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Azotobacter vinelandii strains of disparate origin produce azotobactin siderophores with identical structures

William J. Page[1], S. Karen Collinson[1], Pascal Demange[2], Anne Dell[3], and Mohamed A. Abdallah[2]

[1] Department of Microbiology, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada
[2] Laboratoire de Chimie Microbienne, associé au CNRS, Centre de Recherche de Chimie, Université Louis Pasteur, 1 rue Blaise Pascal, F-67008 Strasbourg, France
[3] Department of Biochemistry, Imperial College, London SW7 2AZ, England

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Summary. The yellow green fluorescent siderophore, azotobactin, was purified from cultures of two *Azotobacter vinelandii* strains. Structural analysis of azotobactin from the North American *A. vinelandii* strains O and its capsule negative variant UW (also called OP) revealed that both strains produced azotobactins with identical structures. Moreover, azotobactin produced by these two strains was structurally identical to azotobactin D, the fluorescent siderophore previously isolated from the European *A. vinelandii* strain D (CCM 289). Unlike strains of fluorescent *Pseudomonas* which produce structurally diverse pyoverdins, strains of *A. vinelandii* of disparate origin produced azotobactins of identical structure. Lactonization of azotobactin did not interfere with the ability of this compound to function as a siderophore.

Key words: *Azotobacter vinelandii* - Siderophores - Pyoverdin - Azotobactin - Iron uptake

Introduction

Azotobacter vinelandii is a large Gram-negative soil bacterium that has a great demand for iron during growth. This obligate aerobe is one of a few genera that can fix nitrogen during vigorous aeration since the oxygen-labile nitrogenase is protected by respiratory consumption of oxygen (Robson and Postgate 1980; Thompson and Skerman 1979). Iron is an essential component of various enzymes required for respiration, nitrogen fixation and protection from the toxic oxygen products generated by these metabolic processes (Neilands 1980). Therefore, it is not surprising that *A. vinelandii* has evolved an efficient siderophore system for the extraction, chelation and transport of iron into the cell (Knosp et al. 1984; Page and Huyer 1984; Page and von Tigerstrom 1988). Even under iron-sufficient growth conditions, *A. vinelandii* strain UW produces 2,3-dihydroxybenzoic acid which solubilizes iron from a variety of natural iron-containing minerals (Page and Huyer 1984). However, if the available iron concentration is less than 7 μM, *A. vinelandii* strain UW produces the catecholate siderophores, azotochelin and aminochelin (Page and Huyer 1984; Page and von Tigerstrom 1988). These two siderophores are produced in abundance, but have a low affinity for Fe(III). Finally, if the prevailing iron concentration is growth-limiting (less than 3 μM), *A. vinelandii* UW produces the yellow-green fluorescent siderophore, azotobactin, which has high affinity for iron (Page and Huyer 1984). This differential control of siderophore biosynthesis by *A. vinelandii* in response to the ambient iron concentration attests to the importance of a continuous supply of iron to this diazotroph and suggests that regulation of siderophore production in *A. vinelandii* will be at least as complex as found in the well studied enteric bacteria (DeLorenzo et al. 1987; Page and Patrick 1988).

The molecular structure of the yellow-green fluorescent siderophore produced by *A. vinelandii* strain UW has not been reported but its pH-dependent absorption spectrum was found to resemble that of the *Pseudomonas* siderophores pyoverdin and pseudobactin (Teintze et al. 1981; Wendenbaum et al. 1983). It was this fact that prompted Knosp and Page (1983) to coin the trivial name 'azotobactin' for the *A. vinelandii* strain UW siderophore. Structural analysis of the fluorescent siderophores from *Pseudomonas* spp. has shown that each species and strain examined produces a pyoverdin siderophore with a different structure (Demange et al. 1986; Leong 1986). This strategy ensures that the chelated iron is used exlusively by the siderophore-producing strain or a strain that produces the corresponding ferrisiderophore receptor protein (Buyer and Leong 1986; Leong 1986).

Recently the structure of the yellow-green fluorescent azotobactin produced by iron-limited *A. vinelandii* strain D (Czechoslovakian Culture Collection CCM 289) was determined (Demange et al. 1988a). Although this compound was not tested for siderophore activity,

Offprint requests to: W. J. Page

its structural similarity to pyoverdin and pseudobactin siderophores leaves little doubt as to its function (Demange et al. 1988a). Like pyoverdin and pseudobactin, azotobactin D possesses a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline. However, the acyl group has been replaced in the latter by a carbonyl group to form an imidazolone ring (Demange et al. 1988a). Despite this structural difference, the decapeptide attached to the chromophore of azotobactin D: chromophore-aspartyl-seryl-homoseryl-glycyl-D-*threo*-β-hydroxyaspartyl-seryl-citrullinyl-homoseryl-N^{δ}-acetyl-N^{δ}-hydroxyornithinyl-homoserine is designed for the octahedral coordination of iron via the two modified amino acids, D-*threo*-β-hydroxyaspartic acid and N^{δ}-acetyl-N^{δ}-hydroxyornithine in conjunction with the catechol group of the chormophore (Demange et al. 1988a).

Azotobactin D possesses a chromophore structurally identical to that of the previously characterized fluorescent peptide produced by iron-limited *A. vinelandii* strain O (Fukasawa et al. 1972). However, the octapeptide sequence reported for azotobactin O: chromophore-aspartyl-homoseryl-seryl-homoseryl-citrullinyl-seryl-glycyl-D-*threo*-β-hydroxyaspartic acid (Fukasawa et al. 1972), reveals only two potential chelating groups, provided by the chromophore and the terminal β-hydroxyaspartic acid, indicating that this structure would be a different type of iron chelator compared to azotobactin D (Demange et al. 1988a), leading to complexes with a 3:2 stoichiometry. Since azotobactin D and the structurally related pyoverdins and pseudobactins always possess three iron-coordinating ligands (Abdallah 1991; Briskot et al. 1989; Demange et al. 1986, 1987, 1988a, 1989, 1990a, b, c; Poppe et al. 1987; Teintze et al. 1981; Teintze and Leong 1981; Weisbeek et al. 1986; Yang and Leong 1984), the structure of azotobactin from *A. vinelandii* strain O needs verification.

A. vinelandii strains O and D are of disparate origin. Strain O is a North American isolate in the University of Wisconsin collection (Monsour et al. 1955) and deposited in the American Type Culture Collection as ATCC 12518. Strain D is a European isolate deposited by H. L. Jensen in the Czechoslovakian Culture Collection as CCM 289 (Thompson and Skerman 1979). Our studies of cell structure, physiology and iron assimilation in *A. vinelandii* have involved the capsule-minus variant of strain O, strain OP (ATCC 13705), originally isolated by P. W. Wilson (Bush and Wilson 1959). The subculture of strain OP used in many laboratories, including ours, is called strain UW (University of Wisconsin). We undertook this study to determine the structure of azotobactin from cultures of *A. vinelandii* strain UW and strain O as an important aspect of our current studies concerning the genetics and regulation of siderophore biosynthesis in *A. vinelandii*.

Materials and methods

Bacteria and culture conditions. Azotobacter vinelandii strain UW was incubated for 24 h in Burk's iron-limited medium (Page and von Tigerstrom 1982) containing 1% glucose, 15 mM ammonium acetate and 60 mg micaceous hematite in 120 ml medium (Page and Huyer 1984). Culture medium in a 500-ml conical flask was inoculated to an initial optical density at 620 nm of 0.08 and shaken in the dark at 260 rpm, 30° C for 24 h. *A. vinelandii* strain O was grown aerobically in the medium described by Demange et al. (1988a), which contained 1 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 1.0 g $CaCO_3$; 0.2 g NaCl; and 10 g mannitol in 1 l distilled H_2O. Cultures were incubated at 30° C for 3 days as described previously (Demange et al. 1988a).

Purification of azotobactin from A. vinelandii strain UW and strain O. A. vinelandii strain UW cells were removed from the culture fluids by centrifugation and the culture supernatant fluid was sterilized by filtration (0.45-μm pore size Millipore filter). The culture fluid (100-ml batches) containing azotobactin was lyophilized and stored at −70° C. To purify azotobactin from cultures of *A. vinelandii* strain O or UW, filter-sterilized culture supernatant fluid was applied to and eluted from a column of octadecylsilane as previously described (Demange et al. 1988a). Fractions containing crude azotobactin were pooled and chromatographed on a DEAE-Sephadex A25 column. Azotobactin recovered from this column was further purified by HPLC as described by Demange et al. (1988a).

Electrophoresis. Electrophoresis was performed on samples spotted on to the center of cellulose acetate film and subjected to electrophoresis at 300 V for 30 min using 0.1 M pyridine/acetic acid pH 5.0. Following electrophoresis, the cellulose acetate membranes were illuminated with 366-nm light to detect fluorescent compounds and then sprayed with a solution of 1% (mass/vol.) $FeCl_3$ in distilled H_2O to detect iron-binding compounds.

Structural analyses. The techniques used for the structural analysis of azotobactin including amino acid analyses, circular dichroism, NMR and fast-atom-bombardment (FAB) mass spectroscopy were performed as previously described (Demange et al. 1988a).

Iron transport assays. Iron uptake assays by iron-limited *A. vinelandii* were performed using ^{55}Fe as described by Knosp et al. (1984).

Results

Production and initial purification of azotobactin from Azotobacter vinelandii *strain UW*

A. vinelandii strain UW and strain O excreted similar levels of the catechol siderophores into iron-limited culture fluids ($A_{310} = 0.190$–$0.200/10^8$ cells). However, *A. vinelandii* strain UW always produced more of the yellow-green fluorescent azotobactin than strain O. After 24 h of growth in iron-limited medium, the culture supernatant from strain UW had an A_{410} of $0.171/10^8$ cells whereas that from strain O had an A_{410} of $0.075/10^8$ cells. The iron-stress conditions of aerobic nitrogen fixation stimulated the production of azotobactin to a greater extent by strain O than by strain UW, but such increases were only realized after cultures were incubated for 3 days. A twofold stimulation of azotobactin by strain UW was caused by the addition of insoluble and limiting amount of mineral iron to the culture medium. Azotobactin production by strain O, however, could not be similarly induced to the same extent.

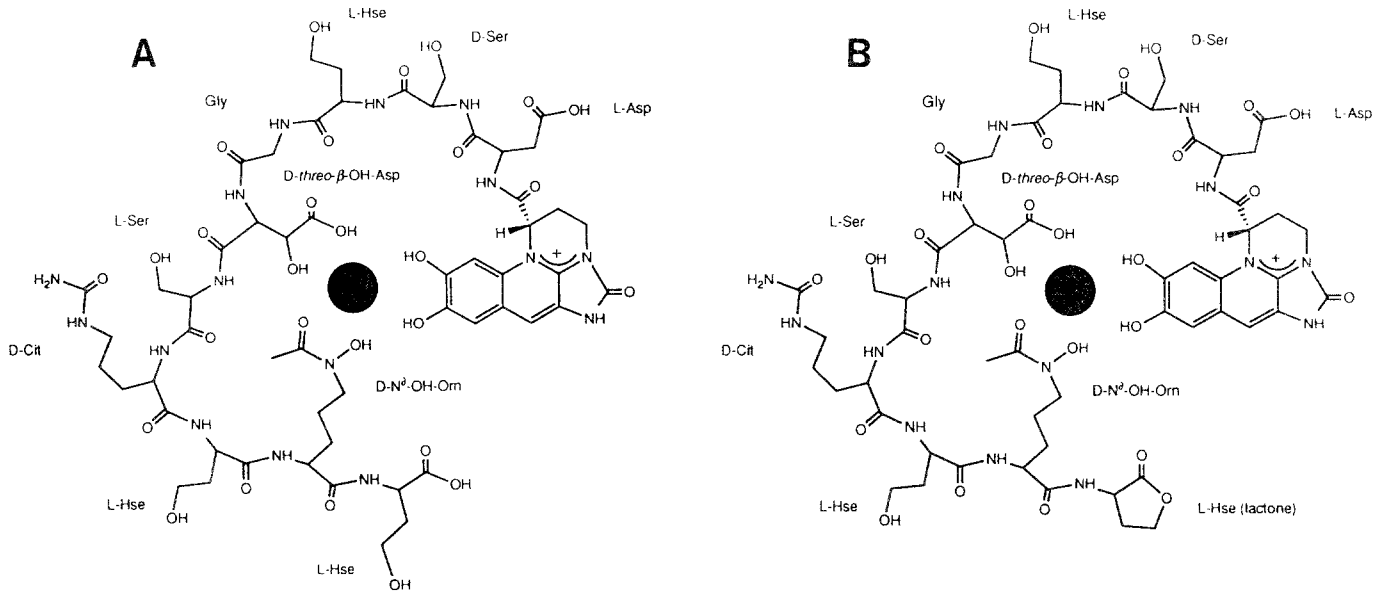

Fig. 1. Structure of azotobactin (**A**) and its lactonized form (**B**) isolated from *Azotobacter vinelandii* strain UW and O. Shaded areas indicate the position of Fe(III) in the ferrated molecule. Abbreviations: Asp, aspartic acid; Ser, serine; Hse, homoserine; Gly, glycine; Cit, citrulline; OH-Orn, hydroxyornithine

Structural analysis of azotobactin from strains O and UW

Cellulose acetate film electrophoresis indicated that the yellow-green water-soluble compound was constituted with approximately equal amounts of two anions, A and B, which fluoresced green and both bound iron. There was slight contamination of this sample with two minor compounds exhibiting blue fluorescence but they did not appear to bind iron. A and B were separated by HPLC and it was found that they had the same retention time and co-eluted with azotobactin δ (lactone) and azotobactin D, respectively. A and B also had the same electronic spectra for deferrated ($\lambda_{max} = 380$ nm at pH 4.0, $\varepsilon = 23\,500\ M^{-1}\ cm^{-1}$) and ferrated ($\lambda_{max} = 412$ nm, $\varepsilon = 23\,000\ M^{-1}\ cm^{-1}$) azotobactin D. Fast atom bombardment (FAB) revealed that fraction A and B had respectively the same molecular ions as azotobactin δ and azotobactin D, at m/z 1393 and m/z 1411, respectively. In addition, the FAB mass spectral fragmentation patterns confirmed that A and B had the same peptide sequence as azotobactin δ and azotobactin D and that A, like azotobactin δ, had a lactonized terminal homoserine (Fig. 1). ^{1}H-NMR spectra were also found to be identical with those previously reported for azotobactins with four signals between 7.82–5.80 ppm, three of them being characteristic of the chromophore and the other, at 5.81 ppm, corresponding to H-11 is a part of an AA'BB'X system. The aliphatic regions of the spectra were also identical to those previously determined for azotobactin D and azotobactin δ (Demange et al. 1988a).

The circular dichroic spectra of both compounds were also identical to those previously reported showing the identity of the configuration (*S*) of the common chromophore. Moreover, gas chromatography on (L)-Chirasil-Val (Demange et al. 1988b) of the derivatized total hydrolyzates of both compounds showed that their amino acids are the same as in azotobactin D and δ and possess the same configuration (Fig. 1).

Azotobactin was also isolated from *A. vinelandii* strain O and structurally analyzed as above. This strain also produced two azotobactin fractions which were determined to be structurally identical to azotobactin δ and azotobactin D.

Iron uptake activity of purified azotobactin

Azotobactin and its lactone form isolated from *A. vinelandii* strain UW were tested for the promotion of ^{55}Fe by iron-limited strain UW cells. Both forms of azotobactin were equal in their ability to promote the uptake of ^{55}Fe (Fig. 2). The uptake activity promoted by the purified siderophores was in both cases greater than that observed in the uptake buffer alone. However, there was significant ^{55}Fe uptake in the uptake buffer, due to the endogenous production of siderophores during the assay. This complicating factor has prevented an accurate estimate of the K_m for azotobactin or its lactone. The lactone form is most stable at acidic pH and will partly convert to azotobactin D at neutral pH. However, this conversion is not instanteneous and it was estimated that the concentration of azotobactin D formed in the azotobactin δ solution during the course of the iron assay was negligible and could not account for the equivalent rates of ^{55}Fe uptake observed. Therefore, both forms of azotobactin had approximately equivalent siderophore activity.

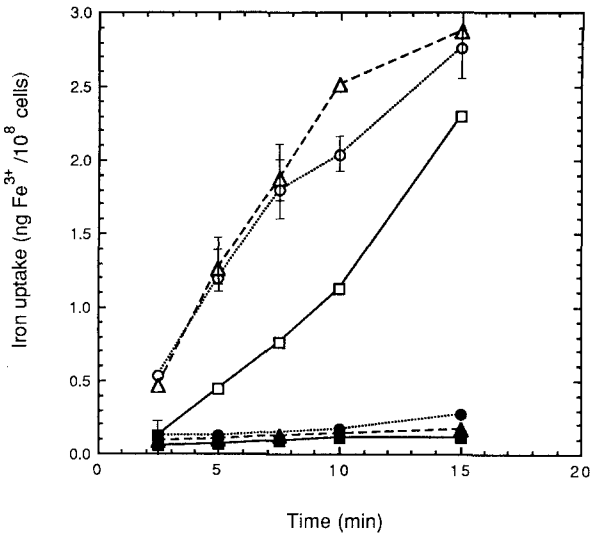

Fig. 2. Iron uptake by iron-limited *A. vinelandii* strain UW. Cells were resuspended in the following solutions to measure the uptake of iron: uptake buffer (□, ■); uptake buffer supplemented with azotobactin (△, ▲); uptake buffer supplemented with azotobactin δ (lactone) (○, ●). (■, ▲, ●) Assay performed on cells held at 0° C; (□, △, ○) cells assayed at 25° C. Error bars are noted where range of duplicate samples exceed 0.15 ng Fe(III)/10^8 cells

Discussion

This study reveals that the yellow-green fluorescent siderophore, azotobactin, isolated from *Azotobacter vinelandii* is structurally conserved among two *A. vinelandii* strains of disparate origin (strain D and strain O) and in strain UW, the capsule-negative variant of strain O. This is in marked contrast with the varied siderophore structures found among the fluorescents pseudomonads. Typically, pyoverdins and pseudobactins have a relative molecular mass of 1000–1500 and are composed of a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline bound to a peptide of 6–10 amino acids (Abdallah 1991; Mohn et al. 1990; Demange et al. 1986, 1987, 1988a, 1989, 1990a, b, c; Persmark et al. 1990; Philson and Lliñas 1982; Poppe et al. 1987; Teintze et al. 1981; Teintze and Leong 1981; Weisbeek et al. 1986; Wendenbaum et al. 1983; Yang and Leong 1984). This peptide varies in composition, even among some strains of the same species, but always contains two modified amino acids designed for the chelation of iron in concert with the catechol group of the chromophore (Demange et al. 1987). In addition, the amino group on the carbon C-3 of this latter is substituted with various acyl groups generally derived from succinate, malate or 2-oxoglutarate (Demange et al. 1987, 1989, 1990b; Poppe et al. 1987). While the structure and function of the various pyoverdins and pseudobactins produced by the fluorescent *Pseudomonas* are well documented, little was known, until now, concerning the structure of azotobactin from various *A. vinelandii* strains. The imidazolone ring of the chromophore obviates chemical modification at the amino group located on carbon C-3 of the chromophore of azotobactin, thereby reducing one site of structural variability normally seen with various pyoverdin structures. Two other factors which may contribute to the conservation of the azotobactin structure include the extreme polyploidy of the *A. vinelandii* cell, which would tend to minimize random mutagenesis leading to altered azotobactin structure (Phandis et al. 1988; Sadoff et al. 1979). Also prolonged iron-limitation can induce the encystment of *A. vinelandii* (Layne and Johnston 1964; Page 1983). In this form *A. vinelandii* can remain dormant for years, until revived by more favorable conditions of growth (Vela 1974).

This study establishes that the structure previously reported for azotobactin isolated from *A. vinelandii* strain O is not correct (Fukasawa et al. 1972). Although the structure of the chromophore was accurately determined, the authors failed to identify the presence of N^{δ}-acetyl-N^{δ}-hydroxyornithine and did not determine the correct number or sequence of amino acids in the peptide. This is very likely due to the acid work-up performed during their purification procedure, which certainly cleaves the peptide chain between the second homoserine and the dipeptide N^{δ}-hydroxyornithinyl-homoserine. These shortcomings likely reflect the difficulty in using solely chemical methods for the determination of peptide sequences containing these unusual amino acids. In fact Bulen and Lecomte (1962) came closer to the actual amino acid content using acid hydrolysis, paper chromatography and an amino acid analyzer to identify the amino acids present in azotobactin from *A. vinelandii* strain O. They observed a nonapeptide composed of serine, homoserine, glycine, citrulline, aspartic acid, β-hydroxyaspartic acid and ornithine in the ratio 2:2:1:1:1:1: < 1. However, they believed that the critical ornithine residue was merely a breakdown product of citrulline. In this current study, the determination of the sequence of the azotobactin peptide was facilitated by the techniques of NMR and FAB mass spectroscopy (Dell et al. 1982; Demange et al. 1988a, b). These techniques are the best methods for the identification of modified amino acids, notably the elusive N^{δ}-acetyl-N^{δ}-hydroxyornithine which is essential for iron chelation by this siderophore.

Azotobactin exists in equilibrium in solution with its lactone form, the former being predominant at physiological pH. Lactonization of the terminal homoserine apparently has no effect on the ability of azotobactin to function as a siderophore when tested under the assay conditions described herein. It is important to note that azotobactin has a constant structure (apart from the variation caused by lactonization) after growth for different periods of time (24 h, strain UW; 3 days, strain O; 6 days, strain D), using different carbon sources (glucose, strain UW; mannitol, strains O and D), or nitrogen sources (N_2, strains O and D; CH_3COONH_4, strain UW). Cultures incubated for more than 24 h contained $CaCO_3$, which was essential to maintain a neutral pH in the medium. Also, both pyoverdin and azotobactin will suffer photodecomposition at alkaline pH (Bulen and Lecomte 1962; Demange et al. 1987).

A. vinelandii does not produce extracellular depolymerases and is not found in as many environments as the *Pseudomonas* spp. *A. vinelandii* grows on a variety of simple carbon and nitrogen sources likely to be in demand in the soil (Thompson and Skerman 1979). It is not a good competitor for space in the rhizosphere or on the rhizoplane and is doubtfully a plant-growth-promoting rhizobacterium (Kloepper et al. 1980; Page and Dale 1986). *A. vinelandii* is a relatively rare isolate from agricultural soils, but is more commonly found in barren mineral soils (Rennie 1980) or oxidized tropical soils (Thompson and Skerman 1979). It is in these latter soils that the sequential pattern of siderophore derepression may provide a competitive advantage, provided nutrients and soil moisture are growth-promoting. Faced with continued iron-limitation, these bacteria become increasingly encapsulated and differentiate into dormants forms. Thus, *A. vinelandii* has developed an efficient but passive survival strategy, rather than the very aggressive stance assumed by *Pseudomonas* spp.

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