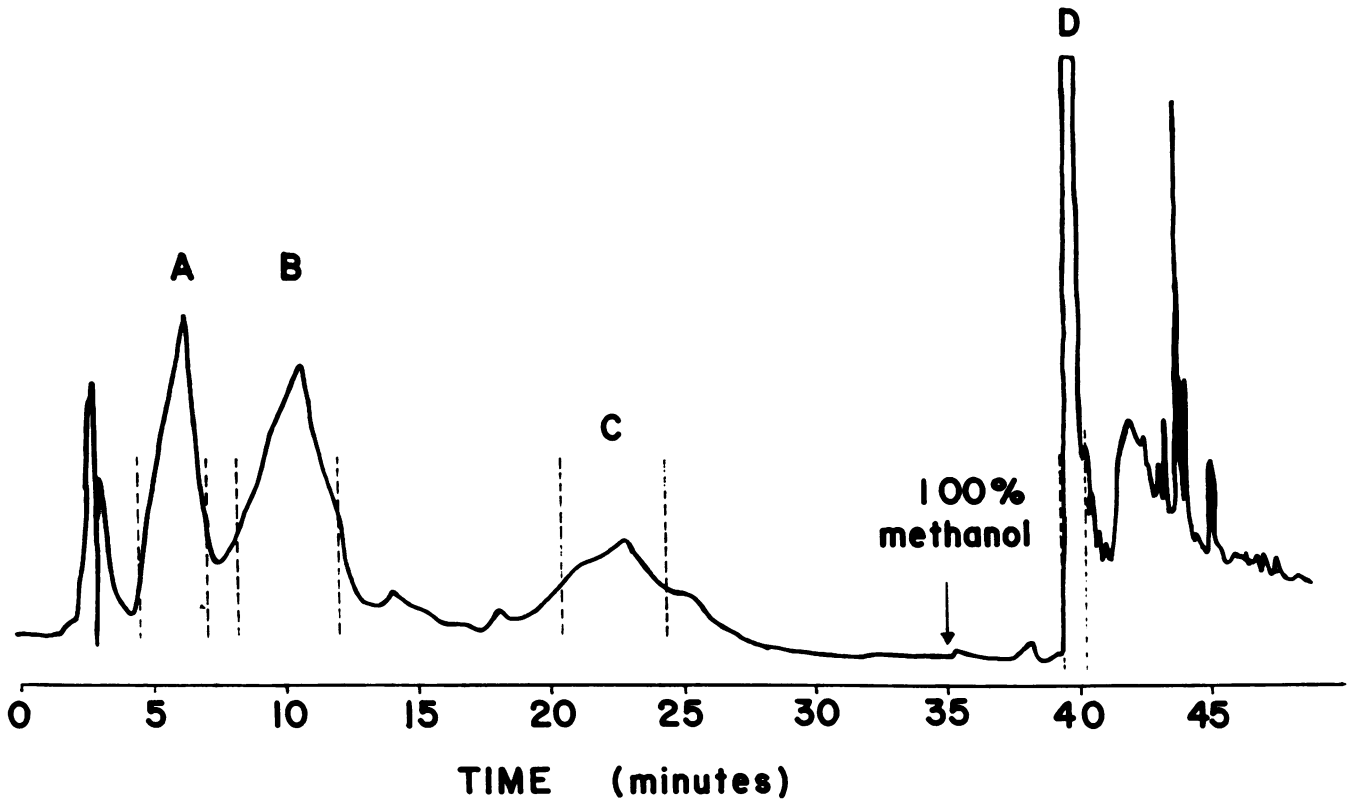

FIG. 1. High-performance liquid chromatogram of ferrated hydroxamate compounds on a reverse-phase semipreparative column, using 30% methanol in water at 4 ml min^{-1}. Letters indicate fractions with UV-visible spectra characteristic of iron-hydroxamate complexes.

fractions A, B, C, and D showed characteristic Fe(III)-hydroxamate siderophore absorption maxima. Figure 2 shows the UV-visible absorption spectrum of fraction A, which was characteristic of the spectra of all four fractions. The major peak in the visible range of each fraction showed very little shift in the absorption maximum over a pH range from 3 to 9, with values ranging from 420 to 423 nm (Fig. 2).

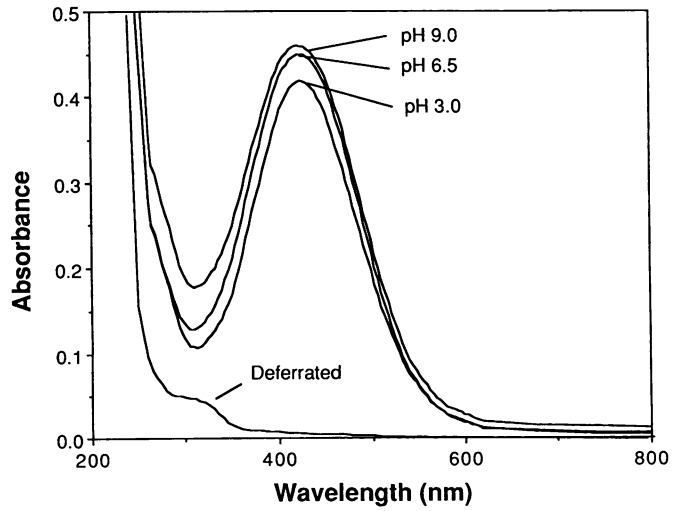

FIG. 2. UV-visible electronic absorption spectra of an aqueous solution of compound A and $FeSO_4 \cdot 7H_2O$ (25 μM) at pH 3.0, 6.5, and 9.0. The spectrum of uncomplexed (deferrated) compound A was obtained at pH 6.5.

Deferration of fractions A, B, C, and D with 8-hydroxyquinoline resulted in the disappearance of the visible peak at 420 to 423 nm, which could then be restored by the addition of Fe(III) (Fig. 2).

FAB MS. The positive-ion FAB mass spectra of HPLC fractions C and D gave several high-mass peaks (between 800 and 900 daltons), which suggested that they contained multiple compounds. Further characterization of the hydroxamate complexes in these fractions will be addressed in a later report (G. A. Mabbott, J. M. Macey, R. N. Hayes, and F. A. Fekete, manuscript in preparation). However, the spectra of fractions A and B were dominated by single intense peaks at *m/z* 801.3198 and 845.3453 daltons, respectively, and were treated as pure for the purposes of this report. Since these peaks represent the $(M+H)^+$ ions, the corresponding calculated exact masses for the neutral molecules were 800.3120 daltons for compound A and 844.3375 daltons for compound B.

Ornithine detection. A hydroxamate based on ornithine is reduced to ornithine by hydriodic acid hydrolysis (3, 18, 19). The presence of a form of ornithine in both compounds A and B was confirmed by gas chromatography-MS of the trifluoroacetyl methyl ester derivatives of the hydriodic acid hydrolysates. Chromatograms for each of the derivatized hydrolysates as well as those from similarly treated rhodotorulic acid and derivatized authentic ornithine contained a peak at 12.7 min. The mass spectrum of the derivative from compound A is compared with that from the authentic L-ornithine derivative in Fig. 3. The spectra for compound B and rhodotorulic acid derivatives were also virtually identical to the spectrum for the ornithine derivative (data not shown).

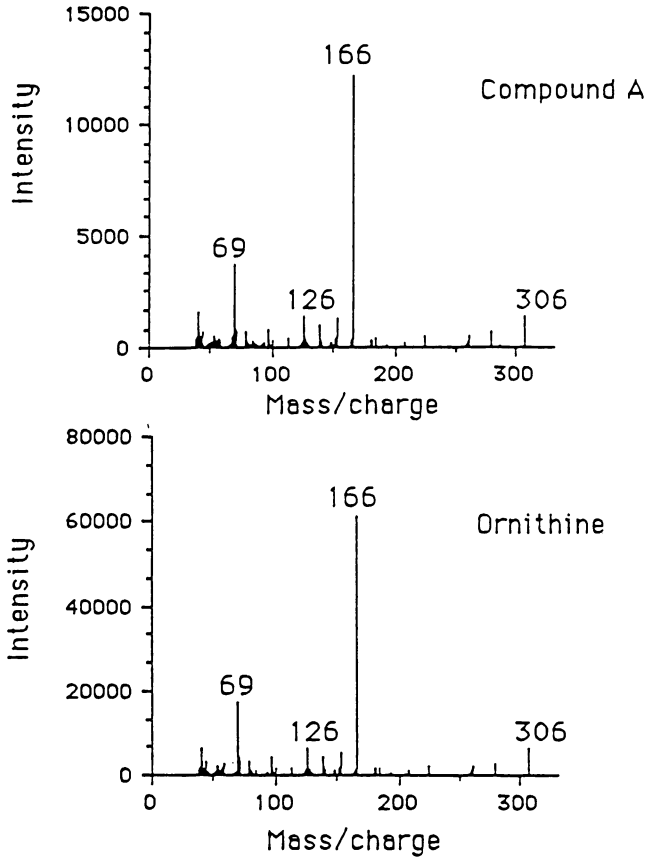

FIG. 3. Mass spectra of the trifluoroacetyl methyl ester derivatives formed from the hydriodic acid hydrolysis of compound A and from authentic ornithine recorded from the gas chromatographic peaks that emerged at 12.7 min. Similar treatment of compound B and of rhodotorulic acid gave the same results.

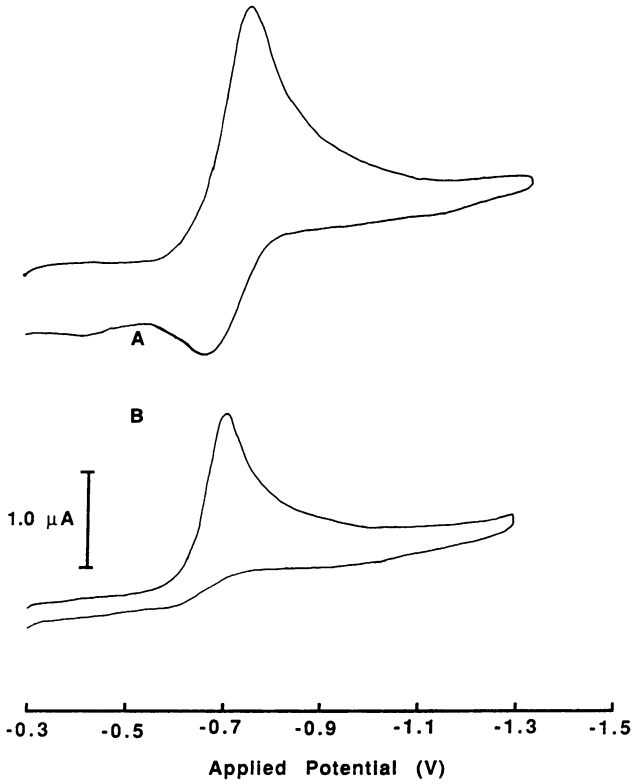

FIG. 4. Cyclic voltammograms of compound A. Scan rate, 100 mV s^{-1}; Ag-AgCl reference electrode in 0.100 M KCl. (A) Compound A in 0.100 M KCl–0.100 M Tris buffer (pH 8.0); (B) same conditions as above, with the addition of excess EDTA.

Electrochemistry. The reliability of formal potential determinations made in the small-volume cell was verified. The cyclic voltammogram for Fe(III)-EDTA obtained by using the special apparatus yielded a reduction potential for the complex of −0.178 V versus the Ag-AgCl reference electrode (or +0.109 V versus normal hydrogen electrode [NHE]) at pH 6.0, which compares very well with recently published values (+0.112 V versus NHE) under similar solution conditions (1, 20). The reduction potentials for the iron-hydroxamate complexes as calculated from the average of the cathodic and anodic cyclic voltammetry peaks were: complex A, −0.714 V (−0.426 V versus NHE); complex B, −0.720 V (−0.432 V versus NHE); complex C, −0.86 (−0.57 V versus NHE); and complex D, −0.730 V (−0.442 V versus NHE). The cyclic voltammograms for A, B, and D were similar in appearance. Work with C and D will be deferred until further purification of these compounds. The formal reduction potentials for complexes A and B shifted to more positive potentials as the pH was lowered (data not shown). For five measurements between pH 7.8 and 6.6, the reduction potential shifted by 57 mV per pH unit for complex A (correlation coefficient, 0.90). Complex B shifted by 80 mV per pH unit in a similar experiment (correlation coefficient, 0.98). A change of 59 mV per pH unit is consistent with a single-electron, single-proton reduction process.

Figure 4A shows a voltammogram for compound A at pH 8.0. The fact that the return peak was much smaller than the peak in the forward scan indicates that the reduction was more complicated than a simple electron transfer process. The ratio of the return peak height to the reduction peak height decreased with increasing scan rate (data not shown). This trend is indicative of a reversible homogeneous chemical step after the electron transfer step (36). Release of the ferrous ion from the complex would be one possible explanation for this observation. Figure 4B shows the same system with the addition of EDTA. The absence of a peak on the return (oxidation) scan in the solution with EDTA is consistent with the hypothesis that the Fe(II)-hydroxamate complex was less stable than Fe(II)-EDTA.

Iron-repressible OM protein detection. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the OM fractions prepared by sarcosyl extraction revealed the hyperproduction of an 85,000-dalton protein in response to iron limitation (Fig. 5). This protein was not observed in OM obtained from nitrogen-fixing cells grown with 25 μM iron. The OM protein preparation contained only 6.0% of the total succinic dehydrogenase activity of the various cell fractions. Because succinic dehydrogenase is a cytoplasmic membrane enzyme, this finding confirmed that the OM protein preparation obtained from *A. chroococcum* B-8 was relatively free of cytoplasmic membrane proteins.

DISCUSSION

Nitrogen-fixing cells of *A. chroococcum* B-8 formed high-affinity Fe(III)-binding hydroxamate compounds when grown under nutritional iron stress conditions (21). In addi-

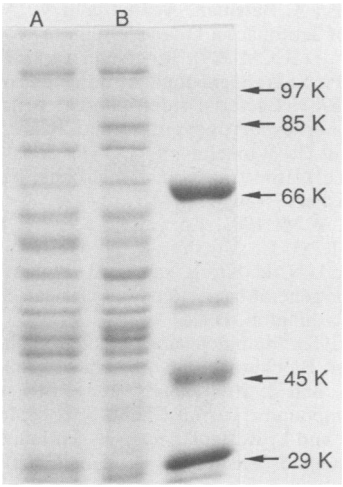

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OM proteins from *A. chroococcum* B-8 grown in iron-sufficient (25 μM; lane A) and iron-limited (lane B) medium. Molecular weight standards (in thousands) and the 85,000-molecular-weight hyperproduced protein are designated on the right.

tion, a major 85,000-dalton OM protein was produced at an iron concentration which induced hydroxamate production (Fig. 5). It is likely that this iron-repressible protein functions as a receptor for one or possibly more of the hydroxamate compounds elicited by *A. chroococcum* B-8. Syntheses of all of the hydroxamate compounds and the 85,000-dalton protein were repressed when the organism was grown with adequate (25 μM) iron (21) (Fig. 5). The presence of both the iron-binding hydroxamate compounds and the iron-repressible OM protein provides evidence suggesting that *A. chroococcum* B-8 utilizes a classic high-affinity iron acquisition system.

The hydroxamates elicited by *A. chroococcum* B-8 have characteristics that are typical of other hydroxamate siderophores. The iron-binding compounds formed red-orange Fe(III) complexes, were readily extractable into benzyl alcohol, and were positive for bound hydroxylamines in the Csaky assay. Furthermore, the isolated compounds promoted the growth of the hydroxamate siderophore auxotroph *Arthrobacter flavescens* JG-9 in the standard hydroxamate bioassay. In most of the known hydroxamate siderophores, the hydroxamate functional group is derived from δ-*N*-hydroxyornithine (32). Similarly, we found ornithine in the hydriodic acid hydrolysates of compounds A and B (Fig. 3).

In contrast, the hydroxamate isolated from the sodium-dependent strain 184 of *A. chroococcum* does not appear to have characteristics of a typical hydroxamate (40). For example, the strain 184 hydroxamate fails to form a red Fe(III) complex and does not partition into benzyl alcohol. Furthermore, culture fluids containing the hydroxamate do not promote the growth of the auxotroph *Arthrobacter flavescens* JG-9 (40).

The spectroscopic behavior of the purified hydroxamates formed by *A. chroococcum* B-8 (Fig. 2) is similar to that of the trihydroxamates, such as malonichrome (18) and the ferrichromes (38), and differs from the large shift with change in pH shown by mono- and dihydroxamate iron chelates, e.g., schizokinen (9) and aerobactin (27), respectively.

The reduction potentials observed for complexes A and B (−0.426 and −0.432 V versus NHE at pH 8.0) are very similar to those obtained for known siderophores such as ferrichrome A (−0.448 V), ferrioxamine B (−0.468 V), and ferric rhodotorulic acid (−0.359 V) (38, 50). Because Fe(II) was much less tightly bound than Fe(III), this reduction appears to be a reasonable mechanism for releasing the iron from the complex. The weak return peak [for the reoxidation of the Fe(II) complex back to the Fe(III) form] in the cyclic voltammogram (Fig. 4) suggests that the reduced form was unstable in this buffer. We can set some limits on the formation constants for the ferric and ferrous hydroxamate complexes by comparing the cyclic voltammograms recorded with and without EDTA in the electrochemical cell. The reduction peak for the Fe(III)-hydroxamate in the presence of EDTA indicates that the EDTA was not capable of removing the iron from the hydroxamate. This observation indicates that the Fe(III)-hydroxamate complex has a formation constant of greater than 7×10^{22}, the binding constant for Fe(III)-EDTA at pH 8.0 (30). The lack of a return peak for the voltammogram recorded for the same complex in the presence of EDTA (Fig. 4B) indicates that the Fe(II)-hydroxamate complex was less stable than the Fe(II)-EDTA complex (which has a binding constant of 10^{15} at this pH) (30). In addition, the shift in the redox potential of the hydroxamate complex compared with that of the Fe(III)-$(H_2O)_6$ complex (E_0 = 0.771 [31]) indicates that the ratio of the formation constant for the Fe(III)-hydroxamate to that for the Fe(II)-hydroxamate must be approximately 10^{20} (36). On the basis of this ratio and the limits on the formation constants inferred from the experiments in the presence of EDTA, the binding constants for the Fe(III)-hydroxamate complexes A, B, and D would be in the range of 10^{22} to 10^{32}, whereas that of the Fe(II)-hydroxamate complexes would be in the range of 10^2 to 10^{12}. The formation constants of known ferric trihydroxamate siderophores include those for ferrioxamine E ($10^{32.5}$), ferrichrome ($10^{29.1}$), and ferrioxamine B ($10^{30.6}$) (32). These formation constants are consistent with the range predicted for the Fe(III)-hydroxamate complexes from *A. chroococcum* B-8.

A number of studies have shown that some plants can utilize microbially produced siderophores for iron assimilation (12, 44). For example, hydroxamate siderophores supply iron to a number of different hydroponically grown plants, including oat, tomato, sunflower, and sorghum (12). It has been established that Fe(III)-hydroxamates are stable in soils over a broad range of pH and that hydroxamates occur in soils at concentrations important for increasing the availability of iron to plants. Thus, it is plausible that plant utilization of microbial siderophore iron occurs in nature (12).

The mechanisms by which plants remove the iron from the tightly bound siderophore complexes is of current interest. Crowley et al. (12) discuss three such mechanisms that involve reduction of the iron to Fe(II). Bienfait (5) has shown that the source of electrons for iron reduction in plants is NADPH, which has a formal potential of −0.320 at pH 7.0 (39). NADPH would be thermodynamically capable of reducing the Fe(III) complexes of compounds A and B from *A. chroococcum* B-8 only if the Fe(II) formed were incorporated into some other stable form or the pH were lowered significantly. Since the $NADP^+$-NADPH couple is a two-electron, single-proton system and the iron-hydroxamate complexes studied here appear to be single-electron, single-proton systems, the projected redox potentials for the two systems would be equal at a pH of between 5 and 6. Under these conditions, significant reduction of the complex could

occur. Interestingly, the extracellular medium pH of iron-stressed *A. chroococcum* B-8 is approximately 5.5 (21).

In summary, our results show that compounds A and B from *A. chroococcum* B-8 have many of the characteristics exhibited by known trihydroxamate siderophores. They have a high affinity for Fe(III), contain ornithine, are stable at pH 3 to 9, and have comparable molecular weights (of 800 and 844, respectively). Complete chemical characterization of these compounds is now in progress.

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