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The effect of manganese oxides and manganese ion on growth and siderophore production by *Azotobacter vinelandii*

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The addition of manganese oxides to iron-limited medium promoted the formation of the pyoverdin siderophore azotobactin by *Azotobacter vinelandii*. When active-MnO_2 was used, there was greatly decreased iron uptake into the cells, hyperproduction of azotobactin and the abiotic, chemical destruction or adsorbtion of the catechol siderophores azotochelin and aminochelin by this strong oxidizing agent. Although the iron content of the cells was the same as iron-limited cells, the growth of cells in medium with active-MnO_2 was increased 1.5- to 2.5-fold over iron-limited controls. This growth promotion was not caused by iron contaminating the oxide or by manganese solubilized from the oxide. Soluble 0.05–4 mM Mn^{2+} inhibited the growth of iron-limited cells and had a minimal effect on iron uptake, but caused hyperproduction of azotobactin. This later effect was caused by the inhibition of soluble ferric reductase, in a manner identical to that previously observed for Zn^{2+}. These results suggest that active-MnO_2 may interfere with a surface-localized iron uptake site, possibly another ferric reductase. The reason for the growth promotion by active-MnO_2 remains unknown, but is most likely related to decreased oxygen toxicity.

Keywords: active-MnO_2, azotobactin, ferric reductase, inhibition, manganese, oxygen, toxicity

Introduction

Azotobacter vinelandii has a complex siderophore system for the accumulation of iron from its native soil environment (Page 1993). This bactreium produces both catecholate and pyoverdin siderophores, which are produced in a sequential fashion as iron becomes more limiting (Page & Huyer 1984). The catechols include N,N^1-bis(2,3-dihydroxybenzoly)-L-lysine (azotochelin), 2,3-dihydroxybenzoyl-putrescine (aminochelin) and 2,3-dihydroxybenzoic acid (DHBA) which are produced in media containing up to 5 μM Fe (Page & von Tigerstrom 1988). Azotochelin and aminochelin are the most abundant catechols and are formed in the ratio of 1:1 to 2:1, while DHBA is a minor ligand (Page & von Tigerstrom 1988). These catechols have a low affinity for Fe^{3+}, but are produced in abundance and are effective in solubilizing iron from insoluble soil minerals (Page & Huyer 1984). When the available iron concentration in the medium drops to 2 μM or below, the pyoverdin siderophore azotobactin is formed (Page & von Tigerstrom 1988, Page *et al.* 1991).

The present study started as a series of growth experiments to determine the effects of metal oxides, other than iron oxides, on *A. vinelandii* growth and siderophore production. The results showed that manganese oxides appeared to suppress catechol siderophore production and interfered with the regulation of azotobactin synthesis. This was interesting, because it has been proposed that Mn^{2+} may substitute for Fe^{2+} in the intracellular control of metabolism (Williams 1982). For example, the transcription of iron-regulated genes is under the negative control of the Fur protein with Fe^{2+} acting as an essential co-repressor (DeLorenzo *et al.* 1987). *In vitro*, Mn^{2+} can substitute for Fe^{2+} as a co-repressor with Fur (DeLorenzo *et al.* 1987). Furthermore, resistance of *Escherichia coli* to high concentrations of Mn^{2+} has been used to select *fur* mutants that are derepressed for siderophore production (Hantke 1987). Therefore, this study has focused on the effects of manganese oxides and soluble manganese on the iron-regulated physiology of *A. vinelandii*.

Materials and methods

Strains and culture conditions

A. vinelandii strain UW (ATCC 13705) was incubated for 24 h in Burk's iron-limited medium (Page & Sadoff 1976) containing 1% glucose, 15 mM ammonium acetate and 1 μM ferric citrate. Additional ferric citrate was added to

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concentrations noted in the Results. The medium (100 ml) was contained in a 500 ml Erlenmeyer flask and sterilized in the autoclave. The cultures were inoculated with iron-limited cells as previously described (Page & Huyer 1984) and incubated at 30 °C with shaking at 225 r.p.m. Culture growth was measured by the Lowry assay (Page & Huyer 1984) or the dye-binding assay of Bradford (1976).

Metal oxides were all reagent grade chemicals: black cupric oxide (CuO), red cuprous oxide (Cu_2O), yellow lead oxide (PbO), red lead oxide (Pb_3O_4), red mercuric oxide (HgO), yellow mercuric oxide (HgO), manganese monooxide (MnO), manganese dioxide (activated or crystalline powder, MnO_2), manganese sesquioxide (Mn_2O_3) and manganese tetraoxide (Mn_3O_4). These were added at a concentration of 10–50 mg per 100 ml medium as dry powders which had been sterilized in the autoclave. Metal oxides also were included in the medium, but separated from the cells by placing the oxide inside a small dialysis sac containing 2 ml of medium (Page 1993). Soluble manganese was added as $MnCl_2$.

Siderophore analysis

The cells were removed from the culture by centrifugation and the culture fluid was adjusted to pH 1.8 with HCl. Absorbance at A_{310} was used to estimate the catechols azotochelin, aminochelin and DHBA, while absorbance at A_{380} was used to estimate azotobactin (Page & Huyer 1984). The acidified culture fluid was extracted twice with ethylacetate, which removed azotochelin and DHBA, and left aminochelin in the aqueous phase (Page & von Tigerstrom 1988). The amount of catechol also was determined by the Arnow assay (Barnum 1977) and the amount of hydroxamate from azotobactin also was determined by the Csaky (1948) assay. Azotochelin and DHBA from the ethylacetate extracts were identified by thin layer chromatography on silica gel G plates developed in benzene:acetic acid:water (125:72:3). The catechols were detected as pink spots after spraying with dipyridyl:$FeCl_3$ (Krebs *et al.* 1969).

Metal ion analysis

Cells grown with manganese oxide were separated from the mineral before iron or manganese determination. Typically, the cells from 200 ml of culture were collected by centrifugation and resuspended in 15 ml of 8 mM Tris–HCl, pH 7.8. A 2 ml sample of this suspension was layered over a 3 ml Percoll:0.15 M NaCl (2:1) cushion in a 15 ml conical centrifuge tube and centrifuged as described (Page & Huyer 1984) to separate the cells from the oxide. The iron content of the cells was estimated colorimetrically by an ascorbate-bipyridyl assay after digestion of the cells in perchloric acid (Page 1993). The manganese content of the cells was estimated in a colorimetric assay based on the periodate-oxidation of manganese to permanganate (Young 1978). The iron content of MnO_2 was similarly assayed by the ascorbate-bipyridyl assay after solubilization of 50 mg MnO_2 in 20 ml 0.35 N HCl overnight at 37 °C.

Ferric reductase activity

Cells from 100 ml of culture were collected by centrifugation and washed once in a buffer containing 10 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$ and 6 mM 2-mercaptoethanol. The cells were resuspended in 20 ml of the previous buffer and broken by sonication (Braun Sonifier 350, setting 7 for 7 × 20 cycles). Debris was removed by low speed centrifugation (10 min at 3000 *g*) and cell envelope material was removed by high speed centrifugation (1 h at 39000 *g*). The cell extract was made to 10% glycerol and was stored at −20 °C.

Ferric reductase activity was estimated in a modification of the assay described by Huyer & Page (1989). The assay contained (final concentrations) 20 μM FMN, 0.4 mM NADH, 1 mM $MgCl_2$ and 0.16 mM ferric citrate in 10 mM Tris–HCl, pH 7.6 (total volume 630 μl). This mixture was incubated in the dark at 37 °C for at least 10 min before the addition of 20 μl ferrozine (kept in the dark on ice) to 0.8 mM final concentration. The reaction was started by the addition of up to 350 μl cell extract and the increase in absorbance at 650 nm was measured in a Hitachi U2000 recording spectrophotometer for 5 min at 37 °C. Activity was defined as nmol Fe^{2+} formed min^{-1} mg^{-1} protein (Dailey & Lascelles 1977).

Results and discussion

Growth of A. vinelandii with metal oxides

A. vinelandii did not grow in the presence of cuprous oxide, mercurous oxide or mercuric oxide when added at 10 mg per 100 ml culture. However, the cells would grow in the presence of cupric, lead and manganese oxides (Table 1). In iron-limited medium where growth was partially inhibited, there also was a stimulation of one or both siderophores. Catechol siderophore production (Table 1 and as μg catechol mg^{-1} cell protein, data not shown) also was stimulated in iron-sufficient medium. Analysis of the culture fluids indicated that azotochelin, aminochelin and DHBA were being produced under iron-sufficient conditions, but azotobactin was not. These data suggested that catechols may be induced by signals other than iron deficiency.

A different pattern of siderophore production was obtained when active-MnO_2 was added to the media (Table 1). In iron-limited medium, the amount of catechol formed was decreased and azotobactin was increased. The actual A_{380} increased from 1.9 (control) to 3.2 and the A_{310} decreased from 1.7 (control) to 1.0. DHBA was the dominant catechol formed, while aminochelin and azotochelin were present in trace amounts. Azotobactin also was induced in iron-sufficient medium containing active-MnO_2, without a similar derepression of catechols. In iron-sufficient culture, only DHBA was present, as observed in the control. Thus it appeared that active-MnO_2 was suppressing catchol siderophore production but promoting azotobactin production. The over-production of azotobactin has been observed previously in catechol-negative mutants (Sevinc & Page 1992), but selective repression has not been observed before.

Table 1. Growth and siderophore production by *A. vinelandii* with metal oxides[a]

Oxide	Concentration (mg per 100 ml)	Iron-limited culture (1 μM Fe)			Iron-sufficient culture (20 μM Fe)		
		cell protein (% of control)	A_{310} per mg protein	A_{380} per mg protien	cell protein (% of control)	A_{310} per mg protein	A_{380} per mg protein
Control	0	100	4.4	4.8	100	0.3	0
CuO	10	100	3.5	3.6	93	0.6	0
PbO	50	70	4.9	6.3	74	1.4	0
Pb_3O_4	10	42	6.6	8.8	53	1.8	0
MnO_2[b]	50	150	1.7	5.4	110	0.5	1.1

[a]The control culture protein was 300 μg ml^{-1} in iron-limited or 780 μg ml^{-1} in iron-sufficient culture after 24 h incubation
[b]Active-MnO_2.

The effect of manganese oxides on catechol production

A variety of other manganese oxides were similarly added to iron-limited and iron-sufficient medium. Only active-MnO_2 promoted the production of azotobactin in iron-sufficient medium. In iron-limited medium, azotobactin production was stimulated in all cases while catechol production was at control levels or decreased (Table 2). This decrease was most evident with active-MnO_2 [Mn(IV)] > Mn_3O_4 [Mn(III), Mn(II)] > MnO [Mn(II)]. Only DHBA was evident in culture fluids containing active-MnO_2 and Mn_3O_4, while all three catechols were present in culture fluids containing MnO.

The results clearly showed that different types of MnO_2 gave different effects. Different lots of active-MnO_2 and crystalline-MnO_2 gave results typical of those described in Table 2. The difference between these two types of MnO_2 is that active-MnO_2 is a strong chemical oxidant, prepared by precipitation of the oxide from warm aqueous $MnSO_4$ and $KMnO_4$, or other methods (Fatiadi 1976). Oxidation of organic molecules by active-MnO_2 can occur in aqueous suspensions at neutral pH. The mechanisms of oxidation are diverse, but oxidation may require binding of the substrate to MnO_2 surface, followed by oxidation and desorption of the product. Oxidation of hydroxyl groups is commonly observed, with the production of a oxide and soluble manganese. Thus the apparent loss of catechol siderophores could be caused by oxidation of the hydroxyl groups (hence a loss of Arnow reactivity and reactivity with thin layer chromatography spray reagents), by catechol binding to the MnO_2 and failure to desorb or by aromatic ring oxidation (hence loss of A_{310} from the culture fluids).

Table 2. Comparison of the effect of manganese oxides on siderophore production

Oxide	Iron-limited culture (1 μM Fe)		
	cell protein (% of control)[a]	A_{380} per mg protein	μg catechol per mg protein
Control	100	4.3	104
MnO	87	7.2	72
MnO_2(crystalline)	72	5.5	108
MnO_2(active)	208	5.5	9.4
Mn_2O_3	52	10	101
Mn_3O_4	91	7.1	38

[a]The control protein was 336 μg ml^{-1} after 24 h growth.

When *A. vinelandii* was grown in iron-limited medium containing 50 mg of active-MnO_2 contained in a sealed dialysis sac, growth and siderophores production were characteristic of the control culture with no oxide. When 100 ml of the cell-free iron-limited culture supernantant fluid was mixed with 50 mg active-MnO_2 and incubated with shaking at 30 °C, the catechol concentration dropped from 36 to 21 μg ml^{-1} in 2 h. This rate of loss (8 μg catechol ml^{-1} h^{-1}) could easily account for the disappearance of catechols from the cultures containing active-MnO_2 even in the presence of continued biosynthesis. Indeed, after 24 h, all catechols were missing from the cell-free culture fluid. The persistence of DHBA in cultures containing active-MnO_2, therefore, could have been caused by a greater rate of DHBA production relative to aminochelin and azotochelin. In any event, these data show that the loss of catechols from cultures containing active-MnO_2 was the result of an abiotic, chemical event.

Growth promotion by active-MnO_2

The cells in iron-limited medium containing active-MnO_2 grew much better than the control cells. The culture protein was 1.5- to 2.5-fold greater than the control in replicate flasks containing 50 mg oxide per 100 ml culture. This increase in protein was confirmed by the BioRad dye-binding assay, which indicated it was not an artifact caused by manganese interference with the Lowry assay. Increased culture protein was only evident at low iron concentration and was not additive to the growth obtained at higher iron concentrations (Figure 1).

Active-MnO_2 contained 14.9 μg Fe per 50 mg oxide. Therefore, if all of the oxide was solubilized in the process of catechol oxidation, there would be at most 2.7 μM Fe added to the culture. Since hydroxyls reduce MnO_2 in the ratio of 6:1 (Fatiadi 1976), there should have been only 10.9 mmol MnO_2 solubilized by the 32.7 mmol catechol in the 100 ml of culture fluid, or 19% of the added MnO_2.

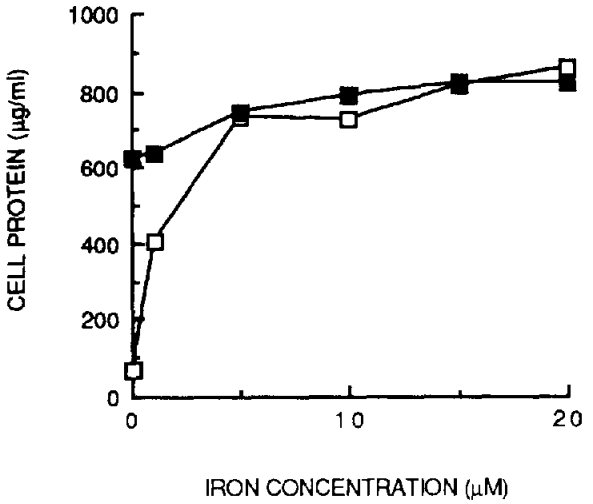

Figure 1. Comparison of growth in medium containg varied iron and active-MnO_2. The cell protein content was measured after 24 h growth in the absence (□) or presence (■) of 50 mg active-MnO_2 per 100 ml culture.

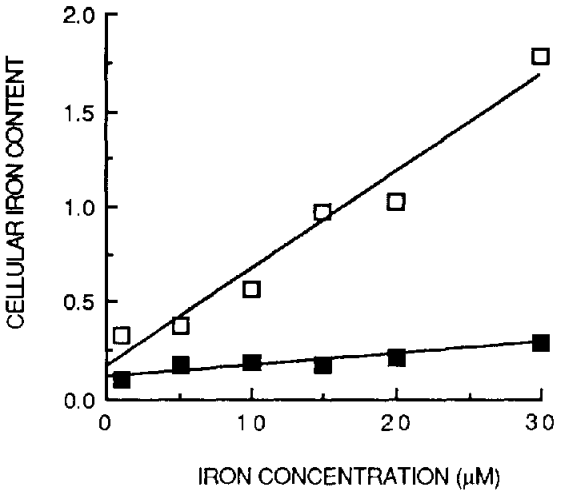

Figure 2. Comparison of iron accumulated by cells grown in medium containing varied iron and active-MnO_2. The iron content of the cells ($\mu g\,mg^{-1}$ cell protein) was measured after 24 h growth in the absence (□) or presence (■) of 50 mg active-MnO_2 per 100 ml culture.

This would contribute only 0.5 μM Fe to the medium, not enough to account for the growth promotion seen.

Cells grown overnight in medium containing 1–30 μM Fe and 50 mg active-MnO_2 (per 100 ml culture) were examined for manganese and iron uptake. In the absence of MnO_2, there was very little manganese in the cells (0.05 $\mu g\,mg^{-1}$ cell protein). With added MnO_2, iron-limited cells contained 0.18 $\mu g\,mg^{-1}$ cell protein and iron-sufficient cells contained 0.66 $\mu g\,mg^{-1}$ cell protein. Thus manganese was being bound to or taken up by the cells grown with MnO_2. However, MnO_2 interfered with iron uptake at all iron concentrations (Figure 2). In medium containing MnO_2 and up to 30 μM Fe, the cellular iron content was not greater than that found in iron-limited cells ($< 0.38\ \mu g\,mg^{-1}$ cell protein). Thus the enhanced production of azotobactin in media containing active-MnO_2 and normally repressive exogenous iron concentrations (Table 1) could be explained by inhibited iron uptake.

Effect of Mn^{2+} on A. vinelandii *growth and siderophore production*

The growth promotion by active-MnO_2 (Figure 1) was hard to reconcile with the fact that these cells were iron-limited (Figure 2). Since manganese appeared to be solubilized in the process it was possible that manganese was substituting for iron as a cofactor in metabolism (Williams 1982). Therefore the effect of soluble manganese on *A. vinelandii* growth and siderophore production was examined.

There was no increase in the growth of *A. vinelandii* in iron-limited medium containing 0.05–4.0 mM Mn^{2+} (Figure 3). Iron-limited cells were inhibited by all concentrations of Mn^{2+} while iron-sufficient cells were unaffected by up to 3.0 mM Mn^{2+}, then inhibited 35% by 3.5 mM Mn^{2+} and 100% by 4.0 mM Mn^{2+} (data not shown). Thus the growth promotion seen with active-MnO_2 did not appear to be caused by solublized manganese.

All the iron-limited cultures containing Mn^{2+} had elevated production of azotobactin. This was demonstrated by very bright-green fluorescence of the culture and increased A_{380} per mg cell protein (Figure 3). Hydroxamate-N values were considered unreliable because Mn^{2+} interfered with color development in the assay. Catechols, on the other hand, remained at control levels (3.0–3.6 A_{310} per mg protein). This Mn^{2+}-promoted

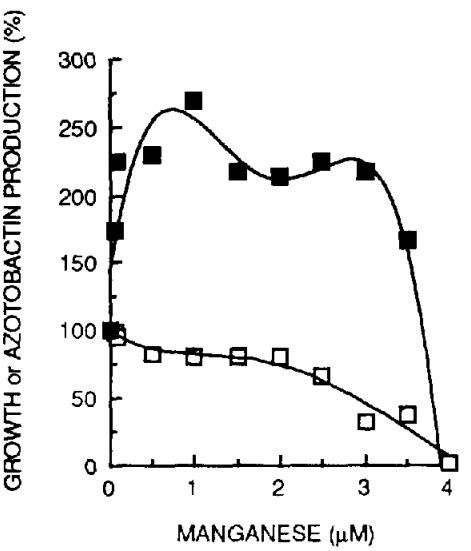

Figure 3. The effect of Mn^{2+} on *A. vinelandii* growth and azotobactin production. All cultures were incubated for 24 h in medium containing 1 μM Fe, then the percent protein ($\mu g\,ml^{-1}$) (□) and the amount of azotobactin (A_{380} per mg cell protein) (■) relative to the control culture containing no Mn^{2+} was measured.

hyperproduction of azotobactin was not evident in medium with no added iron, but appeared to prevent normal repression of azotobactin by 1 μM Fe or more (Figure 4). Mn^{2+} also caused some overproduction of catecols in medium containing 2.5–7.5 μM Fe (Figure 4).

Mn^{2+} interfered with iron uptake by *A. vinelandii*. When 1 mM Mn^{2+} was added to medium with varied iron content, there was no inhibition of iron uptake at 0–1 μM Fe. Cells from medium containing 2.5–10 μM Fe had 27% less iron per cell protein, but the amount of iron found in these cells (0.52–1.68 μg Fe mg^{-1} cell protein) was always more than that found in iron-limited cells (0.30 μg Fe mg^{-1} cell protein). The same kind of results were obtained at higher Mn^{2+} concentrations (data not shown). Therefore, it could not be said with certainty that the cells grown with Fe and Mn^{2+} containing 0.05–4.0 mM were iron-limited and that this was the reason for the hyperproduction of azotobactin seen in Figures 3 and 4.

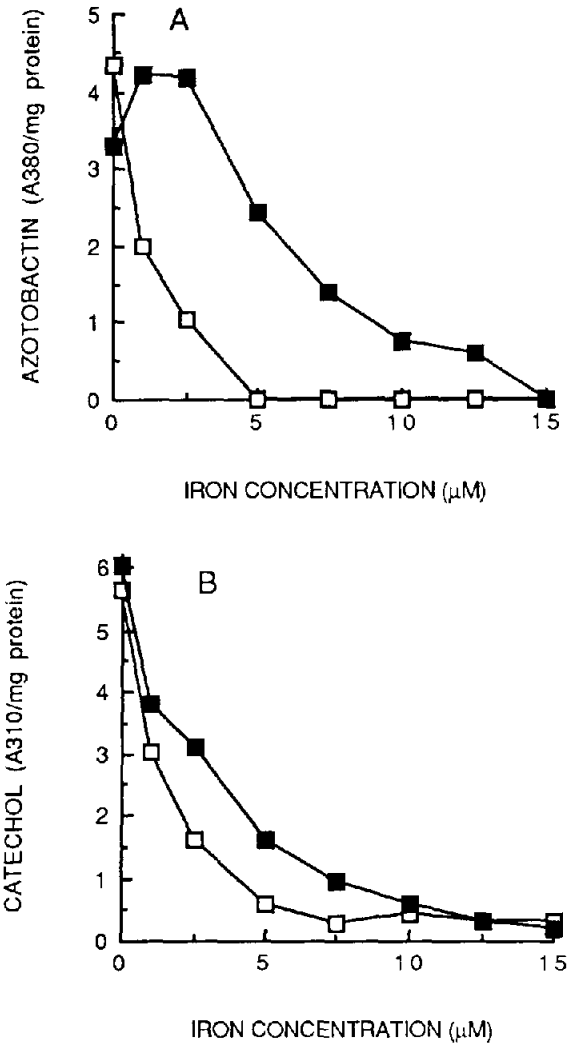

Figure 4. Effect of Mn^{2+} on the iron-promoted repression of *A. vinelandii* siderophores. The repression of (A) azotobactin and (B) catecholates by iron concentration was examined after 24 h growth in the absence (□) or presence (■) of 1 mM Mn^{2+}.

Effect of Mn^{2+} on ferric reductase activity.

The preceeding results obtained with Mn^{2+} were reminiscent of those obtained previously with Zn^{2+} (Huyer & Page 1988). Addition of 20–40 μM Zn^{2+} to *A. vinelandii* in iron-limited medium resulted in a great increase in azotobactin production and a modest effect on increased catechol production, but did not have a significant effect on iron uptake. It was shown that Zn^{2+} inhibited the soluble ferric reductase of *A. vinelandii* (Huyer & Page 1989). Inhibition of ferric reductase appeared to decrease the Fe^{2+} content of the cell, thus affecting Fe^{2+}-regulated repression of azotobactin production (Huyer & Page 1988, 1989). Catechol production is less dramatically effected, presumably because it is less tightly regulated by Fe^{2+} (Page & Huyer 1984).

Before investigating the effect of Mn^{2+} on ferric reductase activity, the assay conditions were optimized further from that reported by Huyer & Page (1989). The activity was found to be optimal (4.5 nmol Fe^{2+} min^{-1} mg^{-1} protein) when assayed by the previous method in 10 mM Tris–HCl buffer, pH 7.6, at 37 °C. The enzyme had a definite preference for NADH and required FMN (Huyer & Page 1989), but enzyme activity increased as FMN was increased beyond the 1 μM used previously in the assay. At least 20 μM FMN was required to saturate the enzyme (at saturating 100 μM Fe and 300 μM NADH) and resulted in a 5-fold increase in enzyme specific activity (Figure 5). The apparent K_m for FMN was 5 μM. Thus, the ferric reductase of *A. vinelandii* may be a flavin reductase that has the ability to reduce a number of ferric chelates (Fontecave *et al.* 1994). The revised assay also contained 0.8 mM ferrozine, twice the concentration used previously (Huyer & Page 1989). These revisions to the assay procedure did not significantly change the kinetics of the

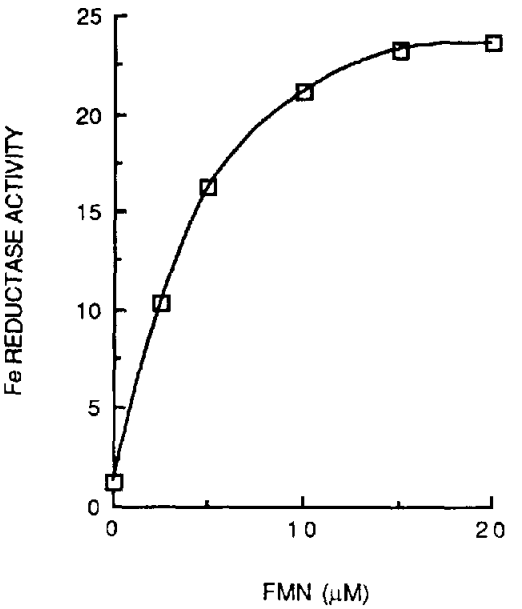

Figure 5. FMN-dependent specific activity of the *A. vinelandii* soluble ferric reductase.

A. vinelandii ferric reductase (data not shown). The apparent K_m for iron was 18 μM (at saturating NADH and FMN) and the apparent K_m for NADH was 20 μM (at saturating iron and FMN).

Ferric reductase activity was assayed in cell extracts from *A. vinelandii* grown in medium containing 0–50 μM Fe. All cells contained the same ferric reductase activity (22.4 nmol Fe^{2+} min^{-1} mg^{-1} protein), despite the initial iron content of the medium. In all cases, ferric reductase activity was inhibited 46% by 30 μM Mn^{2+} and 54% by 100 μM Mn^{2+}, but complete inhibition was not possible. In comparison, ferric reductase activity was inhibited 44% by 30 μM Zn^{2+} (this study and assay conditions) and complete inhibition by Zn^{2+} also was not possible (Huyer & Page 1989). The inhibition of ferric reductase by Mn^{2+} resembled Zn^{2+}, in that both the V_{max} and K_m values were affected by 10–100 μM Mn^{2+}, characteristic of a mixed-type inhibitor (Segal 1975). The apparent K_i for Mn^{2+} was 26 μM with respect to iron, exactly as observed for Zn^{2+} as an inhibitor (Huyer & Page 1989).

Comparison of active-MnO_2 and soluble manganese effects

The loss of catechol siderophore production when *A. vinelandii* was grown in the presence of active-MnO_2 appears to be the result of an abiotic, chemical reaction between this strong oxidizing reagent and the catechols. However, the increased production of azotobactin in the presence of manganese oxides appears to be the result of decreased iron uptake (in the case of active-MnO_2) and inhibition of ferric reductase activity by the solublized manganese. Soluble manganese alone is able to cause the hyperproduction of azotobactin, without greatly reducing the iron content of the cell. Mn^{2+} inhibits a constitutive soluble ferric reductase in a manner almost identical to that observed previously with Zn^{2+} (Huyer & Page 1989). In the previous study, the addition of 20–40 μM Zn^{2+} to the growth medium was needed to effect azotobactin production, but as shown here, the addition of 1 mM Mn was required to achieve the same effect. Thus it is likely that Mn^{2+} is not transported into *A. vinelandii* as efficiently as Zn^{2+}. When added to cell extracts, the ferric reductase (or flavin reductase) activity was inhibited to the same extent by identical micromolar amounts of Mn^{2+} or Zn^{2+}. In the presence of these inhibitors it is expected that the intracellular ratio of Fe^{2+} decreases relative to Fe^{3+} and this affects Fe^{2+}-regualted activities (Huyer & Page 1989). It will be very interesting to attempt to accurately measure the fate and speciation of iron under these growth conditions in the future, possible by Mössbauer spectroscopy (Mielczarek 1993).

We have tried to use high Mn^{2+} concentrations to select *fur* mutants as described by Hantke (1987). However, the interference of Mn^{2+} with ferric reductase activity, resulting in siderophore over-production, has prevented the selection of Mn^{2+}-resistant, siderophore over-producing (*fur*-defective) strains (work in progress).

Active-MnO_2 also resulted in greatly decreased iron uptake (Figure 2), unlike soluble manganese, which suggests that there is an iron uptake system on the cell surface that is interfered with by the insoluble oxide. Since active-MnO_2 is an oxidizing agent, it is likely that it is extracting electrons or protons from the iron uptake site. If this is the case, it would suggest that the surface site is another ferric reductase. Surface localized ferric reductases have been reported in *E. coli* (Fischer *et al.* 1990) and yeast (Dancis *et al.* 1992). While the soluble ferric reductase may be involved in interconversion of cellular Fe^{2+}/Fe^{3+} (Huyer & Page 1989, Fischer *et al.* 1990), the constitutive surface bound reductase may be involved in low affinity iron uptake (Figure 2). The inhibition of iron uptake and inhibition of this proposed surface-localized ferric reductase by MnO_2 requires some means of transmitting reductant across the cell envelope to the insoluble oxide. If the ferric reductase is indeed a flavin reductase, the soluble reductant could be reduced FMN (Fontecave *et al.* 1994). Surface localized reduction of crystalline-MnO_2 also occurs aerobically in a marine pseudomonad and other bacteria, by a mechanism that involves a shuttle of reductant to the cell surface (Ehrlich 1993). Recently, we have detected a surface-bound, FMN-dependent ferric reductase in *A. vinelandii* and are in the process of characterizing it (Rudy & Page, unpublished results).

A question that remains to be answered is why does the inhibition of iron uptake by active MnO_2 result in enhanced growth of *A. vinelandii*? Clues to the solution of this puzzle are that the cells must be iron-limited but not iron-starved, active-MnO_2 must be in contact with the cells and soluble manganese (alone) is not responsible. Decreased growth in iron-limited medium must relate to decreased iron-catalyzed enzyme activities. Certainly a decrease in cytochrome and non-heme iron components of respiration is expected (Rainnie & Bragg 1973), but the components remaining are most likely fully functional in electron transport to O_2 (Ackerell *et al.* 1984; Hubbard *et al.* 1986). Thus decreased protection from toxic oxygen products like $^{\cdot}O_2^-$ and H_2O_2 would lead to severely limited cell growth, especially in the presence of low levels of iron where the iron-catalyzed Haber–Weiss reaction could occur:

$$^{\cdot}O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2-} \quad (1)$$

$$2\,^{\cdot}O_2^- + 2H^- \rightarrow O_2 + H_2O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad (3)$$

The final reaction (3) is also known as the Fenton reaction. This is the basis of toxicity and tissue damage in ischemia, inflammation and many diseases (Halliwell & Gutteridge 1988). Furthermore, iron chelators like EDTA may promote reaction (1) but desferrioxamine may delay it, according to the affinity of the ligand for Fe^{3+}. Thus pyochelin, a *Pseudomonas aeruginosa* siderophore with a relatively low affinity and low specificity for Fe^{3+} (Visca *et al.* 1992), can promote $^{\cdot}OH$ formation, while the higher affinity, Fe^{3+}-specific siderophore pyoverdin does not (Coffman *et al.* 1990). Iron-limited *A. vinelandii* have normal catalase activity, but have 8-fold less superoxide dismutase activity than iron-sufficient cells (Page *et al.*

1988). The catechol siderophores have a low specificity and low affinity for Fe^{3+} (Page & von Tigerstrom 1988) and, therefore, are not expected to hinder $^{\cdot}OH$ formation. Protection of the cells, decreased toxicity and growth promotion should be mediated by the removal of $^{\cdot}O_2^-$ or strong chelation of Fe^{3+}. Thus it is the hyperproduction of the pyoverdin siderophore azotobactin that should act as an antioxidant, as observed with the pyoverdin of *P. aeruginosa* (Coffman *et al.* 1990, Lescoat *et al.* 1992). Removal of $^{\cdot}OH^-$ could be accomplished by the induction of a manganese-dependent superoxide dismutase, but such an enzyme has not been detected in *A. vinelandii* (Moore *et al.* 1984).

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