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ISBN 0-315-55441-X

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THE UNIVERSITY OF ALBERTA

IRON ASSIMILATION BY *AZOMONAS MACROCYTOGENES*

by

S. Karen Collinson 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1989

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TITLE OF THESIS: Iron Assimilation by *Azomonas macrocytogenes*

DEGREE: Doctor of Philosophy
YEAR THIS DEGREE GRANTED: 1989

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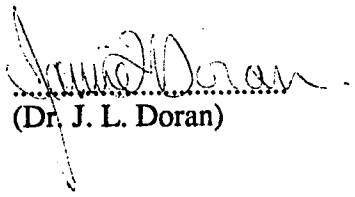
Collinson, K., Doran, J. L. & Page, W. J. (1987). Production of 3,4-dihydroxybenzoic acid by *Azomonas macrocytogenes* and *Azotobacter paspali*. *Canadian Journal of Microbiology* **33**, 169-175.



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Page, W. J. & Collinson, S. K. (1987). Characterization of *Azomonas macrocytogenes* strains isolated from Alberta soils. *Canadian Journal of Microbiology* **33**, 830-833.

Collinson, S. K. & Page, W. J. (1989). Production of outer-membrane proteins and an extracellular fluorescent compound by iron-limited *Azomonas macrocytogenes*. *Journal of General Microbiology* **135**, 1229-1241.

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ABSTRACT

Iron assimilation by the gram-negative, soil diazotroph *Azomonas macrocytogenes* ATCC 12334 was studied. *A. macrocytogenes* was grown in a simple defined medium and was found to excrete two iron-binding compounds. The first compound, 3,4-dihydroxybenzoic acid (3,4-DHBA) was purified and chemically identified from cell-free culture supernatants and found to promote the solubilization of iron from various iron-containing minerals. Moreover, preincubation of iron-limited growth medium with olivine or glauconite in the presence of 3,4-DHBA, supported enhanced growth of *A. macrocytogenes* thus suggesting a role for 3,4-DHBA in mineral-iron solubilization. However, 3,4-DHBA was not a true siderophore since it was also produced by cells growing in iron-sufficient medium and did not enhance $^{55}Fe^{3+}$ assimilation by *A. macrocytogenes*.

A. macrocytogenes also excreted a blue-white fluorescent compound in iron-limited media. This compound, named azoverdin, was purified in the ferrated form to 98% purity. This compound exhibited a pH-independent absorption spectrum which became pH-dependent following deferration with 8-hydroxyquinoline. Preliminary FAB mass spectral results indicated a 1:1 stoichiometry for the iron complex, and a positive reaction in the Csáky assay suggested the presence of bound hydroxylamine. Although elucidation of the exact chemical structure is in progress, these results indicated that azoverdin was chemically related to pyoverdin siderophores.

Electrophoretic analysis of outer membranes of *A. macrocytogenes* revealed that iron-limited cells overproduced two proteins of apparent molecular mass 74 kDa and 70 kDa, which were characteristic of ferrisiderophore receptor proteins isolated from other iron-limited gram-negative bacteria.

Azoverdin acted as a siderophore to enhance $^{55}Fe^{3+}$ assimilation in *A. macrocytogenes* cells. Iron-limited cells were unable to produce azoverdin when grown at 34°C rather than 28°C. However, these cells still expressed the 74 kDa and 70 kDa iron-repressible outer membrane proteins and were capable of azoverdin-mediated iron transport with an apparent Km of 0.2 μM and a Vmax of 0.46 ng Fe^{3+} (min 10^8 cells $)^{-1}$. In the absence of azoverdin, *A. macrocytogenes* cells assimilated iron at a low rate in an azoverdin-independent manner.

ACKNOWLEDGMENTS

I am grateful to Dr. W. J. Page for his support and guidance, without which this thesis would not have been possible. I appreciate the freedom he gave me, allowing me to work independently and experience first hand, the various facets of research. I am also indebted to Dr. M. A. Abdallah who made it possible for me to join his lab in Strasbourg, France for three months, during which time he guided me in the purification of azoverdin. I benefitted from his expertise and gained an appreciation of chemistry I would not have otherwise acquired.

I would like to thank Dr. D. W. S. Westlake and my committee members; Dr. J. N. Campbell, Dr. S. E. Jensen, Dr. M. Peppler and Dr. M. A. Pickard, who offered their support and time away from their busy schedules to see me through various seminars, exams and committee meetings. I also extend my appreciation to my external examiner, Dr. B. R. Byers and to the committee chairman, Dr. R. von Tigerstrom.

Dr. J. L. Doran provided encouragement and useful discussions and Dr. P. M. Fedorak was always willing to field questions concerning chemistry. I thank Margaret von Tigerstrom for sharing her technical expertise. Park Yee of the Department of Soil Sciences, University of Alberta performed the atomic absorption analyses and Dr. Hogg of the Department of Chemistry, University of Alberta did the FAB mass spectral analyses and helped with interpreting the results.

I greatly appreciate my fellow graduate students and colleagues; Dr. W. Bingle, R. Carmichael, J. Cunningham, Dr. J. M. Foght, M. Huyer, W. Lam, B. Leskiw, R. Mah, K. Semple, S. Shivprasad, and X. Wu, who gave me moral support and friendship. I also appreciate the efforts of T. Hantos, J. Hoppenbrouwers, A. McKinnon, F. Pickard, D. Shelmerdine, V. Whitehead and H. Tebelmann who kept so many things running smoothly.

Finally, the funding provided by the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research in the form of scholarships and bursaries is gratefully acknowledged. The University of Alberta Graduate Studies and Research, and the University of Alberta Alma Mater Fund provided me with travel funds to attend scientific meetings and work in Strasbourg.

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ABBREVIATIONS

A_{380}	absorbance at 380 nm
ATCC	American Type Culture Collection
CM	carboxymethyl
DCPIP	2,6-dichlorophenol-indophenol
DEAE	diethylaminoethyl
3,4-DHBA	3,4-dihydroxybenzoic acid
ϵ	molar extinction coefficient
EDTA	ethylenediaminetetraacetic acid
FAB	fast atom bombardment
HPLC	high performance liquid chromatography
kDa	kiloDaltons
NCIB	National Culture Collection of Industrial Bacteria
NMR	nuclear magnetic resonance
OD_{620}	optical density at 620 nm
ODS	octadecyl silane
OM protein	outer-membrane protein
$pyoverdin_{Pa}$	pyoverdin produced by *Pseudomonas aeruginosa*
$pyoverdin_{Pss}$	pyoverdin produced by *Pseudomonas syringae* pv.*syringae*
Sarkosyl	N-lauroylsarcosine, sodium salt
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TMPD	*N,N,N'N'*-tetramethyl-*p*-phenylenediamine
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1

General Introduction

1.1 Microbial Iron Assimilation

Micro-organisms assimilate iron by a variety of mechanisms but two general classifications are in current use. High affinity iron assimilation refers to an efficient and specific iron acquisition strategy whereby iron-limited micro-organisms produce ferric iron chelating compounds called siderophores (Lankford, 1973). Once released into the environment, siderophores specifically bind iron and diffuse back to the cell where the ferrisiderophore complex is recognized by its respective cell surface receptor protein (Neilands, 1981*b*; 1982). The iron is internalized by the cell via a specific transport mechanism and is stored in the cell or used in metabolism but ultimately regulates the expression of the siderophore and cognate receptor protein (Bagg & Neilands, 1987*b*; Neilands, 1984*a*). Low affinity iron assimilation refers to the poorly understood, nonspecific mechanism by which cells obtain iron without high affinity iron transport mechanisms (Neilands, 1977). Mutants unable to produce siderophores have the ability to procure iron unless certain iron-sequestering agents such as citrate (Pollack *et al.*, 1970), 2,2'-dipyridyl (Stuart *et al.*, 1980) or nitrilotriacetate (Frost & Rosenberg, 1973) are incorporated into the medium.

Other strategies of iron assimilation which are not strictly described by these working definitions include pathogens capable of obtaining iron without producing siderophores (Griffiths *et al.*, 1988; Reeves *et al.*, 1983), the utilization of iron from exogenous iron chelates such as citrate (Cox, 1980; Leong & Neilands, 1981; Messenger & Ratledge, 1982; Neilands, 1981*b*) or phenolic acids (Peters & Warren, 1970), accumulation of iron at

the cell surface (Beveridge, 1981; Lankford, 1973) or transport of ferrous iron (Evans *et al.*, 1986; Hantke, 1987). Obviously, the mechanism used by a given micro-organism will depend on several factors including its environment and the nature of the prevailing iron source.

High affinity iron assimilation has received considerable attention during the last 30 years because it is a common strategy by which many micro-organisms acquire iron and it offers a further criterion with which to understand how micro-oganisms interact with each other, their environment and various plant and animal hosts.

1.2 Rationale for Microbial Iron Transport Systems

1.2.1 Biological significance of iron

Most micro-organisms, with the possible exception of lactobacilli (Archibald, 1983), have evolved with an absolute metabolic dependence on iron. The catalytic capabilities inherent in iron have been harnessed by various liganding proteins capable of influencing the relative stabilities between the biologically common oxidation states of iron, Fe^{2+} and Fe^{3+}, and their respective electronic configurations (Aisen, 1977; Hill, 1982; Reed, 1982; Spiro & Saltman, 1974). The interplay between the complex chemistry of iron and these various proteins enables iron to react with oxygen at physiological conditions in a controlled manner or to participate in single electron transfer reactions over the full range of biologically useful redox potentials between dihydrogen (-0.42 v, pH 7, 25°C) and dioxygen (+0.82 v, pH 7, 25°C) (Hill, 1982; Reed, 1982; Spiro & Saltman, 1974; Stryer, 1975; Wrigglesworth & Baum, 1980). While not the sole biologically important transition element (Brill, 1977), iron in iron-containing proteins participates in the widest range of essential biochemical reactions enabling micro-oganisms to survive in most environments (Table 1.1) (Neilands, 1974; 1980).

1.2.2 Microbial iron requirements

The iron content of micro-organisms varies widely from lactobacilli which apparently

Table 1.1 Representative groups of biologically important iron-containing proteins[a]

Iron-containing proteins	Occurrence in micro-organisms	References[b]
Electron transport proteins		
cytochromes	widespread	Slater, 1987
ferredoxins	widespread	Matsubara *et al.*, 1987
nitrogenase	certain genera including: *Azotobacteraceae, Klebsiella,* blue-green bacteria, *Rhizobium Clostridium*	Cammack, 1988; Haaker & Klugkist, 1987
hydrogenases	widespread	Stam *et al.*, 1987
glutamate synthase	wide spread	Miller, 1974
ribonuclotide reductase B2	wide spread	Thelander, 1979
flavoproteins	widespread	Neilands, 1974
Hydroperoxidases		
catalase	aerobes	Yonetane, 1974
superoxide dismutase	aerobes, aerotolerant	Steinman, 1982
Incorporation of molecular oxygen		
mono-, di-oxygenases	widespread	Nozaki & Ishimura, 1974
Iron storage		
bacterioferritin	*Escherichia coli, Azotobacter, Pseudomonas*	Harrison *et al.*, 1987
Oxygen transport		
leghemoglobin	*Rhizobium*	Bisseling *et al.*, 1986; Godfrey *et al.*, 1975

[a] This table was adapted from Neilands (1974; 1980).
[b] Additional reference citations can be found in Neilands (1974; 1980).

have no iron requirements (Archibald, 1983; Neilands, 1981*b*) to magnetotactic bacteria which accumulate up to 1.5% of their dry weight as magnetite (Frankel *et al.*, 1979), but most bacteria contain less than 0.1% iron by dry weight (Lankford, 1973; Neilands, 1974). Iron requirements for the optimum growth of gram-negative bacteria range from approximately 0.36 μM for enterics to 1.6 μM for strict aerobes such as the pseudomonads (Lankford, 1973; Waring & Werkman, 1942) whereas fungi and gram-positive bacteria require 0.4 to 4 μM iron (Weinberg, 1974). These values are at best mere estimates since manipulation of culture conditions can force micro-organisms to use alternate metabolic routes which in turn evoke higher iron demands as has been observed by varying the carbon source (Meyer & Abdallah, 1978; Neilands, 1984*b*) or nitrogen source (Carnahan & Castle, 1958; Esposito, 1956; Subramanian *et al.*, 1968). Moreover, depending on the form of iron in the growth medium and the mode of iron assimilation by a given organism, the concentration of iron required for optimum growth may vary (Pollack *et al.*, 1970). Generally, media supplemented with 10 μM iron are considered iron-sufficient whereas 0.1 μM are iron-limiting (Neilands, 1984*a*).

1.2.3 Bioavailability of iron

Iron is the fourth most abundant element on earth comprising on average, a few percent in soils and generally below 0.05 μM in oceans and rivers (Neilands, 1974; Subcommittee on Iron, 1979). Although iron is present in virtually all microbial habitats, its solution and coordination chemistry is such that in many environments it is not in a form considered readily assimilable by micro-organisms.

In acidic, aqueous environments Fe^{2+} and Fe^{3+} exist as soluble $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$ ions, but an increase in pH causes these ions to hydrolyse resulting in the formation of the insoluble hydroxy-iron compounds $Fe(OH)_2$ and $Fe(OH)_3$ (ideal formulae) (Reed, 1982; Spiro & Saltman, 1974; Subcommittee on Iron, 1979). At pH 7 the solubility of Fe^{2+} and Fe^{3+} is approximately 10^{-1} M and 10^{-18} M, respectively, and

aerobic conditions favor the oxidation of Fe^{2+}. Therefore, free iron is scarce in aerobic environments at physiological pH (Spiro & Saltman, 1974). Furthermore, $Fe(OH)_3$ tends to polymerize in solution forming high molecular mass colloids (Spiro & Saltman, 1969).

A wide variety of inorganic and organic ligands readily replace the H_2O or OH^- ions present in the coordination sphere of Fe^{2+} and Fe^{3+}. Fe^{3+} has a particularly high affinity for ligands which coordinate through oxygen such as phosphates, phenols, diketones and certain sugars (Charley *et al.*, 1963; Spiro & Saltman, 1974) and some of these chelates have a propensity to form aggregates (Spiro & Saltman, 1969). In contrast, Fe^{2+} is preferentially chelated by nitrogen ligand systems such as 2,2'-dipyridyl and o-phenanthroline (Spiro & Saltman, 1974).

In biological environments intracellular iron is invariably coordinated by proteins (Critchton & Charloteaux-Wauters, 1987) and the level of free iron carefully controlled (Harrison *et al.*, 1987; Williams, 1982). This presumably minimizes otherwise uncontrollable reactions of iron with oxygen which generate highly reactive superoxide and hydroxyl radicals (Dunford, 1987; Wrigglesworth & Baum, 1980) capable of damaging essential macromolecules (Wolff *et al.*, 1986; Halliwell & Gutteridge, 1986). In vertebrates, iron is chelated in extracellular fluids by the glycoproteins transferrin or lactoferrin thereby rendering the effective free iron concentration in the order of 10^{-18} M (Griffiths *et al.*, 1988).

Thus, with few exceptions, iron is generally unavailable for direct assimilation by micro-organisms due to its extreme insolubility or to the limitations of biomembranes which are impermeable to iron-proteins, polymeric forms of iron, certain iron chelates or iron-containing minerals (Nikaido & Vaara, 1985; Spiro & Saltman, 1969; Subcommittee on Iron, 1979). Therefore, out of necessity many microbes have evolved specific, high affinity iron assimilatory mechanisms for procuring iron in spite of its varied forms.

1.3 Siderophores

1.3.1 Discovery of siderophores

The field of microbial iron transport grew from the observation that *Ustilago sphaerogena* produced ferrichrome, an organic, iron-containing compound (Neilands, 1952). Ferrichrome was of interest since it acted as a microbial growth stimulant, analogous to mycobactin, coprogen, and "terregens factor", other microbial products being studied at that time (Francis *et al.*, 1949; Garibaldi & Neilands, 1955; Hesseltine *et al.*, 1952). However, the significant finding was that *U. sphaerogena* hyperproduced iron-free ferrichrome when grown in iron-deficient media (Garibaldi & Neilands, 1956). Similar observations of enhanced production of ferric iron-binding agents by iron-limited *Bacillus* spp. and *Aspergillus* spp. prompted Garibaldi and Neilands (1956) to postulate a general role for these compounds in microbial iron sequestration as an important survival mechanism. The possible role of ferrichrome as an iron transferring compound rather than redox catalyst was hypothesized since it had a specific, strong affinity for ferric but not ferrous iron (Neilands, 1957).

With improved culture techniques to overcome the problem of extraneous iron contamination in media, many other organisms such as the enterics were also found to produce siderophores (Lankford, 1973). Genetic confirmation of the role of siderophores in microbial iron sequestration was realized with mutants of *Salmonella typhimurium* defective in the synthesis of their siderophore enterobactin (Pollack *et al.*, 1970). Siderophore-mediated iron assimilation has been firmly established as a common microbial strategy for iron acquisition (Neilands, 1981*a*; 1981*b*).

1.3.2 Microbial distribution of siderophores

The inability of micro-organisms to produce siderophores appears to be the exception rather than the rule. Siderophores have yet to be described for anaerobes or lactic acid bacteria but the fact that the former live at low redox potentials such that Fe^{2+} is relatively

soluble (Spiro & Saltman, 1969; Subcommittee on Iron, 1979) and the latter have no apparent metabolic use for iron (Archibald, 1983), suggests that these organisms do not need siderophores. Also, certain pathogens such as *Neisseria* procure iron directly from transferrin, independent of siderophore production (Griffiths *et al.*, 1988; McKenna *et al.*, 1988).

Otherwise, siderophore production is common among a wide range of aerobic and facultative anaerobic micro-organisms including; phytopathogens (Leong & Neilands, 1982; Enard *et al.*, 1988; Holzberg & Artis, 1983), animal pathogens (Chart & Trust, 1983; Griffiths *et al.*, 1988), nitrogen fixers (Knosp *et al.*, 1984; Fekete *et al.*, 1989), actinomycetes (Hider, 1984), fungi (Neilands, 1981*a*; Winkelman & Huschka, 1987) and cyanobacteria (Simpson & Neilands, 1976).

1.3.3 Siderophore structure

The structures of nearly one hundred siderophores have been described to date (Diekman, 1984; Hider, 1984; Lankford, 1973; Neilands, 1973; Neilands & Ratledge, 1982; Raymond *et al.*, 1984; Winkelman, 1982) and new compounds are constantly being discovered (Actis *et al.*, 1986; Bachhawat & Ghosh, 1987; Demange *et al.*, 1988; Fekete *et al.*, 1989; Page & von Tigerstrom, 1988; Weisbeek *et al.*, 1986). Despite considerable structural diversity, siderophores share a common low molecular mass of approximately 500-1500, a virtual specificity for chelating Fe^{3+} and usually contain hydroxamate and/or catecholate liganding groups in combination with either alpha-hydroxy acid, oxazoline, fluorescent quinolinyl, or thiazoline liganding groups (Fig. 1.1) (Hider, 1984; Neilands, 1984*a*). Rhizobactin is an exception being based on EDTA-like structure (Smith *et al.*, 1985). Other, as yet uncharacterized siderophores also apparently lack the hydroxamate and catecholate groups (Chart & Trust, 1983; Russell & Holmes, 1983).

1.3.4 Siderophore methodology

Techniques for the detection, isolation and structural analysis of siderophores are well established (Neilands, 1984*b*). Generally, siderophores are found in cell-free culture

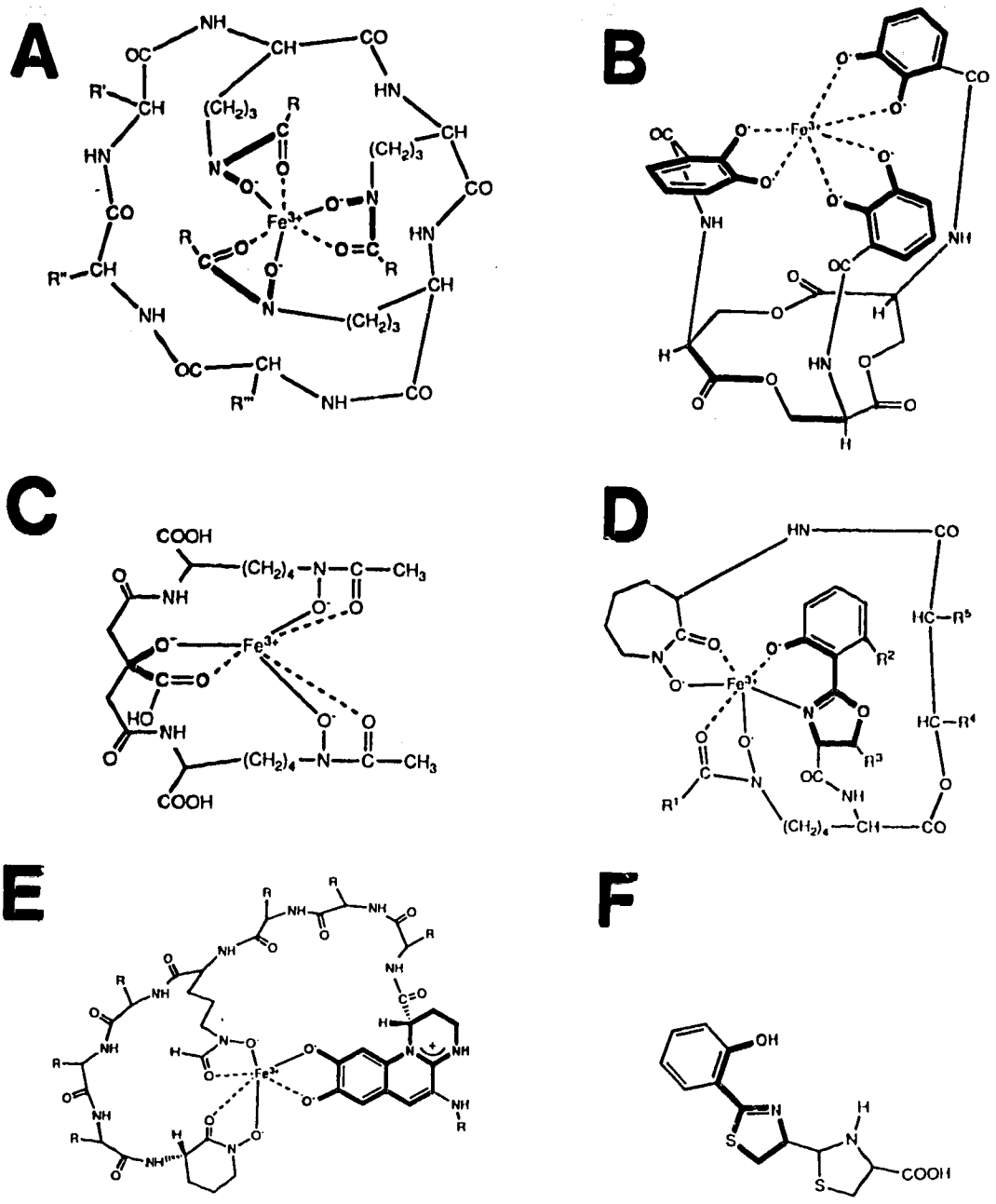

Fig 1.1. Representative siderophore structures showing the various types of liganding groups (bold outline). (A) ferrichromes (hydroxamate); (B) enterobactin (catechol); (C) aerobactin (α-hydroxy acid); (D) mycobactins (2-(*o*-hydroxyphenyl) oxazoline); (E) pyoverdin$_{Pa}$ (fluorescent quinolinyl); (F) pyochelin (2-(*o*-hydroxyphenyl) thiazoline). Structures were reproduced from: (A) Lankford, 1973; (B) Neilands *et al.*, 1981; (C) Neilands, 1984*a*; (D) Ratledge, 1987; (E) Wendenbaum *et al.*, 1983; (F) Cox *et al.*, 1981.

supernatants from iron-limited microbial cultures, unless they are membrane bound as is mycobactin in *Mycobacteria* (Ratledge, 1987). In addition to the formation of coloured complexes with ferric iron (Neilands, 1984*b*), many hydroxamate-containing siderophores are detected by various chemical assays including; periodate oxidation (Atkin *et al.*, 1970), the semiquantitative assay for bound hydroxylamine (Csáky, 1948; Gillam *et al.*, 1981; Tomlinson *et al.*, 1971), or reaction with the folin reagent (Subramanian *et al.*, 1965).

Catecholate siderophores have a characteristic absorption spectrum in the UV range exhibiting pH dependence (Seikel, 1964) and their fluorescence is readily visualized under 254 nm or 366 nm UV light. Many catechol-containing siderophores also form colored complexes with iron but are detected chemically by an assay for aromatic vic-diols (Arnow, 1937; Barnum, 1977) or their ability to reduce iron (Barton, 1965; Krebs, 1969; Rioux *et al.*, 1983).

Alternatively, chemical assays based of the ability of siderophores to remove iron from colored iron complexes (Arnold & Viswanatha, 1983; Schwyn & Neilands, 1987) or bioassays using the siderophore auxotroph *Arthrobacter* JG-9 or enterobactin mutant *S. typhimurium LT-2* (Neilands, 1977*a*; 1984*b*) are functional assays useful for detecting siderophores lacking both hydroxamate and catechol groups.

Once detected, isolation, purification and chemical characterization of siderophores proceeds by a variety of techniques depending on the nature of the siderophore (Neilands, 1984*b*).

1.3.5 Ferrisiderophore complexes

Of the bidentate ligands listed in Fig. 1.1, catechol and hydroxamate ligands have the strongest affinity for Fe^{3+} (Hider, 1984). Siderophores are commonly, although not exclusively, hexadentate molecules comprising three bidentate ligands which coordinate Fe^{3+}, in an octahedral geometry usually via oxygen (Hider, 1984; Raymond *et al.*, 1984). The formation constants of these ferric chelates are significantly higher than a complex derived from three individual bidentate ligands (Hider, 1984). Although the formation

constants of many siderophores are in the range of 10^{30}, enterobactin being the exception at 10^{52} (Neilands 1981*a*; Raymond *et al.*, 1984), the actual formation constant is somewhat lower in biological environments since, by definition, formation constants exclude the concentration of hydrogen ions (Hider, 1984; Raymond *et al.*, 1984). Nevertheless, siderophores solubilize iron, effectively withholding it from insoluble $Fe(OH)_3$ (Hider, 1984) and transferrin or lactoferrin (Cox, 1986; Konopka & Neilands, 1982; Sriyosachati & Cox, 1986; Tidmarsh & Rosenberg, 1981). Since siderophores represent the strongest iron-binding compounds known, their structure and coordination chemistry is of exceptional interest to chemists (Bergeron, 1984; Hider, 1984; Raymond *et al.*, 1984; van der Helm *et al.*, 1987).

Siderophores also chelate other trivalent metals such as Al^{3+} and Ga^{3+} but these chelates are of little biological importance since the later metal is rare, and neither metal is removed from siderophores by reduction and therefore is unlikely to be incorporated into proteins (Hider, 1984; Neilands, 1984*a*). Ga^{3+} siderophore complexes are used in the study of intracellular iron release mechanisms (Emery, 1987) where as the kinetically inert siderophore chelates of Cr^{3+} can be synthesized (Neilands, 1984*a*) and are required for transport studies concerning siderophore isomers (Raymond, 1977; Raymond & Carrano, 1979).

Siderophores also bind other biologically important transition metals such as Cu^{2+} and MoO_2^{2+} (Fekete *et al.*, 1983; Hider, 1984) but their involvement in the transport of these metals into cells, while theoretically possible, has yet to be determined (Hider, 1984).

1.4 Ferrisiderophore Receptor Proteins

1.4.1 Discovery of ferrisiderophore receptor proteins

Most of the information concerning ferrisiderophore receptor proteins comes from studies with gram-negative bacteria, particularly the enterics (Braun, 1981; Neilands,

1982). In the early 1970's, several researchers recognized that siderophores and certain bacterial colicins and *Escherichia coli* phages mutually interfered with the specific function of each other (Guterman, 1973; Hantke & Braun, 1975*a*; Luckey *et al.*, 1975; Wayne & Neilands, 1975). As a result, an outer membrane receptor protein required for ferrichrome transport was identified in *E. coli* (Hantke & Braun, 1975*b*) and later in *S. typhimurium* (Braun *et al.*, 1977; Luckey & Neilands, 1976) thus confirming that both organisms possessed transport mechanisms for ferrichrome, a siderophore they did not synthesize (Luckey *et al.*, 1975). Uemura & Mizushima (1975) noted that the expression of certain high molecular mass outer membrane proteins in *E. coli* was regulated by iron, an observation subsequently confirmed by many laboratories (Neilands, 1982) and shown to be a feature of the enterobactin receptor protein (Hancock & Braun, 1976). Specific binding between enterobactin and its receptor was established (Ichihara & Mizushima, 1977) and confirmed by Hollifield and Neilands (1978). It is firmly established that *E. coli* produces several high molecular mass, iron-repressible outer membrane proteins most of which participate in the specific transport of a given ferrisiderophore (Braun & Hantke, 1981; Braun *et al.*, 1987; Neilands, 1982).

Likewise, many gram-negative bacteria generally produce high molecular mass (65 to 85 kDa) outer membrane proteins in response to iron limitation (Neilands, 1982). These proteins presumably function in siderophore-mediated iron assimilation, but only in a few cases has the role of these proteins as ferrisiderophore receptors been further studied with ferrisiderophore binding assays (Bergeron, 1988; Hohnadel & Meyer, 1988; Saxtena *et al.*, 1986; Sokol & Woods, 1983) or with mutants (Magazin *et al.*, 1986).

Very little information concerning siderophore receptor proteins in fungi and gram-positive bacteria is currently available but iron-repressible envelope proteins ranging from 25 kDa to 180 kDa have recently been identified in *Mycobacterium smegmatis* (Hall *et al.*, 1987).

1.4.2 Ferrisiderophore receptor protein specificity

Ferrisiderophore receptor proteins of *E. coli* specifically mediate transport of iron via their respective ferrisiderophore (Neilands, 1982). They not only distinguish between different molecules such as ferrichrome and enterobactin, but also discriminate between certain structurally similar compounds (Luckey *et al.*, 1975), stereoisomers and coordination isomers (Neilands *et al.*, 1981; Raymond *et al.*, 1984). Strains of *Pseudomonas* which produce structurally similar pyoverdins also discriminate between native and exogenous ferrisiderophores (Hohnadel & Meyer, 1988) at the level of the outer membrane receptor protein (Magazin *et al.*, 1986).

Siderophore selectivity with respect to hydroxamate structure (Emery, 1971) and stereoisomers (Adjimani & Emery, 1988; Winkelman & Braun, 1981) by fungi suggests the involvement of ferrisiderophore receptor proteins. However, little progress concerning the isolation and characterization of such receptor proteins has been made (Emery, 1987; Winkelman & Huschka, 1987).

1.4.3 Ferrisiderophore receptor protein structure and function

Apart from recognition and binding of the ferrisiderophore, receptor proteins are deemed necessary for the translocation of ferrisiderophores through the outer membrane of gram-negative bacteria since the size of many ferrisiderophore complexes exceed the molecular exclusion limit of outer membrane porin proteins (Neilands, 1982; Nikaido & Vaara, 1985). The iron-repressible outer membrane receptor proteins in *E. coli* specifying the transport of iron via ferrichrome (FhuA), enterobactin (FepA), aerobactin (Iut), coprogen and rhodotorulic acid (FhuE) and citrate (FecA), comprise a group of proteins sharing certain physical characteristics with the porin outer membrane proteins such as signal sequence, similar acidic net charge, lack of extended regions of hydrophobicity and lack of α-helical regions (Braun *et al*, 1987; Coulton *et al.*, 1986; Krone *et al.*, 1985; Lundrigan & Kadner, 1986; Nau & Konisky, 1989; Pressler *et al.*, 1988; Sauer *et al.*, 1987). However, the iron repressible-outer membrane proteins share a short region of

homology at their amino terminus not shared by the Omp proteins of *E. coli* (Braun *et al*, 1987; Coulton *et al.*, 1986; Lundrigan & Kadner, 1986; Nau & Konisky, 1984; Pressler *et al.*, 1988) which may be related to their interaction with TonB (Lundrigan & Kadner, 1986), a cytoplasmic protein required for energy-dependent ferrisiderophore transport (Braun *et al.*, 1987). The exact mechanism by which ferrichrome and ferrienterobactin are translocated through the outer and cytoplasmic membrane of *E. coli* (Hartman & Braun, 1980; Leong & Neilands, 1976) is unclear being further complicated by the requirement for additional specific gene products, the roles of which are currently under investigation (Braun *et al.*, 1987; Earhart *et al.*, 1987).

1.5 High affinity iron assimilation

1.5.1 Mechanisms

The kinetics and mechanism of various siderophore-mediated iron assimilation systems have been studied in numerous fungi and bacteria using radiolabelled iron and/or siderophores (Braun *et al.*, 1987; Buyers, 1974; Earhart, 1987; Emery, 1971; Lankford, 1973; Neilands, 1981*b*; 1984*a*; Ratledge, 1987). Despite considerable diversity concerning the details of iron assimilation, siderophore-mediated iron transport either involves receptor-mediated internalization of the ferrisiderophore complex with intracellular iron release by reduction (Neilands, 1984*a*), or recognition of the ferrisiderophore at the membrane surface followed by extracellular release of the iron with subsequent internalization of the iron alone (Emery, 1987). Since the majority of iron assimilation studies use radiolabelled iron alone, the fate of the siderophore is not followed so the mechanism of iron assimilation is not known in these cases (Emery, 1987).

1.5.2 Ferrisiderophore transport

Receptor-mediated ferrisiderophore transport is characterized by its specificity and saturable rate at low siderophore concentrations (Frost & Rosenberg, 1978; Negrin & Neilands, 1978). Iron enters the cell as a ferrisiderophore complex, as indicated by

synchronous uptake of both $^{55}Fe^{3+}$ or $^{59}Fe^{3+}$ and ^{3}[H]- or ^{14}[C]-labelled siderophores (Arceneaux *et al.*, 1973; Emery, 1971; Hartman & Braun, 1980; Leong & Neilands, 1976). It is generally believed that iron is released from the internalized ferrisiderophore via a reduction mechanism (Arceneaux, 1983; Emery, 1987). Mössbauer spectroscopy has been useful for following the oxidation state of iron during transport and readily distinguishes between intracellular iron release or iron reduction at the cell surface (Matzanke *et al.*, 1986; Matzanke, 1987). Once iron is released from the siderophore the deferrisiderophore is expelled from the cell unchanged as with schizokinen in *Bacillus* (Arceneaux *et al.*, 1973), with minor structural modifications resulting in a non-recyclable siderophore with a lower affinity for iron as in ferrichrome of *E. coli* (Hartman & Braun, 1980) or is destroyed by hydrolysis as is enterobactin of *E. coli* (Earhart, 1987).

1.5.3 Siderophore-mediated iron transport

Many fungi and some bacteria use ferrisiderophores as a shuttle which donates iron to the cell surface (Emery, 1987; Hider, 1984; Müller *et al.*, 1985*a*; Ratledge, 1987) with subsequent internalization of the iron without the siderophore (Emery, 1987). It is generally believed that iron is released from the siderophore by reduction (Emery, 1987) although a mechanism of ligand exchange has also been proposed (Müller *et al.*, 1985*a*). Siderophore-mediated iron transport also exhibits specificity with respect to the siderophore and stereochemistry of the ferrisiderophore complex (Müller *et al.*, 1985*b*; Winkelman & Huschka, 1987) thereby implicating a membrane receptor protein in this mechanism as well.

Ferrisiderophore transport and siderophore-mediated iron transport may offer an explanation for microbial iron assimilation which show biphasic uptake kinetics possibly resulting from the saturation of two systems operating simultaneously (Emery, 1987). The extreme lengths to which micro-organisms have gone in order to ensure a constant iron source is reflected in the complexity of various microbial iron-assimilatory systems currently being studied (Braun, 1987; Emery, 1987; Neilands, 1982; 1984*a*; Ratledge,

1987).

1.6 Regulation of Iron Uptake

Micro-organisms invest substantial amounts of energy in the biosynthesis of siderophores (Earhart, 1987; Marugg *et al.*, 1985; Viswanatha *et al.*, 1987) and membrane receptor proteins, therefore it is not surprising that efficient and sensitive regulatory systems are involved in expressing components of iron uptake. *E. coli* detects and responds rapidly to available iron (Klebba *et al.*, 1982) which in turn coordinately regulates the production of siderophores and outer membrane proteins (McIntosh & Earhart, 1977; Fleming *et al.*, 1983). Similarly, *Azotobacter vinelandii* is sensitive to prevailing iron concentrations and sequentially expresses its siderophores as the iron concentration becomes growth-limiting (Page & Huyer, 1984).

1.6.1 Regulation by iron via Fur

The study of how iron mediates regulation of components of iron assimilation was not possible until the isolation of regulatory mutants, the first of which were isolated in *S. typhimurium* (Ernst *et al.*, 1978). These mutants produced iron-repressible outer membrane proteins and enterobactin irrespective of the prevailing iron concentration (Ernst *et al.*, 1978). Subsequent work with *fur* (*f*erric *u*ptake *r*egulation) mutants in *E. coli* (Hantke, 1981) established that *fur* is a chromosomal gene (Hantke, 1984; Bagg & Neilands, 1985) coding for Fur, a protein which binds DNA at a specific consensus sequence located in the promoter region of plasmid and chromosomal iron regulated genes in *E. coli* (Bagg & Neilands, 1987*a*; Calderwood & Mekalanos, 1988; De Lorenzo *et al.*, 1987). This confirmed initial studies indicating that Fur negatively regulates various iron-regulated genes at the transcriptional level (Worsham & Konisky,1981; Fleming *et al.*, 1983; Hantke, 1984). Furthermore, ferrous but not ferric iron acts as a corepressor with Fur to bind DNA and regulate expression of the aerobactin operon as a classical negative repressor (Bagg & Neilands, 1987*a*).

1.6.2 Regulation by temperature

In addition to the direct regulation of iron assimilation systems by iron as a corepressor with Fur, several researchers have observed temperature-regulation of siderophore production (Garibaldi, 1971; 1972; Ismail *et al.*, 1985; Worsham & Konisky, 1984), a phenomenon which appears to manifest itself independently of Fur in *E. coli* (Worsham & Konisky, 1984). While the mechanism by which increased temperature restricts siderophore production is not known, this phenomenon may be pertinent to research concerning the effect of the host febrile response on bacterial virulence (Kluger & Rothenburg, 1979; Kluger *et al.*, 1983).

1.7 Practical aspects of microbial iron transport

1.7.1 Medical applications

The importance of iron in microbial disease has been recognized for some time (Weinberg, 1978) and it is well established that the ability of some pathogens to produce siderophores enhances their virulence *in vivo* (Griffiths *et al.*, 1988). Therefore, additional knowledge concerning iron assimilatory mechanisms of various pathogens not only contributes to our understanding of microbial-host interactions (Bullen, 1981; Finkelstein *et al.*, 1983) but opens up possibilities for new strategies to curb disease (Rogers, 1987). Unfortunately, the practical use of siderophores as antibacterial agents (Hider, 1984) or as carriers for the delivery of toxic metals such as In^{3+} or Sc^{3+} (Rogers *et al.*, 1984) to pathogenic bacteria is presently limited due to their chemical lability, low solubility or low residence time in the body (Hider, 1984; Raymond, 1984) as well as the hazard that a therapeutic siderophore may donate iron to alternate pathogens (Rogers *et al.*, 1984; West & Sparling, 1987).

In spite of the side effects, lack of oral efficacy and expense, the siderophore desferrioxamine B (Desferal®) produced by *Streptomyces pilosus*, is currently used for its iron chelation ability as the drug of choice for treating acute accidental iron overdose in

children and chronic iron overload in patients receiving frequent blood transfusions (Raymond *et al.*, 1984; Subcommittee on Iron, 1979). Proposals also exist to use siderophores in cases of accidental internal poisoning with Al^{3+} or plutonium and to remove trace nuclides of $^{67}Ga^{3+}$ or $^{111}In^{3+}$ currently used in tumour visualization procedures (Hider, 1984). Furthermore, experiments suggest that the iron chelating ability of Desferal® or other siderophores may limit the formation of hydroxy radicals *in vivo* in the event that intracellular iron becomes decompartmentalized, a process considered important in causing some diseases (Willson, 1977; Halliwell & Gutteridge, 1986)

These potential medical applications of siderophores have prompted continued research for synthetic or natural chelators with superior qualities of therapeutic value (Hider, 1984; Raymond *et al.*, 1984).

1.7.2 Agricultural applications

Principles of microbial iron assimilation have equal application in plant pathology and soil ecology with respect to microbial-plant or microbial-microbial interactions. Very little is known about the role of iron in plant pathogenesis (Neilands & Leong, 1986), but it is becoming apparent that some pathogens require a functional iron assimilatory mechanism for virulence (Enard, 1988; Expert & Toussaint, 1985). Other phytopathogens such as *Agrobacterium* apparently have no such siderophore-requirement for virulence (Leong & Neilands, 1981).

Current research has focussed on certain rhizosphere fluorescent pseudomonads of agricultural importance whose ability to promote plant growth is directly related to the production of pseudobactins, fluorescent siderophores which sequester iron in the rhizosphere soil thereby limiting the growth of pathogenic fungi and bacteria (Buyer & Leong, 1986; Kloepper *et al.*, 1980; Schroth & Hancock, 1982). Such micro-organisms are being actively studied as possible biopesticides (Buyer & Leong, 1986; Leong, 1986). Additional research concerning iron assimilation by soil microorganisms will hopefully increase our knowledge as to their role in soil ecology and phytopathology, and may offer

future solutions to various agricultural problems.

1.8 Thesis objectives

1.8.1 Background on *Azomonas macrocytogenes*

Azomonas macrocytogenes is a gram-negative, free-living nitrogen-fixing soil bacterium originally isolated from neutral to alkaline soils in temperate regions (Jensen, 1955; Norris & Baird, 1960; Thompson & Skerman, 1979). *Azomonas macrocytogenes* is thought to be a rare organism, as reflected by the relatively few number of original isolates (Table 1.2), however recent surveys in Canada and Poland suggest that this soil diazotroph may be more prevalent than previously thought (Kole *et al.*, 1988; Page & Collinson, 1987; Szember *et al.*, 1981). Since the initial description of *Azomonas macrocytogenes* as an acid-tolerant azotobacter (Jensen, 1955), only studies concerned with descriptions of the genus *Azomonas* and its taxonomic placement have emerged in the literature. Accordingly, the previous names *Azotobacter macrocytogenes* (Jensen, 1955) and *Azomonotrichon macrocytogenes* (Thompson & Skerman, 1979; 1981) have been dropped in favor of *Azomonas macrocytogenes* (New & Tchan, 1982).

Azomonas macrocytogenes has characteristically large, oval cells (Jensen, 1955) with 1 to 4 polar flagella (Baillie *et al.*, 1962; Jensen, 1955; Norris & Chapman, 1968). *Azomonas macrocytogenes* is a strict aerobe distinguished from *Azotobacter* by its lack of TMPD-dependent oxidase (Jurtshuk *et al.*, 1984) and inability to form cysts (New & Tchan, 1982). Other criteria used to distinguish these genera include antigenic characterization (Norris & Chapman, 1968; Tchan *et al.*, 1980; Tchan *et al.*, 1983), phage sensitivity (Duff & Wyss, 1961; Hegazi & Jensen, 1973), DNA base composition (%G+C) and DNA homology (De Ley & Park, 1966), and production of soluble, fluorescent pigments (Johnstone, 1957; Johnstone *et al.*, 1958; Johnstone *et al.*, 1959; Norris & Chapman, 1968). Recent DNA:rRNA hybridization studies indicate that *Azomonas macrocytogenes* is equally related to *Azotobacter* and the group I fluorescent

Table 1.2 Previously described and catalogued isolates of *Azomonas macrocytogenes* [a]

Strain name[b]	Description and source	Synonymous strain names
ATCC 12334	Capsule-negative variant of ATCC 12335 (Jensen, 1955)	NCIB 8702; WR 124; WR 148; Strain I
ATCC 12335[c]	Original isolate (Jensen, 1955) ATCC 12335 (Jensen, 1955)	NCIB 8700; WR 111; DSM 721 Strain O
ATCC 12336	Colony variant of ATCC 12335 (Jensen, 1955)	NCIB 8701; WR 125; Strain M
NCIB 9128[c]	Original isolate (Norris & Baird, 1960)	WR 45; strain 10 EM(O)
NCIB 9129	Motile variant of NCIB 9128 (Norris & Baird, 1960)	WR 46; DSM 722; strain 10 EM (M)
NCIB 10958[c]	Original isolate (Norris 1973)[d]	strain 2A
NCIB 10959	Motile variant of NCIB 10958 (Norris 1973)[d]	strain 2AM
NCIB 10960	Colony variant of NCIB 10958 (Norris 1973)[d]	strain 2AR
WR 140[c]	Reclassified by Thompson and Skerman (1979)	Cotype *Azotobacter agilis* ssp. *jakutiae*

[a] This table was reprinted from Page and Collinson (1987).
[b] Culture collection abbreviations: ATCC American Type Culture Collection; NCIB National Collection of Industrial Bacteria; WR Queensland Wheat Research Institute; DSM Deutsche Sammlung von Mikroorganismen.
[c] Parent strain.
[d] No citation was found for these isolates, except their listing in the NCIB catalogue (1975).

pseudomonads but forms an independent, distinct branch (De Ley & Park, 1966; De Smedt *et al.*, 1980; De Vos *et al.*, 1989). Other relevant metabolic characteristics have been compiled (Johnstone, 1974; Tchan and New 1984; Thompson & Skerman, 1979). However, previous studies have not addressed iron assimilation in *Azomonas macrocytogenes.*

1.8.2 Objectives

In view of the lack of information concerning iron assimilation in *Azomonas macrocytogenes* the object of my thesis was to investigate iron assimilation in this diazotroph. This study reports the isolation, purification and identification of two iron-binding compounds recovered from culture supernatants of *Azomonas macrocytogenes,* 3,4-dihydroxybenzoic acid and azoverdin. While the former is not apparently regulated by iron, it had the ability to solubilize iron from various mineral sources thereby promoting the growth of *Azomonas macrocytogenes* ATCC 12334. Azoverdin was identified as a novel pyoverdin-like fluorescent compound which functioned as a siderophore to promote $^{55}Fe^{3+}$ assimilation by *Azomonas macrocytogenes.*

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CHAPTER 2

Isolation and purification of 3,4-dihydroxybenzoic acid from *Azomonas macrocytogenes*

2.1 SUMMARY

Four strains of *Azomonas macrocytogenes*, five strains of novel *Azomonas macrocytogenes* isolates from Alberta soils and a strain of *Azotobacter paspali* were found to produce a common extracellular, iron-binding, phenolic compound. This compound was purified from cell-free culture supernatant fluids and chemically identified as 3,4-dihydroxybenzoic acid (protocatechuic acid). 3,4-dihydroxybenzoic acid was produced by cells growing in a simple defined medium containing acetate and glucose or sucrose as sole carbon sources. This compound promoted the solubilization of iron from the minerals olivine, glauconite, pyrite and marcasite. Mineral-free medium prepared by preincubation with olivine or glauconite as the only iron source supported enhanced growth yields of *Azomonas macrocytogenes* (ATCC 12334) when 3,4-dihydroxybenzoic acid was present during the preincubation period. This suggests that 3,4-dihydroxybenzoic acid may play a role in solubilizing iron from some natural iron sources, however, 3,4-dihydroxybenzoic acid can not be considered a true siderophore since it was produced by *Azomonas macrocytogenes* and *Azotobacter paspali* growing in iron-limited and iron-sufficient medium.

A version of this chapter has been published:

Collinson, K., Doran, J. L. & Page, W. J. (1987). Production of 3,4-dihydroxybenzoic acid by *Azomonas macrocytogenes* and *Azotobacter paspali*. *Canadian Journal of Microbiology* **33**, 169-175.

Page, W. J. & Collinson, S. K. (1987). Characterization of *Azomonas macrocytogenes* strains isolated from Alberta soils. *Canadian Journal of Microbiology* **33**, 830-833.

2.2 INTRODUCTION

Virtually all organisms, with the possible exception of lactobacilli (Archibald, 1983), require iron for growth. Although iron is abundant in the environment, its availability to organisms is limited due to its extreme insolubility (Neilands, 1984; Spiro & Saltman, 1969). In response to iron limitation, many micro-organisms produce siderophores which are low molecular weight catechol or hydroxamate compounds that specifically chelate and solubilize iron (Neilands, 1981*b*). The biosynthesis of the siderophores and the respective outer membrane protein receptors, which specifically recognize the ferri-siderophore complex, are regulated in response to the concentration of available iron (Neilands, 1981*a*). Together these components constitute a high affinity iron uptake system. At concentrations of iron sufficient to suppress the expression of high affinity iron uptake systems, microorganisms obtain iron via low affinity iron uptake mechanisms which, at present, are poorly understood (Neilands, 1984).

Azotobacteraceae are gram-negative, non-symbiotic nitrogen fixing, obligate aerobes common to soil. These organisms have a high requirement for iron, not only for respiratory enzymes and the components of nitrogenase, but also for the enzymes that aid in protecting nitrogenase from oxygen (Yates & Jones, 1974). One member of this family, *Azotobacter vinelandii*, is known to benefit from a high affinity iron uptake system. Under conditions of iron limitation, *Azotobacter vinelandii* produces two iron-repressible fluorescent siderophores, azotochelin (2-N,6-N-di-(2,3-dihydroxybenzoyl)-L-lysine), (Corbin & Bulen, 1969) and azotobactin, a yellow-green fluorescent peptide (Fukasawa *et al.*, 1972), that function in iron uptake (Knosp *et al.*, 1984). The catechol 2,3-dihydroxybenzoic acid is produced by *Azotobacter vinelandii* whether or not iron is limiting in the growth medium (Corbin & Bulen, 1969) and has been postulated to play a role in mineral iron solubilization thereby facilitating low affinity iron uptake (Page & Huyer, 1984).

It was our interest to determine whether other members of the *Azotobacteraceae* produce extracellular compounds involved in iron assimilation. This paper reports that four strains of *Azomonas macrocytogenes*, five *Azomonas macrocytogenes* isolates obtained from Alberta soils and a strain of *Azotobacter paspali* have been found to produce an extracellular, iron-binding, phenolic compound under iron-limited and iron-sufficient conditions which has been identified as 3,4-dihydroxybenzoic acid (3,4-DHBA). These studies have indicated that 3,4-DHBA enhances the solubilization of iron from several iron-containing minerals. In two instances, this solubilization of iron was demonstrated to have a growth promoting effect on cultures of *Azomonas macrocytogenes* ATCC 12334 which suggests that 3,4-DHBA may serve as part of a low affinity iron assimilation system. Preliminary results of this study were presented at the 85th Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 3-7 March, 1985 (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, O65, p 246).

2.3 MATERIALS AND METHODS

Organisms and growth conditions. Four *Azomonas macrocytogenes* strains ATCC 12334 (Jenson, 1955), NCIB 10958, NCIB 9129, and NCIB 8700 (ATCC 12335) were routinely grown in nitrogen-free, iron-limited Burk medium (Page & Sadoff, 1976) containing 14 mM-sodium acetate and 1% glucose (autoclaved separately). Five strains of *Azomonas macrocytogenes* (3, 132, 223, 240, 241), obtained from a culture collection of free living, aerobic, nitrogen-fixing soil bacteria isolated from Alberta soils (Page, 1986) and previously identified according to routine taxonomic tests (Tchan & New, 1984; Thompson & Skerman, 1979), were similarly grown. *Azotobacter paspali* WR129, obtained from Dr. B. Terzaghi (Department of Scientific and Industrial Research, Palmerston North, New Zealand), was grown similarly but sucrose was substituted for

glucose in the medium. Iron-sufficient medium contained 90 μM-$FeSO_4$. When required, dry, sterilized, iron-containing minerals (Ward's Natural Science Establishment, Inc., Rochester, N.Y.) were added to iron-limited medium (0.5 g L^{-1}) prior to inoculation. To minimize iron contamination, the distilled water used in media preparation was deionized using the Milli-Q water purification system (Millipore Corp., Bedford, MA) and glassware was washed with 4 M-HCl and 50 mM-EDTA, pH 7, before use.

Cultures grown on slants of solid (1.8% agar), iron-sufficient Burk medium (containing 14 mM-ammonium acetate in place of sodium acetate) for 2 to 4 days at 30°C were used to prepare an inoculum. A cell suspension prepared by washing slant cultures with iron-limited Burk medium was used to inoculate liquid media to an OD_{620} of approximately 0.05. Liquid cultures were incubated in a New Brunswick model G-76 gyrotory water bath shaker at 175 r.p.m. and 30°C.

Identification of iron-binding phenolic compounds in culture supernatants.

The bacterial strains were grown in iron-limited or iron-sufficient Burk medium for 24 h before the cells were harvested by centrifugation (10 400 ***g***, 10 min) and the supernatants were filter-sterilized (0.45 μm pore-sized Millipore filter). The filtrate was acidified to pH 1.8 using HCl and extracted three times with 0.25 volumes of ethyl acetate. Ethyl acetate extracts were combined and reduced to dryness. The residue was dissolved in ethyl acetate or ethanol and spotted on silica gel-G thin layer chromatography (TLC) plates (Redi-plate, Fisher Scientific Co., Pittsburg, PA or Polygram sil-G, Macherey-Nagel, Düren, FRG). Chromatograms were developed in one of six solvent systems: i) butanol:acetic acid:water (4:1:5); ii) benzene:acetic acid:water (125:72:3); iii) benzene:methanol:acetic acid (45:8:4); iv) benzene:acetic acid:water (6:7:3); v) chloroform:methanol:acetic acid (89:10:1); vi) chloroform:acetic acid:water (140:8:52). Solvent systems (i)-(iv) were previously described by Seikel (1964). Spots were located visually by illumination under UV light (366 nm and 254 nm). Iron-binding phenolic compounds were detected using the spray

reagents ferric chloride (0.1 M-$FeCl_3 \cdot 6H_20$ in 0.1 M-HCl), ferricyanide (potassium)-ferric chloride, and 2,2'-dipyridyl-ferric chloride (Krebs *et al.*, 1969). Arnow reagents were sprayed onto chromatograms to locate o-dihydric phenols which react with the nitrite-molybdate reagent to form a yellow complex which changes to red when sprayed with NaOH (Arnow, 1937). A portion of the residue from the ethyl acetate extracts was dissolved in 0.1 M-HCl, 0.1 M-NaOH, or distilled water for UV spectral analysis using a Perkin-Elmer Lambda 3 scanning spectrophotometer.

Purification and identification of 3,4-DHBA. Cell-free, filter-sterilized culture supernatant fluids prepared from 24 h cultures of *Azomonas macrocytogenes* ATCC 12334 or *Azotobacter paspali* WR129 were used to obtain material for the purification and identification of 3,4-DHBA. The culture supernatant from *Azotobacter paspali* was mixed with 1 volume of acetone and 0.03 volumes of 5 M-NaCl to precipitate the capsular polysaccharide which was removed by decanting the supernatant. The acetone was removed from the supernatant by rotary evaporation using a Buchi Rotavapor-R at 30 to 34°C prior to filtration. Otherwise, the culture supernatants were treated in the same manner. Nine litres of culture supernatant fluid were concentrated two-fold by rotary evaporation. This concentrate was acidified and extracted with ethyl acetate. Ethyl acetate extracts were concentrated to approximately 50 mL by rotary evaporation, washed three times with 1 volume of distilled water and back extracted three times with 1 volume of 50 mM-potassium phosphate buffer, pH 7. The phosphate buffer layers were combined, reacidified and reextracted with ethyl acetate. This ethyl acetate extract was reduced to dryness. The yellowish residue was dissolved in a small volume of ethyl acetate to which several volumes of cold hexane (pesticide grade) were added. The resulting white precipitate was collected by vacuum filtration on Whatman 50 filter paper. This material was further purified on preparatory silica gel-G TLC plates developed in benzene:acetic acid:water (125:72:3). The major iron-binding phenolic compound was scraped off the

chromatography plates, eluted from the silica gel using ethyl acetate and reprecipitated with hexane. This purified compound was analyzed by mass spectroscopy using a Kratos model MS-50 instrument for high resolution analysis and a Kratos model MS-12 instrument for the low resolution analysis. Infrared spectroscopy was performed on a methanol cast of the sample which was analyzed on a Nicolet model 7199 FTIR. Nuclear magnetic resonance spectroscopy was performed on the compound dissolved in deuterated methanol using a Bruker model WH400 instrument at 400 MHz. Authentic 3,4-DHBA (Aldrich Chemical Company, Inc. Milwaukee, WI) was used as a standard.

Assay for quantifying 3,4-DHBA in culture supernatants. The concentration of 3,4-DHBA in 24 h cell-free culture supernatant fluids was determined by the Arnow procedure as modified by Barnum (1977). Authentic 3,4-DHBA dissolved in uninoculated growth medium was used as a standard. The A_{497} was read within 30 seconds of the NaOH addition because the red color produced was not stable in culture supernatant fluids.

2,2'-dipyridyl iron assay. For the determination of iron solubilized from minerals, samples of growth medium preincubated under normal growth conditions with iron minerals were centrifuged (1 640 ***g***, 15 min) to remove mineral debris. It should be noted that iron as oxyhydroxide polymers and ferric salts are removed from the medium if filtration is used to remove mineral debris (Knosp *et al.*, 1984). Iron concentrations were determined by the 2,2'-dipyridyl procedure as previously described (Osaki *et al.*, 1971), however, the concentration of ascorbate was increased to 0.5 M to ensure the reduction of Fe^{3+} to Fe^{2+}, the form of iron reactive with 2,2'-dipyridyl. The preincubated media containing detectable soluble iron turned purple due to the complexing of Fe^{3+} by 3,4-DHBA (Migal & Ivanov, 1973) otherwise the preincubated medium was colourless. The ascorbate and HCl added to the 2,2'-dipyridyl iron assay reduced the iron thereby destroying the coloured complex and hence 3,4-DHBA did not interfere in the iron assay.

Bioassay of mineral iron solubility. 50 mL of sterile iron-limited Burk medium was preincubated with minerals in the presence or absence of 3,4-DHBA for 24 h under normal culture conditions. The mineral was allowed to settle for 30 min and then a 20 mL sample of mineral-free growth medium was very carefully removed and transferred to a sterile 50 mL flask for inoculation with *Azomonas macrocytogenes* ATCC 12334. After 24 h incubation the bacterial cells were pelleted, resuspended in 0.1 M NaOH, and incubated for 1 h at 90°C. The protein content of the cell pellets was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

2.4 RESULTS

Identification of 3,4-DHBA in culture supernatants

The ethyl acetate extracts of acidified, cell-free culture supernatant fluids obtained from iron-limited cultures of the *Azomonas macrocytogenes* strains (ATCC 12334, NCIB 10958, NCIB 9129 and NCIB 8700) and *Azotobacter paspali* strain WR129 were examined by TLC for the presence of iron-binding, catechol-related compounds. Each organism produced a prominent compound that comigrated on chromatography plates developed in each of the six solvent systems. The prominent compound from each supernatant reacted positively with ferric chloride (Fig. 2.1A), ferricyanide (potassium)-ferric chloride, 2,2'-dipyridyl-ferric chloride and the Arnow reagents sprayed onto the chromatograms indicating that it was an iron-binding, o-dihydric phenolic compound. On the TLC plates this compound was non-fluorescent under UV illumination at 366 nm but produced deep purple fluorescence under 254 nm UV light. The UV spectrum of the compound from culture supernatants acidified to pH 1.8 produced by these organisms matched the spectrum of the compound eluted from the TLC plates (Fig. 2.1B).

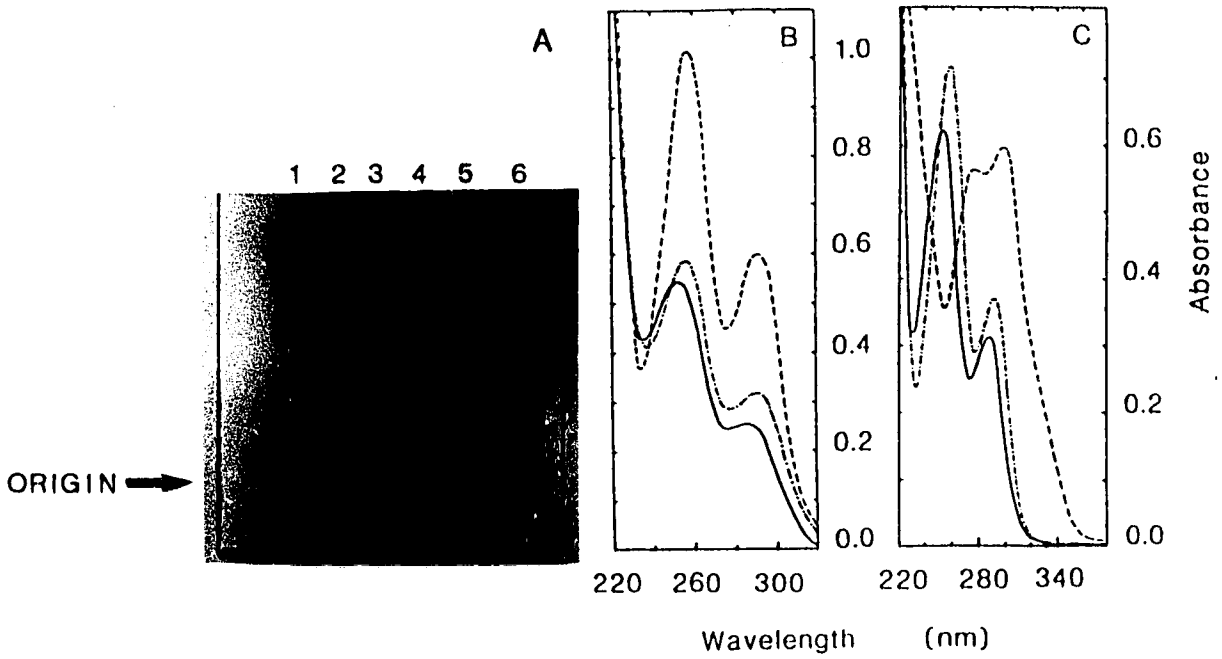

Fig. 2.1. (A) Ethyl acetate extracts from cell-free, filter-sterilized, culture supernatant fluids of *Azomonas macrocytogenes* and *Azotobacter paspali* grown on iron-limited Burk medium were spotted onto silica gel-G plates, developed in benzene:acetic acid:water 125:72:3 and sprayed with 0.1 M-$FeCl_3 \cdot 6H_2O$ in 0.1 M-HCl. (Lane 1) authentic 3,4-DHBA; ethyl acetate extracts of *Azomonas macrocytogenes*: (Lane 2) ATCC 12334; (Lane 3) NCIB 8700; (Lane 4) NCIB 9129; (Lane 5) NCIB 10958; (Lane 6) *Azotobacter paspali* WR129. (B) UV scan of, filter-sterilized culture supernatant fluid of *Azomonas macrocytogenes* ATCC 12334 grown in iron-limited Burk medium (——), acidified culture supernatant, pH 1.8 (·-·-), and a scan of an acidified preparation of the major compound eluted from silica gel (- - -). (C) The major iron-binding, phenolic compound purified from *Azomonas macrocytogenes* ATCC 12334 culture supernatant fluids and dissolved in 0.1 M-HCl, pH 1.2 (·-·-); distilled H_2O, pH 4.6 (——); or 0.1 M-NaOH, pH 12 (- - -).

Furthermore, the UV spectral shifts of this compound at different pH values (Fig. 2.1C) were the same for the compound isolated from all five organisms, indicating that they all produced the same iron-binding, o-dihydric phenolic compound. This compound was also present in the culture supernatant fluids from each of these organisms grown under iron-sufficient conditions. Similarly, the *Azomonas macrocytogenes* strains (3, 132, 223, 240 and 241) also produced the iron-binding o-dihydric phenolic compound (data not shown).

This compound was purified from culture supernatants of *Azomonas macrocytogenes* ATCC 12334 as described in Materials and methods. Mass spectroscopy on the purified compound revealed a molecular formula $C_7H_6O_4$ and a molecular weight of 154. One of the possible isomers, 3,4-DHBA, comigrated with the compound purified from *Azomonas macrocytogenes* ATCC 12334 on chromatograms developed in each of the six solvent systems and demonstrated identical UV spectra at different pH values (Kamath *et al.*, 1975; Pittard *et al.*, 1961). Like authentic 3,4-DHBA, the compound purified from *Azomonas macrocytogenes* ATCC 12334 formed a green coloured complex with $FeCl_3$ below pH 6 and colour shifts through blue to red as the pH was increased (Migal & Ivanov, 1973). Moreover, a mixed melting point determination with authentic 3,4-DHBA and the compound purified from *Azomonas macrocytogenes* ATCC 12334 or *Azotobacter paspali* WR129 gave a sharp melting point at 201-202°C with decomposition indicating that the phenolic compound purified from the culture supernatants was 3,4-DHBA. This conclusion was confirmed by infrared and nuclear magnetic resonance spectroscopy (data not shown).

Production of 3,4-DHBA

Azomonas macrocytogenes ATCC 12334 was able to grow in iron-limited medium (Fig. 2.2). The production of 3,4-DHBA was apparent from the onset of growth and continued after the culture entered stationary phase (Fig. 2.2). The pH of the culture

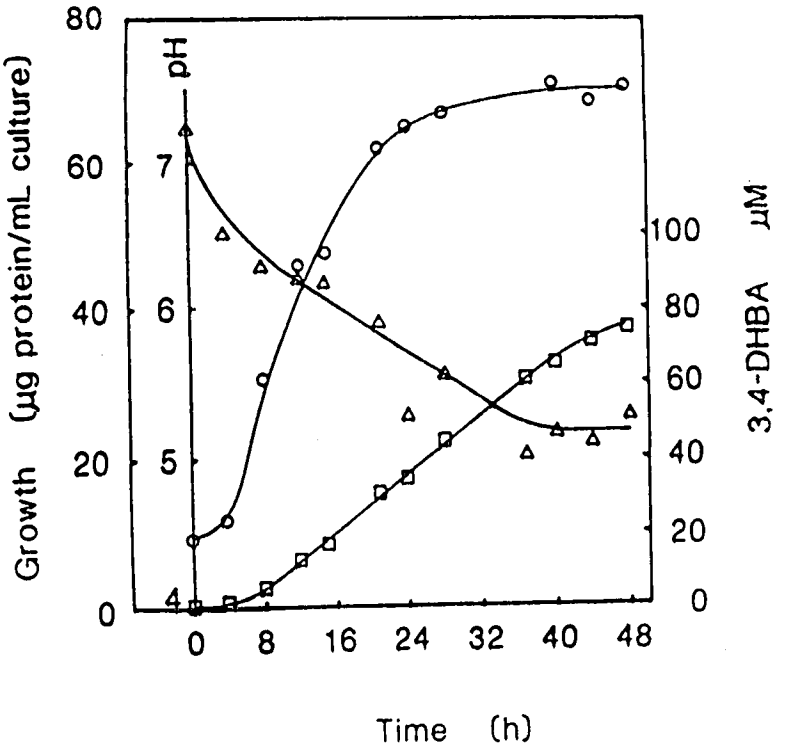

Fig. 2.2. Growth of *Azomonas macrocytogenes* ATCC 12334 in iron-limited Burk medium. At time intervals individual cultures were removed from incubation, centrifuged and the cell pellets assayed for total cell protein (○) using the method of Lowry *et al.* (1951) as described in Materials and methods. The cell-free culture supernatants were assayed for pH (△) and 3,4-DHBA concentration (□) using the method of Barnum (1977) as described in Materials and methods.

decreased with increasing culture age, as noted previously by Jensen (1955). With increased amounts of added iron in the growth medium the total cell protein from 24 h cultures of *Azomonas macrocytogenes* ATCC 12334 increased from a level sustained by residual iron contamination from medium constituents and cell inoculum (Table 2.1). The concentration of 3,4 DHBA assayed in the cell-free culture supernatant fluids was not altered significantly by increasing iron concentration in the growth medium (Table 2.1). Similarly, when *Azomonas macrocytogenes* ATCC 12334 was grown in direct contact with iron-containing minerals as sole iron sources (Table 2.2), the concentration of 3,4-DHBA produced by the cells remained constant while the total cell protein increased in response to the apparent increase in available iron from the different minerals (Page & Huyer, 1984). Whereas the concentration of 3,4-DHBA in the culture supernatants did not vary with the differing concentrations of iron added to the various cultures, the nmol 3,4-DHBA produced per μg of protein decreased as soluble iron became more available.

Solubilization of iron by 3,4-DHBA

The iron-containing minerals were preincubated under normal culture conditions in sterile, iron-limited Burk medium alone, or with increasing additions of authentic 3,4-DHBA. After 24 h preincubation, the sterile media were assayed for pH and for soluble iron using the 2,2'-dipyridyl assay or a bioassay using *Azomonas macrocytogenes* ATCC 12334. The pH of the preincubated media remained between 6.8 and 7.3 despite the addition of up to 1 mM-3,4-DHBA. No soluble iron could be detected with the 2,2'-dipyridyl assay when minerals were incubated alone or with 0.06 mM-3,4-DHBA. Similarly, no soluble iron could be detected when goethite, micaceous hematite, or hematite were incubated in the presence of up to 1 mM-3,4-DHBA. Only trace levels of soluble iron were detected when olivine and pyrite were preincubated with 0.5 to 1.0 mM 3,4-DHBA. Preincubation of glauconite, marcasite, and $FeSO_4$ with 1.0 mM-3,4-DHBA resulted in the

Table 2.1. The effect of iron concentration on 3,4-DHBA production by cultures of *Azomonas macrocytogenes* ATCC 12334.

Iron added[a] μM	Total cell protein[b] μg protein mL^{-1} culture	3,4-DHBA[c] μM	nmol 3,4-DHBA μg protein
0	63	60	0.95
$1.8x10^{-5}$	64	57	0.89
$1.8x10^{-4}$	66	55	0.83
$1.8x10^{-3}$	75	53	0.71
$1.8x10^{-2}$	70	55	0.79
$1.8x10^{-1}$	85	58	0.68
1.8	119	58	0.49
$1.8x10^{1}$	126	59	0.47
$9.0x10^{1}$	128	59	0.46

[a] Iron was added as $FeSO_4$ from a 90 mM stock solution which was freshly prepared and filter-sterilized. The iron concentration of the stock solution was confirmed using the 2,2'-dipyridyl assay prior to serial dilution and addition to the media. Residual iron contamination of the medium was estimated to be 0.5 μM.

[b] Protein determinations were conducted by the method of Lowry *et al.* (1951) as described in Materials and methods.

[c] Determination of 3,4-DHBA concentrations in cell-free culture supernatant fluids was conducted according to Barnum (1977) as described in Materials and methods.

Each value presented is the average of duplicate trials.

Table 2.2. Growth of *Azomonas macrocytogenes* 12334 in iron-limited Burk medium containing mineral iron.

Iron source[a]	Theoretical formula[b]	Total cell protein[c] μg protein mL^{-1} culture[e]	3,4-DHBA[d] μM[e]
none	-	82	43
micaceous hematite	unknown	94	44
goethite	$HFeO_2$	102	44
olivine	$(Mg,Fe)_2SiO_4$	106	44
hematite	Fe_2O_3	116	43
pyrite	FeS_2	120	45
glauconite	[f]	122	46
marcasite	FeS_2	134	50
$FeSO_4$	[g]	136	50

[a] Minerals were added to iron-limited Burk medium at 0.5 g L^{-1}. $FeSO_4$ was added to a final concentration of 90 μM.

[b] Phillips & Phillips (1980).

[c] Protein determinations were conducted by the method of Lowry *et al.* (1951) as described in Materials and methods.

[d] Determination of 3,4-DHBA concentrations in cell-free culture supernatant fluids was conducted according to Barnum (1977) as described in Materials and methods.

[e] Each value presented is the average of duplicate trails.

[f] $K_2(Mg,Fe)_2Al_6(Si_4O_{10})_3(OH)_{12}$

[g] $FeSO_4$ in Burk medium was rapidly converted to insoluble compounds under the culture conditions used (see Discussion).

detection of 0.39, 3.5 and 3.1 μg mL^{-1} soluble iron, respectively, whereas when these minerals were preincubated with 0.5 mM-3,4-DHBA approximately one half as much soluble iron was detected.

The bioassay proved to be a more sensitive indicator of iron availability than the 2,2'-dipyridyl assay. Medium preincubated with minerals without added 3,4-DHBA supported increased total cell protein of *Azomonas macrocytogenes* ATCC 12334 compared to the control without added iron (Table 2.3). However, only media preincubated with olivine or glauconite supported significantly enhanced total cell protein of *Azomonas macrocytogenes* ATCC 12334 when increasing concentrations of 3,4-DHBA were preincubated with these two minerals, suggesting that 3,4-DHBA enhanced iron availability to these cultures (Table 2.3). There was no significant increase in total cell protein of *Azomonas macrocytogenes* ATCC 12334 cultures grown in media preincubated with pyrite, marcasite or $FeSO_4$ despite the presence of increasing amounts of 3,4-DHBA. This suggested that sufficient iron was available from these minerals to support maximal growth without the increased solubilization effect of 3,4-DHBA (Table 2.3). There was no relative increase in total cell protein with increasing 3,4-DHBA in the preincubated medium when pyrite, marcasite or $FeSO_4$ was supplied as sources of available iron. This means that the increase in total cell protein observed with 3,4-DHBA preincubated with olivine and glauconite was due to an increase in available iron and not to the presence of increasing amounts of 3,4-DHBA. The slight increase of total cell protein observed with preincubated medium without added mineral iron suggested that 3,4-DHBA rendered the low levels of residual iron present in the medium components more available to support cell growth (Table 2.3).

Effect of pH on solubilization of iron from minerals

Since *Azomonas macrocytogenes* ATCC 12334 lowers the pH of its growth medium and mineral iron is more soluble under acidic conditions (Subcommittee on Iron, 1979), the

Table 2.3. Bioassay and iron determination of iron-limited Burk medium preincubated with iron-containing minerals and 3,4-DHBA.

Iron source[b]	Bioassay: total cell protein (μg protein mL^{-1} culture)[a]			
	no 3,4-DHBA[c]	0.06 mM 3,4-DHBA	0.5 mM 3,4-DHBA	1.0 mM 3,4-DHBA
none	68	73	83	83
goethite	72	75	84	90
micaceous hematite	75	83	83	91
hematite	75	76	81	97
olivine	88	92	142	135
glauconite	115	123	137	154
pyrite	122	128	133	129
marcasite	134	130	138	131
$FeSO_4$	139	139	133	131

[a] Total cell protein of 24 h *Azomonas macrocytogenes* cultures grown on sterile mineral-free, iron-limited Burk buffer preincubated with iron-containing minerals and 3,4-DHBA. Protein determinations were conducted by the method of Lowry *et al.* (1951) as described in Materials and methods.

[b] Minerals (0.5 g L^{-1}) and $FeSO_4$ (90 μM final concentration) were preincubated in iron-limited Burk buffer. Minerals were removed from the iron-limited Burk buffer prior to inoculation with *Azomonas macrocytogenes* ATCC 12334.

[c] Final concentration of 3,4-DHBA added to iron-limited Burk buffer prior to preincubated with minerals.

Results from a typical experiment are presented.

effect of pH on the solubilization of iron from the minerals was investigated. Iron-limited medium was acidified to pH 6.0, 5.5, 5.0 c. 4.5 with HCl. The pH of the medium did not change after sterilization and preincubation with the minerals. Above pH 5, soluble iron could be detected only in medium preincubated with marcasite whereas at pH 5, 1.7 $\mu g/mL^{-1}$ (30 μM) and 5.4 $\mu g/mL^{-1}$ (97 μM) soluble iron could be detected in medium preincubated with 90 μM $FeSO_4$ and marcasite respectively, using the 2,2'-dipyridyl assay. A trace amount of iron was solubilized from hematite but no measurable release of iron from the other minerals was observed. However, at pH 4.5 soluble iron could be detected in growth medium containing any one of the minerals except goethite and micaceous hematite. A bioassay using iron-limited medium preincubated with the various minerals at pH 6.0 gave results similar to the bioassay in iron-limited medium preincubated with minerals without added 3,4-DHBA at pH 7.3 (data not shown). Bioassays could not be performed on supernatants with an initial pH of 5.5 or less since *Azomonas macrocytogenes* ATCC 12334 would not initiate growth at these low pH values. As the culture approached the end of active growth, the pH of the culture supernatant fluids was just below 6.0, and although the pH continued to decline with increasing culture age, this was not concomitant with an increase in total cell protein (Fig. 2.2).

2.5 DISCUSSION

The purification and characterization of 3,4-DHBA from culture supernatant fluids has identified this compound as a major extracellular product of *Azomonas macrocytogenes* strains ATCC 12334, NCIB 10958, NCIB 9129, NCIB 8700 as well as five isolates from Alberta soils and *Azotobacter paspali* WR129. This confirms the results of Westervelt *et al.* (1985) who first reported the production of 3,4-DHBA by a diazotroph by identifying 3,4-DHBA in extracts of spent solid medium of cultures of *Azomonas macrocytogenes*

ATCC 12335 (NCIB 8700), and extends these results to include 3,4-DHBA production by another diazotroph and a second species of the *Azotobacteraceae*, *Azotobacter paspali*. However, production of 3,4-DHBA by *Azotobacter paspali* may be restricted to this species of *Azotobacter* since *Azotobacter chroococcum* and *Azotobacter vinelandii* have not been found to produce detectable levels of 3,4-DHBA when grown under iron-limited, or iron-sufficient conditions (Collinson & Page, unpublished data). 3,4-DHBA has previously been identified in culture supernatants of *Bacillus cereus*, *Bacillus anthracis* (C. Caggle and R. P. Williams, Abstr. Annu. Meet. Am. Soc. Microbiol. 1969, P185, p.146; Chao *et al.*, 1967) and *Streptosporangium rubrum* (Ratledge & Chaudhry, 1971). However, the functional significance of 3,4-DHBA production by these organisms was not addressed.

Many organisms produce 3,4-DHBA as a transient intermediate in the biodegradation of simple aromatic compounds (Kieslich, 1976) with further degradation of 3,4-DHBA via protocatechuate 3,4-dioxygenases (Hardisson *et al.*, 1969; Kieslich, 1976; Mehler, 1962; Sandmann & Hilgenberg, 1982). Since *Azomonas macrocytogenes* strains and *Azotobacter paspali* WR129 synthesize and excrete 3,4-DHBA when grown in a defined medium containing acetate and glucose or sucrose as sole sources of carbon, 3,4-DHBA production was not a direct result of the biodegradation of aromatic carbon sources. Nor is the excretion of 3,4-DHBA by these organisms the result of aromatic amino acid auxotrophy as has been observed in *Aerobacter aerogenes* [*sic*] (Pittard *et al.*, 1962) and *Neurospora crassa* (Gross, 1958).

3,4-DHBA is a metabolite which has been chemically well-characterized (Buckingham, 1982). The fact that 3,4-DHBA is a catechol which exhibits an affinity for iron (Migal & Ivanov, 1973), suggests that it might function in iron assimilation by organisms which excrete substantial amounts of it. *Azomonas macrocytogenes* and *Azotobacter paspali* are native to neutral to alkaline soils (Jensen, 1955; Norris & Baird, 1960; Thompson & Skerman, 1979) where most of the iron present would be in an

insoluble form (Subcommittee on Iron, 1979). It was of interest to determine, therefore, whether the production of a low-affinity iron solubilizing compound such as 3,4-DHBA could enhance an organism's ability to obtain iron from its environment. Although the concentration of 3,4-DHBA in batch cultures of *Azomonas macrocytogenes* ATCC 12334 rarely exceeded 60 μM 3,4-DHBA under the growth conditions used, up to 1.0 mM-3,4-DHBA was tested in growth medium since higher local concentrations of 3,4-DHBA may occur in nature on colonized soil and mineral particles. Several minerals common to various soils were tested for enhanced iron solubility in the presence of 3,4-DHBA. Whereas iron was more readily solubilized from olivine, glauconite, pyrite, marcasite and $FeSO_4$, the more recalcitrant minerals, micaceous hematite, goethite and hematite did not release detectable levels of iron in the presence of up to 1.0 mM-3,4-DHBA.

Decreasing the local pH of colonized soil particles by *Azomonas macrocytogenes* ATCC 12334 may be an additional strategy for solubilizing iron as has been noted for tomato plants which release hydrogen ions from their roots in response to iron-limitation resulting in a decreased pH in the rhizosphere (Subcommittee on Iron, 1979). Although the pH of batch cultures of *Azomonas macrocytogenes* ATCC 12334 became as low as pH 5.1 as the cells entered stationary phase, *Azomonas macrocytogenes* ATCC 12334 failed to grow at pH values low enough (5.5 or less) to promote detectable solubilization of iron from all of the minerals except marcasite.

More important was the determination of whether the solubilization of iron from these minerals by 3,4-DHBA could have a growth promoting effect on *Azomonas macrocytogenes* ATCC 12334. The availability of iron from pyrite, marcasite and $FeSO_4$ may have been aided by oxidation of Fe^{2+} under the neutral, aerobic conditions in the batch cultures. These iron sources provided sufficient iron and no growth promoting effect by 3,4-DHBA was observed despite the enhanced solubilization of iron. Solubilization of iron from olivine, which can exist in soils in an unweathered form, and glauconite, which is a

major constituent of some clay sediments (Subcommittee on Iron, 1979), resulted in the largest growth enhancement of *Azomonas macrocytogenes* ATCC 12334 cultures. It is very unlikely that soluble Mg or Si released from these two minerals was responsible for enhanced growth since 0.8 mM Mg is present in the growth medium and Si is ubiquitous however, the role of Si as an essential trace element for bacteria has not been established (Krumbein & Werner, 1983). The oxidation of iron from all of the above mentioned minerals would result in the formation of insoluble ferric oxyhydroxides and ferric salts in the growth medium (Spiro & Saltman, 1969; Knosp *et al.*, 1984; Greenwood & Earnshaw, 1984). Whereas the most common forms of iron in aerobic soils are amorphous ferric oxyhydroxides, goethite and hematite (Subcommittee on Iron, 1979), solubilized extracts of goethite and hematite proved to be poor iron sources to cultures of *Azomonas macrocytogenes* ATCC 12334.

While these data suggest a role for 3,4-DHBA in facilitating iron solubilization from olivine and glauconite for utilization by *Azomonas macrocytogenes* ATCC 12334, a role similarly proposed for 2,3-dihydroxybenzoic acid in *Azotobacter vinelandii* (Page & Huyer, 1984) and *Bacillus subtilis* (Peters & Warren, 1970), it is not known whether 3,4-DHBA plays a direct role in the transport of iron for *Azomonas macrocytogenes*. Peters & Warren (1970) have demonstrated that several phenolic compounds related to 3,4-DHBA solubilize iron thereby making iron more available to *Bacillus subtilis* but these compounds are not transported as iron chelates. While 3,4-DHBA can facilitate the solubilization of iron from some minerals, it can not be considered a true siderophore (Neilands, 1981*b*) since cells of *Azomonas macrocytogenes* and *Azotobacter paspali* produce it under conditions of iron sufficiency and stability constants for 3,4-DHBA bound to iron (Migal & Ivanov, 1973) are many orders of magnitude lower than those of conventional siderophores (Raymond *et al.*, 1984). The effectiveness of 3,4-DHBA as an iron chelator in the soil environment is somewhat uncertain since 3,4-DHBA would be competing for iron with several other iron-binding compounds including phenolic

compounds resulting from lignin degradation (Kieslich, 1976), microbial or plant humic substances, as well siderophores and organic acids produced by soil fungi and bacteria (Berthelin, 1983). However, it may be notable that the production of 3,4-DHBA per μg cellular protein was slightly lowered in response to increased added iron.

The production of 3,4-DHBA under iron-limited conditions does not preclude the probable induction of a high affinity iron uptake system in *Azomonas macrocytogenes*. In fact, *Azomonas macrocytogenes* ATCC 12334 produces a blue-white fluorescent compound (Thompson & Skerman, 1979), when grown in medium containing less that 1.8 μM added $FeSO_4$ and iron-limited supernatants containing this compound promote the uptake of ^{55}Fe by *Azomonas macrocytogenes* ATCC 12334 (S. K. Collinson & W. J. Page, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, O65, p. 246). Consistent with this hypothesis, *Azomonas macrocytogenes* ATCC 12334 produces at least two outer membrane iron-repressible proteins when grown in iron-limited medium (Collinson & Page, unpublished results; Westervelt *et al.*, 1985). The induction of a potential high affinity iron uptake system under conditions of iron-limitation would interfere with the study of 3,4-DHBA as a low affinity iron chelating compound and the determination of its role, if any, in low affinity iron acquisition. In the absence of mutants deficient in high affinity iron uptake, a bioassay involving the preincubation of the minerals with 3,4-DHBA in a cell-free system was devised to allow detection of the solubilization of iron by 3,4-DHBA without interference by derepression of siderophore production. Whether 3,4-DHBA production was evolved in *Azomonas macrocytogenes* to enhance iron availability or whether its production is the result of the loss of a protocatechuate 3,4-dioxygenase enzyme, which is induced in some *Azotobacter* spp. growing on simple aromatic compounds (Hardisson *et al.*, 1969), remains a matter of speculation.

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CHAPTER 3

Production of Outer Membrane Proteins and an Extracellular Fluorescent Compound by Iron-limited *Azomonas macrocytogenes*

3.1 SUMMARY

Outer membranes were isolated from iron-limited and iron-sufficient *Azomonas macrocytogenes* ATCC 12334 by sucrose density centrifugation or treatment with Sarkosyl. Analysis of the outer membranes by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that iron-limited cells produced greater amounts of two proteins of apparent molecular mass 74 kDa and 70 kDa than iron-sufficient cells. The enhanced production of these two proteins was evident in media containing less than 9.7 μM added iron with maximum production occurring in media containing less than 1.1 μM added iron. Iron-limited growth conditions also caused *Azomonas macrocytogenes* ATCC 12334 to excrete a visible yellow, blue-white fluorescent compound into culture supernatant fluids with maximum levels being produced in media that contained less than 1.1 μM added iron. This fluorescent compound had a distinctive pH-dependent absorption maximum characteristic of pyoverdin-type siderophores and appeared to bind iron. Furthermore, culture supernatant fluids containing the fluorescent compound promoted the uptake of $^{55}Fe^{3+}$ in *Azomonas macrocytogenes* ATCC 12334 cells. Three other *Azomonas*

A version of this chapter has been published:
Collinson, S. K. & Page, W. J. (1989). Production of outer-membrane proteins by iron-limited *Azomonas macrocytogenes*. *Journal of General Microbiology* **135**, 1229-1241.

macrocytogenes strains NCIB 10958, NCIB 8700, and NCIB 9129 produced iron-repressible outer membrane proteins but only the former two strains produced the iron-repressible fluorescent compound. It is proposed that the iron-regulated proteins and fluorescent compound produced by *Azomonas macrocytogenes* may function in high affinity iron uptake.

3.2 INTRODUCTION

Iron is an essential nutrient playing an important metabolic role in virtually all life forms so it is not surprising that iron regulates the expression of components of high affinity iron uptake systems in various microorganisms (Neilands, 1984). In response to iron limitation, gram-negative microorganisms synthesize iron-chelating compounds called siderophores and certain outer membrane (OM) proteins (Neilands, 1981; 1982). In *Escherichia coli* these iron-regulated OM proteins function as ferrisiderophore receptors which mediate high affinity iron uptake (Neilands, 1982). Many other gram-negative organisms similarly express specific OM proteins in conjunction with the production of extracellular siderophores only when grown under iron limitation (Bachhawat & Ghosh, 1987; De Weger *et al.*, 1986; Hoe *et al.*, 1985; Meyer *et al.*, 1979; Page & Huyer, 1984; Williams *et al.*, 1984; Wright *et al.*, 1986). These responses to iron limitation are also prevalent in pathogenic organisms grown *in vivo* (Brown *et al.*, 1984; Griffiths, 1983; Sciortino & Finkelstein, 1983) as well as in soil organisms grown *in vitro* with natural iron sources (Page & Huyer, 1984). Therefore, high affinity iron uptake appears to be a universal strategy by which microorganisms combat the extreme insolubility and unavailability of iron in their environment (Griffiths, 1983; Spiro & Saltman, 1969).

Of the free-living, nitrogen-fixing *Azotobacteraceae*, *Azotobacter vinelandii* has been studied to an appreciable extent with respect to siderophore production (Page & Huyer, 1984; Page & von Tigerstrom, 1988) and iron-repressible OM proteins (Page & von Tigerstrom, 1982) including their presumed role in the mediation of high affinity iron uptake (Knosp *et al.*, 1984; Page & Huyer, 1984). Also, iron-limited *Azotobacter chroococcum* strain 184 produces iron-repressible OM proteins and a putative hydroxamate siderophore (Page, 1987). *Azomonas macrocytogenes*, another member of the *Azotobacteraceae*, produces 3,4-dihydroxybenzoic acid (3,4-DHBA) (Collinson *et al.*, 1987, Westervelt *et al.*, 1985), an extracellular, low affinity iron-binding compound that may play a role in solubilizing iron from the environment (Collinson *et al.*, 1987). Evidence is accumulating that *Azomonas macrocytogenes* may also benefit from a high affinity iron uptake system. Several strains produce a blue-white fluorescent compound whose synthesis, unlike that of 3,4-DHBA (Collinson *et al.*, 1987), is repressed by iron (Johnstone *et al.*, 1958; Thompson & Skerman, 1979). Moreover, Westervelt *et al.* (1985) isolated outer membranes from *Azomonas macrocytogenes* ATCC 12335 by Sarkosyl treatment and reported that this strain produced an iron-repressible protein with an apparent molecular mass of 83 kDa.

In view of the preliminary nature of the above studies, this study was undertaken to determine the effect of iron on the production of OM proteins and the fluorescent compound by the capsule-negative strain *Azomonas macrocytogenes* ATCC 12334. We found that iron-limited *Azomonas macrocytogenes* ATCC 12334 produced two OM proteins of 74 kDa and 70 kDa and a visible yellow, blue-white fluorescent compound which promoted $^{55}Fe^{3+}$ uptake in these cells.

3.3 MATERIALS AND METHODS

Bacterial strains and growth conditions. *Azomonas macrocytogenes* strains ATCC 12334, NCIB 10958, NCIB 9129 and NCIB 8700 were grown in iron-, nitrogen-, and acetate-free liquid Burk medium (Page & Sadoff, 1976) containing 1% (w/v) mannitol (autoclaved separately). Where indicated, 1% (w/v) glucose replaced mannitol and Burk medium was supplemented with 14 mM-sodium acetate. To minimize extraneous iron contamination the distilled water was deionized and the glassware acid washed before use (Collinson *et al.*, 1987). Iron-sufficient medium contained $FeSO_4$ added as a sterile stock solution to a final concentration of 90 μM unless otherwise specified.

Liquid medium was inoculated from cultures grown on slants of nitrogen- and iron-sufficient Burk medium to an optical density at 620 nm (OD_{620}) of approximately 0.05 as previously described (Collinson *et al.*, 1987). Cultures of 200 or 400 mL medium in 0.5 or 1 litre flasks, respectively, were incubated in the dark at 28 to 29°C on a gyratory shaker at 225 r.p.m. for 16 h.

Isolation of outer and inner membranes. All manipulations were carried out at 0 to 4 °C. Cells were harvested from a 400 mL culture by centrifugation (10 000 *g*, 15 min), washed twice with 10 mM-Tris/HCl, pH 7.5, then resuspended in the same buffer. The cells were broken by sonic disruption then treated for 60 min at room temperature (22°C) with chicken egg white lysozyme (0.1 mg mL^{-1}, final concentration), DNAase and RNAase (0.05 mg mL^{-1}, final concentration each) before the lysate was cleared of cellular debris by centrifugation (12 000 *g*, 10 min). Outer membranes were separated from inner membranes in the cleared lysate by sucrose density centrifugation essentially as described

by Page & von Tigerstrom (1982). The crude membranes recovered from the two-step gradient were layered on a discontinuous sucrose gradient composed of 2 mL each of 72, 60, 50, 45, 40% (w/v) sucrose in 10 mM-Tris/HCl, pH 7.5, before centrifugation (120 000 *g*, 18 h). Gradient fractions (approximately 0.4 mL) were collected by bottom puncture, analyzed for sucrose concentration by refractometry, and stored at -20°C.

Alternatively, outer membranes were isolated from cleared cell lysate which had not been treated with lysozyme by selective solubilization of the inner membranes with N-lauroylsarcosine, sodium salt (sarkosyl, Sigma Chemical Co.) as previously described (Filip *et al.*, 1973; Page & Huyer, 1984). The final outer membrane pellet was resuspended in 0.1 mL 8 mM-Tris/HCl, pH 7.5, containing 0.1% (w/v) sodium dodecyl sulfate (SDS) before storage at -20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS-PAGE was performed essentially as described by Laemmli (1970). However, the stacking and separating gels were polymerized using 0.1% (v/v) and 0.067% (v/v) *N, N, N', N'*-tetramethylethylenediamine (Eastman Kodak Co.), respectively, and 0.05% (w/v) ammonium persulfate. Samples were boiled 5 min in sample buffer (Bingle *et al.*, 1984) and applied to gels prior to electrophoresis. Protein- and carbohydrate- containing material in the polyacrylamide gels was stained with Coomassie brilliant blue-R (Sigma Chemical Co.) and Schiff reagent, respectively, according to Fairbanks *et al.* (1971).

Succinate dehydrogenase assay. Succinate dehydrogenase activity was assayed by a modification of the method of Ells (1959) and Kasahara & Anraku (1974). The reaction mixture contained 50 mM-Tris/HCl, pH 7.5, 4 mM-KCN (freshly prepared), 20 mM-sodium succinate, 0.053 mM-2,6-dichlorophenol-indophenol (DCPIP, Sigma

Chemical Co.), 0.2 mM-phenazine methosulfate (Sigma Chemical Co.), and approximately 5 μg protein from the sucrose gradient fractions. The decrease in absorbance at 600 nm was monitored in a Perkin-Elmer Lambda 3 spectrophotometer for 8 min. The molar extinction coefficient for DCPIP (2.16×10^4 L mol^{-1} cm^{-1}) was calculated according to the formula provided by Armstrong (1964) for pH 7.6, the pH of the assay mixture. The average specific activity of succinate dehydrogenase (nmol DCPIP reduced min^{-1} mg $protein^{-1}$) was calculated from duplicate assays.

Protein determinations. Protein was quantified by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma Chemical Co.) as a standard. Samples of protein from the sucrose gradient fractions or Sarkosyl preparations were assayed directly but sucrose, Tris buffer, and SDS were incorporated into standard curves as required to compensate for the interference of these compounds (Gerhardt & Beevers, 1968).

To determine the total cell protein in cultures, cell pellets recovered from a known volume of cell culture (1 640 ***g***, 15 min) were digested in 0.1 M-NaOH at 90°C for 1 h. After removal of the cellular debris (1 640 ***g***, 15 min) the supernatant was assayed for protein.

Detection and analysis of the fluorescent compound. Cell-free culture supernatant fluids were obtained by removal of the cells by centrifugation (10 000 ***g***, 15 min) followed by filter sterilization of the supernatant fluids (0.45 μm pore size, Millipore Corp.). The fluorescent compound was readily detected in cell-free culture supernatant fluids by illumination with UV light at 366 nm. Absorption spectra of cell-free culture supernatant fluids were recorded using a Perkin-Elmer Lambda 3 spectrophotometer. To quantify the fluorescent compound, cell-free culture supernatants were adjusted to pH 5

with pyridine/acetic acid buffer (0.05 M, final concentration) and the absorbance measured at 380 nm (A_{380}).

^{55}Iron uptake assay conditions. $^{55}Fe^{3+}$ uptake assays were performed essentially as described by Knosp *et al.* (1984) with the following modifications. Cells grown for 16 h in iron-limited or iron-sufficient medium were harvested by centrifugation (10 000 *g*, 15 min, 4°C) and resuspended in 10 mL iron-, nitrogen-, and acetate-free liquid Burk medium containing 0.5% (w/v) mannitol and 10 mM-trisodium citrate (uptake buffer). The cells were layered on a 20 ml Percoll (Pharmacia LKB) cushion composed of Percoll : 0.15 M-NaCl, 2:1 (v/v), and centrifuged (12 000 *g*, 10 min, 4°C). Both iron-limited and iron-sufficient cells were subjected to this step; however, it was done mainly to separate insoluble iron salts from the iron-sufficient cells. The insoluble iron salts were presumably in the form of ferric hydroxide and ferric phosphate which would be expected to form under culture conditions of neutral pH and high aeration (Spiro & Saltman, 1969; Greenwood & Earnshaw, 1984). Cells recovered from the top of the Percoll cushion were washed twice in 20 mL uptake buffer and the final cell pellet resuspended to OD_{620} of approximately 9, from which samples were taken for viable cell counts and protein determinations. The resuspended cells were diluted to an OD_{620} of approximately 3 in the appropriate uptake solution and held on ice until required for the $^{55}Fe^{3+}$ uptake assays.

Uptake solutions consisted of uptake buffer or filter-sterilized culture supernatant fluids recovered from 16 h cultures. Iron-sufficient culture supernatants were made to 0.5% (w/v) mannitol and 10 mM-trisodium citrate then diluted with an equal volume of uptake buffer. Iron-limited culture supernatant fluids, used as a source of the fluorescent compound, were similarly supplemented with mannitol and trisodium citrate then diluted

with an equal volume of uptake buffer or undiluted, supplemented iron-sufficient supernatant giving a final A_{380} of approximately 0.05. The pH of the uptake solutions ranged from 6.9 to 7.4.

Uptake solutions were incubated (25°C, 225 r.p.m., 1.5 h) with $^{55}FeCl_3$ (Amersham) added to a concentration of 0.5 μCi mL^{-1} (18.5 kBq) and 5 μM-Fe^{3+} to allow equilibration of the $^{55}Fe^{3+}$ with the uptake solutions. Five minutes prior to the start of the assay, the cell suspensions and the preincubated uptake solutions were placed at 25 °C (test) or 0 °C (control) at 225 r.p.m. To start the assay, a volume of the cell suspension was added to an equal volume of the respective preincubated uptake solution giving a final OD_{620} of 1.5 with 0.25 μCi mL^{-1} (9.25 kBq) $^{55}Fe^{3+}$ and 2.5 μM-Fe^{3+}. These solutions were incubated at the appropriate temperature, duplicate samples (0.5 mL) withdrawn at the indicated times, cells collected by rapid filtration, and the amount of the cell-associated $^{55}Fe^{3+}$ determined by liquid scintillation counting as previously described (Knosp *et al.*, 1984) using a Beckman LS3801 liquid scintillation counter. Standards for counting efficiency were made by placing a known amount of $^{55}Fe^{3+}$ onto a dried filter on which cells from 0.5 mL of a cell suspension OD_{620} 1.5 had been collected. The amount of the $^{55}Fe^{3+}$ present was determined by liquid scintillation counting as above. The counting efficiency was generally found to be 27 to 30%. Iron associated with *Azomonas macrocytogenes* cells was expressed as ng Fe^{3+} $(10^8$ cells$)^{-1}$ taking into account the specific activity of the $^{55}FeCl_3$ and the level of iron contaminating the medium (0.5 μM) as previously determined by atomic absorption spectroscopy.

Iron determinations. Iron-limited growth medium was reduced to near dryness (Büchi

Rotavapor-R), then resuspended in 1.6 M-HCl to give a 10-fold concentrated medium. This concentrated medium was analyzed in an atomic absorption spectrophotometer (Instrumentation Laboratory Spectrophotometer 751) using $FeCl_3$ in 1.6 M-HCl as a standard. All glassware was acid washed.

To confirm the amount of iron added to growth media, the concentration of iron in a freshly prepared, filter-sterilized stock solution of $FeSO_4$ was determined using 2,2'-dipyridyl (Collinson *et al.*, 1987).

3.4 RESULTS

Isolation of outer and inner membranes from *Azomonas macrocytogenes* ATCC 12334 by sucrose density centrifugation

Sucrose gradient density centrifugation of *Azomonas macrocytogenes* ATCC 12334 crude membrane preparations resulted in two peaks of buoyant density 1.26 (H) and 1.14 (L), each with a shoulder of buoyant density of 1.22 and 1.16, respectively (Fig. 3.1). This was consistent with the separation of outer and inner membranes of other gram-negative bacteria (Bachhawat & Ghosh, 1987; Mizuno & Kageyama, 1978; Osborn *et al.*, 1972; Schnaitman, 1970). Similar results were obtained when crude membranes were isolated from cells grown in iron-sufficient or iron-limited media (Fig. 3.1).

SDS-PAGE analysis of the high density peak (H) revealed a protein profile consisting of a few major proteins and some minor protein bands typical of gram-negative outer membrane (Lugtenberg & van Alphen, 1983) (Fig. 3.2A). Conversely, electrophoresis of proteins from the low density peak (L) showed an array of bands typical

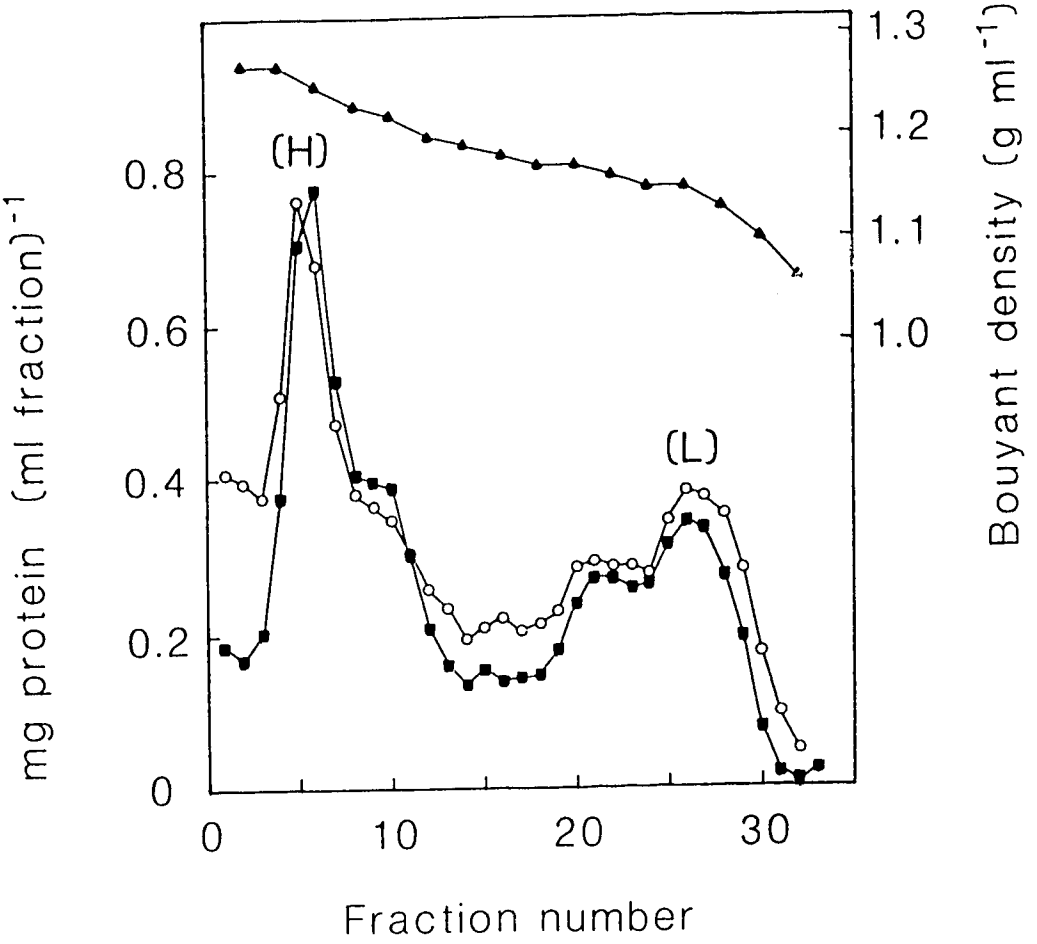

Fig. 3.1. Separation of crude membranes isolated from *Azomonas macrocytogenes* ATCC 12334 by sucrose density centrifugation. Membranes were isolated from cells grown in iron-limited (o) or iron-sufficient medium (■) and subjected to sucrose density centrifugation. (H) high density peak; (L) low density peak. Data from a typical gradient are presented.

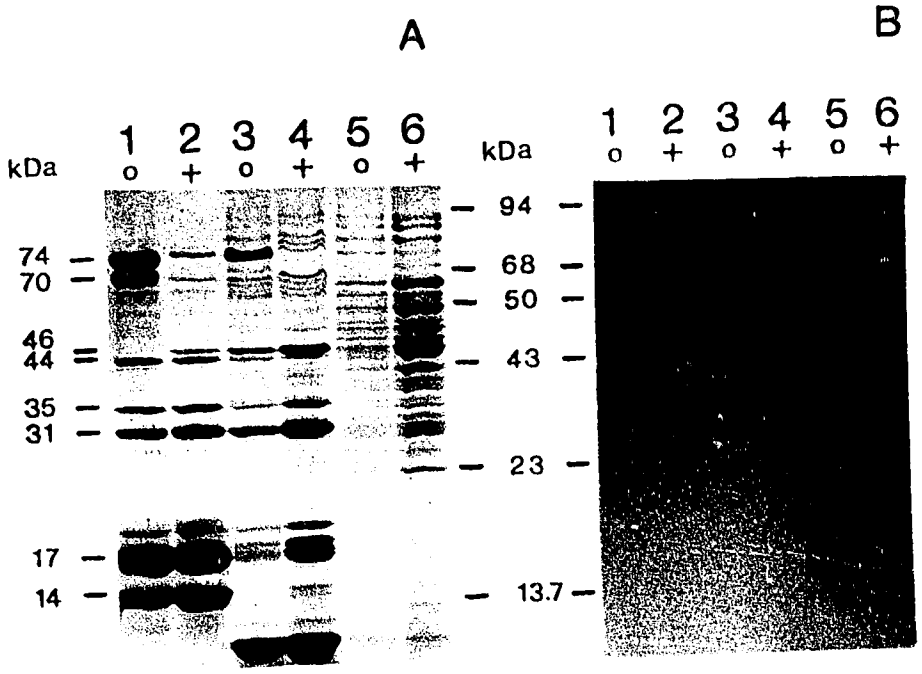

Fig. 3.2. SDS-PAGE of outer membranes isolated from iron-limited (o) or iron-sufficient (+) *Azomonas macrocytogenes* ATCC 12334 isolated by sucrose density centrifugation or treatment with Sarkosyl. Two 12% polyacrylamide gels were loaded with samples from Sarkosyl treated outer membranes (lanes 1,2); fraction 5 of the H peak (lanes 3,4); or fraction 26 of the L peak (lanes 5,6). Each lane received 15 μg protein. One gel was stained for protein with Coomassie blue-R (A), and the other gel was stained for carbohydrate with Schiff reagent (B). The following proteins (all from Sigma Chemical Co.) were used as standards: 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), ribonuclease A (13.7 kDa).

of inner membrane protein profiles of gram-negative bacteria (Fig 3.2A). The H peak did not appear appreciably contaminated with proteins from the L peak. Furthermore, the H peak contained material reactive with the Schiff reagent following periodate oxidation which indicated the presence of carbohydrate material, probably from lipopolysaccharide (Fig 3.2B). The slight amount of Schiff positive material present in the L peak implied minor contamination of this peak with material from the H peak. The minor peaks between H and L had protein profiles similar to that of the adjacent main peak (data not shown). This general distribution of proteins and carbohydrates in the H and L peaks was seen when membranes were recovered from cells grown in iron-limited or iron-sufficient medium.

Succinate dehydrogenase specific activity of the L peak isolated from iron-limited and iron-sufficient cells was 105 and 718 nmol DCPIP min^{-1} mg $protein^{-1}$, respectively, compared to 26 and 74 nmol DCPIP min^{-1} mg $protein^{-1}$ for the H peak. This lower succinate dehydrogenase activity for the H peak was not due to inhibition of enzyme activity by the higher levels of sucrose in these fractions (Hinton *et al.*, 1969) since supplementation of the L peak with sucrose to equivalent levels found in the H peak did not significantly lower the enzyme activity. These results indicated that the H peak was outer membrane and was separated from the L peak which was inner membrane, albeit with some residual inner membrane contamination of the outer membrane.

The succinate dehydrogenase specific activity of cytoplasmic membrane recovered from iron-limited cells was 7 fold lower than that of iron-sufficient cells. While this observation is likely a result of the severe iron stress experienced by iron-limited *Azomonas macrocytogenes* ATCC 12334, as has been observed in other iron-stressed microorganisms (Healy *et al.*, 1955; Winder & O'Hara, 1964), it is not known whether this lower activity reflects a lack of iron in the iron-requiring succinate dehydrogenase

enzyme or a decreased level of enzyme production due to the iron starved status of these cells.

Comparison of OM proteins isolated by sucrose density centrifugation and by Sarkosyl treatment

Since OM proteins of *Azomonas macrocytogenes* have been previously isolated by Sarkosyl treatment of cells recovered from solid media (Westervelt *et al.*, 1985), this simple extraction method was compared to the physical separation of inner membrane from outer membrane by sucrose density centrifugation. OM proteins recovered from *Azomonas macrocytogenes* ATCC 12334 cells grown with or without iron by Sarkosyl extraction gave SDS-PAGE profiles that differed from the respective sucrose gradient preparations (Fig. 3.2A). Firstly, a front running protein seen in the SDS-PAGE of outer membrane isolated by sucrose density centrifugation but not by Sarkosyl treatment was probably lysozyme as this protein had the same mobility as an authentic lysozyme standard. Also, there is a precedent for lysozyme binding to membrane preparations of other gram-negative bacteria (Mizuno & Kageyama, 1978; Osborn *et al.*, 1972). Secondly, the apparent ratios of some of the proteins varied depending on the method used to isolate the outer membrane. Except for the 31 kDa and 46 kDa OM proteins, the major OM proteins were present in higher amounts in Sarkosyl preparations compared to the proteins of the same molecular weight seen on SDS-PAGE of outer membrane isolated by sucrose density centrifugation. In the case of the 70 kDa protein from iron-limited cells, or 14 kDa protein from both cell types, only trace amounts were found in sucrose density centrifugation preparations. Conversely, the 46 kDa protein was lacking in outer membrane prepared from Sarkosyl preparations of iron-limited cells. Finally, the SDS-PAGE of OM proteins isolated by treatment with Sarkosyl lacked some of the minor proteins seen in the

SDS-PAGE of OM proteins isolated by sucrose density centrifugation. This probably reflected the slight contamination of outer membrane with inner membrane in the sucrose preparations and the possible extraction of some minor proteins from the outer membrane by Sarkosyl as has been noted in *E. coli* (Chopra & Shales, 1980). In spite of the few differences mentioned, Sarkosyl treatment resulted in isolation of outer membrane from *Azomonas macrocytogenes* ATCC 12334.

Iron-regulated OM proteins of *Azomonas macrocytogenes* ATCC 12334

Two proteins, 74 kDa and 70 kDa were produced in greater amounts in the outer membrane of iron-limited rather than iron-sufficient *Azomonas macrocytogenes* ATCC 12334 (Fig. 3.2A). Whether cells were grown with 1% (w/v) glucose or mannitol with or without 14 mM-acetate the 74 kDa and 70 kDa proteins were consistently present in outer membrane isolated by Sarkosyl extraction as long as the cultures were iron-limited (data not shown). The 74 kDa iron-repressible OM protein was present when outer membrane was prepared by sucrose density centrifugation but the 70 kDa iron-repressible protein was not usually visible in these preparations. SDS-PAGE of various fractions taken from several regions of the sucrose gradient did not reveal the enhanced production of proteins of either mobility in any other part of the sucrose gradient (data not shown).

Production of an iron-binding fluorescent compound by *Azomonas macrocytogenes* ATCC 12334

Iron-limited *Azomonas macrocytogenes* ATCC 12334 excreted a visible yellow, strongly blue-white fluorescent compound into the culture supernatant fluid which was completely repressed in cells grown in iron-sufficient media (Fig. 3.3A). The amount of the fluorescent compound produced was greater if mannitol was the carbon source rather

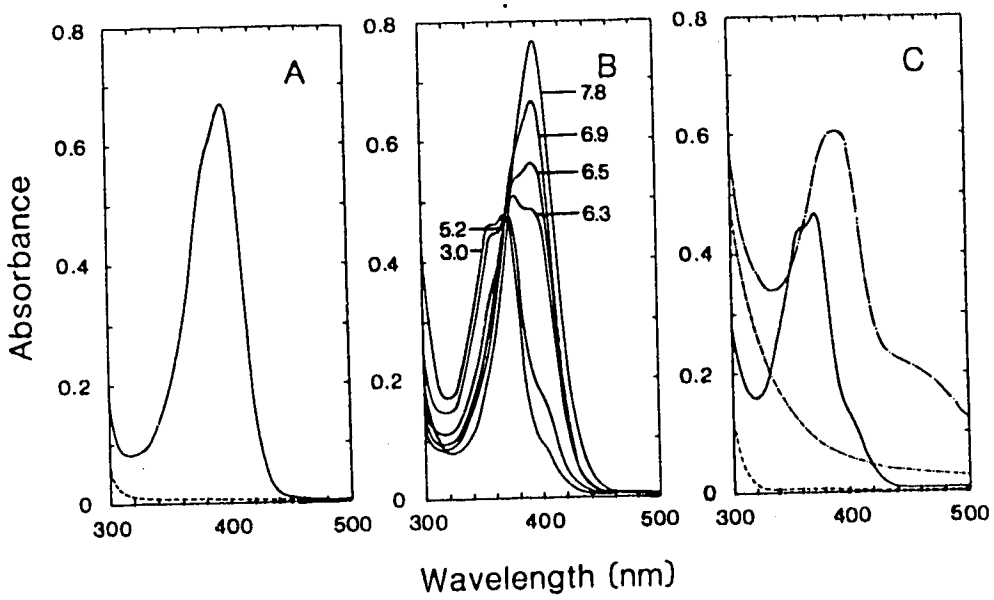

Fig. 3.3. Absorption spectra of filter-sterilized culture supernatant fluids of *Azomonas macrocytogenes* ATCC 12334 grown in iron-limited (——) or iron-sufficient (– – –) Burk medium for 38 h. (A) Iron-limited and iron-sufficient supernatants were diluted with an equal volume of distilled H_2O and the pH determined to be 6.9 and 6.2, respectively. (B). The iron-limited culture supernatant fluid from A was diluted in an equal volume of distilled H_2O, the pH adjusted with concentrated HCl or NaOH, and the spectrum recorded. The total volume change of the adjusted fluids was less than 0.2% (v/v). The pH of each supernatant is noted in the figure. (C). Culture supernatant fluids from A were adjusted to pH 5 with an equal volume of 0.1 M pyridine/acetic acid buffer and the spectrum of the iron-limited supernatant (——) and the iron-sufficient supernatant (– – –) was recorded. Then $FeCl_3$ was added to each supernatant (0.1 mM, final concentration) and the spectrum of the iron-limited (·—— · —— ·) and iron-sufficient supernatants (–·–·–) were recorded. The volume change by adding the iron was less than 0.2% (v/v).

than glucose even though media supplemented with either sugar contained similar levels of contaminating iron as determined by atomic absorption spectroscopy. Iron-limited cultures grown for 20 h with 1% (w/v) mannitol produced levels of the fluorescent compound corresponding to A_{380} of 0.25 to 0.36 and reached an OD_{620} of 0.32 to 0.34. The substitution of glucose for mannitol in these cultures resulted in low production of the fluorescent compound (A_{380} 0.03 to 0.04) even though cultures reached an OD_{620} of 0.22 to 0.28. Similar results were obtained if the cultures containing mannitol or glucose were also supplemented with 14 mM-sodium acetate. Furthermore, cultures grown for 24 h with 1% (w/v) glucose and 14 mM-sodium acetate with reduced aeration (176 r.p.m.) (Collinson *et al.*, 1987) produced lower levels of the fluorescent compound (A_{380} 0.02 to 0.13; OD_{620} 0.15 to 0.28) compared to similar cultures where mannitol replaced glucose (A_{380} 0.20 to 0.34; OD_{620} 0.27 to 0.34).

All cultures containing glucose became pH 5 or less after 24 h growth, and although the cells remained viable, further production of the fluorescent compound did not occur. Cells cultured in the presence of mannitol however, rarely acidified the medium below pH 6 and continued producing the fluorescent compound (Fig. 3.3A). A characteristic shift in the visible spectrum of the fluorescent compound was seen when the pH of the culture supernatant fluids was adjusted (Fig. 3.3B). Moreover, when $FeCl_3$ was added to the supernatants buffered at pH 5, the fluid turned brown, the absorption maximum shifted to 400 nm, and a broad shoulder appeared near 450 nm (Fig. 3.3C).

Iron-regulated production of OM proteins and the fluorescent compound by *Azomonas macrocytogenes*

Azomonas macrocytogenes ATCC 12334 cells were grown for 16 h in media containing decreased amounts of iron. SDS-PAGE of outer membranes isolated from these cells indicated that production of the 74 kDa and 70 kDa proteins was slightly increased when media contained less than 9.7 μM added iron and was at a maximum when cultures contained less than 1.1 μM added iron (Fig. 3.4). Similarly, production of the blue-white fluorescent compound by *Azomonas macrocytogenes* ATCC 12334 increased in culture supernatant fluids as a result of decreased iron availability and became maximal in medium containing less than 1.1 μM added iron (Fig. 3.5). The final pH of these cultures was 6.2 to 6.3. Coincident with enhanced production of the two OM proteins and the blue-white fluorescent compound, was restricted growth of cells when media contained less than 9.7 μM added iron (Fig. 3.5). The growth that occurred in cultures without added iron was a result of contamination of the iron-limited medium with approximately 0.5 μM iron as determined by atomic absorption spectroscopy. Also, cells in the initial inoculum were iron-sufficient as indicated by the lack of the 74 kDa and 70 kDa proteins in their outer membranes and their failure to produce the fluorescent compound (data not shown).

^{55}Iron uptake by *Azomonas macrocytogenes* ATCC 12334

^{55}Iron uptake by iron-limited *Azomonas macrocytogenes* ATCC 12334 cells at 25°C was greatly enhanced only when uptake solutions contained the fluorescent compound (Fig. 3.6A). The lower rate of $^{55}Fe^{3+}$ uptake by cells resuspended in iron-sufficient supernatant fluid was not due to a significant dilution of the $^{55}Fe^{3+}$ specific activity by residual iron in this supernatant. Iron-limited supernatant diluted with an equal volume of

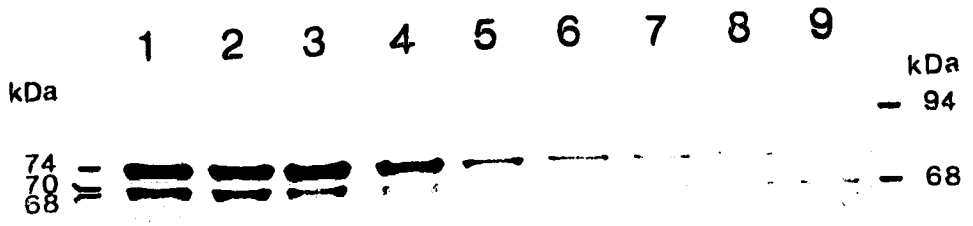

Fig. 3.4. High molecular-mass OM proteins of *Azomonas macrocytogenes* ATCC 12334 isolated from cells grown in Burk medium containing the following concentrations of iron added as $FeSO_4$: no added iron (lane 1); 0.02 μM (lane 2); 0.2 μM (lane 3); 1.1 μM (lane 4); 2.3 μM (lane 5); 9.7 μM (lane 6); 19 μM (lane 7); 95 μM (lane 8); 185 μM (lane 9). The 10% polyacrylamide gel was loaded with 10 μg protein per well. Standard proteins were the same as for Fig. 3.2.

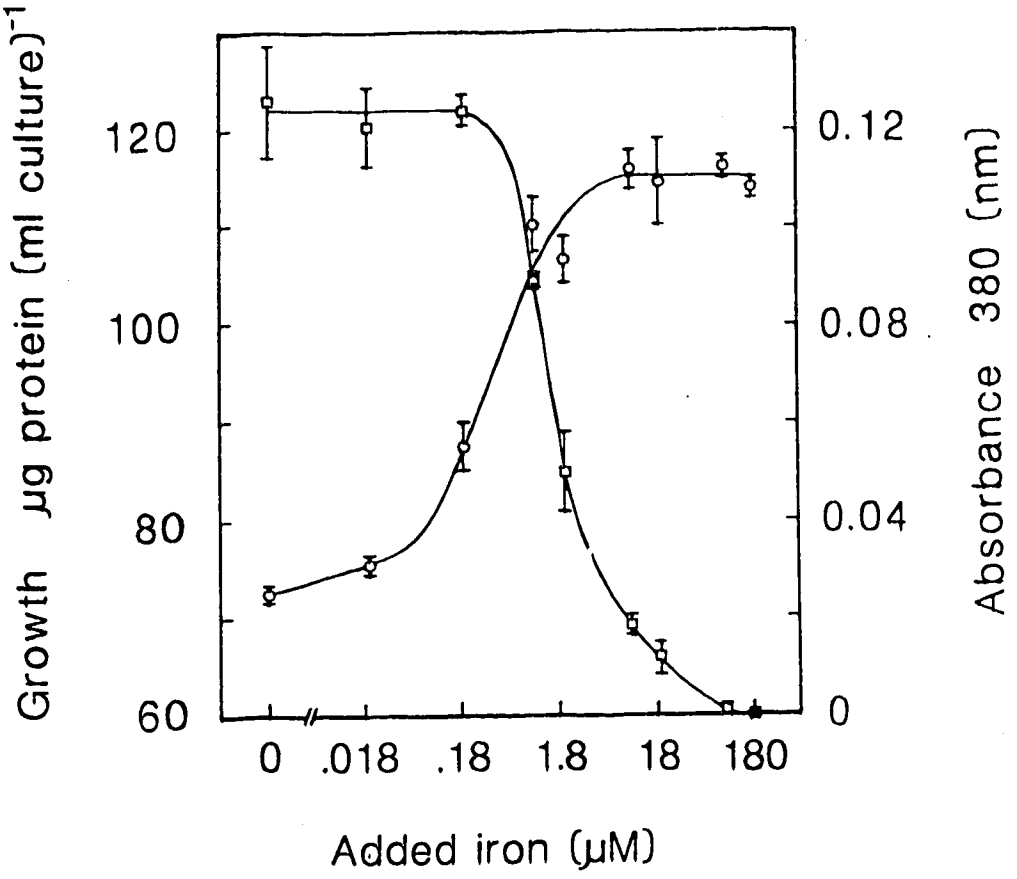

Fig. 3.5. *Azomonas macrocytogenes* ATCC 12334 was grown for 16 h in Burk medium containing various amounts of iron added as $FeSO_4$. The total cellular protein (o), and the absorbance of the cell-free culture supernatant fluid at 380 nm (□) were recorded. Each value represents the average from duplicate cultures. Error bars indicate the range of values for each determination.

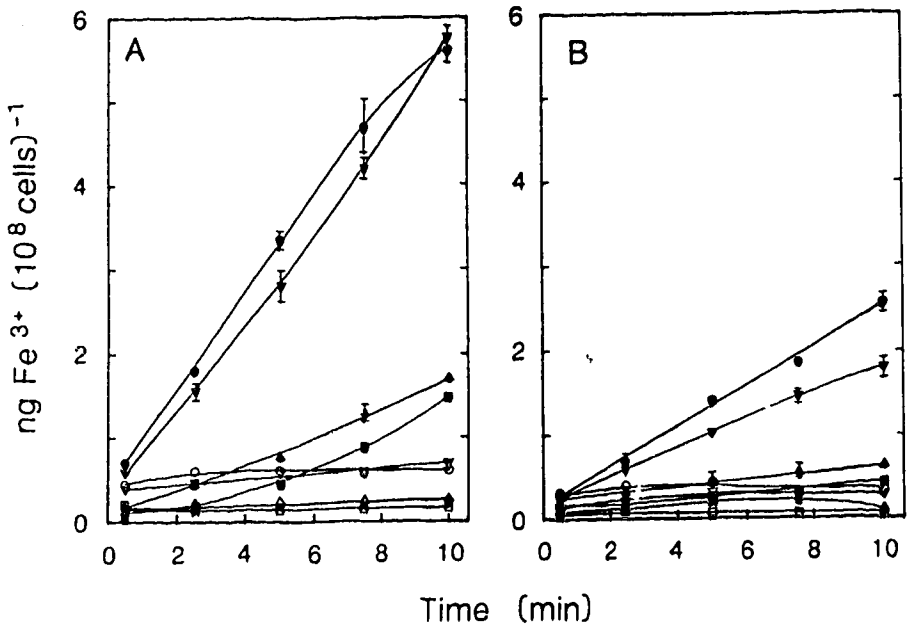

Fig. 3.6. Uptake of $^{55}Fe^{3+}$ by *Azomonas macrocytogenes* ATCC 12334. (A) Iron-limited cells were assayed for $^{55}Fe^{3+}$ uptake at 25°C (closed symbols) or 0°C (open symbols) after resuspension in the following uptake solutions: uptake buffer (■,□); iron-limited culture supernatant supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate then diluted with an equal volume of uptake buffer (●,○); iron-sufficient culture supernatant supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate then diluted with an equal volume of uptake buffer (▲,△); or a 1:1 dilution (v/v) of iron-limited culture supernatant with iron-sufficient culture supernatant both supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate (▼,▽). (B) Iron-sufficient cells were assayed for $^{55}Fe^{3+}$ uptake at 25°C (closed symbols) or 0°C (open symbols) as for iron-limited cells. Symbols for uptake solutions as for Fig. 6A. Error bars indicate when the range of duplicate samples exceeded 0.1 ng Fe^{3+} 10^8 $cells^{-1}$.

iron-sufficient supernatant promoted similar, albeit slightly less, $^{55}Fe^{3+}$ uptake rates in iron-limited cells as iron-limited supernatant diluted with uptake buffer (Fig. 3.6A). It is likely that the rates of $^{55}Fe^{3+}$ uptake by iron-limited cells resuspended in iron-sufficient supernatant or uptake buffer was due to endogenous production of the fluorescent compound by the iron-limited cells. These cells were typically very fluorescent and despite washing of the cells prior to resuspension in uptake buffer, the fluorescent compound was released at levels of A_{380} 0.013 to 0.020 when the cells were held on ice. When these cells were incubated at 25°C for 50 min, the A_{380} increased linearly to 0.055. The possibility of citrate-promoted $^{55}Fe^{3+}$ uptake in these cells was not ruled out and attempts to use other potentially inert chelators such as nitrilotriacetate or ethylenediamine di-(*o*-hydroxyphenyl acetic acid) at 1.4 mM or 10 μM, respectively, to keep the iron soluble in this assay system resulted in unacceptably high backgrounds even when no cells were present in the assay system. $^{55}Fe^{3+}$ associated with iron-limited cells assayed at 0°C was not non-specific binding of $^{55}Fe^{3+}$ to the filters since the ^{55}Fe collected on filters from 0.5 mL samples without cells was 2 to 10 fold less than samples containing cells (data not shown). Iron-limited cells assayed at 0°C in the presence of the fluorescence compound bound approximately 2.5 fold more iron than cells similarly assayed in the absence of the fluorescent compound (Fig 3.6A). Since this binding of iron was almost complete in 0.5 min, it probably represented adventitious binding of iron to the surface of these cells.

The pattern of $^{55}Fe^{3+}$ uptake rates by iron-sufficient cells suspended in the various uptake solutions was similar to the iron-limited cells but the respective rates were 2 to 4.5 fold less over the 10 min assay period than iron-limited cells assayed under identical conditions (Fig. 3.6B).

Effect of iron on the production of OM proteins and the fluorescent compound by various *Azomonas macrocytogenes* strains

Outer membranes of various strains of *Azomonas macrocytogenes* were isolated by treatment of cell lysates with Sarkosyl and the proteins analyzed by SDS-PAGE to compare their OM protein profile to that of *Azomonas macrocytogenes* ATCC 12334. While *Azomonas macrocytogenes* strains NCIB 10958 and NCIB 8700 exhibited iron-repressible OM proteins analogous to those of ATCC 12334, strain NCIB 9129 did not produce the 74 kDa iron-repressible OM protein to the same extent of the other strains (Fig. 3.7). Strain NCIB 9129 produced an additional iron-repressible OM protein of 77 kDa (Fig. 3.7, lane 5). Like strain ATCC 12334, both NCIB 10958 and NCIB 8700 produced a blue-white fluorescent compound under iron-limited conditions however, iron-limited NCIB 9129 failed to produce this compound.

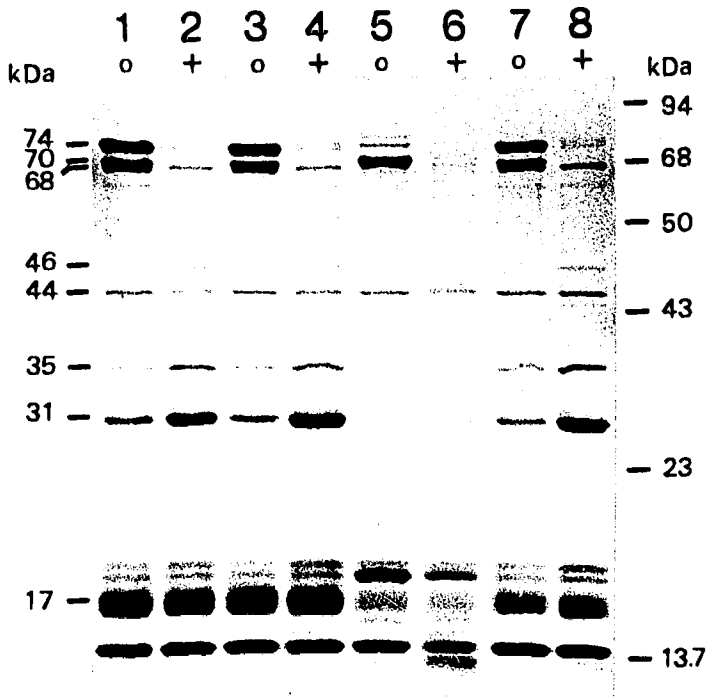

Fig. 3.7. SDS-PAGE of OM proteins isolated from various strains of *Azomonas macrocytogenes* by Sarkosyl extraction. Outer membranes were isolated from strains ATCC 12334 (lane 1, 2); NCIB 8700 (lane 3, 4); NCIB 9129 (lane 5, 6); and NCIB 10958 (lane 7, 8) grown in iron-limited (o) or iron-sufficient (+) medium. The 10% polyacrylamide gel was loaded with 10 μg protein per well. Standard proteins were the same as for Fig. 3.2.

3.5 DISCUSSION

The possible existence of a high affinity iron uptake system in *Azomonas macrocytogenes* was investigated. The characterization of the OM proteins and an extracellular fluorescent compound produced by iron-limited *Azomonas macrocytogenes* ATCC 12334 provided evidence that implicated these molecules as functional components of a high affinity iron uptake system in this organism.

Isolation of outer membranes from *Azomonas macrocytogenes* ATCC 12334 by Sarkosyl treatment of cell lysates resulted in a typical gram-negative OM profile on SDS-PAGE as confirmed by preparations of outer membranes by sucrose density centrifugation. *Azomonas macrocytogenes* ATCC 12334 produced two OM proteins of 74 kDa and 70 kDa when grown in iron-limited but not iron-sufficient media. The minor differences seen in SDS-PAGE profile of OM proteins prepared by Sarkosyl extraction or sucrose density centrifugation was not surprising considering the different techniques involved, but it was unexpected to note the absence of the major, iron-regulated 70 kDa protein in sucrose gradient preparations. The reason for this is not clear but may be explained if this protein were associated with peptidoglycan as is the FhuA iron-repressible OM protein of *E. coli* (Menichi & Buu, 1986). Detailed experimentation of the physiology of *Azomonas macrocytogenes* ATCC 12334 outer membranes will be required to clarify this point, however, it is important to note that the presence of the 70 kDa iron-repressible OM protein is dependent on the procedure used to isolate outer membrane.

Production of high molecular weight, iron-repressible OM proteins is not peculiar to *Azomonas macrocytogenes* ATCC 12334 since two other strains NCIB 10958 and NCIB 8700 produced 74 kDa and 70 kDa OM proteins when grown under iron-limited conditions. Iron-limited NCIB 9129 failed to produce the 74 kDa iron-repressible OM

protein to the extent of that produced by the other strains, but it did produce a 70 kDa as well as a 77 kDa iron-repressible OM protein. In our hands none of the strains produced an iron-regulated OM protein of 83 kDa as was previously isolated from iron-limited *Azomonas macrocytogenes* ATCC 12335 (same strain as NCIB 8700) (Westervelt *et al.*, 1985).

The 74 kDa and 70 kDa OM proteins produced by iron-limited *Azomonas macrocytogenes* ATCC 12334 are possible ferrisiderophore receptor proteins. The iron-regulated production of these two proteins and their molecular mass was similar to ferrisiderophore receptors of *E. coli* and *Pseudomonas* (Magazin *et al.*, 1986; Cody & Gross, 1987; Neilands, 1982) as well as the putative ferrisiderophore receptors of numerous other gram-negative bacteria (Bachhawat & Ghosh, 1987; De Weger *et al.*, 1986; Hoe *et al.*, 1985; Meyer *et al.*, 1979; Neilands, 1982; Page & Huyer, 1984; Williams *et al.*, 1984; Wright *et al.*, 1986). Moreover, iron-limited *Azomonas macrocytogenes* ATCC 12334 cells transported $^{55}Fe^{3+}$ at a higher rate than their iron-sufficient counterparts which agrees with these bacteria having higher levels of ferrisiderophore receptor proteins in the outer membrane.

Consistent with the notion that *Azomonas macrocytogenes* has a high affinity iron uptake system, would be the production of a siderophore by iron-limited cells. Under conditions used in this study, none of the strains produced a yellow-green fluorescent compound analogous to that produced by *Azotobacter vinelandii* as reported by Westervelt *et al.* (1985), however, iron-limited *Azomonas macrocytogenes* ATCC 12334, NCIB 10958, and NCIB 8700 excreted a blue-white fluorescent compound. Our results confirm observations of Johnstone *et al.* (1958) and Thompson & Skerman (1979) who similarly found that iron-limited *Azomonas macrocytogenes* produce a blue-white fluorescent compound. Johnstone studied the fluorescent characteristics of the compound elaborated

by *Azomonas macrocytogenes* and noted that it was distinct from that elaborated by *Azotobacter vinelandii*, so much so that he proposed this as a taxonomic criterion for distinguishing the two species (Johnstone, 1957; Johnstone *et al.*, 1958).

We suspect that the blue-white fluorescent compound produced by *Azomonas macrocytogenes* is a siderophore. Like typical bacterial siderophores (Neilands, 1981), this compound is only produced by iron-limited *Azomonas macrocytogenes* and appears to chelate iron. Interestingly, pH dependent shifts in the absorption maximum of the blue-white fluorescent compound and the iron-induced changes in the absorption spectrum are strikingly similar to the properties of the fluorescent siderophores produced by *Azotobacter vinelandii* (Demange *et al.*, 1988) and the group I fluorescent *Pseudomonas* spp. (Cox & Adams, 1985; Demange *et al.*, 1987; Demange *et al.*, 1986; Meyer & Abdallah, 1978; Philson & Llinás, 1982; Torres *et al.*, 1986). Furthermore, the fluorescent compound promotes $^{55}Fe^{3+}$ uptake in iron-limited *Azomonas macrocytogenes* ATCC 12334 when supplied in a non-purified form found in cell-free iron-limited culture supernatant fluids. The lower, but definite enhancement of $^{55}Fe^{3+}$ uptake in iron-sufficient cells caused by the fluorescent compound is not unexpected since iron-sufficient cells produced basal levels of iron-repressible OM proteins.

Proof of the role of the fluorescent compound as a siderophore must await iron uptake studies with a purified sample since several fluorescent compounds or breakdown products thereof are also present in culture supernatant fluids (Collinson & Page, unpublished results; Johnstone *et al.*, 1958). The purification and chemical characterization of the blue-white fluorescent compound excreted by *Azomonas macrocytogenes* ATCC 12334 is continuing, and preliminary results indicate that this fluorescent compound is structurally related to other fluorescent siderophores (S. K. Collinson, M. A. Abdallah, & W. J. Page, unpublished results). In view of the taxonomic

relationship between *Azomonas, Azotobacter,* and the group I fluorescent pseudomonads as determined by DNA-RNA hybridization experiments (De Smedt *et al.*, 1980), it is not surprising that these groups of organisms have structurally similar siderophores.

At this point it is not known whether the fluorescent compounds produced by *Azomonas macrocytogenes* ATCC 12334, NCIB 10958, or NCIB 8700 are the same structure or variations on a common structure as occurs with the fluorescent compounds elaborated by the various fluorescent pseudomonads (Demange *et al.*, 1987). Nor is it known if either of the two iron-repressible OM proteins functions as a receptor for the fluorescent compound. It is curious that *Azomonas macrocytogenes* NCIB 9129 did not produce the fluorescent compound nor high levels of the 74 kDa OM protein. While it is tempting to speculate that the 74 kDa protein is the receptor for the ferrated fluorescent compound, this question would be better answered using mutants of ATCC 12334 rather than NCIB 9129 whose genetic relationship to the other strains is not known (Table 1.2) (Page & Collinson, 1987).

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CHAPTER 4

Purification and Chemical Characterization of the Fluorescent Compound Produced by Iron-limited *Azomonas macrocytogenes* ATCC 12334

4.1 SUMMARY

Azoverdin, the blue-white fluorescent compound produced by iron-limited *Azomonas macrocytogenes* ATCC 12334 was isolated from cell-free culture supernatants by phenol/chloroform extraction or by filtration through a column of octadecylsilane. Ferrated azoverdin was purified to 98% 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 of azoverdin with 8-hydroxyquinoline. The respective absorption maximum at pH 5 for ferrated and iron-free azoverdin was 400 and 380 nm. A molecular mass of 1143 and 1091 was assigned to ferrated and iron-free azoverdin, respectively, based on preliminary FAB mass spectral results thereby indicating a 1:1 stoichiometry for the chelation of iron by azoverdin. Furthermore, FAB mass spectral data indicated a fragment 302 mass units below the molecular ion which is characteristic of pyoverdin chromophores. The initial

A version of this chapter is in preparation for publication:
Abdallah, M. A., Collinson S. K. & Page, W. J. (1989). Preliminary characterization of azoverdin, the siderophore of *Azomonas macrocytogenes* ATCC 12334. *Biochemical and Biophysical Research Communications.*
Abdallah, M. A., Collinson S. K. & Page, W. J. (1989). Structural determination of azoverdin, the siderophore of *Azomonas macrocytogenes* ATCC 12334. *Biochemistry.*

observation that azoverdin reacted positively in the Csáky assay for bound hydroxylamine was supported by preliminary amino acid analysis of azoverdin which revealed the presence of 6 amino acids, two of which were N^{∂}-acetyl-N^{∂}-hydroxyornithine. Although elucidation of the exact chemical structure is still in progress, preliminary chemical and structural analyses indicate that azoverdin is an iron-binding compound which is chemically similar to pyoverdin siderophores.

4.2 INTRODUCTION

Most micro-organisms respond to iron-limited growth conditions by producing low molecular mass iron-binding compounds, called siderophores, which function to chelate iron from the environment thereby making iron available to the cell for assimilation (Neilands, 1981). While certain functional aspects of siderophore structure are conserved, the chemical structure of siderophores varies widely depending on the producing organism (Hider, 1984; Raymond *et al.*, 1984). Pyoverdins, pseudobactins, and azotobactin comprise a group of structurally similar siderophores which are produced by group I fluorescent *Pseudomonas* spp. or *Azotobacter vinelandii* growing under iron-limitation (Bulen & LeCompte, 1962; Demange *et al.*, 1987; Fukasawa, 1972; Teintze *et al.*, 1981; Yang & Leong, 1984). Pyoverdins have a molecular mass of 1100 to 1400 and are composed of a chromophore, structurally based on 2,3-diamino-6,7-dihydroxyquinoline, to which a peptide of 6 to 10 amino acids is attached via the N terminus (Fig. 4.1A) (Demange *et al.*, 1987; Philson & Llinás, 1982*a*; Poppe *et al.*, 1987; Wendenbaum *et al.*, 1983). In addition, the 3-amino moiety of the chromophore is substituted with various acyl groups derived from succinate, malate, or alpha ketoglutarate (Demange, *et al.*, 1987; Poppe *et al.*, 1987). Pseudobactins, produced by certain plant growth-promoting or plant growth-deleterious fluorescent pseudomonads, are essentially pyoverdins although the

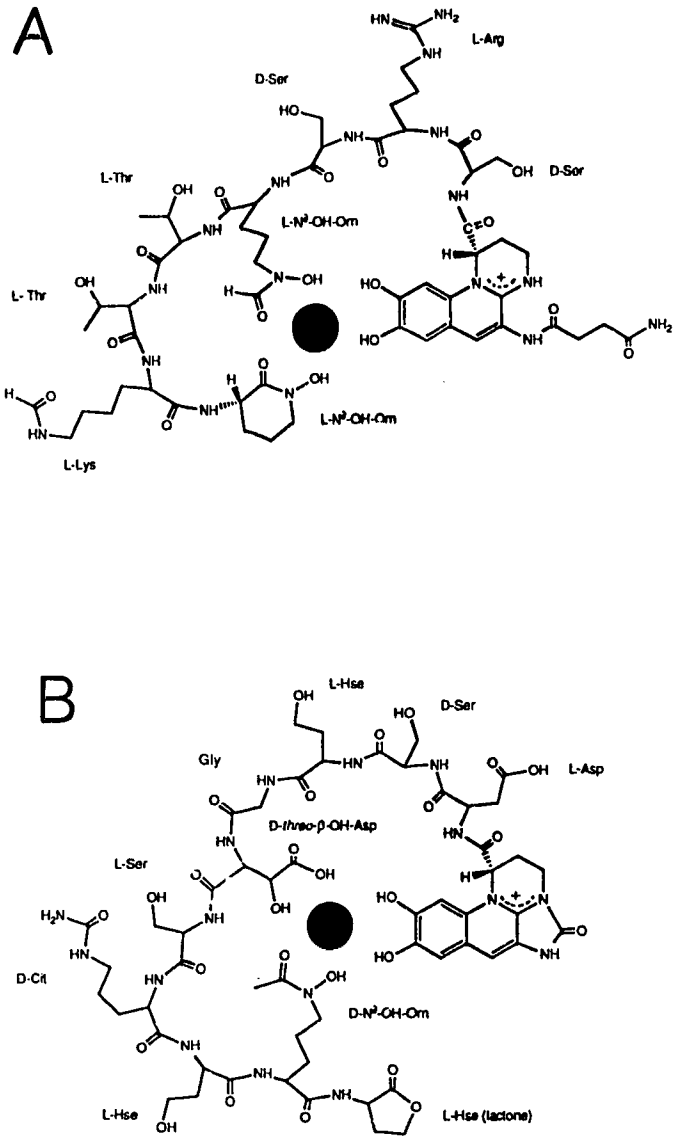

Fig. 4.1. Representative siderophore structures. (A) Iron-free pyoverdin$_{Pa}$ isolated from *Pseudomonas aeruginosa* (adapted from Wendenbaum *et al.*, 1983; Demange *et al.*, 1986) Please see Note Added in Proof for recent revision of this structure. (B) Iron-free azotobactin isolated from *Azotobacter vinelandii* (adapted from Demange *et al.*, 1988). Shaded areas indicate position of Fe^{3+} in ferrated molecule.

nature of the peptide attachment to the chromophore varies (Teintze *et al.*, 1981; Teintze & Leong, 1981; Weisbeek *et al.*, 1986; Yang & Leong, 1984). Finally, azotobactin, produced by *Azotobacter vinelandii*, is "pyoverdin-like" since it has a somewhat different chromophore structure in which the acyl group is replaced by a carbonyl group forming an imidazolone cycle (Fig 4.1B) (Fukasawa *et al.*, 1972; Demange *et al.*, 1988).

Pyoverdins have a strong affinity for ferric iron with an association constant of 10^{24} to 10^{25} at pH 7 (Cody & Gross, 1987; Meyer & Abdallah, 1978; Torres *et al.*, 1986). The three functional groups of pyoverdins, pseudobactins, and azotobactin responsible for the octahedral coordination of one atom of Fe^{3+} are provided by the catechol function of the chromophore and two hydroxamate functions present in the peptide moiety (Fig. 4.1). Generally, N^{∂}-hydroxyornithine (formylated or acetylated) or D-*threo*-β-hydroxyaspartic acid located in the middle of the peptide supplies one hydroxamate group where as the second is provided by a C-terminal cyclized N^{∂}-hydroxyornithine (Demange *et al.*, 1987; Teintze *et al.*, 1981; Teintze & Leong, 1981; Yang & Leong, 1984). Azotobactin differs from pyoverdins and pseudobactins in that the C-terminal N^{∂}-hydroxyornithine is not cyclized but is acetylated and followed by D-homoserine which may or may not be lactonized (Demange *et al.*, 1987; 1988).

Pyoverdins, pseudobactins, and azotobactin are readily detected in culture supernatants of iron-limited *Pseudomonas* spp. and *Azotobacter vinelandii* by virtue of their yellow-green fluorescence and characteristic absorption spectrum owing to the structure of the chromophore (McDonald & Bishop, 1984). These compounds also exhibit a characteristic pH-dependent absorption spectrum and molar extinction coefficient unless they coordinate Fe^{3+}, in which case they lack fluorescence and exhibit a pH-independent absorption spectrum and molar extinction coefficient (Cody & Gross, 1987; Demange *et al.*, 1986; Demange *et al.*, 1987; Meyer and Abdallah, 1978; Philson & Llinás, 1982; Teintze *et al.*, 1981; Torres *et al.*, 1986; Weisbeek, *et al.*, 1986; Yang & Leong, 1984).

These compounds are water soluble and very difficult to extract by solvents, due to the positive charge on the chromophore and the presence of polar amino acids. Purification of these compounds is further complicated since a given strain of *Pseudomonas* produces several pyoverdins which vary with respect to the amino acids present in the peptide or the functional groups attached to the chromophore (Demange *et al.*, 1987). Other minor structural variations include lactonization of the C-terminal D-homoserine in azotobactin (Demange *et al.*, 1988). The structural determination of these compounds relys heavily on fast atom bombardment (FAB) mass spectroscopy, nuclear magnetic resonance (NMR), and circular dichroism (Dell *et al.* 1982; Demange *et al.* 1987; Morris *et al.*, 1981; Williams *et al.*, 1982). In one case, crystals of pseudobactin B10 were obtained which allowed for structural analysis by X-ray crystallography (Teintze, *et al.* 1981).

While the structure and function of various pyoverdins and pseudobactins 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*. *Azomonas macrocytogenes* ATCC 12334, an organism related to *Azotobacter* (Tchan & New, 1984) and the group I fluorescent *Pseudomonas* spp. (De Smedt *et al.*, 1980; De Vos, 1989), also produces an extracellular fluorescent compound when cells are iron-limited (Johnstone, 1957; Thompson & Skerman, 1979). Although Johnstone *et al.* (1958) first suggested similarities between the fluorescent compounds elaborated by *Azomonas macrocytogenes* and those of the fluorescent pseudomonads based on their mutual production by iron-limited cells and their similar absorbtion spectra, no details of these observations were published. Recently, cell-free culture supernatants of *Azomonas macrocytogenes* ATCC 12334 containing a blue-white fluorescent compound were found to exhibit a pH-dependent absorption spectrum analogous to pyoverdins and promote $^{55}Fe^{3+}$ assimilation by *Azomonas macrocytogenes* (Collinson & Page, 1989; Chapter 3). In view of the apparent chemical and functional similarities of the fluorescent compound elaborated

by *Azomonas macrocytogenes* with that of pyoverdins, the name azoverdin has been proposed. This chapter reports the isolation of azoverdin from iron-limited culture supernatants of *Azomonas macrocytogens* ATCC 12334 and its purification to 98% as a prerequisite to its complete structural determination and confirmation of its proposed biological role as a siderophore. Results of preliminary chemical and structural analysis are also presented which indicate that azoverdin is an iron-binding compound structurally related to pyoverdins.

4.3 MATERIALS AND METHODS

Organisms and growth conditions. *Azomonas macrocytogenes* strain ATCC 12334 was grown in liquid, iron-free Burk medium (Page and Sadoff, 1976) containing 14 mM-ammonium acetate and 1% mannitol (w/v) to replace glucose. To reduce iron contamination deionized, distilled water (Collinson *et al.*, 1987) was used but glassware was not acid washed. Liquid media, 400 mL per 1 L flask, was inoculated to an initial optical density at 620 nm (OD_{620}) of approximately 0.1 with *Azomonas macrocytogenes* as previously described (Collinson *et al.*, 1987). Cultures were incubated on a rotary shaker at 225 r.p.m., 28°C for 48 h.

Quantification of azoverdin. The concentration of iron-free or ferrated azoverdin present in various solutions was calculated using the respective absorption maximum (A_{380} or A_{400}) and the respective molar extinction coefficient (ε = 16,500 or ε =19,000) previously determined for iron-free or ferrated $pyoverdin_{P_a}$ at pH 5 (Demange *et al.*, 1987).

Isolation and purification of ferrated azoverdin. Azoverdin was initially isolated

from cell-free culture supernatant fluids as the Fe^{3+} complex by phenol/chloroform extraction and partially purified by ion exchange chromatography as described by Meyer & Abdallah (1978), but to facillitate large-scale purification, azoverdin was recovered directly from cell-free culture supernatant fluids by filtration using octadecylsilane (ODS) (Demange *et al.*, 1988). Bacterial cells were removed from culture supernatant fluids of *Azomonas macrocytogenes* grown for 48 h on iron-limited medium by centrifugation (10 000 ***g***, 15 min, 4°C) and filtration (0.22 μ pore size, Minitan® filtration unit, Millipore). The resulting filtrate was acidified to pH 3.8 with formic acid and pumped (4.5 mL min^{-1}) through a column (3.8 cm x 12.5 cm) of ODS (LiChroprep® RP-18, Merck). The fluorescent material retained by the ODS 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 of 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 x 35 cm) and eluted from this column (1 mL min^{-1}) using a 1 L linear gradient of 0.05 M- to 0.5 M-pyridine/acetic acid. The major compound recovered from this column was ferrated with 6 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+}. Ferrated azoverdin was partially purified by preparative high-performance liquid chromatography (HPLC) and eluted from a diethylaminoethyl (DEAE)-Sephadex A25 anion exchange column with 0.05 M-pyridine/acetic acid prior to final purification by preparative HPLC.

High-performance liquid chromatography conditions. Ferrated azoverdin was purified by preparative reverse-phase HPLC using a column (2.12 cm x 25 cm) containing ODS (10 μ particle size; Zorbax, Dupont) as the bonded-phase. Samples of 2 to 25 mg were eluted with 0.025 M-pyridine/0.016 M-acetic acid containing 2 or 5% (v/v)

acetonitrile (ProAnalisi) at a flow rate of 6 mL min^{-1} at ambient room temperature. Elution of ferrated azoverdin was monitored with a spectrophotometer at an absorbance of 400 nm (A_{400}).

Progression of the purification of ferrated azoverdin was monitored by analytical HPLC using a column (0.5 cm x 26 cm) containing ODS (5 μ particle size; Chromatem). Samples of approximately 0.025 mg were eluted using the the above conditions at a flow rate of 1 mL min^{-1}.

Iron-free azoverdin was analysed by analytical HPLC using a column (0.5 cm x 26 cm) containing ODS (5 μ particle size; Supelco). Samples were eluted with a buffer containing 0.2 M-citric acid, 1.0 mM-EDTA, 1 mM-1-octane sulfonic acid, 0.2 M-Na_2HPO_4, and 10% (v/v) acetonitrile at pH 3. Elution of iron-free azoverdin was monitored with a spectrophotometer at an absorbance of 380 nm (A_{380}).

Deferration of ferrated azoverdin. Iron was removed from ferrated azoverdin using 8-hydroxyquinoline as described by Meyer and Abdallah (1978). Ferrated azoverdin (100 mg) was dissolved in 10 ml distilled H_2O, the solution was adjusted to pH 4 with 10% (v/v) acetic acid, then stirred vigorously for about 1 h with 30 ml 3% (w/v) 8-hydroxyquinoline in chloroform. The extraction procedure was repeated with fresh layers of 8-hydroxyquinoline/chloroform until virtually all of the iron was removed from the aqueous layer. Additional extractions of the aqueous layer with chloroform removed most of the residual 8-hydroxyquinoline.

Deferrated azoverdin was lyophilized, resuspended in 0.05 M-pyridine/acetic acid, applied to and eluted from a CM-Sephadex C25 ion exchange column with a 1 L gradient of 0.05 M- to 0.5 M-pyridine/acetic acid to remove any traces of ferrated azoverdin and 8-hydroxyquinoline from the purified azoverdin. The presence of 8-hydroxyquinoline in column fractions also containing iron-free azoverdin was determined using the ratio of the

absorption of 8-hydroxyquinoline at A_{310} over the absorption maximum for azoverdin at A_{380}. Purified azoverdin was lyophilized and stored desiccated in the dark at -20°C.

Molecular mass determination of azoverdin. The apparent molecular mass of ferrated azoverdin was determined by gel filtration chromatography. Crude, ferrated azoverdin isolated from culture supernatant fluids of iron-limited *Azomonas macrocytogenes* by phenol/chloroform extraction was resuspended in 2 mL distilled H_2O and eluted from a Bio-Gel® P-2 (Bio-Rad, Richmond, CA) column (2.5 cm x 40 cm) equilibrated in distilled H_2O precalibrated with the following molecular weight markers: glutathione, 300; vitamin B_{12}, 1350; bacitracin, 1400; and cytochrome C, 13,000 (all from Sigma Chemical Co.).

Initial FAB mass spectroscopy was performed on partially purified iron-free azoverdin obtained by a modified phenol/chloroform extraction procedured in which culture supernatant fluids were not pretreated with iron. In this case a Kratos model MS9 FAB mass spectrometer was used (Department of Chemistry, University of Alberta). Subsequent FAB mass spectral analysis was performed on purified ferrated and iron-free azoverdin (Department of Biochemistry, Imperial College of Science and Technology, London; M. A. Abdallah, unpublished results).

Electrophoresis. Samples of approximately 0.05 mg were applied to the midpoint of cellulose acetate membranes (5.6 cm x 14 cm) then subjected to electrophoresis (300 V, 30 min, 4°C) using 0.1 M-pyridine/acetic acid as the 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.

Determination of bound hydroxylamine. A chemical assay for bound hydroxylamine was performed on ferrated azoverdin according to the method of Csáky (1948) using hydroxylamine/HCl (Sigma Chemical Co.) as the standard. When required, $FeCl_3$ was incorporated into the standard curve to compensate for the interference of iron in this assay.

Iron determination. Iron present in ferrated azoverdin was determined with 2,2'-dipyridyl as previously described (Collinson *et al.*, 1987; Chapter 2).

4.4 RESULTS

Isolation of azoverdin from cultures of *Azomonas macrocytogenes*

Two methods were used to isolate azoverdin from iron-limited culture supernatant fluids of *Azomonas macrocytogenes* ATCC 12334. Phenol/chloroform treatment of culture supernatants resulted in the extraction of ferrated azoverdin as indicated by the absorption spectrum of the extracted material which was characterisic of the iron-limited culture supernatant fluids treated with $FeCl_3$ prior to extraction (Fig. 4.2A). Alternatively, iron-free azoverdin was recovered from large volumes of culture supernatant fluids on a column of ODS which retained virtually all of the visible yellow, blue-white fluorescent material approximately 90% of which was eluted 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. 4.2B).

Purification of azoverdin

Initial attempts to purify ferrated azoverdin, isolated from cell-free culture supernatants by phenol/chloroform extraction, involved cation exchange chromatography which resulted in 80

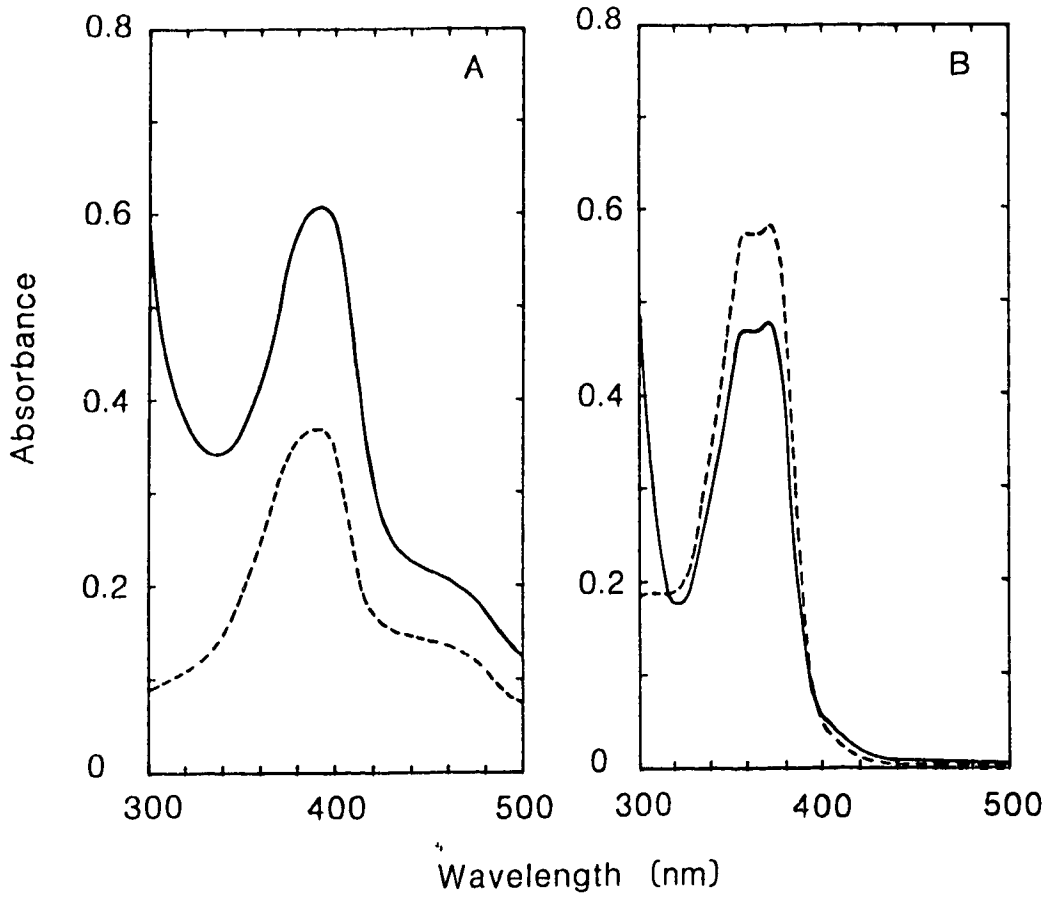

Fig. 4.2. Absorption spectra of ferrated (A) and iron-free (B) azoverdin. (A) Absorption spectrum of cell-free culture supernatant fluids of iron-limited *Azomonas macrocytogenes* with added $FeCl_3$ (0.1 mM, final concentration) (———) compared to the spectrum of azoverdin extracted from culture supernatant fluids with phenol/chloroform (- - - -). (B) Absorption spectrum of above cell-free culture supernatant fluids without added iron (——) compared to the spectrum of iron-free azoverdin recovered from culture supernatant fluids using ODS (- - - -). Culture supernatant fluids and isolated azoverdin (ferrated and iron-free) were adjusted to pH 5 with pyridine/acetic acid (0.05 M final concentration) prior to analysis.

to 90% of the azoverdin eluting from a CM-Sephadex C25 ion exchange column as a single peak preceded by a minor peak (Fig. 4.3). Material from both peaks had an absorption spectrum characteristic of ferrated azoverdin (results not shown) but electrophoresis indicated that the minor peak was composed of two anionic compounds whereas the major compound was a single cationic species (Fig. 4.3, inset). However, analytical HPLC analysis of the major peak revealed the presence of five compounds in addition to the major compound indicating that this ferrated azoverdin preparation was not pure.

In an attempt to avoid the tedious chloroform/phenol extraction procedure and to purify iron-free azoverdin directly without prior ferration, a second purification method was used. Iron-free azoverdin, recovered from culture supernatants with ODS, was eluted through a CM-Sephadex C25 ion exchange column as a major compound comprizing approximately 60% of the A_{380} material effectively separated from several minor compounds (Fig. 4.4). The absorption spectrum of this main peak F, as well as that of peaks C, D, E, and G, was characteristic of iron-limited culture supernatant fluids 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 (Fig. 4.5). Similarly, with the exception of peak A, the other minor peaks comprised at least one iron-binding, fluorescent compound in addition to several minor fluorescent compounds (Fig. 4.5).

Unfortunately, azoverdin in peak F apparently complexed with Fe^{3+} during HPLC analysis as indicated by the presence of additional peaks in HPLC profiles which were unaccounted for in the eletrophoresis results and by inconsistent HPLC profiles resulting from multiple injections of the same sample. The injection of 0.1 M EDTA prior to sample injection did not improve the situation although complexation of Fe^{3+} by azoverdin during HPLC analysis was reduced when azoverdin was eluted with a buffer containing citric acid, EDTA, and 1-octanesulfonic acid (pH 3). However, the cost of the 1-octanesulfonic acid, the long duration of sample elution (3 h), and the additional purification steps which

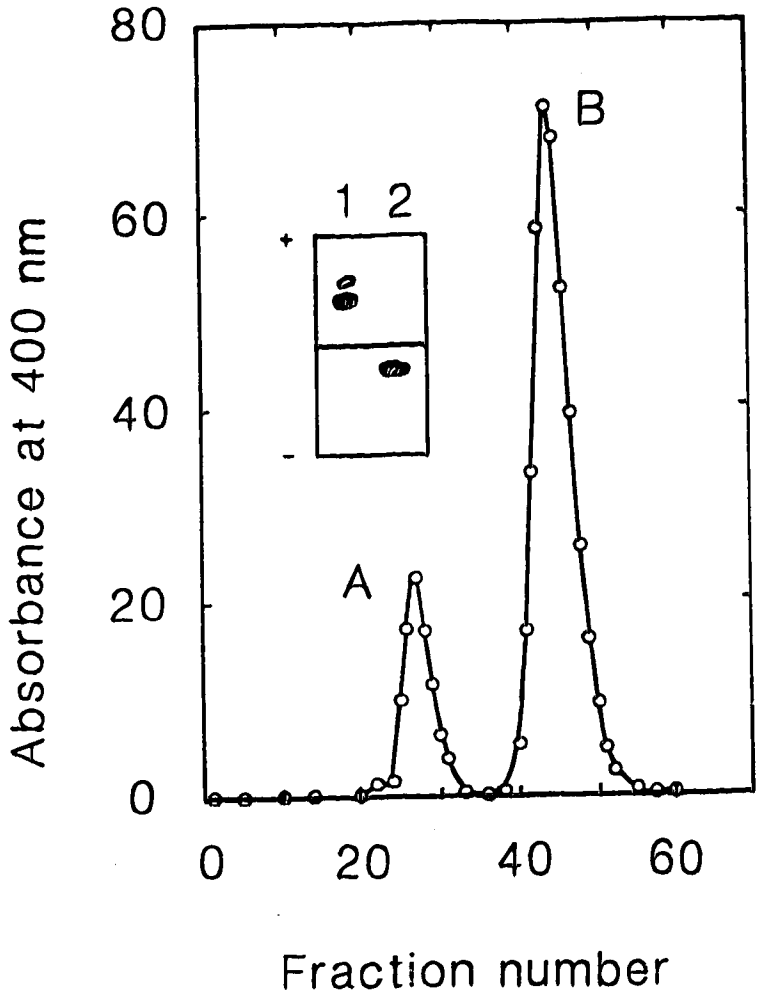

Fig. 4.3. Ion exchange chromatography of ferrated azoverdin. Ferrated azoverdin, recovered from culture supernatant fluids by phenol/chloroform extraction, was eluted from a CM-Sephadex C25 ion exchange column with 0.05 M-pyridine/acetic acid. Fractions of approximately 3 mL were collected at a flow rate of 1 mL min^{-1} and the absorbance at 400 nm measured. Inset shows results of samples from the peak fractions subjected to electrophoresis. (Lane 1) fraction 27 of peak A; (lane 2) fraction 45 of peak B. Visible brown spots (shaded) and fluorescent spots (open) were recorded to scale. Representative results are presented.

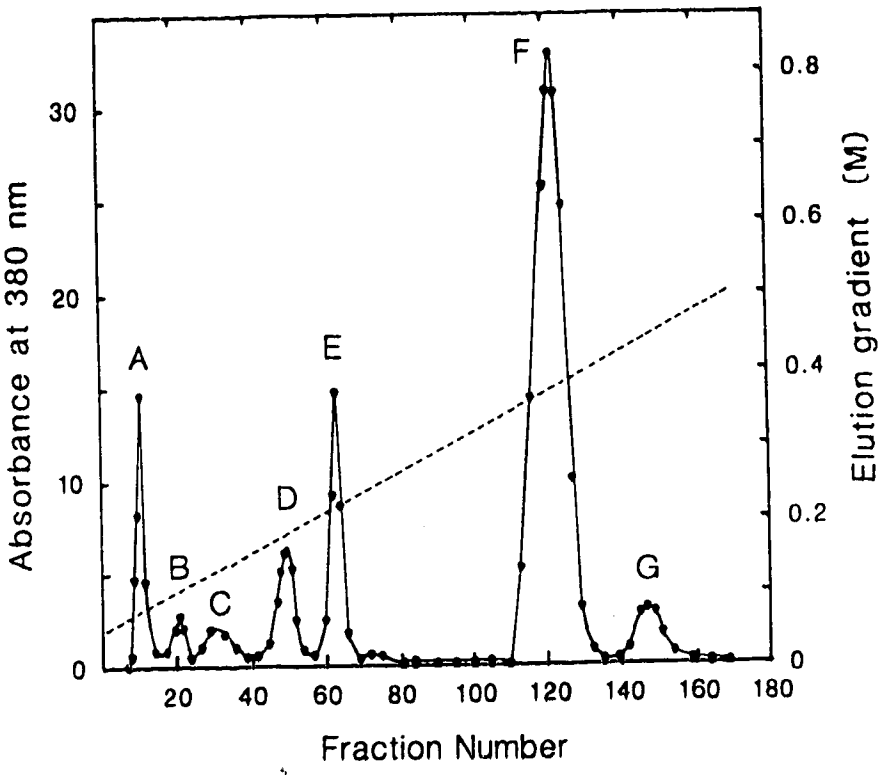

Fig. 4.4. Cation exchange chromatography of the fluorescent material produced by *Azomonas macrocytogenes*. The fluorescent material recovered from *Azomonas macrocytogenes* culture supernatant fluids using ODS was applied to a CM-Sephadex C25 column and eluted with a 1 litre gradient of 0.05 M- to 0.5 M-pyridine/acetic acid buffer pH 5 (- - - -). Approximately 6 mL fractions were collected at a flow rate of 1 mL min^{-1}. The absorbance at 380 nm was used to follow elution of the fluorescent material. Results from a typical profile are presented.

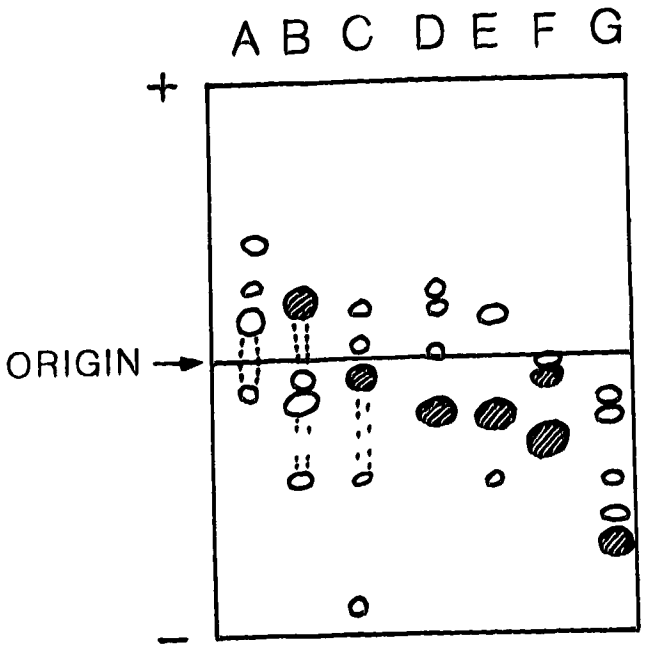

Fig. 4.5. Electrophoresis of the peak fractions obtained from cation exchange chromatography of fluorescent material produced by *Azomonas macrocytogenes*. Approximately 0.01 mg of material from peaks A through G in Fig. 4.4 were spotted at the origin in lanes A to G. respectively, then subjected to electrophoresis as outlined in the materials and methods. The fluorescent spots which reacted with $FeCl_3$ (shaded), fluorescent spots unable to react with $FeCl_3$ (open), and smeared fluorescent material (dotted lines) were recorded to scale. Representative results are presented.

would be required to remove the buffer components from azoverdin made it impractical to further purify the iron-free azoverdin by HPLC. Therefore, azoverdin had to be ferrated prior to further purification by HPLC.

As with partially purified ferrated azoverdin recovered by phenol/chloroform extraction above, analytical HPLC analysis of ferrated azoverdin recovered from peak F revealed the presence of five compounds in addition to the major compound (Fig. 4.6A). Subsequent purification steps involving ion exchange chromatography and preparative HPLC resulted in the purification of the ferrated azoverdin from 55 to 98% (Fig. 4.6). Purified, ferrated azoverdin was deferrated to the yellow, iron-free form by extraction with 8-hydroxyquinoline in chloroform. The residual Fe^{3+} complex and 8-hydroxyquinoline were separated from the purified iron-free azoverdin by cation exchange chromatography (Fig. 4.7).

Original, cell-free culture supernatant fluids were estimated to contain approximately 70 to 90 mg L^{-1} azoverdin based on conversion of A_{380} values measured at pH 5. Of this approximately one third comprised the major compound isolated by this purification scheme. The final yield of purified, iron-free azoverdin was approximately 10 mg L^{-1} original culture supernatant fluid.

Spectral properties of purified azoverdin

Purified, iron-free azoverdin exhibited a characteristic pH-dependent absorption spectrum (Fig 4.8A). In contrast, the absorption spectrum of ferrated azoverdin was pH-independent and at pH 5 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 a shoulder at 450 nm (Fig 4.8B).

Molecular mass of azoverdin

The approximate molecular mass of ferrated azoverdin, isolated by phenol/chloroform

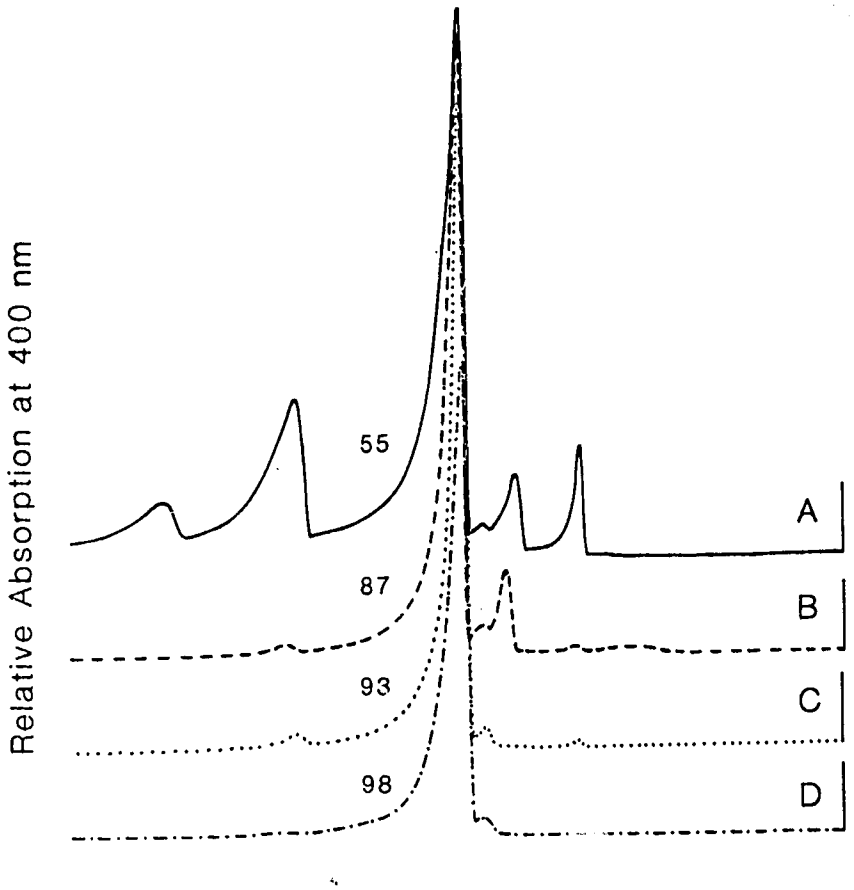

Fig. 4.6. Analytical HPLC profiles of ferrated azoverdin at various stages of purfication. Analytical HPLC profiles of ferrated azoverdin were recorded after the following treatments: (A) Peak F from Fig. 4.5 was ferrated with $FeCl_3$ then eluted from a cation exchange column to remove excess Fe^{3+}; (B) Ferrated azoverdin from A was partially purified by preparative HPLC; (C) Ferrated azoverdin recovered from B was eluted from a DEAE-Sephadex A25 column; (D) Ferrated azoverdin from C was subjected to final preparative HPLC purification. The percent purity of azoverdin in each sample was calculated from the peak area using triangulation and is noted in the figure.

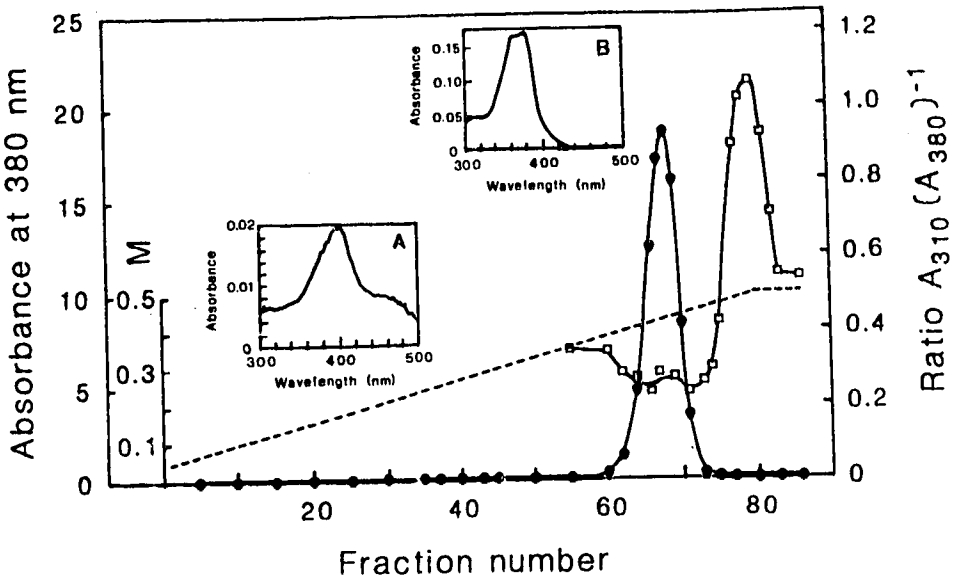

Fig. 4.7. Cation exchange chromatography of purified, deferrated azoverdin. Elution of deferrated azoverdin from a CM-Sephadex C25 ion exchange column using a gradient of pyridine/acetic acid (- - - -), was monitored at A_{380} (●). Approximately 12.5 mL fractions were collected at a flow rate of 1.2 mL min^{-1}. The presence of 8-hydroxyquinoline was determined by the ratio of A_{310} to A_{380} (□). Insets show the absorption spectrum of: (A) trace ferrated azoverdin (fraction 20, undiluted) and (B) iron-free azoverdin (fraction 67, diluted 100 fold).

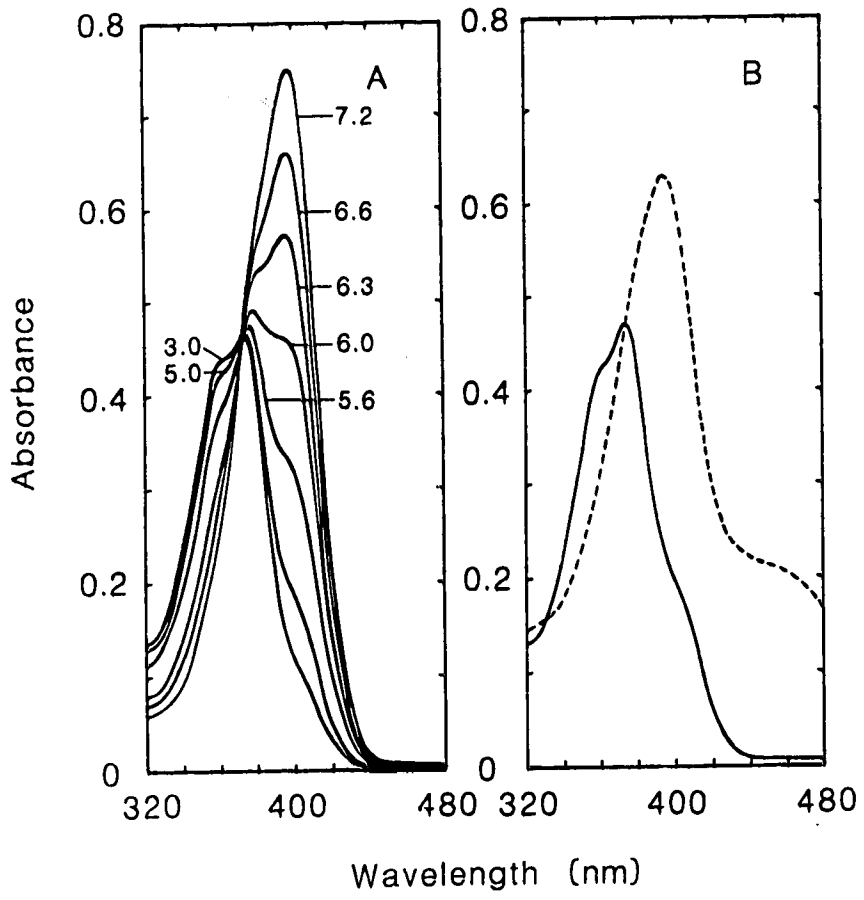

Fig. 4.8. Absorption spectra of purified azoverdin. (A) Iron-free azoverdin was resuspended to a concentration of 28 μM in 0.05 M pyridine, adjusted to various pH with acetic acid, then the absorption spectrum was recorded. The pH of each solution is noted in the figure. (B) Purified iron-free azoverdin was resuspended in 0.05 M-pyridine/acetic acid, pH 5 at a concentration of 28 μM and the absorption spectrum recorded (———). The absorption spectrum was rerecorded following the addition of 28 μM-$FeCl_3$ (final concentration) (- - - - -). The volume change by the various additions was less than 0.5%.

extraction, was initially estimated using gel filtration chromatography. Approximately 85% of the preparation eluted from a Bio-Gel® P-2 column as a single peak preceded by a minor component. Both compounds had an absorption spectrum typical of ferrated azoverdin and the major compound had an estimated molecular mass approximating 1300 (Fig. 4.9). This result was corroborated by preliminary FAB mass spectral analysis performed on a sample of iron-free azoverdin obtained using a modification of the phenol/chloroform extraction procedure. Although the yield of azoverdin was low under these conditions it was possible to obtain a tentative molecular mass of 1091 mass units for the molecular ion and a mass of 1113 for the sodium salt (Fig 4.10). Furthermore, FAB mass spectral data revealed the presence of a fragment 302 mass units below the molecular mass of the ion (Fig. 4.10). The molecular mass for purified, iron-free azoverdin was confirmed to be 1091 by further FAB mass spectral analysis (M. A. Abdallah, unpublished results).

Preliminary chemical characterization of azoverdin

Cultures containing azoverdin reacted positively in the Csáky assay which indicated the presence of compounds with bound hydroxylamine. Partially purified ferrated azoverdin present in the major peak in Fig 4.3 was eluted from a second cation exchange column as a single peak and the concentration of bound hydroxylamine and iron present in the fractions was determined. The average molar ratios of bound hydroxylamine to azoverdin, iron to azoverdin, and bound hydroxylamine to iron were calculated and estimated to be 2.1, 0.8, and 2.4, respectively, which approximate the ratios 2, 1, 2.

Analysis of purified azoverdin indicated the presence of a peptide attached to the chromophore. Further amino acid analysis of this peptide indicated that it was composed of 6 amino acids, two of which were N^{∂}-acetyl-N^{∂}-hydroxyornithine (M. A. Abdallah, unpublished results). This confirmed the results of the Csáky assay which indicated 2 moles of bound hydroxylamine per mole of azoverdin. Moreover, FAB mass spectral analysis of ferrated azoverdin indicated a molecular mass of 1143 thereby proving the 1:1

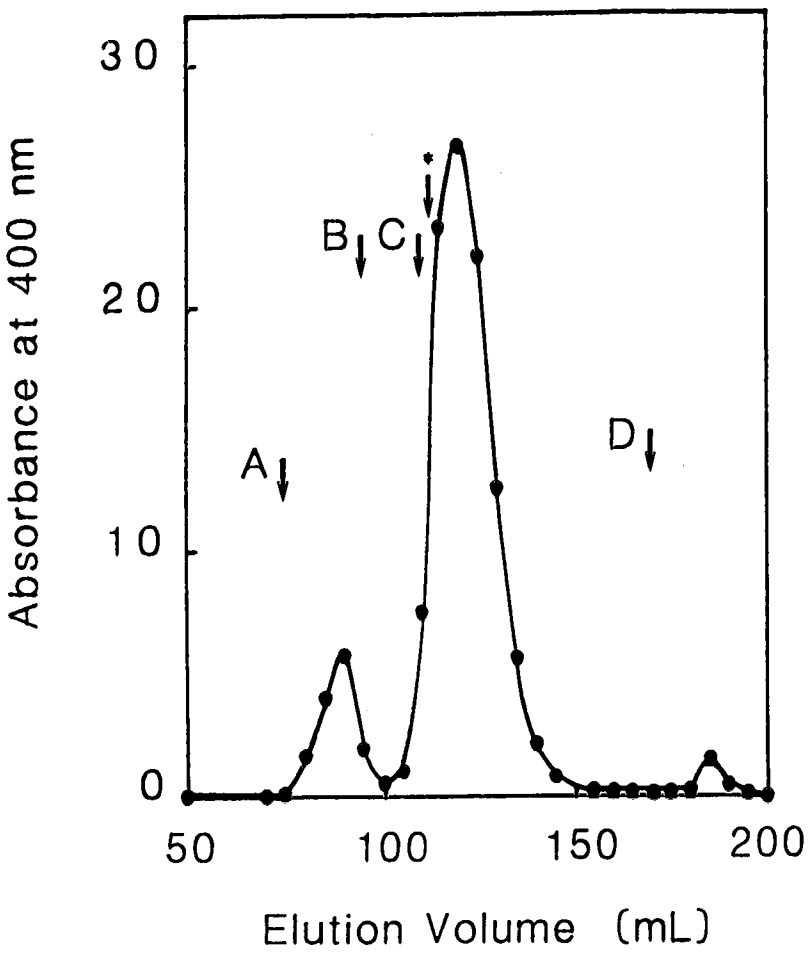

Fig. 4.9. Gel filtration chromatography of ferrated azoverdin. Ferrated azoverdin, isolated from culture supernatant fluids of iron-limited *Azomonas macrocytogenes* by phenol/chloroform extraction was eluted from a Bio-Gel® P-2 column. Fractions of approximately 5 mL were collected at a flow rate of 0.5 mL min^{-1}. The elution volume of azoverdin is indicated (*) along with the elution volume of the standards used to calibrate the column: (A) cytochrome C, 13,000; (B) bacitracin, 1400; (C) vitamin B_{12}, 1350; (D) glutathione, 300. Results of typical profile are presented.

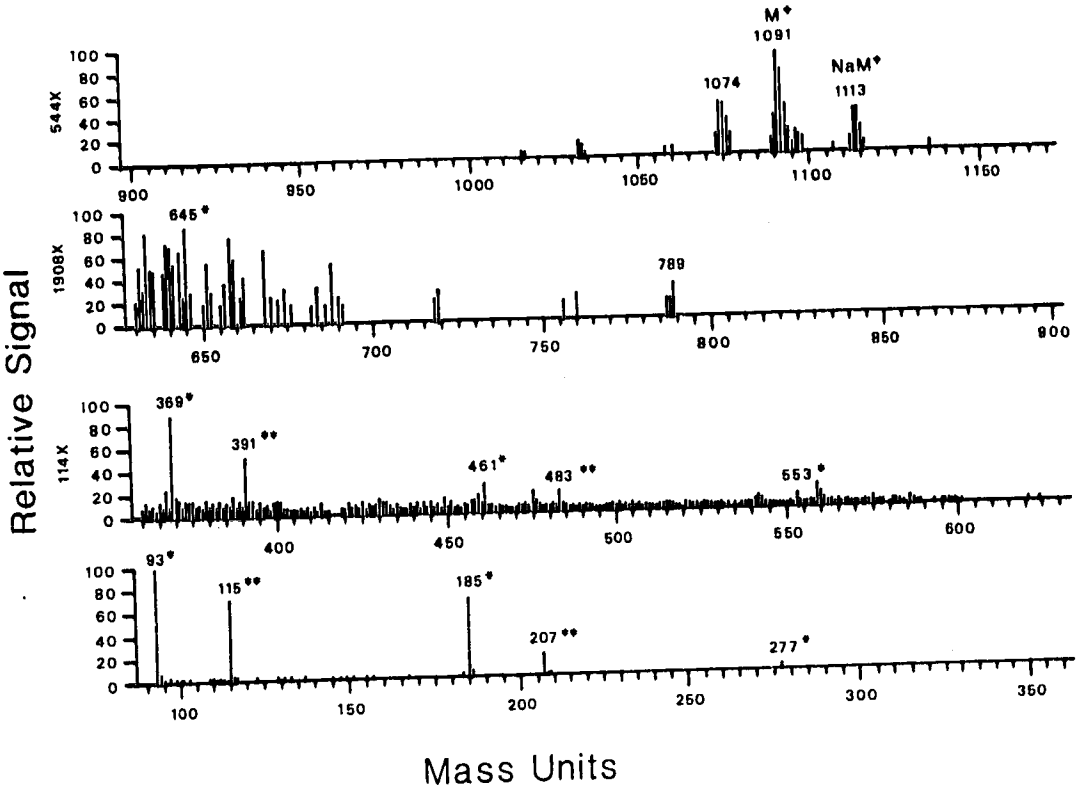

Fig. 4.10. Positive ion FAB mass spectrum of iron-free azoverdin. Iron-free azoverdin, isolated by modified phenol/chloroform extraction as noted in Materials and methods, was prepared with glycerol and subjected to FAB mass spectral analysis. The molecular mass of the molecular ion (M^+) and sodium salt (NaM^+) as well as the mass of standard gycerol oligomers in the protonated (*) and sodium form (**) are noted.

stoichiometry of Fe^{3+} bound by azoverdin (M. A. Abdallah, unpublished results).

4.5 DISCUSSION

The purification and partial characterization of azoverdin, a blue-white fluorescent compound elaborated by iron-limited *Azomonas macrocytogenes* ATCC 12334 has identified this pigment as a major, extracellular, pyoverdin-like compound. *Azomonas macrocytogenes* is only the third genus, apart from *Pseudomonas* and *Azotobacter* known to produce iron-binding, fluorescent compounds of this class. *Azomonas macrocytogenes* NCIB 8700 (ATCC 12335), NCIB 10958, and *Azomonas agilis*, similarly produce a blue-white fluorescent compound when grown under iron-limitation (Thompson & Skerman, 1979; Collinson & Page, 1989; Chapter 3). This is in contrast to the yellow-green fluorescent pigment which Westervelt *et al.* (1985) observed in cultures of *Azomonas macrocytogenes* ATCC 12335. Although the blue-white fluorescent compounds have yet to be chemically characterized, they likely represent additional pyoverdin-like compounds.

Techniques previously used to recover pyoverdins, pseudobactin and azotobactin were successfully applied to the isolation of azoverdin. Ferrated azoverdin was isolated from cell-free culture supernatant fluids of iron-limited *Azomonas macrocytogenes* by phenol/chloroform extraction, a technique used previously to isolate ferrated pyoverdin from cultures of various *Pseudomonas* spp. (Demange *et al.*, 1986; Meyer and 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 *Azomonas macrocytogens.*

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 cultures of *Azomonas 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 chromatrography 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; Hohnadel & Meyer, 1988; Torres *et al.*, 1986).

Further studies will be required to determine whether the various iron-binding, fluorescent compounds produced by *Azomonas macrocytogenes* represent forms of azoverdin which have the same chromophore but varied peptide composition as occurs with pyoverdins produced by various *Pseudomonas* species (Demange *et al.*, 1987), whether the variation is due to various acyl groups attached to the chromophore (Demange *et al.*, 1987; Poppe *et al.*, 1987), or whether variation results from conversions such as lactonization of the terminal amino acid as occurs in azotobactin (Demange *et al.*, 1988). Preliminary evidence suggests ferrated azoverdin converts from one form to another upon storage, whereas iron-free azoverdin does not (M. A. Abdallah, unpublished results). Such variations in structure may account for the multiple peaks seen on HPLC analysis of partially purified azoverdin.

Preliminary characterization of azoverdin indicated that this fluorescent compound is chemically related to pyoverdins and pseudobactins. Since the spectral characteristics of these compounds are due primarily to the chromophore (MacDonald & Bishop, 1983), the fact that the absorption spectrum of iron-free or ferrated azoverdin closely resembles that of iron-free and ferrated pyoverdin, respectively (Cody & Gross, 1987; Cox & Adams, 1985; Demange *et al.*, 1987; Meyer & Abdallah, 1978; Philson & Llinás, 1982*a*; Teintze *et al.*, 1981; Torres *et al.*, 1986) suggests that the chromophore of azoverdin is also based on 2,3-diamino-6,7-dihydroxyquinoline. This is further exemplified by the distinctive pH

dependent absorption spectrum exhibited by iron-free azoverdin which is characteristic for pyoverdins (Cody & Gross, 1987; Meyer & Abdallah, 1978; Philson & Llinás, 1982*a*; Torres *et al.*, 1986). The chromophore of azoverdin is distinct from that of azotobactin since the absorption coefficient for iron-free azoverdin is lower than that of its ferrated counterpart. The opposite holds for azotobactin (Demange *et al.*, 1988). Further evidence to the nature of the azoverdin chromophore was provided by FAB mass spectral data which demonstrated a fragment at 789 mass units indicating a loss of 302 mass units. Such a result is considered diagnostic of pyoverdin since it represents a typical cleavage across the saturated ring of the chromophore (Demange *et al.*, 1987; Wendenbaum *et al.*, 1983). However, azoverdin exhibits blue-white fluoresence at neutral pH in contrast to the yellow-green fluorescence of pyoverdin, pseudobactin, and azotobactin and the pigment produced by *Azomonas macrocytogenes* ATCC 12335. Complete details of the FAB mass spectra and ^{3}H and ^{13}C NMR analysis will confirm the structure of azoverdin and provide insight into the fluorescent character of this compound.

In keeping with a typical pyoverdin structure, azoverdin contains a short peptide of six amino acids attached to the chromophore. The peptide contains two amino acids identified as N^{∂}-acetyl-N^{∂}-hydroxyornithine which are probable ferric chelating groups in addition to the hydroxyl groups of the presumed chromophore. This is consistent with the observed changes in the absorption spectrum when azoverdin becomes ferrated, and is reminiscent of the absorption spectra of ferrated pyoverdins and pseudobactins (Cody & Gross, 1987; Cox & Adams, 1985; Demange *et al.*, 1987; Meyer & Abdallah, 1978; Philson & Llinás, 1982*a*; Teintze *et al.*, 1981; Torres *et al.*, 1986). Furthermore, FAB mass spectral analysis confirmed that azoverdin complexed iron in a 1:1 molar ratio. Likely, azoverdin chelates iron in a manner analagous to pyoverdins and azotobactin thereby providing chemical evidence suggesting that azoverdin is a siderophore. Preliminary results in which the uptake of iron by *Azomonas macrocytogenes* ATCC 12334 was enhanced in the presence of azoverdin supplied in the impure form found in culture supernatant fluids (Collinson *et*

al., 1989; Chapter 3) can now be verified using purified azoverdin.

NOTE ADDED IN PROOF:

Recently, Briskot *et al.* (Briskot, G., Taraz, K. & Budzikiewicz, H. (1989). Pyoverdin-type siderophores from *Pseudomonas aeruginosa*. *Liebigs Annalen der Chemie* 1989, 375-384.) have reported a revised structure for the pyoverdin$_{P_a}$ in which three amino acids of the peptide chain are cyclized: chromophore-D-ser-L-arg-D-ser-L-orn(N$^{\delta}$-OH-N$^{\delta}$-CHO)-L-lys-L-orn(N$^{\delta}$-OH-N$^{\delta}$-CHO)-L-thr-L-thr . These authors have also proposed an alternative nomenclature scheme to the present use of genus/species single letter subscripts following pyoverdin which follows closely to the IUPAC conventions. Under this scheme pyoverdin$_{P_a}$ would be: pyoverdin-Q-SRSO'K*TTO'*-SUCA.

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CHAPTER 5

Azoverdin-mediated iron assimilation in *Azomonas macrocytogenes* ATCC 12334

5.1 SUMMARY

Azoverdin, a blue-white fluorescent compound produced by iron-limited *Azomonas macrocytogenes* ATCC 12334 acted as a siderophore to enhance $^{55}Fe^{3+}$ assimilation in iron-limited *Azomonas macrocytogenes* cells. Iron-limited cells were unable to produce azoverdin when grown at 34°C rather than 28°C. However, these cells still expressed the 74 kDa and 70 kDa iron-repressible outer membrane proteins and were capable of azoverdin-mediated iron transport. The use of *Azomonas macrocytogenes* cells grown at 34°C eliminated endogenous azoverdin production thereby allowing determination of azoverdin-mediated $^{55}Fe^{3+}$ transport rates which proceeded with an apparent Km of 0.2 μM and a Vmax of 0.46 ng Fe^{3+} (min 10^8 cells)$^{-1}$. In the absence of azoverdin, *Azomonas macrocytogenes* cells assimilated iron at a lower rate (0.1 to 0.2 ng Fe^{3+} (min 10^8 cells)$^{-1}$) in an azoverdin-independent manner.

A version of this chapter is in preparation for publication:
Collinson S. K., Abdallah, M. A. & Page, W. J. Azoverdin-mediated iron assimilation by *Azomonas macrocytogenes* ATCC 12334. *Journal of Bacteriology.*

5.2 INTRODUCTION

Many gram-negative bacteria respond to growth-restricting levels of free iron in their environment by producing siderophores, low molecular mass iron-chelating compounds which solubilize iron thereby enhancing the availability of this metal to the cells for subsequent assimilation via specific ferrisiderophore receptor proteins (Lankford, 1973; Neilands, 1981; 1984). The group I fluorescent *Pseudomonas* spp. also procure iron by siderophore-mediated, high affinity iron assimilation. They produce fluorescent siderophores, some of which have been purified and chemically identified as pyoverdins or pseudobactins (Demange *et al.*, 1987; Teintze *et al.*, 1981; Weisbeek *et al.*, 1986; Wendenbaum *et al.*, 1983; Yang & Leong, 1984). These compounds specifically enhance the uptake of radioactive iron by the *Pseudomonas* strain producing a given siderophore (Cox & Adams, 1985; De Weger *et al.*, 1988; Hohnadel & Meyer, 1986; 1988; Meyer *et al.*, 1987; Meyer & Hornsperger, 1978). *Pseudomonas* spp. may have multiple iron assimilation mechanisms, as exemplified by *Pseudomonas aeruginosa* which employs two siderophores, pyoverdin and pyochelin, as well as ferric citrate to obtain iron (Cox, 1980; Cox & Adams, 1985).

Similarly, *Azotobacter vinelandii* has a relatively complex high affinity iron assimilatory mechanism involving the pyoverdin-like siderophore, azotobactin (Demange *et al.*, 1988; Knosp *et al.*, 1984) as well as two other iron-binding compounds, azotochelin (Knosp *et al.*, 1984) and aminochelin (Page & von Tigerstrom, 1988), which similarly stimulate iron transport.

Azomonas macrocytogenes, a soil bacterium related to *Azotobacter* (Tchan *et al.*, 1984) and the group I fluorescent *Pseudomonas* spp. (De Smedt *et al.*, 1980; De Vos, 1989) also produces a fluorescent compound when faced with iron limitation (Johnstone, 1957; Johnstone *et al.*, 1958; Thompson & Skerman, 1979). Initial studies suggest that this fluorescent compound functions as a siderophore since it enhances the uptake of $^{55}Fe^{3+}$

by *Azomonas macrocytogenes* when supplied in an impure form (Collinson & Page, 1989; Chapter 3). Recent purification and preliminary characterization of the iron- binding fluorescent compound produced by *Azomonas macrocytogenes* has established its structural resemblance to pyoverdins thereby justifying its proposed name, azoverdin (Collinson, Abdallah & Page, unpublished results). This study describes the use of purified azoverdin to evaluate its function as a siderophore for *Azomonas macrocytogenes*.

5.3 MATERIALS AND METHODS

Bacterial strains and growth conditions. *Azomonas macrocytogenes* ATCC 12334 was routinely grown in iron-, nitrogen-, and acetate-free liquid Burk medium (Page & Sadoff, 1976) containing 1% (w/v) mannitol. 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; Chapter 2), this medium contained 0.5 μM iron as determined by atomic absorption spectroscopy (Collinson & Page, 1989; Chapter 3) and no further steps were taken to remove it. Iron-sufficient medium contained 90 μM iron added from a sterile stock solution of $FeSO_4$.

Cultures were inoculated as previously described (Collinson & Page, 1989; Chapter 3) and incubated on a rotary shaker (225 r.p.m., 28°C or 34°C) for 16 or 48 h.

Quantification of azoverdin. The Csáky assay for bound hydroxylamine was used to detect the presence of azoverdin in cell-free culture supernatant fluids, but due to the semi-quantitative nature of this assay (Tomlinson *et al.*, 1971) and the lengthy procedure involved, azoverdin was quantified using the absorption of its chromophore.

Cell-free culture supernatants, obtained by removal of *Azomonas macrocytogenes* cells by centrifugation (10 000 ***g***, 15 min, 4°C) and filter sterilization (0.45 μm pore size

Millipore filter), were adjusted to pH 5 with pyridine/acetic acid (0.05 M, final concentration) and the absorption determined at 380 nm (A_{380}). The concentration of purified azoverdin present in various solutions was calculated by converting the A_{380} values determined at pH 5 using the molar extinction coefficient previously determined for pyoverdin$_{Pa}$ (ε=16,500 L mol^{-1}, pH 5) (Demange *et al.*, 1986).

Purification of azoverdin. Azoverdin was isolated from cell-free culture supernatant fluids of iron-limited *Azomonas macrocytogenes* using octadecylsilicic acid and was subsequently purified by ion exchange chromatography and high-performance liquid chromatography (HPLC) (Chapter 4).

Analysis of outer membrane proteins. Outer membrane proteins of *Azomonas macrocytogenes* were isolated from iron-limited cells grown at 28 or 34°C for 16 h by Sarcosyl extraction then subjected to SDS-polyacrylamide gel electrophoresis as previously described (Collinson & Page, 1989; Chapter 3).

Protein determinations. Total cell protein in cultures was determined as previously described (Collinson & Page, 1989; Chapter 3).

Quantification of 3,4-dihydroxybenzoic acid. The approximate concentration of 3,4-DHBA in iron-limited cell-free culture supernatants of *Azomonas macrocytogenes* was estimated using a standard curve prepared by measuring the absorption at 285 nm (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 fluids). For culture supernatants also containing azoverdin, the A_{285} was corrected to exclude the absorbance contributed by azoverdin at this wavelength.

Iron uptake assay conditions. Iron uptake by *Azomonas macrocytogenes* was assayed using $^{55}Fe^{3+}$ as described by Knosp *et al.* (1984) with modifications (Collinson & Page, 1989; Chapter 3).

5.4 RESULTS

Azoverdin-mediated iron assimilation by *Azomonas macrocytogenes*

Iron-assimilation by iron-limited *Azomonas macrocytogenes* cells grown at 28°C and assayed at 25°C was enhanced only when iron uptake solutions contained azoverdin. Whether azoverdin was supplied in the non-purified form found in iron-limited culture supernatant fluids or added to uptake buffer as a purified compound, the rate of iron uptake was stimulated approximately 3.5-fold compared to cells similarly assayed in uptake buffer or iron-sufficient culture supernatant fluids not containing azoverdin (Fig. 5.1).

Azoverdin also enhanced the level of cell-associated iron for cells assayed at 0°C although the values were very low; 0.40 to 0.74 ng Fe^{3+} $(10^8$ cells$)^{-1}$ compared to 0.12 to 0.26 ng Fe^{3+} $(10^8$ cells$)^{-1}$ for cells assayed without azoverdin. Less than 15% of this cell-associated Fe^{3+} represented non-specific association of $^{55}Fe^{3+}$ 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 ng Fe^{3+} (min 10^8 cells$)^{-1}$.

Endogenous production of azoverdin by iron-limited *Azomonas macrocytogenes*

Iron-limited *Azomonas macrocytogenes* cells accumulated iron at a slower rate in the absence of added azoverdin than in its presence (Fig. 5.1). Occasionally these cells

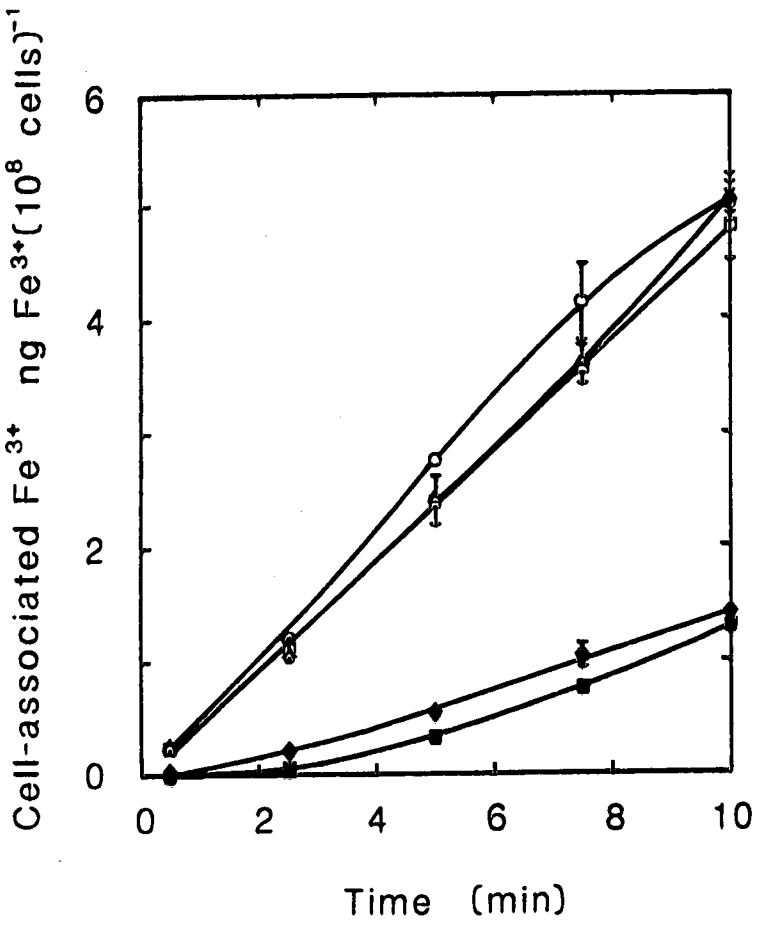

Fig. 5.1. The effect of azoverdin on iron assimilation by *Azomonas macrocytogenes* ATCC 12334. Cells were resuspended in the following uptake solutions: uptake buffer (■); iron-limited culture supernatant fluid supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate then diluted with an equal volume of uptake buffer (○); iron-sufficient culture supernatant fluid supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate then diluted with equal volume of uptake buffer (◆); a 1:1 dilution (v/v) of iron-limited culture supernatant with iron-sufficient culture supernatant both supplemented with 0.5% (w/v) mannitol and 10 mM-trisodium citrate (△); and uptake buffer supplemented with 3.0 μM purified azoverdin (□). Curves represent iron assimilation by cells grown at 28°C and assayed at 25°C from which values determined at 0°C were subtracted. Error bars indicate when the range of duplicate samples exceeded 0.1 ng Fe^{3+} $(10^8$ cells$)^{-1}$.

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 were 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 *Azomonas macrocytogenes* and the rate of iron uptake by cells assayed in uptake buffer without added azoverdin. When the endogenous level of fluorescence was approximately A_{380} 0.013 to 0.022 in the assay system, the rate of iron uptake was approximately 2.5- to 3.5-fold lower than that measured for iron-limited cells assayed in the presence of 3 μM added azoverdin (A_{380}, 0.05). However, if the level of endogenous fluorescence was higher, (A_{380}, 0.030 to 0.037), the iron assimilation rates approximated that of cells assayed in the presence of 3 μM 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 *Azomonas macrocytogenes*.

Measurement of the Km of azoverdin-mediated iron uptake by iron-limited *Azomonas macrocytogenes* cells therefore was hampered by the unpredictable levels of endogenous azoverdin produced by various cell preparations. Cells unable to produce azoverdin, but still able to transport ferriazoverdin would be required to estimate the Km of azoverdin-mediated iron assimilation.

Effect of temperature on growth of *Azomonas macrocytogenes*

Initial observations indicated that iron-limited *Azomonas 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 *Azomonas macrocytogenes* NCIB 8700 and NCIB 10958. *Azomonas macrocytogenes* ATCC 12334

incubated at 28°C and 34°C demonstrated similar growth rates at both temperatures, although cells grown at 34°C produced slightly less cellular protein and did not produce any detectable fluorescence for 48 h (Fig. 5.2). These results were corroborated by the semiquantitative Csáky assay which detected background levels of bound hydroxylamine in culture supernatants of cells grown for 16 h at 34°C compared to approximately 10 μM bound hydroxylamine for culture supernatants of cells grown at 28°C.

The slight increase of absorption at 380 nm seen for cells grown at 34°C for 48 h (A_{380} 0.029) was not due to the production of fluorescent compounds since uninoculated medium supplemented with purified azoverdin to an equivalent A_{380} (1.7 μM) was definitely fluorescent when visualized under 366nm UV light where as the cell-free cultures supernatant of cells grown at 34°C were not fluorescent.

This inability for *Azomonas macrocytogenes* cells to produce azoverdin was not due to a prolonged lag period prior to onset of pigment production since cells initially grown at 34°C only resumed production of azoverdin when shifted to 28°C (Fig 5.3A). Conversely, cells initially grown at 28°C ceased production of azoverdin after being transferred to the higher temperature (Fig 5.3B). 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 to 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.

The increased growth temperature did not significantly affect the production of 3,4-DHBA since both cell types produced similar levels of this compound at either temperature. Approximately 70 to 80 μM or 300 to 350 μM 3,4-DHBA was produced by cells incubated at both temperatures for 16 h or 48 h, respectively.

Azomonas 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

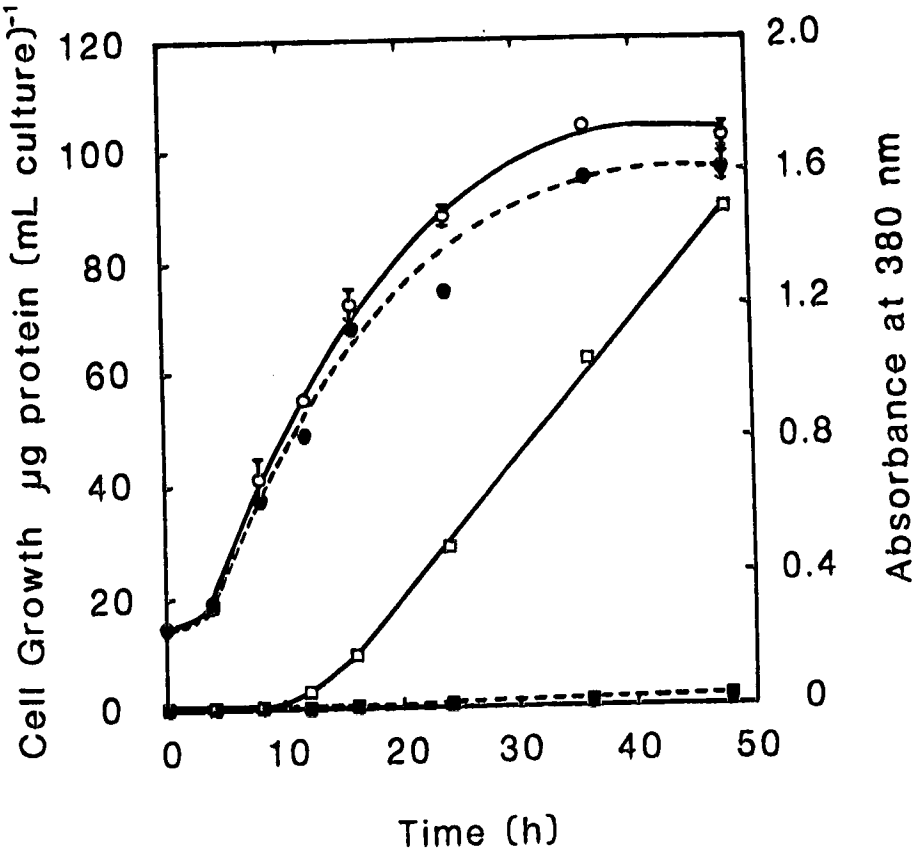

Fig. 5.2. Comparison of cell growth and fluorescence production by iron-limited *Azomonas macrocytogenes* ATCC 12334 incubated at 28 °C (———) and 34 °C (- - - -). Cell growth was measured as total cell protein (○, ●) as described in Materials and methods. The presence of azoverdin was measured spectrophotometrically at 380 nm (□, ■) on samples of cell-free culture supernatant adjusted to pH 5 with pyridine/acetic acid buffer pH 5 (0.05 M, final concentration). Error bars indicate the range of duplicate samples where the range exceeds the diameter of the symbols.

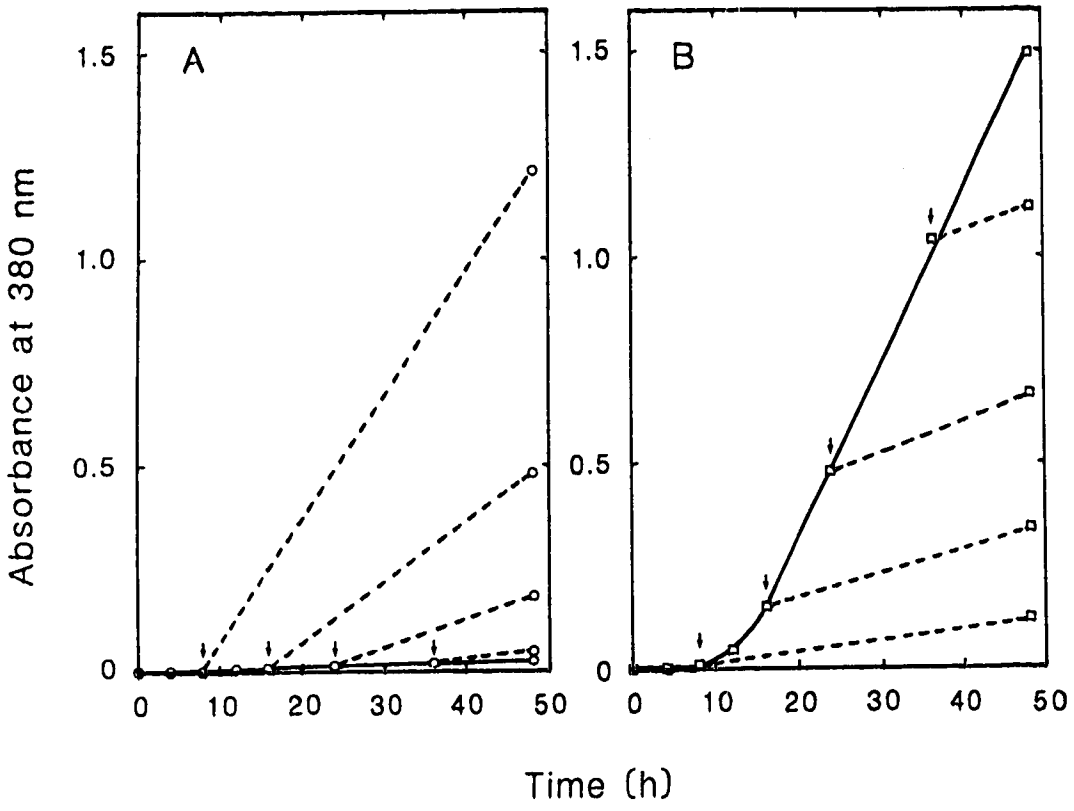

Fig. 5.3. Production of fluorescence by iron-limited *Azomonas macrocytogenes* ATCC 12334 grown at 28°C and 34°C. (A). *Azomonas macrocytogenes* cultures grown at 34°C and the fluorescence produced was assayed (———). At various time intervals (arrows) cultures were transferred to 28°C and at the end of 48 h the A_{380} of the cell-free culture supernatant fluid was recorded. (B). *Azomonas macrocytogenes* was grown at 28°C and the fluorescence produced was assayed (———). At various time intervals (arrows) cultures were transferred to 34°C and at the end of 48 h the A_{380} of the cell-free culture supernatant fluid was recorded. The ranges for duplicate samples were within the width of the symbols.

although relatively less of the 74 kDa protein was present in cells grown at 34°C (Fig. 5.4).

Effect of temperature on iron assimilation by *Azomonas macrocytogenes*

Azomonas 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 Fe^{3+} (min 10^8 cells)$^{-1}$, respectively, when assayed at 25°C in uptake buffer supplemented with 5 μM purified azoverdin (Fig. 5.5A). In the absence of added azoverdin, initial iron assimilation rates were 0.17 and 0.35 ng Fe^{3+} (min 10^8 cells)$^{-1}$ for cells grown at 34°C and 28°C, respectively (Fig. 5.5A). The high rate of iron transport by cells grown at 28°C in the absence of added azoverdin was undoubtedly influenced by the endogenous level of azoverdin produced by these cells. During the 60 min assay period 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 *Azomonas macrocytogenes* cells grown at 28°C and 34°C when assayed for iron assimilation at the increased temperature of 34°C (Fig. 5.5B). The main effect of the increased assay temperature was to increase the initial 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 in the absence of added azoverdin demonstrated a lower rate of iron transport [0.27 ng Fe^{3+} (min 10^8 cells)$^{-1}$] (Fig. 5.5B). The lower levels of endogenous azoverdin (A_{380}, 0.016 to 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 *Azomonas macrocytogenes* cells grown at 34°C when assayed at 0 °C. These cells accumulated 0.62 to 0.84 ng Fe^{3+} (10^8 cells)$^{-1}$ in the presence, and 0.18 to 0.25 ng Fe^{3+} (10^8 cells)$^{-1}$ in the absence of added azoverdin.

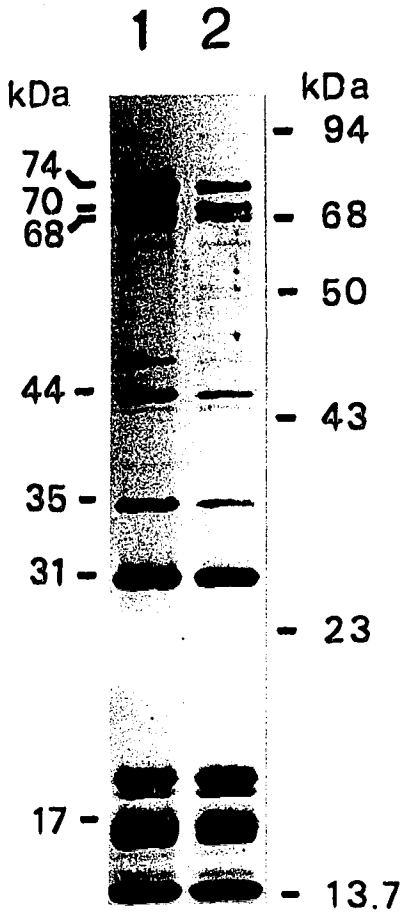

Fig. 5.4. SDS-PAGE of Sarkosyl-extracted outer membranes isolated from iron-limited *Azomonas macrocytogenes* grown at 28°C (Lane 1) or 34°C (Lane 2). Each lane of the 10% polyacrylamide gel was loaded with 10 μg protein. The gel was stained with Coomassie blue-R following electrophoresis. The standard proteins used are the same as for Fig. 3.2 and their molecular masses are noted on the right of the figure.

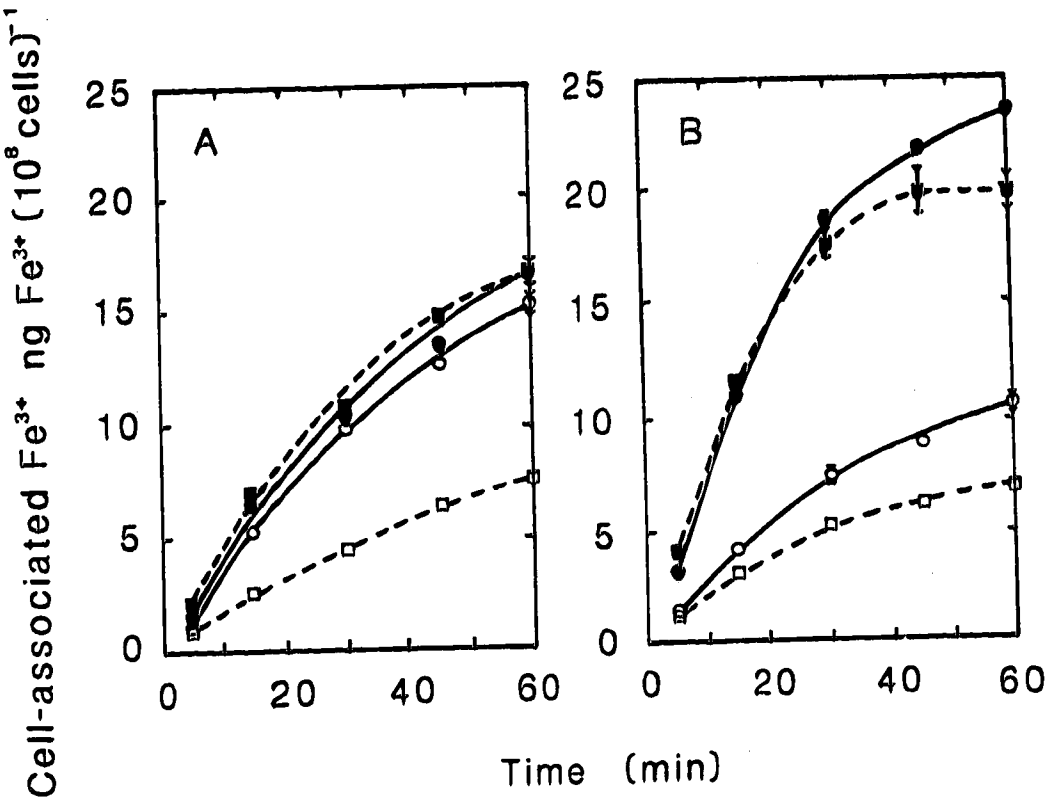

Fig. 5.5. Effect of temperature on azoverdin-mediated iron assimilation by *Azomonas macrocytogenes* ATCC 12334. (A) Iron-limited cells grown at 28°C (○, ●) or 34°C (□, ■) were assayed at 25°C in the absence (open symbol) or presence (closed symbol) of 5 μM purified azoverdin. (B) Iron-limited cells grown at 28°C (○, ●) or 34°C (□, ■) were assayed at 34°C in the absence (open symbol) or presence (closed symbol) of 5 μM purified azoverdin. Control values, obtained from cells similarly assayed at 0°C were subtracted from the respective test values. Error bars represent the range of duplicate assays where the range exceeded 0.1 ng Fe^{3+} (10^8 cells)$^{-1}$. Results of a typical experiment are presented.

Similarly, the iron accumulation under these conditions was rapid and did not increase over the 60 min assay period. The low levels of non-specific $^{55}Fe^{3+}$ accumulation by the filters previously noted were not increased by using an assay temperature of 34°C, an indication that the solubility of the iron in the assay system was not affected by this increase in temperature.

Determination of Km of azoverdin-mediated iron uptake

The initial rate of iron assimilation by *Azomonas macrocytogenes* cells grown at 34°C was dependent on the added azoverdin concentration in the uptake buffer. Increased azoverdin concentrations from 0 to 0.7 μM (A_{380} 0 to 0.014) caused enhanced $^{55}Fe^{3+}$ assimilation by *Azomonas macrocytogenes* cells (Fig. 5.6). These azoverdin concentrations were within the range of endogenous fluorescent compounds released into the uptake buffer by cells grown at 28°C. Azoverdin concentrations exceeding 0.7 μM 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 μM, the observed saturation in the rate of iron transport was not due to limiting iron. Although the molar extinction coefficient for pyoverdin was used for these calculations, preliminary measurements of the molar extinction coefficient for azoverdin place its value within 10% of that measured for pyoverdin.

The double reciprocal plot of the iron assimilation rate vs azoverdin concentration revealed an apparent Km of approximately 0.2 μM and a Vmax of 0.46 ng Fe^{3+} (min 10^8 cells)$^{-1}$ for azoverdin-mediated iron assimilation by *Azomonas macrocytogenes* cells grown at 34°C and assayed at 25°C (Fig 5.6, inset).

Azoverdin-independent iron assimilation by *Azomonas macrocytogenes*

Cell-free culture supernatants of iron-limited *Azomonas macrocytogenes* cells grown for 16 h at 28°C or 34°C contained 70 to 80 μM 3,4-DHBA. Iron assimilation by

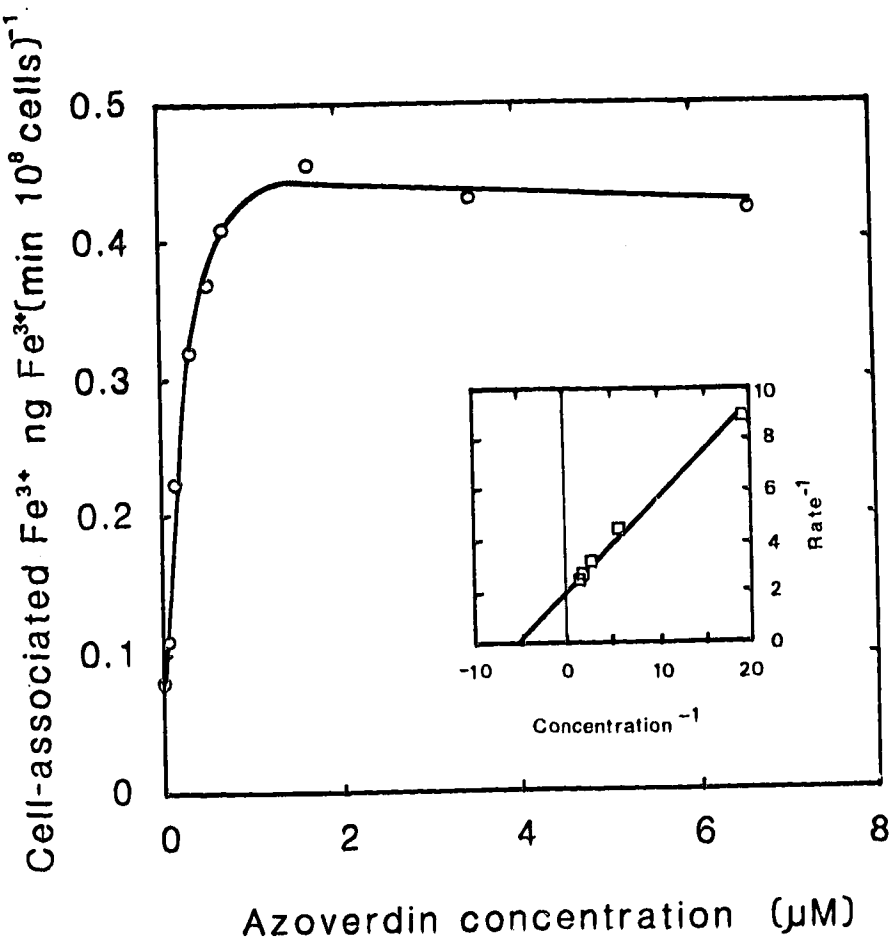

Fig. 5.6. Determination of Km for azoverdin-mediated iron uptake by *Azomonas macrocytogenes*. Iron-limited *Azomonas macrocytogenes* ATCC 12334 cells grown at 34°C were assayed for initial iron uptake rates at 25°C in uptake buffer supplemented with various concentrations of purified azoverdin. Controls were run for each azoverdin concentration at 0°C and the rates, which were less than 0.01 ng Fe^{3+} (min 10^8 cells)$^{-1}$ were subtracted from the test values. Inset shows the double reciprocal plot of rate of iron assimilation [ng Fe^{3+} (min 10^8 cells)$^{-1}$]$^{-1}$ vs azoverdin concentration μM^{-1}. Results of a typical experiment are presented.

Azomonas macrocytogenes was not enhanced by the presence of 3,4-DHBA under the present assay conditions. Cells grown at 34°C and assayed in uptake buffer or in an uptake solution prepared from equal volumes of uptake buffer and cell-free culture supernatant fluids of *Azomonas macrocytogenes* cells grown at 34°C for 16 h which contained 3,4-DHBA (35 to 40 μM, final concentration) assimilated iron at a similar low rate (0.1 to 0.2 ng Fe^{3+} (min 10^8 cells)$^{-1}$). This low level, azoverdin-independent iron assimilation was not further investigated.

5.5 DISCUSSION

The present study shows that purified azoverdin promotes the uptake of $^{55}Fe^{3+}$ by iron-limited *Azomonas macrocytogenes* ATCC 12334 thus confirming preliminary iron assimilation experiments which used culture supernatant fluids from of iron-limited *Azomonas macrocytogenes* as a source of impure azoverdin (Collinson & Page, 1989; Chapter 3). Azoverdin functions as a siderophore to promote iron uptake in a concentration-dependent manner with an apparent Km of 0.2 μM and Vmax of 0.46 ng Fe^{3+} (min 10^8 cells)$^{-1}$ under the present assay conditions. The low azoverdin concentration required for maximum iron uptake by *Azomonas macrocytogenes* is comparable to values obtained for pseudobactin-mediated iron uptake in *Pseudomonas putida* (Km 0.23 μM) (De Weger *et al.*, 1988), pyochelin-mediated iron assimilation by *P. aeruginosa* (Km 0.08 μM) (Cox, 1980) and siderophore-mediated iron uptake by other gram-negative bacteria (Km 0.1 to 0.25 μM) (Bachhawat & Ghosh, 1987; Frost & Rosenberg, 1978; Negrin & Neilands, 1978). *Azomonas macrocytogenes* is only the third genus, apart from *Azotobacter vinelandii* (Knosp *et al.*, 1984) and *Pseudomonas* spp. (De Weger, 1988; Hohnadel & Meyer, 1986; 1988; Magazin *et al.*, 1988; Meyer & Hornsperger, 1978)

known to produce a siderophore of the pyoverdin or pseudobactin class of compounds.

The saturable nature of azoverdin-mediated iron assimilation by *Azomonas macrocytogenes* suggests a rate-limiting step during transport, possibly at the cell membrane. Azoverdin also enhanced the binding of $^{55}Fe^{3+}$ to cells incubated at 0°C. One interpretation of this would include the presence of a protein in the outer membrane of *Azomonas macrocytogenes* capable of binding ferriazoverdin but which does not function at 0°C in iron transport. Consistent with this notion is the observation that cells grown at 34°C, which contained relatively less of the 74 kDa iron-repressible outer membrane protein than cells grown at 28°C, demonstrated saturation of iron uptake sooner than cells grown at 28°C (Fig. 5.5B). The high molecular mass and iron-regulated expression of the 74 kDa and 70 kDa outer membrane proteins of *Azomonas macrocytogenes* are reminiscent of ferrisiderophore receptor proteins identified in the outer membrane of *Pseudomonas* spp. (Cody & Gross, 1987; Hohnadel & Meyer, 1988; Magazin *et al.*, 1986) which bind their cognate ferrisiderophore, confer ferrisiderophore specificity on the cell and are required for siderophore-mediated high affinity iron assimilation. Additional experiments are required to ascertain the role of the 74 kDa and 70 kDa outer membrane protein in azoverdin-mediated iron assimilation by *Azomonas macrocytogenes* ATCC 12334.

Mechanistic details of siderophore-mediated iron-assimilation in *Pseudomonas*, *Azotobacter*, or *Azomonas* have yet to be addressed so it is not known whether fluorescent siderophores are transported into cells as the ferri-complex or if iron is released from the siderophores at the cell surface by reduction or some other mechanism. Recent studies indicate the presence of ferrisiderophore reductase activity in cell-free extracts of *Pseudomonas* spp. (Hallé *et al.*, 1989) and *Azotobacter vinelandii* (Huyer & Page, 1989) suggesting a mechanism for iron release from internalized ferrisiderophores.

The inability of micro-organisms to produce siderophores at slightly increased growth temperatures is a phenomenon previously encountered (Ankenbauer *et al.*, 1986; Garibaldi, 1971; 1972; Ismail *et al.*, 1985; Marugg *et al.*, 1985; Worsham & Konisky, 1984) but it

has not been previously reported for a nitrogen-fixing bacterium. The mechanism of temperature-regulated siderophore biosynthesis is not yet understood, although in *S. typhimurium*, temperature-regulated siderophore production appears to function independently of *fur* (Worsham & Konisky, 1984). The *fur* gene in *E. coli* encodes the Fur protein which regulates the high affinity iron assimilation systems in this bacterium (Bagg & Neilands, 1987). The inability of *Azomonas macrocytogenes* to produce azoverdin at the slightly increased growth temperature of 34°C solved the problems created by the release of endogenous azoverdin by cells grown at 28°C and allowed analysis of high affinity iron transport mediated by azoverdin.

Growth of *Azomonas macrocytogenes* at the elevated temperature did not inhibit production of the iron-repressible outer membrane proteins, a result previously noted with iron-deficient *P. aeruginosa* (Mizushima & Kageyama, 1978). However, *Azomonas macrocytogenes* appeared to produce relatively less of the 74 kDa protein at the increased growth temperature. Similarly, Worsham and Konisky (1984) observed a decrease in the expression of the iron-repressible outer membrane Cir protein of *E. coli* and *S. typhimurium* grown at slightly elevated growth temperatures.

There was little apparent effect on iron assimilation by *Azomonas macrocytogenes* whether cells were grown at 28 or 34°C and assayed for iron uptake at 25°C or 34°C. Again, similar results were reported for *E. coli* unless cells were severely starved for iron from the onset of growth (Worsham & Konisky, 1984).

Current results suggest that *Azomonas macrocytogenes* also assimilates iron in an azoverdin-independent manner since cells grown at 34°C still accumulate iron at a low rate. The presence of 35 to 40 μM 3,4-DHBA did not significantly enhance iron uptake in cells above this basal level suggesting that 3,4-DHBA does not act as a siderophore. However, the high concentrations of citrate (10 mM) employed in the assay system to keep Fe^{3+} soluble (Spiro & Saltman, 1969) may have masked possible 3,4-DHBA-promoted iron assimilation under present assay conditions. This would not preclude a possible role for

3,4-DHBA as an iron solubilizing compound used to facilitate iron assimilation by *Azomonas macrocytogenes* in other situations (Collinson *et al.*, 1987; Chapter 2). Likely, the observed azoverdin-independent iron assimilation was mediated by citrate as occurs in numerous other bacteria including; *Azotobacter chroococcum* (Shivprasad & Page, unpublished results), *E. coli* (Frost & Rosenberg, 1973; Pressler *et al.*, 1988), *Pseudomonas* (Cox, 1980) and *Mycobacterium* (Messenger & Ratledge, 1982). Citrate is not a carbon source for *Azomonas macrocytogenes* (Jensen, 1955; Thompson & Skerman, 1979) and it is not yet known whether *Azomonas macrocytogenes* produces a citrate-inducible outer membrane protein required for citrate-mediated iron transport as in *E. coli* (Hancock *et al.*, 1976).

In addition to azoverdin-mediated iron assimilation, *Azomonas macrocytogenes* apparently has other strategies for iron assimilation the details of which may become apparent upon further investigation .

NOTE ADDED IN PROOF:

Bachhawat and Ghosh (Bachhawat, A. K. & Ghosh, S. (1989). Temperature inhibition of siderophore production *Azospirillum brasilense*. *Journal of Bacteriology* 171,4092-4094.) have recently demonstrated that the diazotroph *Azospirillum brasilense* grown at 42°C instead of 32°C is unable to produce high levels of its siderophore, spirilobactin. However, *Azospirillum brasilense* still assimilates ^{59}Fe via ^{59}ferrispirilobactin at both temperatures and produces iron-repressible, high molecular mass outer membrane proteins. Therefore, the effect of temperature on iron assimilation in *Azospirillum brasilense* is analogous to that seen with *Azomonas macrocytogenes* ATCC 12334.

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CHAPTER 6

General Discussion

6.1 Iron assimilation by *Azomonas macrocytogenes* ATCC 12334

Prior to this study, virtually nothing was known concerning iron assimilation by *Azomonas macrocytogenes*. The current work has established that this gram-negative soil diazotroph possesses a mechanism for siderophore-mediated iron assimilation. In this study, the conspicuous blue-white fluorescent compound produced by iron-limited, but not iron-sufficient *Azomonas macrocytogenes* (Johnstone *et al.*, 1958; Thompson & Skerman, 1979) was purified and found to possess attributes typical of siderophores. It is a low molecular mass, ferric iron-binding, hydroxamate-containing compound chemically related to pyoverdin and pseduobactin siderophores produced by *Pseudomonas* spp. (Demange *et al.*, 1987; Meyer & Abdallah, 1978; Poppe *et al.*, 1987; Teintze *et al.*, 1981; Wendenbaum *et al.*, 1983). Consequently, the trivial name azoverdin has been proposed for this compound. As well as its siderophore-like chemical characteristics, azoverdin promoted $^{55}Fe^{3+}$ assimilation by *Azomonas macrocytogenes* thereby establishing is functional role as a siderophore.

Consistent with this model of azoverdin-mediated iron assimilation is the production of putative ferrisiderophore outer membrane receptor proteins by *Azomonas macrocytogenes*, whose high molecular mass and iron-regulated expression are typical of proven ferrisiderophore receptors in other gram-negative bacteria (Braun *et al.*, 1987; Cody & Gross, 1987; Hohnadel & Meyer, 1988; Magazin *et al.*, 1986). Thus, azoverdin-mediated iron assimilation is reminiscent of siderophore-mediated iron transport demonstrated in the related bacteria, *Pseudomonas* spp. (De Weger *et al.*, 1988; Hohnadel & Meyer, 1988) and *Azotobacter vinelandii* (Knosp *et al.*, 1984) as well as other gram-negative bacteria.

The inhibition of azoverdin biosynthesis by *Azomonas macrocytogenes* grown at slightly elevated temperatures is an intriguing aspect of regulation of iron assimilatory systems especially in view of the fact that the increased temperature did not inhibit azoverdin-mediated iron assimilation nor the production of the iron-repressible outer membrane proteins. Although recent evidence suggests that slightly elevated temperature is an alternate regulatory mechanism to the *fur* gene product in *S. typhimurium* (Worsham & Konisky, 1984), nothing is known about this regulatory mechanism in in other bacteria.

Finally, this study established that *Azomonas macrocytogenes* produces 3,4-dihydroxybenzoic acid (3,4-DHBA) when grown in defined medium which confirms the study by Westervelt *et al.* (1985). While the current data indicate a possible role for this phenolic compound in iron solubilization, a role similarly proposed for 2,3-dihydroxybenzoic acid produced by *Azotobacter vinelandii* (Page & Huyer, 1984), 3,4-DHBA is not a siderophore.

Azoverdin-independent iron assimilation by *Azomonas macrocytogenes* indicates that this bacterium has multiple iron assimilatory mechanisms, a feature common to many gram-negative bacteria (Cox, 1980; Cox & Adams, 1985; Neilands, 1984; Page & von Tigerstrom, 1988).

6.2 Future studies

The relative dearth of information concerning *Azomonas macrocytogenes* provides a virtual open door for possible future studies. From a practical standpoint, the fact that *Azomonas macrocytogenes* is a nitrogen-fixing bacterium should make it of potential agricultural interest. Its presumed rarity may be more a function of the attention given other prominent nitrogen fixers such as *Azotobacter* spp. and *Rhizobium* spp. since recent, albeit limited, surveys indicate that *Azomonas macrocytogenes* may be more prevalent in temperate soils than previously assumed (Kole *et al.*, 1988; Page & Collinson, 1987; Szember *et al.*, 1981). Additional studies concerning the distribution and soil ecology of

Azomonas macrocytogenes are required.

In view of the production of azoverdin by *Azomonas macrocytogenes*, and its chemical similarity to fluorescent siderophores elaborated by significant pathogenic and phytopathogenic group I fluorescent *Pseudomonas* spp., it would be of considerable interest to explore the possible role of azoverdin as an iron sequestration agent capable of interfering with the growth of these deleterious bacteria. A role for *Azomonas macrocytogenes* as a plant-growth promoting bacterium may be limited compared to the plant-growth promoting ability of pseudomonads which actually colonize plant roots and deprive pathogenic fungi of iron via production of their siderophores (Schroth & Hancock, 1982). However, azoverdin, or a chemical derivative thereof may have potential as an antimicrobial agent.

From an academic perspective, the mechanism of temperature regulation of azoverdin production is of considerable interest. It is intriguing that diverse micro-organsims have their siderophores regulated in this manner (Ankenbauer *et al.*, 1986; Garibaldi, 1971; 1972; Ismail *et al.*, 1985; Worsham & Konisky, 1984). Moreover, it has been hypothesized that the host febrile response could deprive pathogens of iron by repressing siderophore biosynthesis (Kluger *et al.*, 1983; Weinberg, 1979). In view of the apparent widespread nature of this phenomenon and the possible medical implications, *Azomonas macrocytogenes* may prove to be a useful model for studying temperature-regulated siderophore biosynthesis due to the ease and low cost of culturing this non-pathogenic bacterium. Presently, there is a general lack of knowledge concerning genetic manipulation of *Azomonas macrocytogenes*, however, once some of the basic research is completed, this bacterium may be worthy of additional attention.

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