Siderophores Produced by Nitrogen-Fixing *Azotobacter vinelandii* OP in Iron-Limited Continuous Culture

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Azotobacter vinelandii requires a high complement of iron and an efficient iron acquisition system to support nitrogen fixation. To circumvent problems inherent in batch culture trace metal studies, continuous cultures were used to measure the response of *A. vinelandii* to iron stress. Iron was found to be growth limiting for nitrogen-fixing *A. vinelandii* at a concentration as high as 12.5 μM; iron was growth sufficient at 25 μM. Iron-stressed *A. vinelandii* in continuous culture formed 2,3-dihydroxybenzoic acid (DHB), 2-N,6-N-di-(2,3-dihydroxybenzoyl)-L-lysine (DHBL), and a chromophoric yellow-green fluorescent peptide (YGFP). At a fixed dilution rate of 0.1 h^{-1}, steady-state growth occurred at growth-limiting iron concentrations. DHB and DHBL were quantitatively measured during iron-limited steady states and iron-sufficient states by Arnow colorimetric assays. YGFP was determined by absorbance measurements taken at 380 nm, and the concentration was calculated from the reported specific absorption coefficient. Biomass increased and DHBL, DHB, and YGFP concentrations decreased as the concentration of growth-limiting iron was increased in the culture vessel and medium reservoirs. DHBL was the major siderophore and YGFP was the minor siderophore species produced during iron-limited equilibrium growth. A low level of DHB and YGFP, but no DHBL, was formed under iron-sufficient conditions. These results provide further physiological evidence that DHB, YGFP, and especially DHBL may function as siderophores in nitrogen-fixing *A. vinelandii*.

Iron is essential for the growth of almost all microorganisms (10). Although iron is abundant in nature, it is not readily available to microorganisms because of its extreme insolubility in aerobic water and soil environments. To sequester iron from the environment, microorganisms excrete high-affinity ferric chelating compounds, termed siderophores, which solubilize and transport the metal (11).

Nitrogenase and other proteins involved in nitrogen fixation require a high complement of iron (2). Therefore, it is likely that nitrogen-fixing organisms evolved efficient iron-acquisition mechanisms. Iron-deficient nitrogen-fixing cultures of *Azotobacter vinelandii* produce 2,3-dihydroxybenzoic acid (DHB), 2-N,6-N-di-(2,3-dihydroxybenzoyl)-L-lysine (DHBL) (5), and a yellow-green fluorescent peptide (YGFP) (3). Although the chemical nature of these molecules and their physiological response to iron make them strong candidates for siderophores (12), it has yet to be demonstrated that these compounds serve as siderophores in *A. vinelandii*.

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However, *A. vinelandii* does induce the production of four high-molecular-weight outer membrane proteins in response to iron stress (13).

Batch culture procedures may be unsuitable for defining microbial iron requirements and measuring the response of a microorganism to iron privation since these systems are always in transient states (1). In contrast, continuous cultures simplify the system and facilitate the elucidation of the reaction of an organism to its environment. For example, Archibald and De-Voe obtained a variety of basic data on the iron requirements and uptake of iron by *Neisseria meningitidis* with iron-limited continuous cultures (1). Similarly, Strachan et al. used a Teflon chemostat to measure trace metal requirements of *Streptococcus mutans* and *Bacillus subtilis* (16).

In the present study, the response of *A. vinelandii* to nutritional iron stress was effectively measured in continuous cultures during three steady states of various iron limitation at a fixed dilution rate. The reported steady-state system demonstrated the high iron requirement of the organism and provided further physiological evidence that DHB, DHBL, and YGFP may func-

tion as siderophores in nitrogen-fixing A. vinelandii.

MATERIALS AND METHODS

Organism and media. Starter and continuous cultures of A. vinelandii OP (ATCC 13705) were grown on medium B_6 as described by Dalton and Postgate (7), with 20 g of sucrose per liter (grade I; Sigma Chemical Co.) in place of mannitol. $FeSO_4 \cdot 7H_2O$ was omitted from the stock trace element solution and added as appropriate. All medium components were made with double-glass-distilled water.

To prevent salt precipitation due to autoclaving, medium B_6 salt solution was sterilized by membrane filtration with 0.4-μm polycarbonate filters (Nuclepore Corp.). Sucrose, iron, and trace element solutions were sterilized separately by autoclaving (121°C) and added later.

To reduce iron contamination, all glassware used in medium preparation and the entire chemostat culture system, including medium reservoirs and lines, was soaked in 0.025 M EDTA tetrasodium salt for 6 h, followed by rinsing with double-distilled water.

Cell growth. An A. vinelandii OP stock slant culture was used to inoculate 50 ml of medium B_6 plus iron (25 μM) in a 500-ml baffled Erlenmeyer flask. This starter culture was incubated at 30°C and 200 rpm on a Gyrotory incubator (model G 76; New Brunswick Scientific Co.) until mid-exponential phase.

For continuous cultures, a Teflon chemostat as described by Strachan et al. (16) was used with the following modifications. Gas input consisted of compressed air, a flowmeter (Rotameter model 603; Matheson Gas Products), an in-line glass fiber filter (8 by 1 cm), and a sintered glass air sparger. The modifications provided high sterile air flow rates so that in conjunction with vigorous magnetic stir bar agitation, oxygen absorption rates of 2 mmol of O_2 liter^{-1} min^{-1} (determined by the sulfite oxidation method [4]) were attained in 300-ml chemostat working volumes. The pH was monitored, but no pH control was necessary owing to the buffering capacity of medium B_6, which maintained a constant pH value of 6.8 (7). The culture vessel, medium reservoirs, and all influent and effluent lines were sterilized by autoclaving (121°C, 30 min). A working volume of 300 ml of medium B_6 plus 25 μM iron was added. Incubation temperature was 30 ± 0.2°C, and air flow was 0.6 liter min^{-1}. The culture vessel was inoculated with a 5% (vol/vol) inoculum from the starter culture described above, and after ca. 18 h (0.4 mg [dry weight] of cells ml^{-1}), low-iron medium flow at a dilution rate of 0.1 h^{-1} was started. Reservoir volume was limited to 1 liter of iron-limited medium B_6 to prevent precipitation of metal phosphates and ferric hydroxide with time.

Equilibrium under iron-limited conditions was maintained for at least four chemostat culture generation times (40 h) before siderophore measurements were done and the iron-limiting conditions were changed.

Analytical procedures. Biomass concentration was determined by dry weight measurements by the method of Dalton and Postgate (6) with 0.45-μm cellulose acetate membrane filters (type HA; Millipore Corp.). Quantitative determinations of DHB and DHBL were done by high-voltage paper electrophoresis of ethyl acetate extracts from 50-ml samples of culture super-

natant fluids by the method of Corbin and Bulen (5). DHB and DHBL concentrations were reported as averages of three 50-ml culture samples taken during steady-state growth periods. All sampling, including biomass determinations, was done from the pump-out effluent line to avoid equilibrium growth perturbation.

Quantitation of YGFP was determined from the specific absorption coefficient reported by Bulen and LeComte (3). Samples (2 ml) from the aqueous fractions of the above DHB and DHBL ethyl acetate extractions were dried in air and dissolved in 2 ml of 0.1 N HCl, and absorbance measurements were taken at 380 nm ($\epsilon = 1.595 \times 10^4$ M^{-1} cm^{-1}). Spectrophotometric (Varian Cary 219 recording spectrophotometer) analyses of acidic and neutral aqueous samples at all sampling periods were done as a control and reproduced the reported YGFP electronic absorption spectra (3).

Total chelate assay, based on the chelin assay method of Ketchum and Owens (9), was taken as a measure of the combined DHB, DHBL, and YGFP concentration in chemostat culture supernatant fluids. Consistent with other reports (13, 15), electronic absorption spectra of iron-deficient Azotobacter culture supernatant fluids showed that binding of Mo(VI), added as MoO_4^{2-}, occurred with an absorption maximum at 400 nm. Total chelate assay consisted of adding 0.1 ml of a 0.01 M Na_2MoO_4 solution to 2 ml of chemostat culture supernatant fluid and reading the absorbance at 400 nm.

RESULTS AND DISCUSSION

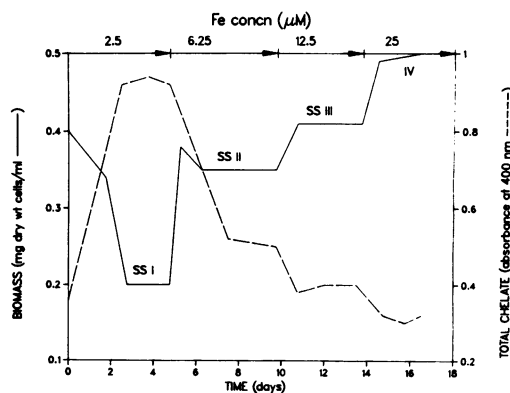Figure 1 illustrates the increase in A. vinelandii biomass and subsequent decrease in total

FIG. 1. Effect of iron on growth and total chelate produced by A. vinelandii in continuous culture. A batch culture was started in the chemostat at 30°C in medium B_6 containing 25 μM Fe. At a biomass of 0.40 mg (dry weight) of cells ml^{-1}, medium B_6 containing 2.5 μM Fe was started at a dilution rate of 0.1 h^{-1}. After growth equilibrium was reached, stepwise increases in Fe concentration were made at the indicated concentrations and times by simultaneous addition to both the culture vessel and medium reservoir. Total chelate was measured by absorbance at 400 nm of the molybdate-reactive YGFP, DHB, and DHBL in chemostat culture supernatant fluids.

chelate produced as the limiting iron concentration was increased in the chemostat system. This pattern was obtained in five A. vinelandii chemostat runs with reproducibility of biomass concentrations within 5%. The relation between total chelate and biomass as a function of iron limitation suggests that much of the metabolic energy of A. vinelandii was directed to siderophore synthesis at the expense of cell growth.

Our continuous-culture data are in agreement with data obtained with A. vinelandii batch systems on the accumulation of DHBL and YGFP in the culture medium; i.e., there is an inverse correlation of growth with the production of the catecholate siderophores under conditions of iron deficiency (15). The total chelate assay was useful as a relative measure of the three catecholates in chemostat culture supernatant fluids, since all were molybdate reactive and formed stable yellow complexes with absorption maxima at 400 nm (15).

The total chelate data became more meaningful when the three compounds were measured separately during iron-limited steady states (Fig. 2; SS I, SS II, and SS III) and under iron-sufficient conditions (Fig. 2, IV). The major siderophore species produced by nitrogen-fixing A. vinelandii continuous cultures during all three steady states was DHBL. During iron-sufficient conditions, low levels of DHB and YGFP, but not DHBL, were formed. These data are consistent with the study of Corbin and Bulen on the effect of iron on the formation of DHB and DHBL in nitrogen-fixing A. vinelandii closed systems (5). They also found that DHBL is produced in larger yields than DHB under iron-deficient conditions and that DHB, but not DHBL, is formed under iron-sufficient growth conditions. Furthermore, the microbial siderophore assay developed in this laboratory also confirms the formation of DHB, and especially DHBL siderophore species, by iron-stressed nitrogen-fixing A. vinelandii (8). YGFP was below detectability under the conditions used in the microbial siderophore assay, and it also proved to be the minor siderophore species produced in the present study (Fig. 2). There are no other reports in the literature concerning relative yields of YGFP, DHBL, and DHB.

The high requirement of A. vinelandii for iron needed to support nitrogen fixation was demonstrated, since suboptimal growth was attained with limiting iron concentrations as high as 12.5 μM (Fig. 1); furthermore, culture wash-out occurred readily at iron concentrations below 2.5 μM in this chemostat system (data not shown).

The Teflon chemostat was highly adaptable to A. vinelandii iron physiology studies, since chemostat runs of up to 3 weeks in duration were achieved. Contamination in medium reservoirs
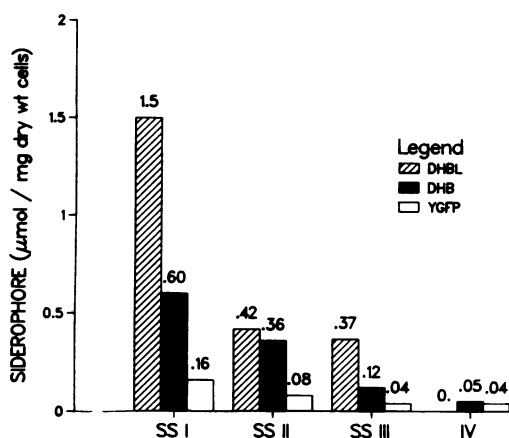

FIG. 2. Quantitative determinations of DHBL, DHB, and YGFP during Fe-limited steady states (SS I, SS II, and SS III) and Fe-sufficient growth (IV). Steady-state growth was maintained for 40 h before siderophores were measured on 50-ml chemostat culture samples. Siderophore determinations were done at 40 h after the initiation of Fe-sufficient conditions (IV).

and input lines did not occur since medium B_6 was free of fixed nitrogen. Also, wall growth was minimal due to high culture turbulence in the chemically inert, nonsticking Teflon culture vessel.

Since the siderophores of A. vinelandii complex Mo in addition to iron (F. A. Fekete, T. Emery, and J. T. Spence, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K88, p. 152; 15), attempts were made to elicit a possible Mo-coordinating compound from Mo-deficient, Fe-sufficient A. vinelandii cultures. All attempts to attain steady-state conditions under various continuous-culture parameters of oxygen absorption and dilution rates where Na_2MoO_4 served as the limiting nutrient were unsuccessful. The efficient Mo accumulation and storage mechanism of A. vinelandii were possible reasons for failure (14).

The reported iron-limited continuous-culture system provided substantial physiological evidence that YGFP, DHB, and, in particular, DHBL may function as siderophores in nitrogen-fixing A. vinelandii.

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