The catecholate siderophores of *Azotobacter vinelandii*: their affinity for iron and role in oxygen stress management

Anthony S. Cornish and William J. Page

Author for correspondence: William J. Page. Tel: +1 403 492 4782. Fax: +1 403 492 2216.
e-mail: bill.page@ualberta.ca

Department of Biological
Sciences, University of
Alberta, Edmonton,
Alberta, Canada T6G 2E9

In iron-limited medium, *Azotobacter vinelandii* strain UW produces three catecholate siderophores: the tricatecholate protochelin, the dicatecholate azotochelin and the monocatecholate aminochelin. Each siderophore was found to bind Fe^{3+} preferentially to Fe^{2+} , in a ligand:Fe ratio of 1:1, 3:2 and 3:1, respectively. Protochelin had the highest affinity for Fe^{3+} , with a calculated proton-independent solubility coefficient of $10^{43.9}$, comparable to ferrioxamine B. Iron-limited wild-type strain UW grown under N_2 -fixing or nitrogen-sufficient conditions hyper-produced catecholate siderophores in response to oxidative stress caused by high aeration. In addition, superoxide dismutase activity was greatly diminished in iron-limited cells, whereas catalase activity was maintained. The ferredoxin I (Fdl)-negative *A. vinelandii* strain LM100 also hyper-produced catecholates, especially protochelin, under oxidative stress conditions, but had decreased activities of both superoxide dismutase and catalase, and was about 10 times more sensitive to paraquat than strain UW. Protochelin and azotochelin held Fe^{3+} firmly enough to prevent its reduction by $\cdot\text{O}_2^-$ and did not promote the generation of hydroxyl radical by the Fenton reaction. Ferric-aminochelin was unable to resist reduction by $\cdot\text{O}_2^-$ and was a Fenton catalyst. These data suggest that under iron-limited conditions, *A. vinelandii* suffers oxidative stress caused by $\cdot\text{O}_2^-$. The catecholate siderophores azotochelin, and especially protochelin, are hyper-produced to offer chemical protection from oxidative damage catalysed by $\cdot\text{O}_2^-$ and Fe^{3+} . The results are also consistent with Fdl being required for oxidative stress management in *A. vinelandii*.

Keywords: *Azotobacter vinelandii*, siderophore, iron affinity, oxygen stress

INTRODUCTION

Iron in aerobic, neutral-pH environments has very low solubility, in the order of 10^{-17} M, with most iron present as insoluble hydroxides and oxyhydroxides. This is far below the minimal concentration required for the optimal growth of micro-organisms and necessitates the production of iron chelators called siderophores which promote high-affinity iron transport (Guerinot, 1994). We have been studying the growth and survival of the obligately aerobic, Gram-negative soil organism

Azotobacter vinelandii under various stress conditions. *A. vinelandii* produces three catecholate siderophores under iron-limited conditions: azotochelin (Corbin & Bulen, 1969), aminochelin (Page & von Tigerstrom, 1988) and protochelin (Cornish & Page, 1995) (Fig. 1). Protochelin is formed from equimolar amounts of azotochelin and aminochelin; for unknown reasons, only very small quantities of protochelin are normally observed unless millimolar molybdate also is present in the growth medium (Cornish & Page, 1995). These catecholate siderophores are potentially excellent iron chelators at neutral pH (Hider, 1984).

A. vinelandii also has the ability to fix N_2 to survive in nitrogen-limited environments. The nitrogenases formed may have either Fe-Mo, Fe-V, or Fe-only active

Abbreviations: 2,3-DHBA, 2,3-dihydroxybenzoic acid; Fdl, ferredoxin I; SOD, superoxide dismutase; Fe-SOD, iron-dependent SOD; Mn-SOD, manganese-dependent SOD.

Fig. 1. Catecholate siderophores produced by *A. vinelandii*: [A] azotochelin, [B] aminochelin, [C] protochelin (Cornish & Page, 1995).

centres and are very sensitive to inactivation by O_2 (Bishop, 1993). To protect the nitrogenase under aerobic conditions, *A. vinelandii* has a branched cytochrome chain with high-affinity terminal oxidases that 'waste' O_2 by the formation of water without ATP synthesis (respiratory protection; Kolonay *et al.*, 1994). This gives *A. vinelandii* the highest respiratory rate of any known organism (Jurtshuk & Yang, 1980). Unfortunately, this respiratory activity also forms superoxide radical ($\cdot O_2^-$) and H_2O_2 . Furthermore, hydroxyl radicals ($\cdot OH$) may form spontaneously in the Fenton reaction by the interaction of H_2O_2 and Fe^{2+} , which is formed by the reduction of Fe^{3+} in the presence of $\cdot O_2^-$ (Cohen, 1994).

These toxic oxygen species can cause mutagenic events, membrane peroxidation and the disintegration of ribosomes, and in general lead to cellular damage collectively referred to as oxidative stress (DiGuseppi & Fridovich, 1984). The response to oxidative stress is most dramatic when facultative anaerobes, like *Escherichia coli*, make the transition from anaerobic to aerobic environments. Under these conditions, *E. coli* induces the *oxyR* regulon in response to H_2O_2 and the *soxRS* regulon in response to $\cdot O_2^-$ (Dempse, 1996). It remains an open question whether oxidative stress management exists in an obligate aerobe like *A. vinelandii*. However, it is likely that as oxygen availability in the soil fluctuates, N_2 -fixing growth would increase the demand for protection from toxic O_2 products, and the limited availability of iron in aerobic soil would decrease the cell's ability to provide respiratory protection and active catalase or iron superoxide dismutase (SOD) enzymes. It also has been proposed that ferredoxin I (FdI) may be part of a SoxRS-like system in *A. vinelandii* (Isas *et al.*, 1995). In addition, siderophores have been shown to decrease oxidative stress in the aerobe *Pseudomonas aeruginosa* by preventing the formation of $\cdot OH$ by chelating and thus removing soluble Fe^{3+} from the Fenton reaction (Coffman *et al.*, 1990). This activity is dependent on the affinity that the siderophore has for Fe^{3+} and hence the ability of the complex to resist reduction by $\cdot O_2^-$. Thus, it was the purpose of this study to observe the interplay of iron limitation and oxidative stress management in wild-type and an isogenic FdI-negative strain of *A. vinelandii*.

METHODS

Bacterial strains and growth conditions. The strains used in this work were wild-type *Azotobacter vinelandii* UW (OP, ATCC 13705) and the isogenic FdI-negative (*fdxA*) mutant strain LM100 (Morgan *et al.*, 1988). The basal medium used for all studies was Burk's medium (Cornish & Page, 1995). This medium was made nitrogen-sufficient by the addition of 15 mM ammonium acetate, while nitrogen-free medium contained 15 mM sodium acetate. Iron-sufficient medium contained 20–75 μM ferric citrate; iron-limited medium contained 1 μM ferric citrate and all glassware was acid-washed. Strains were maintained on slants of Burk's medium and liquid cultures were inoculated and incubated at 28 °C with shaking at 225 r.p.m.

Growth and siderophore production under conditions of oxygen stress. Progressively smaller volumes of medium were incubated with shaking (225 r.p.m.) in 500 ml Erlenmeyer flasks to increase the aeration of the cultures. In some experiments, paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) was added at concentrations of 0.25–30 μM to increase oxidative stress (Korbashi *et al.*, 1986).

The cells were removed from the growth medium by centrifugation and total cellular protein was determined by the Lowry method. Siderophores were detected either by the colorimetric assay of Barnum (1977) or by measuring the A_{310} of acidified (pH 1.8 with 12 M HCl) culture supernatant fluid using a Hitachi U-2000 recording spectrophotometer. Catecholate siderophore concentrations in stock solutions were quantified by using the absorption coefficient for 2,3-dihydroxybenzoic acid (2,3-DHBA) in 80% methanol of $3.2629 \times 10^{-3} A_{310} \text{ cm}^{-1} \mu M^{-1}$, or in buffer of $3.2455 \times 10^{-3} A_{310} \text{ cm}^{-1} \mu M^{-1}$, corrected for the number of 2,3-DHBA moieties per siderophore. Azotobactin in acidified culture supernatant fluid was quantified by measuring A_{380} (Page & Huyer, 1984).

Production and identification of siderophores. Culture supernatant fluid of strain LM100 was used as a source of all catecholate siderophores, as protochelin was formed without the addition of 1 mM molybdate. Iron-limited medium (400 ml in a 2 litre flask) was inoculated with iron-limited strain LM100 (4%, v/v) and after 24 h incubation cells were removed by tangential-flow filtration using dual 0.22 μm pore size Pellicon (Millipore) filters. The culture fluid volume (9.6 litres) was reduced to 400 ml under vacuum in the dark at 37 °C using a Buchi RE111 rotary evaporator.

Siderophores were isolated according to the following protocol. The culture fluid was acidified to pH 1.8 with 12 M HCl and extracted twice with an equal volume of ethyl acetate to

concentrate azotochelin, protochelin and 2,3-DHBA (Cornish & Page, 1995). The remaining acidic aqueous layer contained aminochelin (Page & von Tigerstrom, 1988) and was neutralized to pH 7.0 with 12 M NaOH, reduced in volume to 50 ml and extracted five times with an equal volume of butanol. This extract was then evaporated to dryness and the resulting residue was dissolved in 5 mM ammonium acetate (pH 7.0) for application to a 2.5 cm $\times$ 30 cm CM-Sephadex (Pharmacia) column equilibrated in 5 mM ammonium acetate (pH 7.0). Fractions (5 ml) were eluted with a 400 ml gradient of 5–2000 mM ammonium acetate (pH 7.0) and A_{310} was used to detect catecholates. Fractions containing aminochelin were confirmed with TLC by comparison to authentic aminochelin isolated by the methods of Page & von Tigerstrom (1988). Fractions containing aminochelin were pooled, reduced in volume to 25 ml or less and extracted five times with butanol. The butanol extract was then evaporated to dryness under N_2 and the resulting residue was suspended in 80% methanol and stored at -20°C .

The ethyl acetate fractions were pooled and reduced in volume to 200 ml. The concentrate was washed twice with an equal volume of 100 mM sodium phosphate buffer (pH 7.0), which removed azotochelin and 2,3-DHBA. Protochelin remained in the ethyl acetate layer and was concentrated and dissolved in 80% methanol. Purity was confirmed by TLC (Cornish & Page, 1995) and the fraction was stored at -20°C .

The aqueous phase containing azotochelin and 2,3-DHBA was reduced to 200 ml, acidified to pH 1.8 with 12 M HCl, and extracted twice with an equal volume of ethyl acetate. The solvent was evaporated and the residue was resuspended in a minimum volume of 80% methanol for application to a 2.5 cm $\times$ 30 cm Sephadex LH-20 (Pharmacia) column equilibrated with 80% methanol. Azotochelin and 2,3-DHBA were separated by elution with 80% methanol. Catecholates in fractions were detected by A_{310} and identified by TLC (Cornish & Page, 1995). Azotochelin-containing fractions were pooled, evaporated under N_2 , resuspended in a minimal volume of 80% methanol, and stored at -20°C .

Molar iron-binding ratios and iron-affinity determination. Molar binding ratios of the three catecholate siderophores and Fe^{3+} were determined by the continuous-variation method of Job (Chaberek & Martell, 1959). Each catecholate siderophore was mixed with Fe^{3+} (as ferric nitrate) in 100 mM MOPS buffer (pH 7.0) at various ratios and the absorbance of these mixtures was measured at 300–700 nm until each solution reached equilibrium. The ratio of siderophore to Fe^{3+} that gave the maximum absorbance represented the molar binding ratio. The wavelength of the maximum absorbance at the molar binding ratio was used in all further work.

The affinity of each siderophore for Fe^{3+} and Fe^{2+} (as ferrous sulfate), was determined by competition with EDTA as described by Reid *et al.* (1993). The iron-siderophore complex (0.1 mM final concentration) was allowed to form and equilibrate for 72 h before being mixed with 0.05–2.0 mM (final concentration) EDTA and incubated for 96 h at room temperature. Conversely, solutions containing 0.05 mM to 2.0 mM iron-EDTA complex were incubated with 0.1 mM siderophore for 96 h. The concentration of the iron-siderophore complex in each reaction at equilibrium was determined spectrophotometrically by comparison to a standard curve. Protochelin was added to these assays in 80% methanol because of the low solubility of the uncomplexed ligand in aqueous solution, as described by Harris *et al.* (1979). Aminochelin and azotochelin stocks were dried under a N_2 stream and dissolved in 100 mM MOPS buffer (pH 7.0) before use. All reactions (250 μl volume) were done in 96-well

microtitre plates and absorbance was measured with a Biotek Instruments EL311 microplate reader, with a minimum of two duplicate series containing six replicates of each reaction mixture.

Cell-free extract preparation and enzyme activity. Strains UW and LM100 were grown under standard conditions in 200 ml iron-limited or iron-sufficient (75 μM ferric citrate) medium. Cells were harvested by centrifugation (4000 g, 10 min) and the cell pellet was washed with 50 mM potassium phosphate buffer (pH 7.6) containing 2 mM DTT. Cells were resuspended in buffer and lysed in a French press as described by Page & von Tigerstrom (1982). The cell extract was cleared by centrifugation (40000 g, 1 h) and the resulting cell-free extract was stored at -20°C .

Catalase activity was determined by following the inhibition of H_2O_2 -mediated reduction of dichromate Cr(VI) to chromic Cr(II) acetate (Sinha, 1972). One unit (U) of catalase activity was defined as the degradation of 1 μmol H_2O_2 min^{-1} (ml enzyme^{-1}). SOD activity was measured by its ability to inhibit the reduction of nitro blue tetrazolium (NBT) by $\cdot\text{O}_2^-$ (Oberley & Spitz, 1985). One unit of SOD activity was defined as the amount of protein required to inhibit the maximal rate of NBT reduction by 50%, which was obtained graphically. Values reported are means calculated from at least duplicate assays in which the standard deviation was not greater than 10%.

Catalase and SOD species were visualized after non-denaturing PAGE of cell-free extract protein. The proteins were separated in 7.5% acrylamide (100 cm $\times$ 80 cm Hoeffer mini-gel apparatus) under a 15–25 mA current. Catalase activity was demonstrated as described by Clare *et al.* (1984) except that the gel was soaked in 10 U horseradish peroxidase ml^{-1} for 105 min and 5.0 mM H_2O_2 for 45 min. SOD activity was located as described by Beauchamp & Fridovich (1971). The iron-dependent SOD (Fe-SOD) was inhibited by soaking the gel in 5 mM H_2O_2 for 20 min before the normal SOD activity stain was applied (Allgood & Perry, 1985).

Oxygen radical generation. The ability of ferric-siderophore complexes to withhold iron from the Fenton reaction was tested as follows. Siderophore from an 80% methanol stock was concentrated by evaporation under N_2 , resuspended in 60 mM potassium phosphate buffer (pH 7.6) at 37°C and mixed with 10 mM ferric nitrate in the appropriate molar ratio to obtain a ferric-siderophore that was 100% saturated with Fe^{3+} . Ferric-siderophore complexes were allowed to form for at least 72 h before use. Iron released from a siderophore after its reduction to Fe^{2+} by $\cdot\text{O}_2^-$ was followed with ferrozine (Coffman *et al.*, 1990), except that ferric-siderophore complexes containing a potential free Fe^{2+} concentration of 15 to 45 μM were used. Each reaction contained 100 mU xanthine oxidase ml^{-1} ; 500 U catalase ml^{-1} or 30 U SOD ml^{-1} was also added to some reactions. The molar absorption coefficient for the Fe^{2+} -ferrozine complex of 27900 $\text{A}_{562} \text{ cm}^{-1} \text{ M}^{-1}$ (Stookey, 1970) was used to calculate the amount of Fe^{2+} released.

The detection of $\cdot\text{OH}$ generation by the Fenton reaction was based on Coffman *et al.* (1990). Ferric-siderophore complexes were used which contained potential free Fe^{2+} concentrations of 5–30 μM . Ferric-EDTA in the same concentration range was used as a positive control for $\cdot\text{OH}$ generation. Xanthine oxidase was used at a final concentration of 100 mU ml^{-1} in reactions (1.0 ml) containing 6 mM potassium phosphate buffer (pH 7.6) and 4.5 mM deoxyribose. Catalase (500 U ml^{-1}) and SOD (30 U ml^{-1}) were added to some reactions. Reactions were allowed to proceed for 15 min at room temperature and were stopped by the addition of 250 μl 14.4%

(w/v) trichloroacetic acid. Deoxyribose that had reacted with $\cdot\text{OH}$ formed a coloured complex with the addition of 250 μl 1% (w/v) thiobarbituric acid in 0.1 M NaOH. After heating for 15 min at 100 $^{\circ}\text{C}$, the resulting A_{532} values were expressed as a percentage of the ferric-EDTA control value.

RESULTS

Oxidative stress and catecholate siderophore production

Growth of wild-type *A. vinelandii* strain UW increased in iron-limited, nitrogen-free and nitrogen-sufficient medium as aeration increased until toxicity was reached at the highest aeration rate (Fig. 2a). Growth of N_2 -

fixing cells was adversely affected by iron limitation and increased aeration. Growth of strain LM100 in nitrogen-free medium was essentially the same as that of the wild-type, confirming that the mutation in FdI did not affect N_2 fixation ability (Morgan *et al.*, 1988). Growth of strain LM100 in nitrogen-sufficient medium was also like that of the wild-type, except at the highest aeration values (Fig. 2a). Iron-limited strains UW and LM100 demonstrated enhanced production of catecholate siderophores per cell protein as the aeration rate was increased (Fig. 2b). There was no apparent response of azotobactin production to increased aeration (data not shown), so this siderophore was not examined further in this study. Catecholate siderophore overproduction was most evident in N_2 -fixing cultures, which formed more catecholate than the corresponding cells grown with NH_4^+ , even at low aeration (Fig. 2b).

Fig. 2. (a) Effect of increased aeration on growth of *A. vinelandii*. Growth was estimated as cell protein after 22 h incubation of iron-limited nitrogen-sufficient medium with strain UW (●) or LM100 (■), and of iron-limited nitrogen-free medium with strain UW (○) or LM100 (□). (b) Effect of increased aeration on the formation of catecholate siderophores. Production of catecholates was measured in culture fluids from (a) (symbols are the same).

Culture supernatants were fractionated with ethyl acetate and sodium phosphate and the catecholates present in each fraction were identified by TLC and quantified by A_{310} measurements. In both nitrogen-sufficient and nitrogen-free cultures of strain UW, aminochelin and azotochelin were present in a 1:0.9 ratio and accounted for about 95–97% of the catecholates present, regardless of aeration rate. Protochelin was barely detectable on TLC and accounted for only about 4–5% of total catecholates (3–4 μM in culture fluid). Similarly, in nitrogen-free cultures of strain LM100, aminochelin and azotochelin were present in a 1:0.8 ratio and accounted for about 92% of the catecholates present. Protochelin was again barely visible on TLC and accounted for about 6% of total catecholates, regardless of aeration rate. Similar abundance of the three catecholates were found in nitrogen-sufficient cultures of strain LM100 at low aeration, but protochelin became very definite on TLC and accounted for 17% of total catecholates (17 μM in culture fluid) as aeration was increased (100 ml per flask in Fig. 2a). At the next two higher aeration rates, which were growth inhibitory (Fig. 2a), protochelin declined to 13% and 6% of total catecholates.

Affinity of catecholate siderophores for Fe^{3+} and Fe^{2+}

Assay of the molar iron-binding ratio showed that each purified siderophore bound either Fe^{3+} or Fe^{2+} in a ratio consistent with siderophore structure (Fig. 1): protochelin 1:1, azotochelin 3:2, and aminochelin 3:1. In all cases, the ferric- and ferrous-siderophore complexes exhibited broad spectral peaks; the A_{max} at equilibrium was at 490 nm with protochelin and at 570 nm with azotochelin. Only aminochelin demonstrated different A_{max} wavelengths with Fe^{3+} (490 nm) or Fe^{2+} (540 nm). A stable complex with Fe^{3+} was formed most rapidly by protochelin (18 h), followed by aminochelin (40 h) and by azotochelin (68 h).

Using the determined molar binding ratios, the proton-dependent solubility constant for each siderophore with Fe^{3+} and Fe^{2+} was determined by competition with

Table 1. Catecholate siderophore solubility constants with Fe³⁺ and Fe²⁺

Reaction tested*	log ₁₀ K _{Fe-Sid}	Mean log ₁₀ K _{Fe-Sid}	Reaction tested*	log ₁₀ K _{Fe-Sid}	Mean log ₁₀ K _{Fe-Sid}
PcFe ³⁺ + EDTA	24.33	24.14	PcFe ²⁺ + EDTA	13.39	13.44
Pc + Fe ³⁺ EDTA	23.48		Pc + Fe ²⁺ EDTA	13.85	
AzCFe ³⁺ + EDTA	53.55	53.47	AzCFe ²⁺ + EDTA	31.64	31.67
AzC + Fe ³⁺ EDTA	53.42		AzC + Fe ²⁺ EDTA	31.75	
AmCFe ³⁺ + EDTA	30.13	30.61	AmCFe ²⁺ + EDTA	19.19	19.28
AmC + Fe ³⁺ EDTA	31.25		AmC + Fe ²⁺ EDTA	19.51	

* Ligands: Pc, protochelin; AzC, azotochelin; AmC, aminochelin; all were used at appropriate molar binding ratios.

Table 2. Enzyme activities and electromorph relative mobilities from cells grown under iron-sufficient and iron-limited conditions

Strain	Iron content of medium (μM)	SOD electromorph (R _F)*	SOD activity [U (mg protein) ⁻¹]	Catalase electromorphs (R _F)†	Catalase activity [U (mg protein) ⁻¹]
UW	1	0.9	1.2	0.2; 0.5; 0.6	38.4
	75	0.9	47.0	0.2; 0.5; 0.6	38.6
LM100	1	0.9	1.9	(0.2)‡; 0.5; 0.6	20.2
	75	0.9	18.4	(0.2)‡; 0.5; 0.6	18.5

* 30 μg cell-free extract protein was used for PAGE.

† 100 μg cell-free extract protein was used for PAGE.

‡ The electromorph at R_F 0.2 was very faintly present.

EDTA (Table 1). Equilibrium was approached from either direction as the iron-siderophore complex was challenged with EDTA or the Fe-EDTA complex was challenged with siderophore. The values of K_{Fe-Sid} were very close to each other, indicating that equilibrium could be approached from either direction. However, because of the different stoichiometry of each of the iron-binding reactions it was impossible to directly compare the solubility constants generated for each siderophore, since each value had different units. Thus it was only possible to directly compare the solubility constants that each siderophore had for Fe³⁺ or Fe²⁺ (Table 1). From this comparison it was clear that each siderophore had a higher affinity for Fe³⁺ than for Fe²⁺, which is one of the defining characteristics of a siderophore (Neilands 1981).

To directly compare the ability of different siderophores to bind Fe³⁺, the amount of free Fe³⁺ in a theoretical iron-siderophore system at pH 7.4 was calculated, as described by Harris *et al.* (1979). Comparison of the free Fe³⁺ concentration was thus representative of the affinity that each siderophore had for Fe³⁺. This required the calculation of the proton-independent solubility coefficients for each siderophore using the proton-dependent solubility constants (pH 7.0) for each siderophore and pK_a values from the model compound N,N-dimethyl-2,3-dihydroxybenzamide (Loomis &

Raymond, 1991) as shown by Reid *et al.* (1993). From this value a proton-dependent solubility constant at pH 7.4 for each siderophore was calculated. This value was used to determine the free Fe³⁺ concentration in the hypothetical system. This value was expressed as pFe³⁺ (−log₁₀[Fe³⁺]); thus, the larger the pFe³⁺ value, the lower the concentration of free Fe³⁺ in solution, indicating that the siderophore has a higher affinity for iron. Protochelin was found to have a pFe³⁺ value of 27.5, which was approximately the same as that of ferrioxamine E (27.7) and about eight orders of magnitude lower than that of enterobactin (35.6). The pFe³⁺ value for azotochelin was calculated to be 23.1, which is slightly lower than that of aerobactin (23.3) and one order of magnitude higher than that of aminochelin (22.0).

Siderophores and oxygen radical formation

The ability of each catecholate siderophore to retain iron in the presence of ·O₂[−] was examined. Only ferric-aminochelin released a significant amount of Fe²⁺ in the presence of ·O₂[−]. As the amount of potential free Fe²⁺ in the assay was increased from 5 to 50 μM, ferric-aminochelin continued to be the only siderophore that released Fe²⁺ (data not shown). Ferric-aminochelin released Fe²⁺ at a rate of 1.24 μM Fe²⁺ min^{−1} while ferric-protochelin and ferric-azotochelin released Fe²⁺

at rates of 0.07 and 0.13 $\mu\text{M Fe}^{2+} \text{ min}^{-1}$, respectively. Addition of SOD to the iron-aminochelin reaction decreased the rate of Fe^{2+} release by 30% to 0.95 $\mu\text{M Fe}^{2+} \text{ min}^{-1}$, indicating that Fe^{2+} release was $\cdot\text{O}_2^-$ mediated, while the lack of the xanthine oxidase $\cdot\text{O}_2^-$ -generating system prevented the release of Fe^{2+} altogether (data not shown).

The ability of each siderophore to participate in the formation of $\cdot\text{OH}$ via the Fenton reaction was also studied. Ferric-aminochelin complexes were equal to the ferric-EDTA control in their ability to catalyse the formation of $\cdot\text{OH}$. Ferric-protocochelin and ferric-azotochelin, on the other hand, generated 70% less $\cdot\text{OH}$ under identical conditions. Addition of catalase and SOD blocked $\cdot\text{OH}$ formation in this assay.

Activities of enzymes involved in oxygen stress management

The activities of catalase and SOD were measured in cell-free extracts prepared from cells grown under standard iron-sufficient and iron-limited conditions (Table 2). The activity of SOD was notably decreased by iron limitation of the wild-type strain UW and mutant strain LM100. However, even in iron-sufficient medium, strain LM100 had only 39% of the SOD activity of strain UW. Similarly, catalase activity in strain LM100 was only 45–50% of that found in strain UW. Catalase activity was unaffected by iron limitation in both strains. The cell-free extracts from these cells were fractionated by non-denaturing PAGE and stained to identify the individual electromorphs of catalase and SOD (Table 2). Both strains had an identical single species of SOD, which was inactivated by 5% H_2O_2 , indicating that it was an Fe-SOD. Three catalase electromorphs were observed in strain UW, but strain LM100 produced significantly less of the catalase electromorph with an R_F of 0.2.

Strain UW was much more resistant than strain LM100 to paraquat and the intracellular generation of $\cdot\text{O}_2^-$ (Korbashi *et al.*, 1986). In iron-sufficient medium, nitrogen-sufficient strain UW was inhibited completely by 40 μM paraquat, whereas N_2 -fixing cells were inhibited by 30 μM paraquat. In parallel iron-limited cultures, strain UW was more sensitive to paraquat (20 μM and 10 μM , respectively). However, strain LM100 was extremely sensitive to inhibition by paraquat, consistent with an inability to deal effectively with $\cdot\text{O}_2^-$. Inhibition of growth of strain LM100 was complete at 5 μM paraquat in iron-sufficient cells and at 3 μM in iron-limited cells, regardless of the nitrogen source.

DISCUSSION

In order to bring about oxygen stress, *A. vinelandii* cells were grown under increasingly aerobic conditions in iron-limited medium. These cells had greatly decreased Fe-SOD activity as compared to iron-sufficient cells and thus $\cdot\text{O}_2^-$ was expected to be the main effector of oxidative stress. Decreased SOD activity could arise from the production of iron-deficient apoenzyme (Beyer

& Fridovich, 1991) or could indicate that SOD transcription is activated by iron, as reported for *E. coli* and *P. aeruginosa* (Niederhoffer *et al.*, 1990; Hassett *et al.*, 1996). However, unlike the latter cells (Demple, 1996), *A. vinelandii* has no alternative Mn-SOD to replace the missing Fe-SOD (this study; Jurtshuk *et al.*, 1984). Unchecked $\cdot\text{O}_2^-$ can promote the Fenton reaction, but the formation of $\cdot\text{OH}$ may be limited by the availability of iron and H_2O_2 . Thus, the presence of undiminished catalase activity in iron-limited *A. vinelandii* strain UW may substantially decrease the impact of decreased SOD activity. Such is not the case in the isogenic FdI⁻ strain LM100 (Morgan *et al.*, 1988), which has decreased activity of both SOD and catalase even under iron-sufficient growth conditions. This strain's decreased capacity to deal with $\cdot\text{O}_2^-$ stress also was shown by its extreme sensitivity to paraquat as compared to strain UW.

N_2 -fixing cells may be more sensitive to oxidative stress since $\cdot\text{O}_2^-$ can directly oxidize metal-containing enzymes or uncouple redox reactions, as well as participate in the Fenton reaction (Fridovich, 1986). When nitrogen limitation was coupled with iron limitation and high aeration, *A. vinelandii* overproduced catecholate siderophores. This response was not as pronounced in nitrogen-sufficient cultures, but there was still the trend towards greater catecholate production at higher aeration. Thus we questioned whether catecholate siderophores could play an additional role in limiting oxidative damage by sequestering iron.

Of particular interest was the tricatecholate protocochelin, which was overproduced by oxygen-stressed strain LM100. The proton-independent solubility coefficient for protocochelin was calculated to be very high ($10^{43.9}$). This value can be compared with that of other siderophores that bind Fe^{3+} in a 1:1 ratio since the units for the equilibrium coefficients are the same. Thus, protocochelin has an affinity for Fe^{3+} that is about five orders of magnitude less than that of enterobactin (10^{49}), but it is a superior chelator compared to ferrioxamine B (10^{31}), pyoverdine PaA ($10^{30.8}$), ferrichrome ($10^{29.1}$) and aerobactin ($10^{22.5}$) (Reid *et al.*, 1993). Although both protocochelin and enterobactin are tricatecholates, enterobactin has a much higher affinity for Fe^{3+} (Loomis & Raymond, 1991). The most likely reason for this difference is that enterobactin contains a benzyl-serine triester ring that forms a rigid base for the attachment of the catechol moieties. This conformation makes it possible for enterobactin to easily align the catechols to form six coordinate bonds with Fe^{3+} (Hider, 1984). Protocochelin, on the other hand, is a linear molecule that may form tetradentate coordination of Fe^{3+} quite easily, but a longer time may be needed to move the catechol on the putrescine arm into the final hexadentate coordination structure (Fig. 1).

Iron chelated by protocochelin or azotochelin was not reduced by $\cdot\text{O}_2^-$ and limited $\cdot\text{OH}$ formation by the Fenton reaction. Only iron chelated by aminochelin was unable to limit the Fenton reaction; it was readily reduced by $\cdot\text{O}_2^-$ and generated as much $\cdot\text{OH}$ as the

ferric-EDTA control. These results were consistent with the calculated proton-independent binding constants for the three siderophores. The affinity of protochelin for iron was higher than that of pyoverdine PaA, a siderophore shown to prevent Fenton-mediated $\cdot\text{OH}$ formation (Coffman *et al.*, 1990). Ferric-azotochelin coordinated iron in a 3:2 ratio, bridging two iron molecules with three siderophore molecules in a manner similar to the interaction of rhodotorulic acid with Fe^{3+} (Carrano & Raymond, 1978). Azotochelin had an affinity for Fe^{3+} that was at least four orders of magnitude less than that of protochelin, but it was still a better chelator of Fe^{3+} than aminochelin.

In a mixture of all three catecholates, as formed in iron-limited cultures of *A. vinelandii*, protochelin probably has the greatest effect in limiting the Fenton reaction. Protochelin is likely to form a stable iron complex first owing to its higher affinity for Fe^{3+} and faster reaction time. Previously, we questioned why wild-type *A. vinelandii* would normally form so little protochelin, when this tricatecholate was expected to be a superior iron chelator and siderophore (Cornish & Page, 1995). However, a small amount (about 4–5 μM) of protochelin may be sufficient if one of its major functions is to prevent iron-catalysed oxidative damage under the low- Fe^{3+} growth conditions (6 μM or less) where catecholate siderophores are formed (Page & von Tigerstrom, 1988). Thus the overproduction of protochelin by strain LM100 may well be an attempt to compensate for the decrease in catalase activity observed in iron-limited cells. This is another example of how *Azotobacter* spp. may use a chemical defence system when enzymic protection fails in iron-limited cells. It has been previously shown that *Azotobacter salinestris*, which has very low catalase activity, uses a catechol melanin to bind iron and minimize oxidative damage (Page & Shivprasad, 1995; Shivprasad & Page, 1989).

Collectively, these results suggest that catecholate biosynthesis in *A. vinelandii* may be under the dual control of iron repression (Cornish & Page, 1995) and oxidative stress induction. It has been shown that *A. vinelandii* has a homologue of the Fur repressor (Mehrotra, 1997), so negative regulation by iron may follow the classic regulatory mechanism described by de Lorenzo *et al.* (1987). Recently it was proposed that the *A. vinelandii* FdI is part of a SoxRS-like system (Isas *et al.*, 1995). It also has been proposed that FdI could be a redox sensor and it may bind to DNA directly to regulate gene expression (Thompson, 1991). Our results are certainly consistent with the hypothesis that FdI is involved in the $\cdot\text{O}_2^-$ stress response in *A. vinelandii*. Future studies on the regulation of the transcription of catecholate siderophore biosynthetic genes may help to resolve some of these issues.

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