Temperature-sensitive production of azoverdin, the pyoverdin-like siderophore of *Azomonas macrocytogenes* ATCC 12334

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Azoverdin, a visible yellow, blue–white fluorescent compound produced by iron-limited *Azomonas macrocytogenes* ATCC 12334, was isolated from cell-free culture supernatant fluid and purified in the ferrated form to 98% purity by ion-exchange chromatography and reverse-phase high-performance liquid chromatography, thereby separating it from several related iron-binding fluorescent compounds. Purified ferrated azoverdin exhibited a pH-independent absorption spectrum which became pH-dependent following deferration, typical of a pyoverdin-like siderophore. Azoverdin enhanced ⁵⁵Fe³⁺ assimilation by iron-limited *A. macrocytogenes* and therefore functioned as a siderophore. Iron-limited cells were unable to produce azoverdin when grown at 34 °C rather than 28 °C. However, these cells still expressed a 74 kDa and a 70 kDa iron-repressible outer membrane protein and were capable of azoverdin-mediated iron transport. The use of *A. macrocytogenes* cells grown at 34 °C eliminated endogenous azoverdin production during iron uptake assays, which made it possible to accurately determine azoverdin-mediated ⁵⁵Fe³⁺ transport rates. Azoverdin-mediated ⁵⁵Fe-uptake proceeded with an apparent K_m of 0.2 µM and a V of 0.46 ng Fe³⁺ (10⁸ cells)^{−1} min^{−1}.

Introduction

Many Gram-negative bacteria respond to growth-restricting levels of free iron in their environment by producing low-molecular-mass iron-chelating compounds (siderophores). The siderophores promote the assimilation of this metal ion into the cells via specific iron-repressible ferrisiderophore receptor proteins (Neilands, 1984). While certain functional aspects of siderophore structure are conserved, the chemical structure of siderophores varies widely depending on the producing organism (Neilands, 1984). The group I fluorescent *Pseudomonas* spp. and *Azotobacter vinelandii* produce a group of structurally similar fluorescent siderophores composed of a chromophore, structurally based on 2,3-diamino-6,7-dihydroxyquinoline, to which a peptide of 6–10 amino acid residues is attached (Briskot *et al.*, 1989; Demange *et al.*, 1987, 1988, 1989; Poppe *et al.*, 1987). These siderophores, the pyoverdins (or pseudobactins)

and azotobactin, are readily detected in culture supernatant fluids of iron-limited *Pseudomonas* spp. or *Azotobacter vinelandii*, respectively, by virtue of their yellow-green colour, their fluorescence and the characteristic absorption spectrum of the chromophores (Demange *et al.*, 1987; MacDonald & Bishop, 1984). These fluorescent siderophores specifically enhance the uptake of iron by the bacterial strain producing a given siderophore (Cox & Adams, 1985; De Weger *et al.*, 1988; Hohnadel & Meyer, 1986, 1988; Knosp *et al.*, 1984; Meyer *et al.*, 1987).

While the structure and function of various pyoverdins produced by the group I fluorescent pseudomonads are well documented, little is known about the fluorescent compounds elaborated by members of the *Azotobacteraceae* other than azotobactin, which is produced by *Azotobacter vinelandii* (Demange *et al.*, 1988). *Azomonas macrocytogenes* ATCC 12334, an organism related to *Azotobacter* spp. (Tchan & New, 1984) and the group I fluorescent *Pseudomonas* spp. (De Smedt *et al.*, 1980; De Vos *et al.*, 1989), also produces an extracellular fluorescent compound when cells are cultured under iron-limited conditions (Johnstone, 1957; Thompson & Skerman, 1979). Although D. B. Johnstone and co-

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Abbreviations: CM-Sephadex, carboxymethyl-Sephadex; 3,4-DHBA, 3,4-dihydroxybenzoic acid; ODS, octadecylsilane.

workers first suggested similarities between the fluorescent compounds elaborated by *A. macrocytogenes* and those of the fluorescent pseudomonads, based on their mutual production by iron-limited cells and their similar absorption spectra (Johnstone *et al.*, 1958), no details of these observations were published. Recently, cell-free culture supernatant fluids of *A. macrocytogenes* ATCC 12334 containing a visible yellow, blue-white fluorescent compound were found to exhibit a pH-dependent absorption spectrum analogous to the pyoverdins and to promote $^{55}\text{Fe}^{3+}$ assimilation by *A. macrocytogenes* (Collinson & Page, 1989). In view of the apparent chemical and functional similarities of the fluorescent compound elaborated by *A. macrocytogenes* with that of the pyoverdins, we have named this compound azoverdin.

This study reports the isolation and purification of azoverdin from iron-limited culture supernatant fluid of *A. macrocytogenes* ATCC 12334 and demonstrates that azoverdin functions as a siderophore for *A. macrocytogenes*. Azoverdin production was found to be temperature-sensitive and exploitation of this fact has permitted an accurate determination of the kinetics of azoverdin-mediated ^{55}Fe uptake. The chemical structure of azoverdin will be published elsewhere.

Methods

Organisms and growth conditions. *Azomonas macrocytogenes* strain ATCC 12334 was routinely grown in iron-, nitrogen- and acetate-free Burk medium containing 1% (w/v) mannitol to replace glucose (Collinson & Page, 1989). Cultures for large-scale azoverdin purification also contained 14 mM-ammonium acetate. Although extraneous iron contamination of the medium was minimized through the use of acid-washed glassware and deionized, distilled H_2O (Collinson *et al.*, 1987), this medium contained 0.5 μM -iron as determined by atomic absorption spectroscopy (Collinson & Page, 1989). No further steps were taken to remove this iron. Liquid medium was inoculated with *A. macrocytogenes* as previously described (Collinson *et al.*, 1987). Cultures were incubated on a rotary shaker at 225 r.p.m., at 28 or 34 °C for 24 or 48 h, according to the experiment (see Results).

Quantification of azoverdin. The Csáky assay for bound hydroxylamine (Csáky, 1948) was initially used to detect the presence of azoverdin in cell-free culture supernatant fluids. However, due to the semi-quantitative nature of this assay and the lengthy procedure involved, azoverdin was quantified using the absorption of its chromophore, which is structurally similar to pyoverdin (M. A. Abdallah, unpublished results). The concentration of iron-free or ferrated azoverdin present in various solutions was calculated using the absorption maximum and the molar absorption coefficient previously determined for iron-free ($\lambda_{\text{max}} = 380 \text{ nm}$ and $\epsilon = 16500 \text{ M}^{-1} \text{ cm}^{-1}$) or ferrated ($\lambda_{\text{max}} = 400 \text{ nm}$ and $\epsilon = 19000 \text{ M}^{-1} \text{ cm}^{-1}$) pyoverdin at pH 5.0 (Demange *et al.*, 1988).

Isolation and purification of ferrated azoverdin. Initially azoverdin was isolated from iron-limited *A. macrocytogenes* culture supernatant fluid as an Fe^{3+} complex by phenol/chloroform extraction as described by Meyer & Abdallah (1978). However, to facilitate large-scale purification,

azoverdin was recovered directly from cell-free culture supernatant fluid by filtration using octadecylsilane (ODS) (Demange *et al.*, 1988). Bacterial cells were removed from iron-limited cultures grown for 48 h by centrifugation (10000 g, 15 min, 4 °C) and filtration (0.22 μm pore size, Minitan filtration unit, Millipore). The resulting culture supernatant fluid (2–5 litres) was acidified to pH 3.8 with formic acid and pumped (4.5 ml min⁻¹) through a column (3.8 cm $\times$ 12.5 cm) of ODS (LiChroprep RP-18, Merck). The fluorescent material retained in the top 2–3 cm of the ODS column was rinsed with at least 5 bed volumes of acidified distilled H_2O (adjusted to pH 3.8 with acetic acid), then eluted from the column with 400 ml 50% (v/v) methanol in 0.05 M-pyridine/acetic acid. This fluorescent eluate was concentrated, lyophilized, resuspended in 0.05 M-pyridine/acetic acid, applied to a carboxymethyl (CM)-Sephadex C25 cation-exchange column (2 cm $\times$ 35 cm) and eluted from this column (1 ml min⁻¹) using a 1 litre linear gradient of 0.05–0.5 M-pyridine/acetic acid. The major compound recovered from this column was ferrated with sixfold molar excess Fe^{3+} (added as FeCl_3) and eluted from a second cation-exchange column with 0.05 M-pyridine/acetic acid to separate the unchelated Fe^{3+} . The ferrated azoverdin was purified by preparative high-performance liquid chromatography (HPLC) by elution from a DEAE-Sephadex A25 anion-exchange column with 0.05 M-pyridine/acetic acid. Progression of the purification of ferrated azoverdin was monitored by analytical HPLC using an ODS column (4.2 mm $\times$ 25 cm) eluted isocratically with 5% (v/v) acetonitrile in 25 mM-pyridine/acetic acid buffer, pH 5.0.

Deferration of ferrated azoverdin. Iron was removed from ferrated azoverdin using 8-hydroxyquinoline as described by Meyer & Abdallah (1978). Deferrated azoverdin was lyophilized, resuspended in 0.05 M-pyridine/acetic acid, and applied to and eluted from a CM-Sephadex C25 column with a 1 litre gradient of 0.05–0.5 M-pyridine/acetic acid to remove any traces of ferrated azoverdin and 8-hydroxyquinoline from the purified azoverdin. Purified azoverdin was lyophilized and stored desiccated in the dark at -20°C .

Electrophoresis of siderophore. Samples of approximately 0.05 mg were applied to the midpoint of cellulose acetate membranes (5.6 cm $\times$ 14 cm), then subjected to electrophoresis (300 V, 30 min, 4 °C) using 0.1 M-pyridine/acetic acid as buffer. Following electrophoresis, the cellulose acetate membranes were illuminated with UV light (366 nm) to detect fluorescent compounds and then sprayed with a solution of 1% (w/v) FeCl_3 in distilled H_2O to detect iron-binding compounds.

Quantification of 3,4-dihydroxybenzoic acid (3,4-DHBA). The approximate concentration of 3,4-DHBA in iron-limited cell-free culture supernatant fluid of *A. macrocytogenes* was estimated from a standard curve prepared by measuring the A_{285} of known amounts of 3,4-DHBA added to uninoculated media adjusted to pH 6.5 (the final pH of the culture supernatant). For culture supernatant fluid also containing azoverdin, the A_{285} value was adjusted to exclude the absorbance contributed by azoverdin at this wavelength.

Analysis of outer-membrane proteins. Outer membrane proteins of *A. macrocytogenes* were isolated from iron-limited cells grown at 28 or 34 °C for 16 h by Sarkosyl extraction then subjected to SDS-PAGE as previously described (Collinson & Page, 1989). The molecular mass standards used were phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa), gamma globulin II H-chain (50 kDa), ovalbumin (43 kDa), gamma globulin II L-chain (23 kDa), and ribonuclease A (13.7 kDa), all obtained from Sigma. Protein concentration was determined as previously described (Collinson & Page, 1989).

Iron uptake assay conditions. Iron uptake by *A. macrocytogenes* was assayed using $^{55}\text{Fe}^{3+}$ as described by Knosp *et al.* (1984) with the modifications of Collinson & Page (1989).

Results

Isolation of azoverdin from cultures of *A. macrocytogenes*

Two methods were used to isolate azoverdin from iron-limited culture supernatant fluids of *A. macrocytogenes* ATCC 12334. Phenol/chloroform treatment of culture supernatant fluids resulted in the extraction of ferrated azoverdin, which had an absorption spectrum similar to the unextracted iron-limited culture supernatant fluid treated with FeCl_3 (Fig. 1a). Alternatively, iron-free azoverdin was recovered from a large volume of culture supernatant fluid on a column of ODS, which retained virtually all of the visible yellow, blue-white fluorescent material. Approximately 90% of the fluorescent material was eluted from ODS with 50% (v/v) methanol in 0.05 M-pyridine/acetic acid. The absorption spectrum of this material was characteristic of the iron-limited culture supernatant fluid not treated with Fe^{3+} (Fig. 1b).

Purification of azoverdin

Ferrated azoverdin isolated by phenol/chloroform extraction was eluted from a CM-Sephadex C25 column as a single peak (80–90% of the azoverdin) preceded by a minor peak (results not shown). Although the major peak had an absorption spectrum characteristic of ferrated azoverdin and electrophoresis indicated that it was a single cationic species, analytical HPLC revealed the presence of five minor compounds in addition to a major compound (data not shown).

Iron-free azoverdin, recovered from culture supernatant fluids by adsorption to and elution from ODS, was eluted from a CM-Sephadex C25 column as a major compound (accounting for approximately 60% of the A_{380} material) which was effectively separated from several minor compounds (Fig. 2). The absorption spectrum of the main peak F, as well as that of peaks C, D, E and G, was characteristic of the iron-limited culture supernatant fluid containing the fluorescent compound (results not shown). Electrophoretic analysis of material from peak F revealed one major and one minor cationic species, both of which fluoresced white and reacted with FeCl_3 to give a brown complex. Similarly, with the exception of peak A, the other minor peaks were comprised of at least one iron-binding, fluorescent compound in addition to several minor fluorescent compounds (data not shown).

The azoverdin in peak F readily complexed with trace amounts of Fe^{3+} during HPLC analysis, resulting in additional peaks in HPLC profiles which were unaccounted for in electrophoresis results and generated inconsistent results between replicate HPLC injections of the same sample. Therefore, the material in peak F

Fig. 1. Absorption spectra of ferrated (a) and iron-free (b) azoverdin. (a) Absorption spectrum of iron-limited culture supernatant fluid of *A. macrocytogenes* treated with FeCl_3 (0.1 mM, final concentration) (—) compared to the spectrum of ferrated azoverdin extracted from culture supernatant fluids with phenol/chloroform (---), (b) Absorption spectrum of iron-limited culture supernatant fluid (—) compared to the spectrum of iron-free azoverdin recovered from ODS (---). The culture supernatant fluid and isolated azoverdin (ferrated and iron-free) were adjusted to pH 5.0 with pyridine/acetic acid (0.05 M final concentration) prior to analysis.

Fig. 2. Cation-exchange chromatography of the fluorescent material produced by *A. macrocytogenes*. The fluorescent material recovered from culture supernatant fluid using ODS was applied to a CM-Sephadex C25 column and eluted with a 1 litre gradient of 0.05–0.5 M-pyridine/acetic acid buffer pH 5 (---). Fractions (approximately 6 ml) were collected and the A_{380} was used to follow elution of the fluorescent material. Results from a typical profile are presented.

was ferrated before further purification. Peak F was found to be comprised of five minor compounds in addition to a major compound (Fig. 3, A). Subsequent purification steps involving ion-exchange chromatography resulted in the purification of the major com-

Fig. 3. Analytical HPLC profiles of ferrated azoverdin before and after purification. The profiles were recorded after the following treatments: A, peak F from Fig. 2 was ferrated with FeCl_3 then eluted from a cation-exchange column to remove excess Fe^{3+} ; B, ferrated azoverdin following final preparative HPLC purification. The relative absorbance at 400 nm was recorded and the percentage purity of azoverdin in each sample was calculated from the peak area using triangulation; this is noted in the figure.

pound to 98% purity (Fig. 3, B). The purified, ferrated azoverdin was then deferrated to the yellow, iron-free form.

The original culture supernatant fluid was estimated to contain approximately 70–90 mg azoverdin l^{-1} . Of this, approximately one-third was present as the major compound according to this purification scheme. The final yield of purified, iron-free azoverdin was approximately 10 mg per litre of original culture supernatant fluid.

Spectral properties of purified azoverdin

Purified, iron-free azoverdin exhibited a characteristic pyoverdin-like pH-dependent absorption spectrum (Fig. 4a). In contrast, the absorption spectrum of ferrated azoverdin was pH-independent and at pH 5.0 the spectrum shifted from an absorption maximum at 380 nm for iron-free azoverdin to a new absorption maximum at 400 nm with the appearance of shoulders at 450 nm (Fig. 4b) and 540 nm (not shown in Fig. 4b).

Azoverdin-mediated iron assimilation by *A. macrocytogenes*

Iron assimilation by iron-limited *A. macrocytogenes* cells grown at 28 °C and assayed at 25 °C was enhanced only

Fig. 4. Absorption spectra of purified azoverdin. (a) Iron-free azoverdin was dissolved to a concentration of 28 μM in 0.05 M-pyridine, adjusted to various pH values (as noted in the figure) with acetic acid, then the absorption spectrum was recorded. (b) Purified iron-free azoverdin was dissolved in 0.05 M-pyridine/acetic acid, pH 5.0, at a concentration of 28 μM and the absorption spectrum was recorded (—). The absorption spectrum was recorded again following the addition of 28 μM - FeCl_3 (final concentration) (----). The volume change by the various additions was less than 0.5%.

when the iron uptake solutions contained azoverdin. Whether azoverdin was supplied in the unpurified form found in iron-limited culture supernatant fluid or added to uptake buffer as a purified compound, the rate of iron uptake [$0.5 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1} \text{ min}^{-1}$] was stimulated approximately 3.5-fold compared to cells similarly assayed in uptake buffer or in iron-sufficient culture supernatant fluid which did not contain azoverdin.

Azoverdin also promoted the association of iron with cells assayed at 0 °C, although the values were very low: $0.40\text{--}0.74 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1}$ total compared to $0.12\text{--}0.26 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1}$ total for cells assayed without azoverdin. Less than 15% of this cell-associated Fe^{3+} represented non-specific association of $^{55}\text{Fe}^{3+}$ with the filters as determined by filtration of a representative volume of the uptake solution incubated without added cells. Association of iron with the cells assayed at 0 °C was virtually complete at 0.5 min, with the subsequent rate of iron assimilation under these conditions ranging from 0 to $0.03 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1} \text{ min}^{-1}$.

Endogenous production of azoverdin during iron uptake assay

Iron-limited *A. macrocytogenes* cells accumulated iron at a lower rate [$0.14 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1} \text{ min}^{-1}$] in the absence of added azoverdin than in its presence. Occasionally these cells accumulated iron at higher rates despite the absence of added azoverdin, and in two experiments, the rates of iron assimilation were similar with or without the addition of purified azoverdin. Iron-limited cells grown at 28°C were washed three times in uptake buffer prior to iron uptake assays, but the cells remained highly fluorescent and released varied amounts of endogenous fluorescent compounds into the uptake medium during the assay. There was a positive correlation between the level of endogenous fluorescence produced by *A. macrocytogenes* and the rate of iron uptake by cells assayed in uptake buffer without added azoverdin. When the endogenous level of fluorescence was approximately $0.013\text{--}0.022$ at 380 nm in the assay system, the rate of iron uptake was approximately 2.5–3.5-fold lower than that measured for iron-limited cells assayed in the presence of $3 \mu\text{M}$ added azoverdin ($A_{380} = 0.05$). However, if the level of endogenous fluorescence was higher ($A_{380} = 0.030\text{--}0.037$) the iron assimilation rates approximated that of cells assayed in the presence of $3 \mu\text{M}$ added azoverdin. This suggested that the rate of iron assimilation was dependent on the prevailing azoverdin concentration and that relatively low concentrations of azoverdin were required for maximum rates of iron uptake by *A. macrocytogenes*.

The measurement of the K_m of azoverdin-mediated iron uptake by iron-limited *A. macrocytogenes* cells was virtually impossible because of the unpredictable levels of endogenous azoverdin produced by various cell preparations. Therefore, cells unable to produce azoverdin, but still able to transport ferriazoverdin, would be required to estimate the K_m of azoverdin-mediated iron assimilation.

Effect of temperature on azoverdin production

Initial observations indicated that iron-limited *A. macrocytogenes* ATCC 12334 grown on solid or liquid medium failed to produce fluorescence when grown at the slightly elevated temperature of 34°C . This was also the case for *A. macrocytogenes* NCIB 8700 and NCIB 10958. *A. macrocytogenes* ATCC 12334 incubated at 28°C and 34°C demonstrated similar growth rates, although cells grown at 34°C produced slightly less cellular protein and did not produce any detectable fluorescence for 48 h (Fig. 5). These results were corroborated by the Csáky assay, which indicated background levels of bound hydroxylamine in culture supernatants of cells grown for

Fig. 5. Fluorescence production by iron-limited *A. macrocytogenes* ATCC 12334 incubated at 28°C (—) and 34°C (---). Cell growth was measured as total cell protein ($\circ$, $\bullet$) and the presence of azoverdin in the culture supernatant was measured spectrophotometrically at 380 nm ($\square$, $\blacksquare$) on samples adjusted to pH 5.0 with pyridine/acetic acid buffer, pH 5.0 (0.05 M , final concentration). Error bars indicate the range of duplicate samples where the range exceeds the diameter of the symbols.

16 h at 34°C compared to approximately $10 \mu\text{M}$ bound hydroxylamine for culture supernatants of cells grown at 28°C .

The slight increase of absorption at 380 nm seen after 48 h at 34°C ($A_{380} = 0.029$) was not due to the production of fluorescent compounds. Addition of purified azoverdin to an equivalent A_{380} of 0.029 ($1.7 \mu\text{M}$) to uninoculated medium gave definite fluorescence under 366 nm UV light, whereas the supernatant fluid from the culture incubated at 34°C was not fluorescent.

The increased growth temperature did not significantly affect the production of 3,4-DHBA, since cells grown at either temperature produced similar levels of this compound. Approximately $70\text{--}80 \mu\text{M}$ or $300\text{--}350 \mu\text{M}$ 3,4-DHBA was produced by cells incubated at either temperature for 16 h or 48 h, respectively. 3,4-DHBA can promote iron uptake in *A. macrocytogenes* (Collinson *et al.*, 1987) and presumably it was this chelator that scavenged iron from the iron-limited medium and promoted the growth of the cells at 34°C (Fig. 5).

The inability of *A. macrocytogenes* cells to produce azoverdin at 34°C was not due to a prolonged lag period prior to onset of pigment production because cells initially grown at 34°C only resumed production of azoverdin when shifted to 28°C (data not shown).

Fig. 6. SDS-PAGE of outer-membrane isolated from iron-limited *A. macrocytogenes* grown at 28 °C (lane 1) or 34 °C (lane 2). Each lane of the 10% (w/v) polyacrylamide gel was loaded with 10 µg protein. The gel was stained with Coomassie brilliant blue R following electrophoresis. The molecular masses of the standard proteins are noted on the right of the figure.

Conversely, cells initially grown at 28 °C ceased production of azoverdin after being transferred to the higher temperature (data not shown). The fluorescence was stable in culture supernatant fluids incubated at 34 °C, indicating that the pigment was not destroyed by these conditions. The transfer of cells from 28 °C to 34 °C, and vice versa, did not adversely affect growth rate or final cell protein yield, which remained 93–116 µg protein (ml culture)⁻¹ for cultures harvested at 48 h regardless of the time at which they were transferred to the other temperature.

A. macrocytogenes ATCC 12334 grown at 34 °C also produced the 74 and 70 kDa iron-repressible outer-membrane proteins previously identified in cells grown at 28 °C (Collinson & Page, 1989), although relatively less of the 74 kDa protein was present in cells grown at 34 °C (Fig. 6).

Fig. 7. Effect of temperature on azoverdin-mediated iron assimilation by *A. macrocytogenes* ATCC 12334. (a) Iron-limited cells grown at 28 °C (○, ●) or 34 °C (□, ■) were assayed at 25 °C in uptake buffer alone (open symbols) or uptake buffer containing 5 µM purified azoverdin (filled symbols). (b) Iron-limited cells grown at 28 °C (○, ●) or 34 °C (□, ■) were assayed at 34 °C in uptake buffer alone (open symbols) or uptake buffer containing 5 µM purified azoverdin (filled symbols). Control values, obtained from cells similarly assayed at 0 °C were subtracted from the respective test values. Uptake buffer consisted of Burk medium salts containing 0.5% mannitol and 10 mM trisodium citrate (Collinson & Page, 1989). Error bars represent the range of duplicate assays where the range exceeded 0.1 ng Fe³⁺ (10⁸ cells)⁻¹. Results of a typical experiment are presented.

Effect of temperature on iron assimilation by *A. macrocytogenes*

A. macrocytogenes cells grown at 34 °C, and therefore unable to produce azoverdin, still demonstrated azoverdin-mediated iron assimilation. Cells grown at 34 °C or 28 °C exhibited initial rates of iron assimilation of 0.46 or 0.44 ng Fe³⁺ (10⁸ cells)⁻¹ min⁻¹, respectively, when assayed at 25 °C in uptake buffer supplemented with 5 µM purified azoverdin (Fig. 7a). In the absence of added azoverdin, initial iron assimilation rates were 0.17 and 0.35 ng Fe³⁺ (10⁸ cells)⁻¹ min⁻¹ for cells grown at 34 °C and 28 °C, respectively (Fig. 7a). The higher rate of iron transport by cells grown at 28 °C in the absence of added azoverdin was caused by the endogenous production of azoverdin by these cells. During the 60 min assay period with cells grown at 28 °C the *A*₃₈₀ increased from 0.016 to 0.041. Conversely, cells grown at 34 °C produced no detectable fluorescence during the 60 min assay period at 25 °C.

Similar trends were noted for *A. macrocytogenes* cells grown at 28 °C and 34 °C when assayed for iron assimilation at 34 °C (Fig. 7b). The main effect of the increased assay temperature was to increase the rates of iron assimilation 1.2- to 1.8-fold compared to iron assimilation rates measured for the respective cells

assayed at 25 °C. Cells grown at 28 °C and assayed at 34 °C in the absence of added azoverdin demonstrated a lower rate of iron transport [$0.27 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1} \text{ min}^{-1}$] than those assayed at 25 °C (Fig. 7a, b). The lower levels of endogenous azoverdin ($A_{380} = 0.016\text{--}0.025$) released by these cells during the course of the assay at 34 °C probably accounted for this decrease.

As previously noted for cells grown at 28 °C and assayed at 0 °C, azoverdin also enhanced the level of iron associated with *A. macrocytogenes* cells grown at 34 °C when assayed at 0 °C. These cells accumulated $0.62\text{--}0.84 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1}$ total in the presence, and $0.18\text{--}0.25 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1}$ total in the absence of added azoverdin. Similarly, the iron accumulated rapidly under these conditions and did not increase thereafter over the 60 min assay period. The low levels of non-specific $^{55}\text{Fe}^{3+}$ accumulation by the filters (previously noted) were not increased by using an assay temperature of 34 °C.

Determination of the K_m of azoverdin-mediated iron uptake

The initial rate of iron assimilation by *A. macrocytogenes* cells grown at 34 °C was dependent on the added azoverdin concentration in the uptake buffer. Increased azoverdin concentrations up to $0.7 \mu\text{M}$ ($A_{380} = 0.014$) caused enhanced $^{55}\text{Fe}^{3+}$ assimilation by *A. macrocytogenes* cells (data not shown). These azoverdin concentrations were within the range of those of endogenous fluorescent compounds released into the uptake buffer by cells grown at 28 °C. Azoverdin concentrations exceeding $0.7 \mu\text{M}$ did not further increase the rate of iron assimilation. Since azoverdin forms a 1:1 complex with iron (M. A. Abdallah, unpublished results) and iron was present in the assay system at $3 \mu\text{M}$, the observed saturation in the rate of iron transport was not due to limiting iron.

A double reciprocal plot of the iron assimilation rate versus azoverdin concentration revealed an apparent K_m of approximately $0.2 \mu\text{M}$ and a V of $0.46 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1} \text{ min}^{-1}$ for azoverdin-mediated iron assimilation by *A. macrocytogenes* cells grown at 34 °C and assayed at 25 °C (data not shown).

3,4-DHBA-promoted iron uptake in *A. macrocytogenes*

The culture fluids of iron-limited *A. macrocytogenes* contained $70\text{--}80 \mu\text{M}$ 3,4-DHBA after 16 h growth at 28 °C or 34 °C. However, iron assimilation by *A. macrocytogenes* was not enhanced by 3,4-DHBA under the present assay conditions. It is likely that the presence of 10 mM-sodium citrate added to the uptake buffer as a

low-affinity iron chelator to keep the iron soluble in these assays (Knosp *et al.*, 1984), competed with 3,4-DHBA and thereby made it impossible to measure 3,4-DHBA-promoted iron uptake by this method.

Discussion

The purification and partial characterization of azoverdin, a visible yellow, blue-white fluorescent compound elaborated by iron-limited *Azomonas macrocytogenes* ATCC 12334, has identified this pigment as a major, extracellular, pyoverdin-like compound. *Azomonas* is only the third genus, the others being *Pseudomonas* and *Azotobacter*, known to produce iron-binding, fluorescent compounds of this class. *A. macrocytogenes* NCIB 8700 (ATCC 12335) and NCIB 10958, and *Azomonas agilis*, similarly produce a blue-white fluorescent compound when grown under iron limitation (Collinson & Page, 1989; Thompson & Skerman, 1979). These compounds have yet to be chemically characterized.

Techniques previously used to recover pyoverdins and azotobactin were successfully applied to the isolation of azoverdin. Ferrated azoverdin was isolated from cell-free culture supernatant fluids of iron-limited *A. macrocytogenes* by phenol/chloroform extraction, a technique used previously to isolate ferrated pyoverdin from cultures of various *Pseudomonas* spp. (Demange *et al.*, 1987; Meyer & Abdallah, 1978). Alternatively, iron-free azoverdin was recovered using a modification of the procedure used for isolating azotobactin from culture supernatant fluids of *Azotobacter vinelandii* strain D (CCM 289) (Demange *et al.*, 1988). The latter procedure was the method of choice due to the ease with which azoverdin could be isolated, with good recovery, from culture supernatant fluids of *A. macrocytogenes*.

Ion-exchange chromatography, electrophoresis and analytical HPLC revealed that in addition to the main compound azoverdin, which comprised an estimated one-third of the total fluorescent compounds present in the culture medium of *A. macrocytogenes*, several iron-binding compounds related to azoverdin were also present. The difficulty in separating these related compounds was illustrated by gel filtration and ion-exchange chromatography, in which several compounds with identical absorption spectra eluted as a single peak only to be resolved as different compounds by HPLC. In fact, some researchers may have overlooked this aspect of pyoverdin purification (Cody & Gross, 1987; Hohndel & Meyer, 1988; Torres *et al.*, 1986). The chemical structure of azoverdin is presently under investigation.

Azoverdin functions as a siderophore to promote iron uptake in a concentration-dependent manner with an apparent K_m of $0.2 \mu\text{M}$ and V of $0.46 \text{ ng Fe}^{3+} (10^8 \text{ cells})^{-1}$

min^{-1} under the present assay conditions. These values indicate that azoverdin functions in high-affinity iron uptake since low azoverdin concentrations promote maximum iron uptake and the apparent K_m for azoverdin-mediated iron uptake is comparable to values obtained for pseudobactin-mediated iron uptake in *Pseudomonas putida* ($K_m = 0.23 \mu\text{M}$) (De Weger *et al.*, 1988), pyochelin-mediated iron assimilation by *P. aeruginosa* ($K_m = 0.08 \mu\text{M}$) (Cox, 1980) and siderophore-mediated iron uptake by other Gram-negative bacteria ($K_m = 0.1\text{--}0.25 \mu\text{M}$) (Bachhawat & Ghosh, 1987; Frost & Rosenberg, 1973; Negrin & Neilands, 1978).

The saturable nature of azoverdin-mediated iron assimilation by *A. macrocytogenes* suggests a rate-limiting step during transport, possibly at the cell membrane. Azoverdin also enhanced the binding of ^{55}Fe to cells incubated at 0°C . One interpretation of this would include the presence of a protein in the outer membrane of *A. macrocytogenes* capable of binding ferriazoverdin but which does not function at 0°C in iron transport. Further studies will establish whether the 74 kDa or the 70 kDa iron-regulated outer-membrane proteins of *A. macrocytogenes* function as ferrisiderophore receptor proteins analogous to those identified in the outer membrane of *Pseudomonas* spp. (Cody & Gross, 1987; Hohnadel & Meyer, 1986; Magazin *et al.*, 1986).

The inability of micro-organisms to produce siderophores at slightly increased growth temperatures is a phenomenon previously encountered (Ankenbauer *et al.*, 1986; Bachhawat & Ghosh, 1989; Garibaldi, 1971, 1972; Ismail *et al.*, 1985; Marugg *et al.*, 1985; Torres *et al.*, 1986). The mechanism of temperature-regulated siderophore biosynthesis is not yet understood, although in *Salmonella typhimurium*, temperature-regulated siderophore production appears to function independently of the *fur* gene (Worsham & Konisky, 1984), suggesting the existence of another regulatory mechanism for siderophore production. Growth of *A. macrocytogenes* at the elevated temperature of 34°C did not inhibit production of the iron-repressible outer-membrane proteins, a result previously noted with iron-deficient *P. aeruginosa* (Mizuno & Kageyama, 1978) and *Azospirillum brasilense* (Bachhawat & Ghosh, 1989). However, there appeared to be relatively less of the 74 kDa protein at the increased growth temperature. Worsham & Konisky (1984) also observed a decrease in the expression of the iron-repressible Cir protein in the outer membrane of *Escherichia coli* and *S. typhimurium* grown at slightly elevated growth temperatures. Temperature had little apparent effect on iron assimilation by *A. macrocytogenes*, whether cells were grown at 28°C or 34°C and assayed for iron uptake at 25°C or 34°C , again supporting similar findings reported for *Azospirillum brasilense* (Bachhawat & Ghosh, 1989) and *E. coli*

(Worsham & Konisky, 1984). An explanation for this phenomenon is being sought.

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