ROLE OF MICROBIALLY PRODUCED SIDEROPHORES IN INCREASING AVAILABILITY OF MICRO-NUTRIENT CATIONS IN SOILS

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1992

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ROLE OF MICROBIALLY PRODUCED SIDEROPHORES IN INCREASING AVAILABILITY OF MICRONUTRIENT CATIONS IN SOILS

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Department of Soil Science

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

Yadvinder Singh Deol

October, 1992

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ABSTRACT

Siderophores are low molecular weight organic compounds secreted into the environment by bacteria and fungi in response to iron stress. Significant levels of siderophores have been found in the rhizosphere of crop plants; however, their potential significance in terms of mobilizing iron and other micronutrient cations has been overlooked. This research project studied the occurrence of siderophores in some Saskatchewan soils, and their importance in complexing and increasing availability of Cu, Fe, Mn, and Zn.

Twenty four soils were surveyed for siderophore levels using a bioassay which employed the siderophore-requiring bacterium, *Arthrobacter flavescens* JG-9. Hydroxamate type siderophores (HS) in the range of 2.1 to 27.3 x 10^{-9} M deferrioxamine methanesulfonate (DFOM) equivalents were detected in these soils. There was no simple correlation between HS levels and soil pH, % organic carbon, % clay, or DTPA-extractable Cu, Fe, Mn, and Zn.

A total of 210 bacteria were isolated from the rhizosphere of wheat roots grown in three soils (Medstead, Smeaton, and Choiceland), which contained the highest HS levels. Bacteria were screened for siderophore production using the *Arthobacter flavescens* JG-9 bioassay and the Chrome Azurol-S chemical method. Out of the 210 isolates, 29 were found to be producing siderophore. The 29 siderophore-producing bacteria and 9 plant growth-promoting rhizobacteria (PGPR) were further screened for the most efficient siderophore-producing bacteria. Three bacterial strains, CH 16, R 111 and KC 21, were selected and identified as *Pseudomonas fluorescens*. The efficient strains were grown in broth culture and their siderophores extracted and purified by Bio-Gel P2 filtration. The purified siderophores were used to study the mechanisms by which siderophores could increase availability of micronutrient cations. It was shown that siderophores produced by

these bacterial strains were able to complex not only with Fe, but also with Cu. Another way by which these siderophores increased the availability of micronutrient cations was through dissolution of iron-hydroxide, thereby releasing specifically adsorbed and co-precipitated Cu, Mn, and Zn.

The qualitative characterization of bacterial siderophores indicated that some components of these compounds are heat labile, and have maximum absorbance between 375 to 425 nm. The scanning of these siderophores in the infrared region indicated the presence of carboxyl and amino groups.

My research shows that Saskatchewan soils contain siderophore levels that are high enough to increase the availability of Cu, Fe, Mn, and Zn. However, the ability of siderophore-producing bacteria to colonize plant roots under field conditions may be limited. Therefore, the use of siderophore-producing bacteria as seed inoculants might increase the level of siderophores in the rhizosphere and facilitate micronutrient nutrition of crops.

ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to thank Dr. J. J. Germida for his supervision and encouragement throughout the course of this study.

Thanks are also extended to the members of the supervisory committee, Dr. D. W. Anderson, Dr. R. E. Karamanos, Dr. C. van Kessel.

The friendship of other graduate students, staff and faculty of the Saskatchewan Institute of Pedology throughout the period of this study was greatly appreciated.

Financial assistance from the College of Graduate Studies and Research, the Natural Sciences and Engineering Council of Canada is greatly acknowledged.

Finally, I wish to thank all my family members and friends for their continuous encouragement and support.

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1. INTRODUCTION

The micronutrient cations* Cu, Fe, Mn, and Zn play an important role in crop growth and metabolism as enzyme co-factors and regulators of chlorophyll synthesis (Price et al., 1972). Soil is the main source for these micronutrient cations, and their availability is controlled by chemical, biochemi[l]cal and biological transformations. Chemical transformations of these elements have been studied extensively (Lindsay, 1979; Mattigod et al., 1981) and chemical tests have been developed to predict the response of crops to micronutrient fertilization. However, micronutrient fertilizer applications to soils deficient in micronutrients sometimes do not result in predicted yield increases (Kruger et al., 1984; Brar et al., 1986). Such results indicate the need to better understand biochemical and biological transformations of micronutrients in soils.

Complexing of micronutrient cations with organic ligands are important in controlling the availability of these cations in soil. It has been shown that 98 to 99% of Cu, 84 to 99% of Mn, and up to 75% of Zn in displaced soil solutions occur as organic complexes (Mortenson, 1963; Gerring et al., 1969). The organic compounds in a soil solution that form stable complexes with micronutrient cations are of two main types: (1) low molecular weight biochemicals such as organic acids, amino acids, and siderophores which are known to occur in living organisms, and (2) a series of complex polymers such as humic and fulvic acids which are formed by secondary reactions and bear no resemblance to the natural products (Stevenson and Fitch, 1986). The role of humic and fulvic acids in controlling the availability of micronutrient cations to crops has been studied extensively (Schnitzer and Skinner, 1966; Stevenson and Ardakani, 1972; Stevenson and Fitch, 1986). However, little attention has been given to the importance of

* Throughout this thesis I use the abbreviation- Cu, Fe, Mn and Zn for micronutrient cations. Strictly speaking it is the ionic form of these elements to which I refer.

low molecular weight organic compounds in the micronutrient nutrition of crops (Elagwhary et al., 1970).

Siderophores are low molecular weight organic compounds produced by bacteria and fungi in response to iron stress (Neilands, 1973a). Siderophore contains either hydroxamic or phenolic groups and form stable complexes with iron (Fe^{3+}), with stability constants as high as 10^{32} (Powell et al., 1980). It has been shown that siderophores play an important role in the iron nutrition of some crops (Powell et al., 1982; Cline et al., 1983; Jurkevitch et al., 1989). Significant quantities of siderophores have been found in localized soil zones (such as in the rhizosphere) where biological activity is intense (Reid et al., 1981). Siderophores may also form stable complexes with other micronutrient cations such as Cu, Mn, and/or Zn and affect their availability to plants.

In spite of the metal chelating ability of siderophores, little attention has been given to the role of siderophores in Cu, Fe, Mn, and/or Zn nutrition of crops in Saskatchewan soils. The objective of my thesis research was to evaluate the ability of siderophores to increase the availability of micronutrient cations in some Saskatchewan soils. The objective was accomplished by studies designed to:

(1) determine the occurrence of siderophores in some Saskatchewan soils,

(2) isolate and screen bacteria capable of producing siderophores,

(3) isolate and qualitatively characterize siderophores, and

(4) study the mechanisms by which siderophores could increase availability of Cu, Fe, Mn, and/or Zn.

2. REVIEW OF LITERATURE

2.1 What are siderophores?

Siderophores are low molecular weight (500 to 1000 dalton) organic molecules secreted into the environment by bacteria and fungi in response to iron stress. Although iron is one of the most abundant (3.8 % w/w) elements in soils, iron deficiency occurs mainly due to the low solubility of iron minerals ($K_{sp} = 10^{-36}$). In the pH range of 7 to 9, the soluble ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions in soil solution do not exceed 10^{-10} M, which is far below the minimum requirement for the normal growth of microbes and plants (Lindsay, 1979; Lindsay and Schwap, 1982). This low level of available iron is a constant stress on microbes and plants, inducing them to produce powerful sequestering agents (Figure 2.1) which complex iron and increase its availability. The siderophores are specific for Fe^{3+}, and have a characteristic absorption band between 400 to 420 nm.

The criteria for preliminary classification of a natural product as a siderophore are (a) suppression of its formation by Fe^{3+}, and (b) a high specificity to bind Fe^{3+} compared to Fe^{2+} (Neilands, 1973a). Microorganisms also need specific membrane receptor proteins for the transport of the iron-laden form of the siderophores across the cell membrane. These receptors are usually selective for a particular siderophore to be transported. The microorganisms which do not have the specific membrane receptors cannot utilize iron complexed by that siderophore (Emery, 1977; Neilands, 1981).

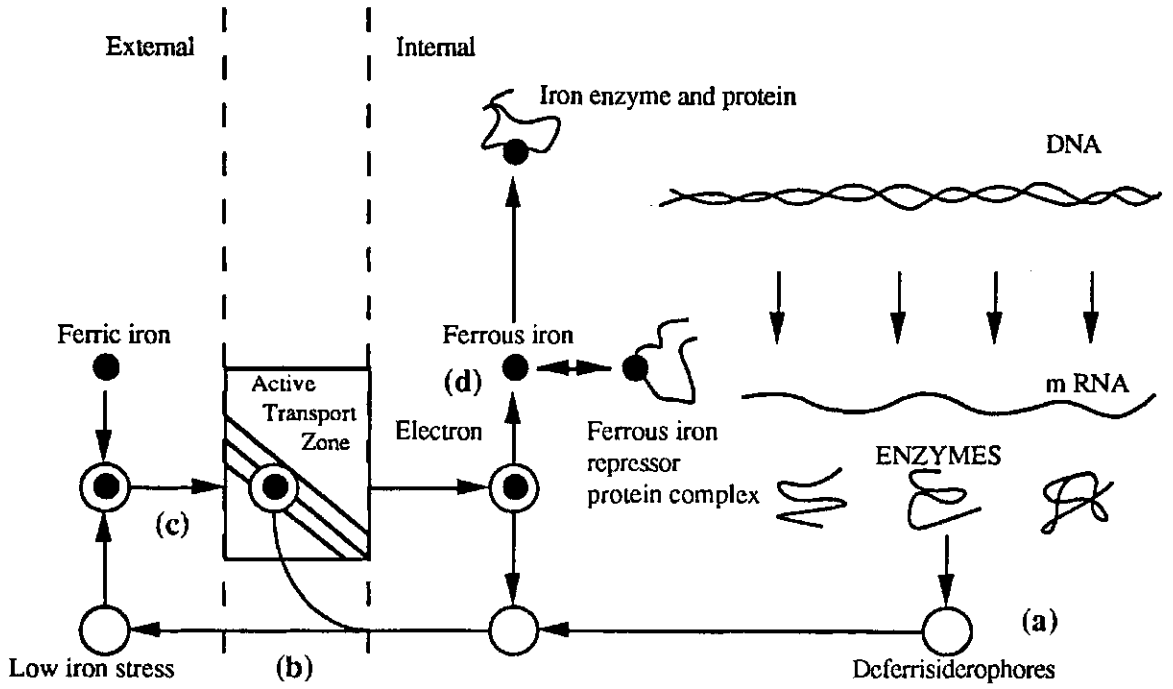


Figure 2.1 Schematic illustration of a microbial iron transport system. The features of this scheme are the following: (a) biosynthesis of a Fe^{3+} specific ligand by a sequence of enzymic reactions; (b) diffusion of the ligand to the membrane cell surface, or medium; (c) complexing of Fe^{3+} and active transport of the metal ion into the cell; (d) retention of the Fe^{3+} complex with in the cell, followed by reduction and delivery of the iron atoms at the point of demand by the biosynthetic machinery of the cell (adapted from Neilands, 1973a).

2.2 Classification and structure of siderophores

Two classes of siderophores are distinguished by the chemical groups that bind Fe^{3+}: (1) hydroxamates, and (2) phenolates (Figure 2.2) (Neilands, 1973a; Emery, 1977). Both of these ligands are weak acids ($pK_a \sim 9$), and only oxygen atoms are in the coordination sphere of the bound metal ion. Typically three hydroxamate or phenolate groups occur in the same molecule and provide very stable hexadentate ligation for Fe^{3+} with formation constants of about 10^{32} for the trihydroxamates (Table 2.1) and on the order of 10^{40} for triphenolates (Neilands, 1973a). The specificity for Fe^{3+} is relatively high, and iron is presumed to be released from the complex following its reduction to the Fe^{2+} state.

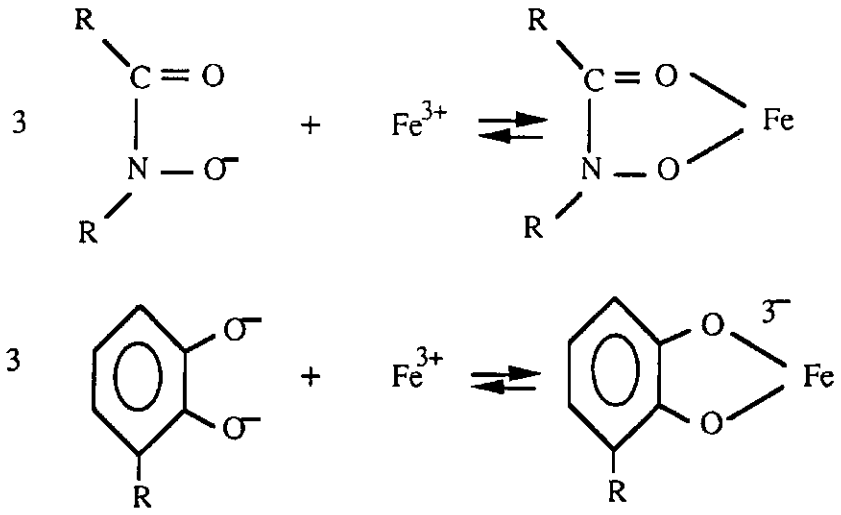


Figure 2.2 Types of Fe^{3+}-hydroxamate (upper) and Fe^{3+}-phenolate (lower) complexes found in microbial iron transport compounds (adapted from Neilands, 1973a).

Table 2.1 Stability constants of Fe^{3+} trihydroxamate siderophores (adapted from Neilands, 1973a).

Ligand	Log K
Deferriferrichrome	29.1
Deferriferrichrysin	30.0
Deferriferrioxamine B	30.5
Deferriferrioxamine D_1	30.8
Deferriferrichrome A	32.0
Deferriferrioxamine E	32.4

2.2.1 Hydroxamate type siderophores

The research into the discovery of hydroxamate type siderophores (HS) was initiated by Neilands (1952). He was studying the metabolism of a fungus, *Ustilago sphaerogena*, in iron deficient media and found that under iron stress conditions large amounts of a compound were excreted into the medium. The compound was isolated in the form of a crystalline, brownish-yellow, iron complex which Neilands named "ferrichrome". In the same year, Hesseltine et al. (1952) found that *Pilobolus kleinii* needed the culture filtrate of a *Penicillium* spp. for growth but the nature of the growth-promoting compound(s) was not known. Similarly, Lochhead et al. (1952) showed that the soil bacterium, *Arthrobacter terregens*, needed the culture filtrate of *Arthrobacter pascans* to grow. These growth-promoting compounds from the *Penicillium* spp and *A. pascans* were eventually isolated, shown to chelate iron, and called "coprogen" and "terregenes factor", respectively. Later the terregens factor was named "arthrobactin". Several years later, Keller-Schierlein et al. (1964) (cited from Keller-Schierlein, 1975)

isolated a family of at least three closely related, iron-containing substances which they called ferrioxamine A, B, and C.

The hydroxamate type siderophores are divided into families based on the number of peptide linkages. Examples are ferrichrome, rhodotorulic acid, citrate-hydroxamates, aerobactin, mycobactin, fusarinine, and ferrioxamine. The characteristic structural feature of the ferrichrome family is a cyclic hexapeptide containing a tripeptide sequence of δ-N-acyl-L-δ-N-hydroxyornithine and a tripeptide composed of small, neutral amino acids. The latter may be glycine, serine or alanine, alone or in some particular combination. Thus ferrichrome, an important siderophore in the ferrichrome family, is composed of three contiguous residues of glycine and three residues of hydroxamate derivative of ornithine, δ-N-acyl-L-δ-N-hydroxyornithine. Ornithine is the most common hydroxylamino acid found in natural hydroxamates, and it occurs not only in compounds of the ferrichrome family, but also in the fusarinine and rhodotorulic acid families (Atkin and Neilands, 1968). The sequence seryl-seryl-glycine is common to ferrichrome A (Emery and Neilands, 1961). Representative ferrichromes have been isolated from cultures of *Aspergillus*, *Penicillium*, *Neurospora*, *Ustilago*, and smut fungi (Table 2.2) (Neilands, 1981).

The rhodotorulic acid family is closely related to the ferrichrome family in that they contain a common dipeptide of δ-N-acyl-L-δ-N-hydroxyornithine. Rhodotorulic acid, dimerum and coprogen are the important compounds belonging to this family. These compounds have been isolated from iron-starved cultures of *Rhodotorula pilimanae*, *Fusarium dimerum*, *Neurospora* and other fungal species (Atkin and Neilands, 1968; Atkin et al., 1970). The citrate-hydroxamate are also dihydroxamates, but the citric acid acts as a backbone holding the metal-binding hydroxamate groups. Three siderophores in this family are aerobactin, schizokinen, and arthrobactin.

Table 2.2 A list of siderophores found in nature (adapted from Hider, 1984; Neilands, 1984).

Siderophore	Microorganism
I. Hydroxamate	
Ferrichrome	*Penicillium spp.*
Ferrichrome A	*Ustilago sphaerogena*
Ferrichrome C	*Cryptococcus melibiosum*
Ferrichysin	*Aspergillus melleus*
Ferricrocin	*Aspergillus fumigatus*
Ferrirubin	*Paecilomyces varioti*
Ferrirhodin	*Aspergillus nidulans*
Pseudobactins	*Pseudomonas fluorescens*
Pyoverdines	*Pseudomonas fluorescens*
Rhodotorulic acid	*Rhodotorula pilimanae*
Dimerum acid	*Fusarium dimerum*
Coprogen	*Neurospora crassa*
Triornoicin	*Epicoccum purpurascens*
Schizokinen	*Bacillus megaterium*
Terregens Factor (Arthrobactin)	*Arthrobacter terregens*
Aerobactin	*Escherichia coli* ColV
Fusarinine (Fusigen)	*Fusarium roseum*
Fusarinine A, B, C	*Fusarium roseum*
Triacetyl fusarinine	*Fusarium roseum*
II. Phenolate	
Enterobactin	Enteric bacteria
2,3-dihydroxy-N-benzoyl lysine	*Azotobacter vinelandii*
2,3-dihydroxy-N-benzoyl serine	*Escherichia coli*
2,3-dihydroxy-N-benzoyl glycine	*Bacillus subtilis*
2,3-dihydroxy-N-benzoyl threonine	*Klebsiella oxytoca*
Agrobactin	*Agrobacterium tumefaciens*
Parabactin	*Paracoccus denitrificans*
Vibriobactin	*Vibrio cholerae*
Pyochelin	*Pseudomonas aeruginosa*

Siderophores of the ferrioxamine class contain units of acetate, succinate and α-amino-ω-hydroxyaminoalkane and are either linear or cyclic. Certain derivatives of the ferrioxamine display antibiotic activity and have been designated as ferrimycins. Deferrioxamine methanesulfonate (DFOM) is the trade name of the mesylate salt of deferrioxamine B.

2.2.2 Phenolate type siderophores

All phenolate-containing siderophores display fluorescence in ultraviolet light. They all contain 2,3-dihydroxybenzoic acid, which is acylated to the amino group of an amino acid such as glycine, lysine, or serine. One member of the series, itoic acid (defined as 2,3-dihydroxybenzoylglycine), contains glycine and was the first phenolate siderophore to be described (Ito and Neilands, 1958). It is secreted by *Bacillus subtilis* when grown under low iron conditions. Production of itoic acid was shown to be completely repressed in *B. subtilis* at an iron concentration of 3×10^{-6} g L^{-1} (Peters and Warren, 1968). Other phenolate type siderophores include 2,3-dihydroxybenzoylserine and 2,3-dihydroxybenzoyl derivatives of lysine (Brot et al., 1966). Enterobactin is the cyclic trimer of 2,3-dihydroxybenzoylserine. It was obtained from low iron cultures of *Salmonella typhimurium* LT2, *Escherichia coli*, *Aerobacter aerogenes*, and *Enterobacter cloacae* (Pollack and Neilands, 1970; Neilands, 1981). It appears to be a common siderophore of the enteric bacteria. It is essentially insoluble in water, but dissolves readily in acetone, dioxane, or methanol. Ferric ion complexes to enterobactin with the displacement of six protons and the resulting chelate has three negative charges per mole (Neilands, 1973a). A list of phenolate type siderophores is presented in Table 2.2.

2.3 Methods of analysis of siderophores

The study of siderophores has recently become an area of active interest because several of these molecules have been shown to be growth factors, iron carriers, or antibiotics for a number of microbial and plant species (Kloepper et al., 1980a; Powell et al., 1982; Elad and Misaghi, 1984; Hider, 1984; Bakker et al., 1987; Misaghi et al., 1988). A number of methods have been developed to detect and quantify siderophores. These methods are grouped into two categories: (1) chemical assays, and (2) biological assays using siderophore auxotrophs (Neilands, 1984).

2.3.1 Chemical methods

In 1926, Blom (cited from Neilands, 1984) proposed a method for the determination of bound hydroxylamine (structural component of hydroxamic acids) which involved the oxidation of hydroxylamine to nitrite. Later, this method was modified by Csaky (1948) (cited from Neilands, 1984) and it involved digestion of the sample in sulfuric acid and oxidation with iodine to nitrous acid. This test is sensitive since the nitrous acid is diazotized and coupled to 1-naphthylamine to yield a highly conjugated, intensely colored dye. However, several conflicting descriptions of the reagent and conditions have been described (Lee and Roughan, 1971). Thus numerous modifications of the original Csaky test are available, resulting in an extensive spread of values for the concentrations of bound hydroxlamine or hydroxamates. Moreover, this method can only be regarded as semiquantitative in nature (Neilands, 1984).

As an alternative to the Csaky method, Atkin et al. (1970) introduced a method whereby siderophores react with ferric perchlorate in dilute acid. Hydroxamic acids form di, tetra, and sexadentate derivatives with Fe^{3+}. The 1:1 complex is produced in acid solution and is purple in color with maximum absorbance between 500-520 nm. The 3:1

complex is generated in neutral pH and is orange in color with maximum absorbance between 420-450 nm. Neilands (1975) proposed another method which involved oxidation of hydroxamic acid with periodic acid and then measurement of absorption at 264 nm. This method was effectively employed by Holzberg and Artis (1983) to detect the presence of hydroxamate siderophores in the cell free supernatant of a number of fungal pathogens.

Fekete et al. (1983) described a method for the detection of siderophore excreted into agar surfaces. The colony is grown up on a low iron medium prepared with purified agar and after growth a segment adjacent to the colony is cut out and placed on filter paper. After electrophoretic separation, the paper is viewed in ultraviolet light for detection of phenolate and sprayed with an iron solution for detection of hydroxamate siderophores. Schwyn and Neilands (1987) developed a chemical assay for the detection and quantification of siderophores. This method involved the use of a dye, Chrome Azurol-S (CAS), which when complexed with iron at pH 5.6 to 6.8 gives a blue color. Addition of siderophores to the CAS-Fe complex, scavenges iron from the CAS-Fe complex and the CAS color changes to orange. This method can be used both in agar or in broth culture, but can not distinguish between hydroxamate and phenolate type siderophores.

2.3.2 Siderophore auxotrophs

Siderophore auxotrophs are those microorganisms which do not produce their own siderophores, but are dependent upon an exogenous source of siderophores for growth (Table 2.3). Bioassays for siderophores are many orders of magnitude more sensitive than the best chemical methods. Lochhead et al. (1952) observed that certain strains of *Arthrobacter* would grow on a defined medium only if supplemented with a soil extract. The active component of the soil extract was purified, named "terregens factor", and found

Table 2.3 Siderophore dependent auxotrophic microorganisms (adapted from Neilands, 1973a).

Source	Active factors	Reference
I. Natural		
Pilobolus kleinii	Dung extract, coprogen, ferrichrome	Hesseltine et al. (1952)
Arthrobacter terregens	Terregens factor,coprogen, ferrichrome, aspergillic acid soil and liver extracts, synthetic chelating agents, mycobactin	Burton and Lochhead (1953)
Arthrobacter flavescens JG-9 (ATCC 25091)	Terregens factor, ferrioxamine B, aspergillic acid, coprogen, mycobactin, rhodotorulic acid, acethydroxamic acid, and synthetic chelating agents	Burnham and Neilands (1961)
Microbacterium lacticum (ATCC 8181)	Ferrichrome, coprogen, terregens factor	Hendlin and Demain (1959)
Mycobacterium johnei	Mycobactins	Snow (1970)
II. Induced		
Bacillus megaterium (ATCC 19213) (Sk mutants)	Schizokinen, ferrioxamine B, ferrimycin A, ferrichrome, mycobactin, 2,3-dihydroxybenzoic acid, natural and synthetic chelating agents.	Arceneaux and Lankford (1966)
Escherichia coli (Chr2 mutant)	2,3-dihydroxybenzoylserine, citrate	Wang and Newton (1969)
Salmonella typhimurium LT2 (Enb mutants)	Dihydroxybenzoylserine, enterobactin, ferrichrome, schizokinen, deferrioxamine E, rhodotorulic acid, ascorbate and EDTA.	Pollack and Neilands (1970)

to contain iron. Later it was observed that ferrichrome, a siderophore from *Ustilago sphaerogena*, would support the growth of many terregens factor-requiring strains (Lochhead, 1958). The terregens factor or ferrichrome requiring strain was identified as *Arthrobacter flavescens* JG-9 (Burnham and Neilands, 1961), and now is the most commonly used method for the assay of hydroxamate type siderophores. This strain does not respond to phenolate type siderophores. The *A. flavescens* JG-9 bioassay involves: (1) spread plating the bacteria (0.1 ml of 10^5 cfu per ml) on agar plates (Appendix 6.1.1); (2) placing a 13 mm sterile paper disc (S & S # 740-E) in the middle of each plate; (3) treating the paper disc with 20 µl of a range of standard siderophore solutions (deferrioxamine methanesulfonate, DFOM, from Ciba-Geigy Pharmaceutical Co., Summit, NJ) or an unknown extract (soil extract or cell free microbial suspension); and (4) incubating the plates at 25°C for 48 h before assessing bacterial growth. The growth diameter of JG-9 around the disc in unknown sample is measured and then related to a standard curve. This method has been effectively used to quantify hydroxamate type siderophores in soil extracts (Powell et al., 1980; Reid et al., 1984), and cell free extracts of bacteria and fungi (Szaniszlo et al., 1981; Fekete et al., 1989). This assay can also be performed in broth culture.

As an alternative to the use of *A. flavescens* JG-9 bioassay, two mutants of *Salmonella typhimurium* LT-2 (*enb*-1 and *enb*-7) have also been used (Luckey et al., 1972; Akers, 1981). The *enb* mutants respond to enterobactin as well as to a range of hydroxamate compounds (Akers, 1981). In 1976, Wayens and co-workers developed two useful *E. coli* K 12 mutants, RW193 and RWB18. Both are *ent* A and hence neither can synthesize enterobactin. The RW193 strain, however, contains the ferric-enterobactin transport protein whereas the strain RWB18 lacks this outer membrane transport protein. Therefore simultaneous use of the strains in a bioassay confirms enterobactin activity.

Recently, other mutants strains of *E. coli* K-12 have also been developed to detect and quantify five different types of siderophores; ferrichrome and its analogs, enterobactin, coprogen, rhodotorulic acid, and ferrioxamine (Powell et al., 1983; Nelson et al., 1988).

2.4 Occurrence of siderophores in nature

2.4.1 Occurrence in soil

Lochhead and co-workers (1952) were the first to show that soil extracts contain active compounds which are essential for the growth of certain strains of *Arthrobacter,* but they did not quantify the active compound. Powell et al. (1980) made an attempt to quantify hydroxamate type siderophores in soils. Using the *A. flavescens* JG-9 bioassay they found levels in the range of 0.3 to 2.7 nM DFOM equivalents in 57 surface soils, representing a wide range of organic carbon and soil types. Concentrations as high as 378 nM DFOM equivalents were found in the rhizosphere of pine (*Pinus contorta* L.) stands at Pingreer Park, Colorado, USA (Reid et al., 1984). Estep et al. (1975) found levels of hydroxamate siderophores up to 33.4 nM DFOM equivalents in a near shore mud. Akers (1981; 1983) used a siderophore requiring *S. typhimurium* strain enb-7 and detected 26.6 to 697.7 nM rhodotorulic acid equivalents in rice field soils.

Efforts have also been made to detect and quantify various kind of siderophores in soils. Powell et al. (1980) used the mutant strain RW193, to quantify ferrichrome type hydroxamate siderophores in mineral soils. The ferrichrome concentration in soil : water (1:1) extract was estimated to be 78.0 nM. Nelson et al. (1988) also used the mutant strains of *E. coli* K-12, BN3306, 3AN, and 22H1, to quantify enterochelin, coprogen (including rhodotorulic acid and ferrioxamine), and ferrichrome, respectively. The results showed that there was 1.5 to 3.5 times more siderophore in the rhizosphere soil of sorghum (*Sorghum bicolor* L. moench) as compared to the bulk soil.

2.4.2 Production of siderophores by plants

Crop plants require a continuous supply of iron for chlorophyll biosynthesis. The amount of Fe^{3+} available in aqueous solutions of most calcareous soils is insufficient for normal plant growth, and plants develop iron chlorosis. A number of theories have been put forward to explain the absorption of iron by plant roots (Chaney et al., 1972; Brown 1978; Bienfait, 1988) and these are summarized below:

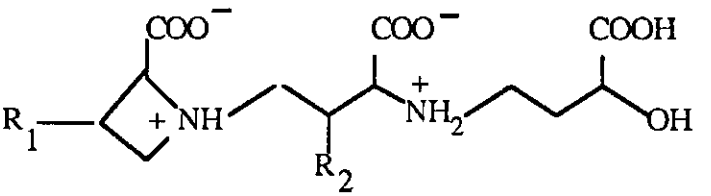
(1) release of hydrogen ions from the roots.

(2) release of reducing compounds from the roots.

(3) reduction of ferric iron to ferrous ion at the roots, and

(4) increase of organic acid (particularly citric acid) in the roots.

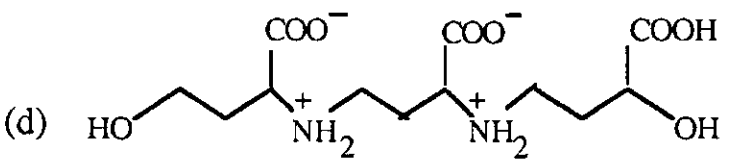
In 1976, Takagi showed that washings from rice roots had a capacity to dissolve Fe^{3+}, and the plants secreted more of the substances under iron-deficient conditions (Takagi, 1976). This new compound (Figure 2.3a) was later isolated from barley (*Hordeum vulgare* L.) and named mugineic acid (Takemoto et al., 1978). Subsequently, iron-chelating amino acids were isolated from some species of gramineous plants: 2'-deoxymugineic acid (2.3b) from wheat (*Triticum aestivum* L.), 3-hydroxymugineic acid (2.3c) from rye (*Secale cereale* L.), avenic acid A (2.3d) from oat (*Avena sativa* L.), and distichonic acid (2.3e) from malt barley (*H. vulgare* L.) (Sugiura and Nomoto, 1984). All of these compounds are good chelator of Fe^{3+} and belong to one family of organic compounds termed "Mugineic acid family-phytosiderophores" (MAS). Only grasses have been shown to excrete MAS and it is still a mystery why the dicotyledons do not have this capacity (Bienfait, 1988). The quality and quantity of MAS secreted by plants roughly correlates with the degree of resistance of plant to Fe-chlorosis (Kawai et al., 1988). In this regard Romheld (1987) showed that Fe-deficient barley plants excreted higher amount



(a) R_1 = H, R_2 = OH

(b) R_1 = R_2 = H

(c) R_1 = R_2 = OH



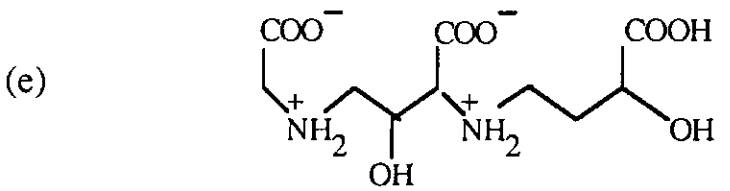


Figure 2.3 Structure of mugineic acid family phytosiderophores. (a) mugineic acid, (b) 2'-deoxymugineic acid, (c) 3-hydroxymugineic acid, (d) avenic acid, and (e) distichonic acid (adapted from Sugiura and Nomoto, 1984).

of MAS compounds as compared to barley plants supplied with iron chelate.

2.5 Importance of siderophores in nature

2.5.1 Iron nutrition of agricultural crops

The potential significance of siderophores to plants in terms of iron nutrition could be great since siderophore-producing bacteria and fungi are very active in the rhizosphere. For the siderophores to be effective suppliers of Fe^{3+} to plants roots, they should be able to diffuse into soil, chelate with iron and then move towards the root along a concentration gradient. Powell et al. (1982), using simulated roots to study this process, found that DFOM (at an concentration of 10^{-4} M) increased the amount of Fe (^{55}Fe) removed from a soil by 2-fold. On the contrary, citrate, and EDTA at 10^{-3} M did not significantly increase the movement of ^{55}Fe (Powell et al., 1982). The ability of siderophores to chelate Fe^{3+} and increase its availability in soils was further tested using chelation computer modelling. The results indicated that the three siderophores tested (DFOM, a mixture of unknown siderophores from *Boletus edulis*, and ferrichrome A from *Ustilago sphaerogena*) were able to complex 100% of the iron in a nutrient solution and soil system over the entire pH range of 4 to 10 (Cline et al., 1982; 1983). The organic acids, oxalate, malate, succinate, α-ketoglutarate, and pyruvate did not chelate iron above pH 6. Recently Treeby et al. (1989) showed that DFOM complexed Fe^{3+} and increasesd its availability in soil. In contrast, Takagi and co-workers (1988) found that DFOM was not an effective Fe^{3+} chelator in soils, whereas DTPA (diethylenetriamine pentaacetic acid), an important extractant used in many soil testing laboratories to extract Fe^{3+} and other micronutrients, effectively solubilized and removed Fe^{3+} from soils.

Neilands (1978) was the first to study the role of siderophores in iron nutrition using tomato as a test crop. He found that ferrioxamine B was superior to $FeCl_3$ as a

source of iron. Similar observations were recorded by Millar et al. (1985) using rhodotorulic acid for tomato plant in hydroponic culture. Reid et al. (1984) also demonstrated that oat plants could take up ferrichrome-chelated iron in nutrient solution, and then transport that iron to the shoots.

Siderophores may behave differently in soil systems compared to nutrient solutions as soils contain many reactive components such as organic matter, clay minerals, and oxides of aluminum and iron. Jurkevitch et al. (1989) found that the ferrated yellow-green fluorescent siderophores, isolated from *Pseudomonas putida*, prevented chlorosis in peanut plants growing on a highly calcareous soil. However, the amount of the yellow-green fluorescent siderophores required to prevent chlorosis was 19 mg as compared to 1.2 mg of Fe-EDDHA [ethylenediamine di(O-hydroxyphenylacetic acid)]. Similar results were obtained by Romheld (1987). This low efficiency might be due to the adsorption of siderophores on soil components such as organic matter (Powell et al., 1980).

Phytosiderophores have also been shown to be involved in increasing the availability of Fe^{3+} in soils. Takagi et al. (1988) and Treeby et al. (1989) demonstrated that mugineic acid is very effective in solubilizing Fe^{3+} from soils, especially soils of alkaline pH. Similarly, Awad et al. (1988) found 10 to 20 times more release of ^{59}Fe from soils by a phytosiderophore (10^{-5} M) compared to DTPA. In a nutrient culture study, sorghum was shown to absorb Fe^{3+} from ferrated-phytosiderophore (Clark et al., 1988).

The use of siderophores as a foliar spray or direct soil application to ameliorate iron deficiency does not seem to be economical, as a number of synthetic iron chelators, EDTA (ethylenediamine tetraacetic acid), EDDHA, and DTPA are cheaper to apply. However, seed inoculation with siderophore-producing bacteria or fungi may be an economical, and alternate way for iron nutrition of crops.

2.5.2 Enhancement of plant growth by suppressing deleterious microorganisms

Beneficial and deleterious microorganisms constantly interact with each other as well as with plant roots in the rhizosphere, and plant health often depends on the outcome of such interactions. Plants suffer when their roots are attacked by pathogens; they thrive when certain growth-promoting microorganisms manage to colonize their roots (Elad and Misaghi, 1984). Therefore, rhizosphere microorganisms should be manipulated in ways that favour root colonization by beneficial microorganisms. This approach to disease control is called biological control. Biological control in the rhizosphere could be due to antibiosis, competition for nutrients, and/or parasitism. Siderophores can act as antimicrobial agents as well as chelators of iron in the rhizosphere. Therefore siderophores enhance the ability of their producer to colonize root surfaces and are an effective means to compete with other microbes for the limited supply of iron (Emery, 1980).

Microbes which benefit from siderophore-mediated Fe^{3+} nutrition should possess a special kind of transporting protein in their cell membrane to transport the siderophore-Fe complex. Microbes which lack transporting proteins cannot utilize the complexed Fe^{3+}. Therefore these microbes, some of them pathogenic in nature, may be suppressed in the rhizosphere by either adding the siderophores or inoculating the seeds with siderophore-producing bacteria or fungi.

Attempts have been made to inoculate crop seeds with certain non-pathogenic non-nitrogen fixing bacteria to promote plant growth. Among different plant growth-promoting rhizobacteria (PGPR) tested, the *P. fluorescens* and *P. putida* groups seem to be the most effective in promoting plant growth and increasing yield. These psuedomonads have been shown to rapidly colonize plant roots of potato, sugerbeet and radish, and caused

statistically significant yield increases (up to 144%) in field tests (Kloepper, 1980a, 1980b).

The siderophore producing PGPR strains exhibited *in vitro* antibiosis against *Erwinia carotovora*, which causes potato soft root and seed piece decay (Kloepper and Schroth, 1981), *Escherichia coli* K-12 AN193 (Kloepper et al., 1980b), *Pythium ultimum* (Howell and Stipanovic, 1980), *Geotrichum candidum, Rhizoctonia solani, Sclerotinia sclerotiorum, Pythium aphanidermatum* (Misaghi et al., 1982, 1988), and *Fusarium spp.* (Scher and Baker, 1982). In all of these studies the inhibition was counteracted by adding iron to the medium. In some cases, not only the bacteria but their isolated siderophores such as pseudobactin inhibited pathogen growth (Howell and Stipanovic, 1980; Kloepper et al., 1980a; Kloepper and Schroth, 1981).

In soil studies, the addition of Fe-EDTA prevented PGPR from enhancing potato yield (Kloepper et al., 1980a). The effect of pseudobactin on plant growth and rhizosphere fungal colonization was determined in a green houseassay. Both *P. fluorescens* B10 suspension and pseudobactin at 10 μM doubled the weight of potato plant, and this was attributed to a 74% decrease in the number of pathogenic fungi found in the root region (Kloepper and Schroth, 1981). Based on these findings it was proposed that PGPR inoculation rapidly colonize roots of the developing plant, produce siderophores which sequester iron in the root zone, making it unavailable to certain pathogenic rhizoplane microorganisms, and thus a more favorable environment for root growth is created.

3. EXPERIMENTAL

3.1 Survey of Saskatchewan soils for siderophores

3.1.1 Materials and Methods

3.1.1.1 Extraction of siderophores from Saskatchewan soils

Soil samples were collected from the surface layer (0 to 15 cm) of the five major soil zones (Brown, Dark Brown, Black, Dark Gray, and Gray) of Saskatchewan (Figure 3.1.1). The soil samples were air-dried at room temperature and ground (using mortar and pastle) to pass through a 2 mm polyethylene sieve. Soil samples were analyzed by the Saskatchewan Soil Testing Laboratory for various physico-chemical properties (Table 3.1.1) as follows: The pH was determined in 1:1 soil to water suspension using a standard pH meter (SSTL, 1990); particle size with the pipette method (McKeague, 1978); organic carbon was determined by a wet oxidation procedure (McKeague, 1978). Micronutrient cations (Cu, Fe, Mn and Zn) were extracted with a solution of 0.005 M diethylenetriaminepentaacetic acid (DTPA), 0.1 M triethanolamine and 0.01 M calcium chloride, and the concentration of Cu, Fe, Mn and Zn in the extract measured using inductively coupled plasma spectrometry (ARL 3560 AES) (Lindsay and Norvell, 1978; SSTL, 1990).

Siderophores were extracted from the soil samples with deionized water (soil to water ratio; 1:1). The mixture was shaken for 2 h at room temperature and centrifuged at 5000 x g for 10 min. The supernatant was filtered through a Whatman # 2 filter paper, adjusted to pH 7.4 using 1 N KOH, and filter-sterilized using a sterile 0.22 μm GSWP membrane filter (Millipore Corporation, Bedford, MA) placed in a Swinnex-25 filter holder (Millipore Corporation, Bedford, MA). The sterilized filtrate was stored at 5°C until analyzed for siderophores (Powell et al., 1980; Perry et al., 1984).

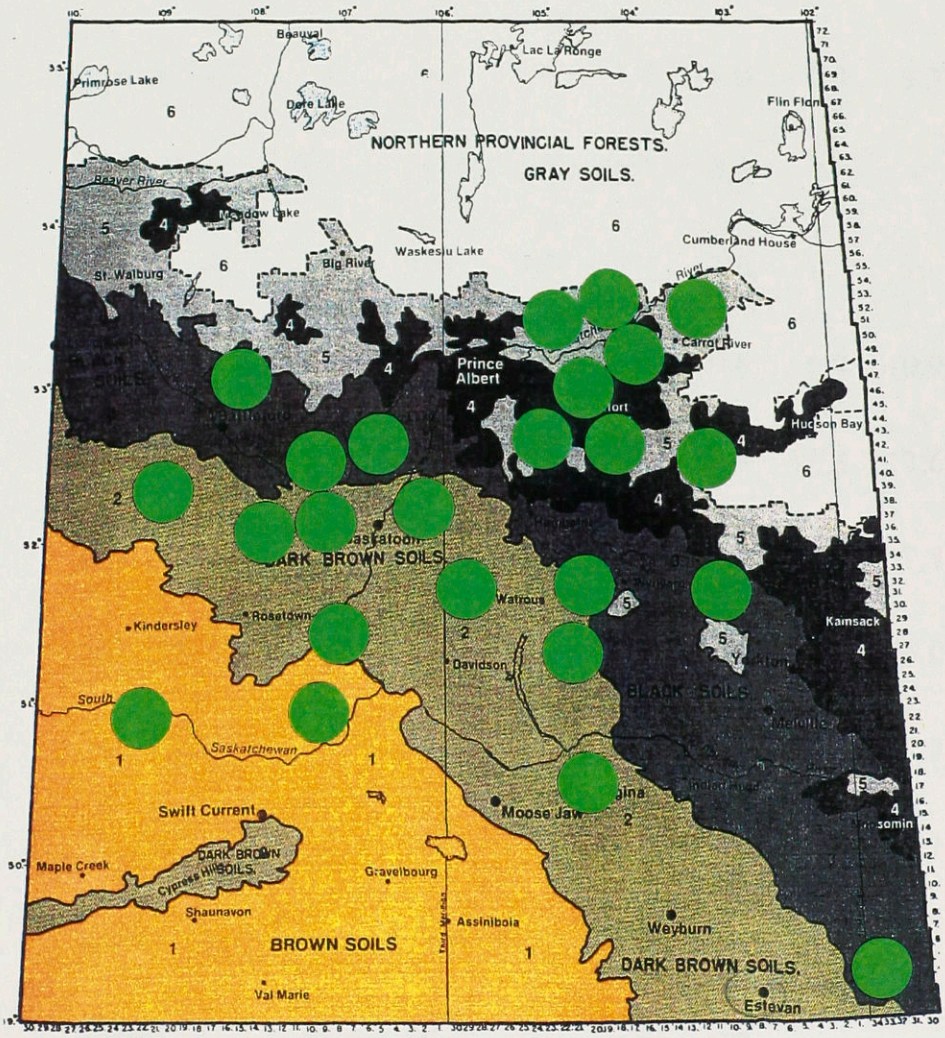


Figure 3.1.1 Map of Saskatchewan showing soil sampling sites ( ).

Table 3.1.1 Physico-chemical properties of some Saskatchewan soils.

Soil association (Soil sampling site)	Soil zone	pH	Clay (%)	Organic carbon (%)	Zn	Cu	Mn	Fe
					-------- $\mu g\ g^{-1}$ ----------			
Haverhill (Lucky Lake)	Brown	7.0	32.5	0.9	1.3	2.6	17.4	24.8
Sceptre (Sceptre)	Brown	7.5	44.6	1.7	1.5	1.5	-	-
Elstow (Scott)	Dark Brown	5.9	20.7	1.5	1.2	0.9	-	-
Elstow (Outlook)	Dark Brown	7.6	-	1.4	1.4	1.0	8.3	9.7
Weyburn (Biggar)	Dark Brown	7.6	8.9	1.2	1.1	0.3	-	-
Regina (Regina)	Dark Brown	8.0	72.0	1.4	0.9	2.0	-	-
Elstow (Elstow)	Dark Brown	7.1	16.3	1.1	1.0	0.6	6.9	10.9
Asquith (Bradwell)	Dark Brown	7.1	21.2	1.4	1.4	0.9	11.3	17.6
Weyburn (Watrous)	Dark Brown	7.7	22.6	1.5	3.2	1.2	8.7	17.8
Asquith (Asquith)	Dark Brown	7.7	18.4	1.3	1.8	1.3	9.8	6.0
Weyburn (Semans)	Dark Brown	7.3	16.2	2.3	2.7	1.2	10.9	12.3
Weyburn (Dunblane)	Dark Brown	7.0	22.3	1.1	1.3	1.0	10.6	16.3
Melfort (Melfort)	Black	7.1	29.3	1.9	1.0	0.8	-	-
Oxbow (Cut Knife)	Black	7.2	30.3	1.8	1.5	0.9	-	-

continued on p. 24 ...

Table 3.1.1 continued

Blaine Lake (Laird)	Black	6.5	31.9	2.9	4.4	0.7	19.0	37.4
Meota (Borden)	Black	7.3	18.0	1.6	1.8	0.9	-	-
Oxbow (Glenavon)	Black	7.4	-	2.4	2.9	0.9	11.6	11.8
Whitewood (Medstead)	Black Gray	7.6	18.6	4.6	-	-	-	-
Kelsey (Choiceland)	Black Gray	7.9	18.2	0.3	2.6	0.2	7.7	13.1
Kelsey (Garrick)	Black Gray	8.2	17.5	2.6	1.2	0.7	-	-
Carrot River (Carrot River)	Black Gray	7.5	10.9	1.0	0.4	0.3	-	-
Nipawin (Nipawin)	Black Gray	7.2	18.3	2.6	1.8	0.9	-	-
Waitville (Waitville)	Gray	6.9	26.4	2.3	1.2	0.8	-	-
Smeaton (Smeaton)	Gray	7.1	9.4	0.7	0.3	0.4	-	-

- not analyzed

3.1.1.2 Quantification of siderophores in soil extracts

The concentration of siderophores in soil extracts was quantified by the *A. flavescens* JG-9 (ATCC 20591) bioassay. This bacterium is incapable of producing its own siderophore and it requires hydroxamate type siderophores (HS) for normal growth. Inoculum was prepared by growing *A. flavescens* JG-9 in 6 ml (15 x 150 mm test tube) of siderophore assay broth (SAB) (Appendix 6.1.1) containing 10 mg ml^{-1} of desferrioxamine methanesulfonate (DFOM, a synthetic siderophore purchased from Ciba-Geigy Pharmaceutical Co., Mississauga, ON) on a G-24 environmental gyratory shaker (New Brunswick Scientific Co. Inc., Edison, NJ) at 135 rpm and 28°C. The bacterial cells were harvested at 48 h by centrifugation for 5 min at 800 x g. The cells were washed twice with sterile deionized water, and the cell suspension in sterile deionized water adjusted to 50 Klett units (optical density 0.1) on a Klett-Summerson colorimeter (Klett Manufacturing Co. Inc., NY. USA) fitted with a blue filter # 42.

To carry out the bioassay, 1 ml of concentrated (6 x) SAB was added to a series of test tubes (15 x 150 mm). Each tube also received a range (0 to 1.5 ml) of a standard DFOM solution (0.006 mg ml^{-1}) and the final volume was adjusted to 6 ml using deionized water. This provided assay tubes with final DFOM concentrations of 0, 0.25, 0.50, 0.75, 1.0, or 1.5 μg ml^{-1}. Siderophore assay broth tubes were also prepared containing 0.5 or 1.0 ml of soil extracts (3.1.1.1). The tubes amended with soil extracts showed some precipitates, therefore a modification to SAB was devised. In the modified SAB (MSAB-1) (Appendix 6.1.2), K_2HPO_4 and $(NH_4)_2HPO_4$ were replaced by KCl and $(NH_4)_2SO_4$, respectively. This modification did not effect the growth of *A. flavescens* JG-9. The MSAB-1 tubes, containing either the soil extract or DFOM, were inoculated with 0.1 ml of the inoculum (optical density 0.1) and shaken on a G-24 environmental gyratory shaker (135 rpm) at 28°C. After 72 h, the growth of *A. flavescens* JG-9 was determined in Klett

units in a Klett-Summerson colorimeter (blue filter # 42). A standard curve of the growth of *A. flavescens* JG-9 vs DFOM concentration was prepared. The growth of *A. flavescens* JG-9 in a given soil extract was compared with the standard curve and siderophore concentration in the soil extract was reported in nM DFOM equivalents. The results presented correspond to hydroxamate type siderophores (HS) levels, since *A. flavescens* JG-9 responds only to HS.

3.1.2 Results and Discussion

3.1.2.1 Hydroxamate type siderophore levels in Saskatchewan soils

The relationship between the growth of *A. flavescens* JG-9 and DFOM concentration is presented in Figure 3.1.2. A linear growth response ($r^2 = 0.99$) was obtained with DFOM concentrations between 0 to 1.2 ng ml^{-1}. The growth curve levelled off after DFOM concentration of 1.2 ng ml^{-1}, therefore, the portion of the growth curve between 0.2 to 1.2 ng ml^{-1} was used to calculate the concentration of siderophres in soil extracts. Twenty four soils representing a wide range of physico-chemical properties were tested for HS levels. The HS levels in these soils ranged from 2.1 to 27.3 nM DFOM equivalents (Table 3.1.2). Powell et al. (1980) assayed 57 soils with a wide range of organic carbon levels using the *A. flavescens* JG-9 bioassay and found HS levels in the range of 0.3 to 2.7 nM DFOM equivalents. Reid et al. (1984) also obtained similar results using JG-9 bioassay; however, they detected much higher HS levels (8 to 56 x) in the rhizosphere soils of pine forest stands.

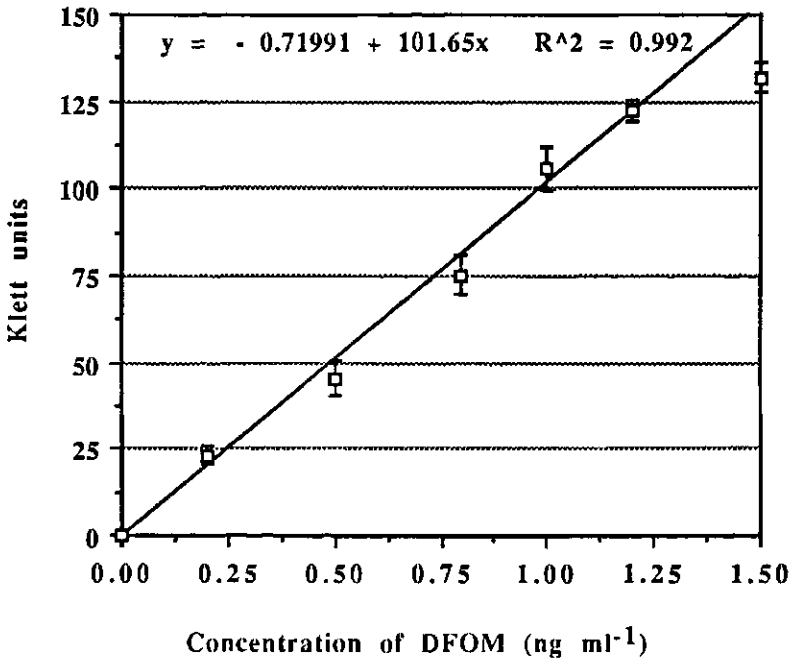


Figure 3.1.2 The relationship between the growth of *Arthrobacter flavescens* JG-9 and deferrioxamine methanesulfonate (DFOM) concentration.

In order to understand whether any soil property could be controlling the production of HS, correlation analysis between soil properties and HS levels was conducted. No simple relationship was evident between HS levels and soil pH, % organic carbon, % clay , or DTPA-extractable Cu, Fe, Mn, or Zn. Powell et al. (1980) also failed to find any correlation between HS levels and soil properties such as pH, % clay, or DTPA-extractable Fe, whereas a high correlation was observed for organic matter(r = 0.83). Furthermore, Powell et al. (1980) showed 82% to 99% adsorption of the added DFOM to a soil. Adsorption of siderophores on reactive materials such as clay minerals, organic matter, and hydroxides of aluminum and iron may mask correlations.

Table 3.1.2 Hydroxamate type siderophore (HS) levels in some Saskatchewan soils.

Soil association (Soil sampling site)	HS levels[a] (nM)	Soil association (Soil sampling site)	HS levels (nM)
Whitewood (Medstead)	27.3±2.9[b]	Weyburn (Biggar)	10.7±1.0
Kelsey (Choiceland)	24.4±2.7	Regina (Regina)	10.3±1.6
Smeaton (Smeaton)	22.1±1.9	Carrot River (Carrot River)	9.9±1.3
Elstow (Scott)	21.8±3.1	Elstow (Elstow)	8.4±1.2
Kelsey (Garrick)	21.1±2.4	Asquith (Bradwell)	8.3±1.0
Melfort (Melfort)	20.5±2.4	Weyburn (Watrous)	7.4±0.8
Oxbow (Cut Knife)	20.2±1.8	Asquith (Asquith)	6.5±0.7
Blaine Lake (Laird)	18.7±2.0	Sceptre (Sceptre)	6.4±0.9
Meota (Bordon)	14.6±2.8	Weyburn (Semans)	5.6±1.0
Elstow (Outlook)	12.1±1.8	Nipawin (Nipawin)	4.8±0.7
Haverhill (Lucky Lake)	11.5±1.7	Waitville (Waitville)	4.7±0.7
Oxbow (Glenavon)	11.3±1.5	Weyburn (Dunblane)	2.1±0.4

[a] deferrioxamine methanesulfonate (DFOM) equivalents.
[b] n = 3

The initial screening of Saskatchewan soils showed the widespread distribution of siderophores, and these organic molecules could be of importance in increasing the availability of Fe as well as other micronutrient cations. However, a constant supply of siderophores would be needed in the rhizosphere. One way to approach this problem would be to isolate siderophore-producing bacteria from the rhizosphere, and use these bacteria as seed inoculant.

3.2 Isolation and screening of siderophore-producing bacteria

3.2.1 Materials and Methods

3.2.1.1 Isolation of bacteria from the rhizosphere of wheat roots

Twenty-four Saskatchewan soils were surveyed for HS levels. Three soils, Medstead, Choiceland and Smeaton (Table 3.1.2), were found to contain the highest levels. Therefore these three soils were selected for isolating siderophore-producing bacteria. Two hundred grams of the air-dried soils were placed into styrofoam cups (300 ml capacity). Soils were brought to field capacity by adding deionized water and then maintained at field capacity (Medstead 27% w/w; Choiceland 24% w/w; Smeaton 20% w/w) for three days. Seeds of spring wheat (*Triticum aestivum* cv. Columbus), the most commonly used wheat variety in Saskatchewan, were surface sterilized by soaking in 70% ethanol for 1 min and then in 20% Javex solution (1.5% NaOCl) for 5 min. The sterile seeds were washed 8 times with sterile deionized water. Five sterile seeds were sown in each cup. After germination, seedlings were thinned (two plants per cup) and placed in a growth chamber at 24°C under a 14 h light/10 h dark cycle. The soil was maintained at 75% of moisture held at field capacity.

After six weeks of growth, wheat roots were recovered from the cups and the excess soil was shaken off. The roots and the associated rhizosphere soil (10 g) were shaken in 90 ml of sterile deionized water for 10 min. This suspension was serially diluted (ten fold), and 0.1 ml of the appropriate dilutions spread-plated onto trypticase soy agar (TSA, Appendix 6.1.3) (Martin, 1975) and King's B agar (KB, Appendix 6.1.4) (King et al., 1954). The plates were incubated at 28°C for 3 days for colony development. Individual bacterial colonies were selected on the basis of color, shape and size, and

streaked on respective media. Pure colonies were selected and maintained at 5°C on TSA and KB slants.

3.2.1.2 Screening of bacterial isolates for siderophore production

Two methods were employed for screening of bacterial isolates for siderophore production: (1) a bioassay using *A. flavescens* JG-9, and (2) a chemical assay using Chrome Azurol-S (Schwyn and Neilands, 1987).

(1) *Arthrobacter flavescens* JG-9 was grown for 48 h in 6 ml of SAB containing 10 mg ml^{-1} of DFOM (3.1.1.2). A loopful of this inoculum was streaked in the middle of SAB agar plates and an isolate streaked perpendicular to the JG-9 (Figure 3.2.1); care was taken that the two streaks did not touch each other. The plates were incubated at 28°C for 2 to 5 days. *Arthrobacter flavescens* JG-9 needs an exogenous source of siderophores to grow. Therefore the plates which showed the growth of *A. flavescens* JG-9 were selected and the corresponding isolates considered to be siderophore producers.

(2) Chrome Azurol-S assay uses a dye, Chrome Azurol-S (CAS), which reacts with iron giving a blue color to the medium. Siderophores form more stable complexes with iron than does CAS. Therefore, when siderophores are produced by an isolate on a medium containing CAS, the color of the medium changes from blue to orange. Chrome Azurol-S agar plates (pH 6.8) (Appendix 6.1.5) were prepared. Isolates were streaked on the CAS plates and incubated at 28°C for 3 days. The appearance of orange pigmentation around colonies indicated putative siderophore production and the corresponding bacteria were selected.

3.2.1.3 Quantification of siderophores produced by bacterial isolates

Twenty-nine putative siderophore-producing bacteria were selected in the initial screening and nine plant growth-promoting rhizobacteria (PGPR) [R 55, R 61, R 75, R 80, R 85, R 92, R 104, R 105 and R 111 (DeFreitas and Germida, 1990)] were assessed for siderophore production in broth cultures.

The three culture media evaluated were the rhizosphere medium (RSM) (Appendix 6.1.6) of Buyer et al. (1989), SAB (Estep et al., 1975), and a modified SAB (MSAB-2, containing 1/10 strength of casamino acid and yeast extract as compared to SAB). The 29 bacterial isolates and 9 PGPR strains were grown in 6 ml (16 x 150 mm test tube) of the media by inoculating each tube with a loopful of an isolate, and shaking (135 rpm) on a G-24 environmental gyratory shaker at 28°C for 72 h. The bacterial cultures were centrifuged at 4000 x g for 10 min, and the supernatant was filter sterilized using a 0.45 µm HAWP membrane filter. Filtrates were stored at 5°C until analyzed for siderophores. Chrome Azurol-S assay was used to quantify the production of siderophore in broth cultures. One ml of the CAS broth was added into each well of a 24-well microtiter plate (Flow Laboratories, Inc., Mississauga, ON). Each well of the microtiter plate also received either 1 ml of a standard DFOM solution (0, 4, 8, 12, 16, or 20 µg DFOM ml^{-1}) or an appropriate volume (0.1 to 0.5 ml) of sterile bacterial supernatant (described above). The microtiter plates were incubated at 26°C for 24 h and then the solution in each well was analyzed for absorbance at 630 nm using a Beckman DU-60 Spectrophotometer (Beckman Instruments, Inc., Irvine, CA). A standard curve was prepared of the absorbance of the CAS-DFOM complex and DFOM concentration. Siderophores in the bacterial supernatants were calculated from this standard curve and concentration reported as DFOM equivalents.

3.2.1.4 Identification of siderophore-producing bacteria

Bacterial identification was based on the analytical profile index (API, 1988). Isolated colonies from King's B medium were suspended in 5 ml sterile saline solution (0.85% NaCl, pH 7.0), and API 20E (Analytab product, Plainview, NY) strips were then inoculated according to the manufacturer's instructions. The API strips were incubated at 36°C and then viewed for various reactions after 24 to 48 h. Gram staining was conducted according to Benson's manual (Benson, 1981). Cellular morphology was observed in wet mounts under phase contrast microscopy. To assess pigment production, isolates were streaked on King's B medium and the plates incubated at 28°C for 48 h. Colonies were exposed to UV light and the production of fluorescent pigment determined.

3.2.1.5 Effect of iron on siderophore production by siderophore-producing bacteria

Iron is the key element which control the production of siderophore. The presence of iron at μM levels completely suppress siderophore production. Therefore, this test is rather a confirmation that the organic molecule secreted by the isolates was in fact a "siderophore". Test tubes of the MSAB-2 medium were prepared containing 0, 2.5, 5.0, 7.5, or 10.0 μM $FeCl_3 \cdot 6H_2O$. Tubes were inoculated with the bacterial isolate CH 16, R 111, or KC 21 (selected on the basis of their consistent siderophore production in the three media, Table 3.2.2) and incubated at 28°C on a G-24 environmental gyratory shaker (135 rpm). After 72 h, the bacterial cells were separated by centrifugation and siderophore levels in the supernatants were determined by the CAS method. The supernatants were also tested for production of yellow-green fluorescent pigments under UV light.

3.2.2 Results and Discussion

3.2.2.1 Isolation of bacteria from the rhizosphere of wheat roots

A total of 210 bacteria were isolated from the rhizosphere of wheat roots grown in Medstead, Choiceland and Smeaton soils (Table 3.2.1). Twenty-nine of the 210 isolates were found to be putative siderophore producers; this accounts for 13.8% of the total isolates. This indicated that only a limited number of the rhizobacteria produced siderophores. Therefore the potential benefits from siderophores without seed inoculation with siderophore-producing bacteria may be limited. Futhermore, any siderophore produced might be adsorbed onto the reactive materials in soils (Powell et al., 1980). The populations of siderophore-producing bacteria could be exploited, however, if they aggressively colonize plant roots. Such inoculants might produce siderophores at levels sufficient to benefit plants in term of iron nutrition and disease suppression.

Table 3.2.1 Number of bacteria isolated from the rhizosphere of wheat (cv. Columbus) plants grown in Medstead, Choiceland and Smeaton soils.

Soil name	Media used	Total bacteria	Siderophore producers
Medstead	TSA	42	3
	King's B	27	6
Choiceland	TSA	39	4
	King's B	35	7
Smeaton	TSA[a]	43	4
	King's B	24	5

[a] trypticase soy agar

The assay methods used for initial screening of isolates effectively separated out siderophore producers from non-producers. The growth enhancement of the strain JG-9 (Figure 3.2.1), or orange zone production on the CAS medium (Figure 3.2.2) was observed as a result of siderophore production by siderophore-producing bacteria. Schwyn and Neilands (1987) and Buyer et al. (1989) also used the CAS assay and clearly showed the usefulness of this chemical assay to identify siderophore-producing bacteria and fungi.

3.2.2.2 Quantification of siderophores produced by bacterial isolates

The relationship between the absorbance of the CAS-DFOM complex and DFOM concentration is presented in Figure 3.2.3. A linear relationship from 0 to 10 μg DFOM ml^{-1} was observed ($r = 0.99$). Schwyn and Neilands (1987) obtained similar standard curves for DFOM, enterobactin, aerobactin and rhodotorulic acid.

Siderophore levels produced by various bacterial isolates in the three different media are presented in Table 3.2.2. The three strains, CH 16, R 111, and KC 21, produced siderophores in the range of 19.4 to 30.4 μg ml^{-1} and production was not affected by the growth medium used. On the other hand, siderophore production by other strains was not consistent and varied depending on the type of medium used. Thus I propose that these three strains CH 16, R 111 and KC 21 would produce siderophores once they colonize plant roots, and these bacteria were selected for further studies.

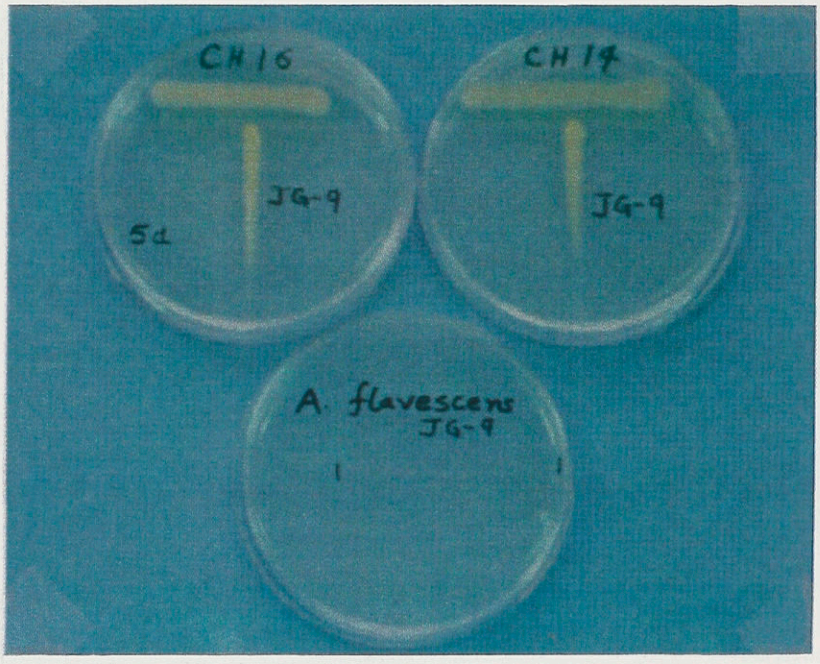


Figure 3.2.1 Growth enhancement of *Arthrobacter flavescens* JG-9 by siderophore-producing bacteria.

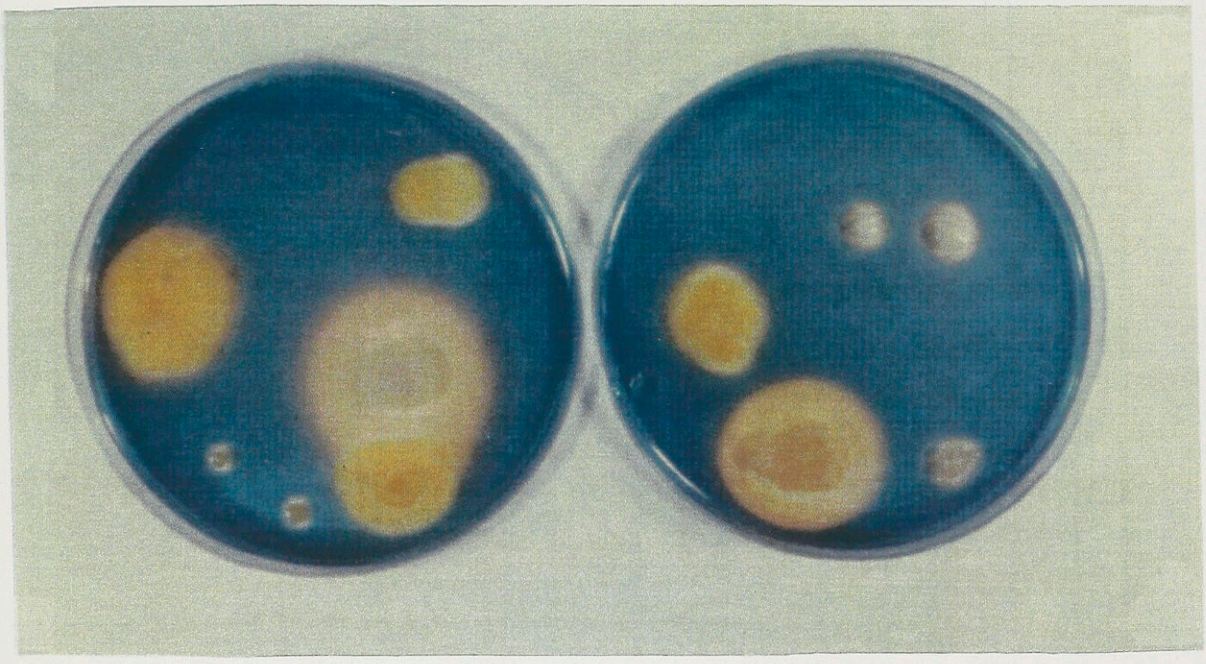


Figure 3.2.2 Change in color of Chrome Azurol-S from blue to orange around bacterial colonies as a result of siderophore production.

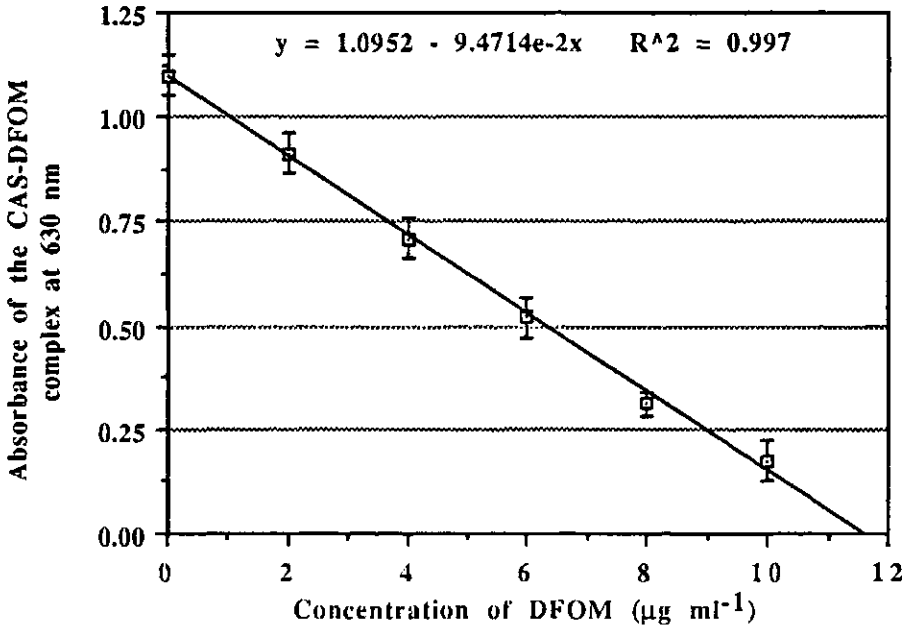


Figure 3.2.3 The relationship between the absorbance of CAS-DFOM complex and DFOM concentration.

Table 3.2.2 Production of siderophores by bacterial isolates in three growth media.

Isolate #	Growth Media RSM[a]	SAB[b]	MSAB-2[c]
	Siderophore concentration (μg ml^{-1})[d]		
R 111	24.0±1.9[e]	30.4±2.6	21.6±1.8
CH 16	21.0±1.6	24.3±1.9	22.5±1.6
KC21	23.0±2.0	21.7±1.9	19.4±1.5
R 75	20.5±2.1	16.1±1.6	ND[f]
KC 630	31.2±2.8	17.3±1.8	48.6±3.4
KS 630	45.4±4.1	ND	ND
KabS 65	ND	4.3±1.0	31.1±1.7
KC 66	27.8±2.1	15.3±1.4	37.0±2.5
KM 68	5.6±0.8	21.6±0.9	ND
KS 622A	3.0±0.4	5.3±0.7	32.8±1.9
TC 62	ND	8.8±1.6	ND
TS 610	ND	12.0±0.9	ND
KM 628	9.6±1.1	ND	ND
TM 622	4.8±0.8	3.9±1.1	ND
TC 630	ND	ND	14.0±1.2
KM 611	26.2±2.4	11.2±1.0	ND
R 105	12.8±0.9	ND	ND
R 104	4.2±0.7	ND	ND
R 92	28.5±1.5	ND	ND
R 80	17.2±1.8	ND	ND

[a] rhizosphere medium
[b] siderophore assay medium (SAB)
[c] modified SAB
[d] expressed as deferrioxamine methanesulfonate (DFOM) equivalents
[e] $n = 2$
[f] not detectable

3.2.2.3 Bacterial identification

The general characteristics and API profiles of the three bacterial strains, CH 16, R 111 and KC 21, are shown in Table 3.2.3. The three strains were Gram negative and produced fluorescent pigments on King's B medium. Two isolates, CH 16 and KC 21, were positive for gelatin liquefaction. All of the isolates were positive for arginine dehydrolase, citrate utilization, and acetoin production. None of the isolates produced H_2S from thiosulfate. Based on these tests and the API profile, the three isolates were tentatively identified as *P. fluorescens*. A large number of siderophore-producing bacteria belong to the genus *Pseudomonas*. Kloepper et al. (1980a and 1980b) and Buyer and Leong (1986) also identified siderophore-producing bacteria as *P. fluorescens*. Jurkevitch et al. (1989) isolated a bacterium from the rhizosphere of peanut (*Arachis hypogaea* L.) which increased Fe^{3+} uptake in the peanut plant and found it to be a strain of *P. putida*. Predominance of the genus *Pseudomonas* in the rhizosphere of spring and winter wheat was also found by Elliott Juhnke et al.(1987) and DeFreitas and Germida (1990). The natural occurrence of *Pseudomonas* in the rhizosphere and their siderophore-producing ability make them well-suited for use as seed inocula for crop production.

Table 3.2.3 General characteristics and API profiles of bacterial isolates CH 16, R 111 and KC 21.

Test	Isolate		
	CH 16	R 111	KC 21
General characteristics			
Gram stain	-	-	-
Morphology	Rod	Rod	Rod
Pigment: on King's B medium	Greenish	Greenish	Yellowgreen
API Profile			
ONGP: hydrolysis of O-nitrophenyl β-galactosidase	-	-	-
ADH: arginine dehydrolase	+	+	±
LDC: lysine decarboxylase	-	-	-
ODC: ornithine decarboxylase	-	-	-
CIT: citrate utilization (Simmons)	+	+	+
H2S: hydrogen sulfide production from thiosulfate	-	-	-
URE: urease activity	-	-	-
TDA: tryptophane deaminase	-	-	-
IND: indole formation from tryptophane (tryptophanase)	-	-	-
VP: acetoin production from Na-pyruvate (Voges-Proskauer)	+	+	±
GEL: liquefication of gelatin	+	-	+
GLU: glucose utilization	-	-	-
MAN: mannitol utilization	-	-	-
INO: inositol utilization	-	-	-
SOR: sorbitol utilization	-	-	-
RHA: rhamnose utilization	-	-	-
SAC: sucrose utilization	-	-	-
MEL: melibiose utilization	-	-	-
AMY: amygdalin utilization	-	-	-
ARA: (L^+) arabinose utilization	-	-	-
API profile #	2203000	2201000	2203000
Identification	*P. fluorescens*	*P. fluorescens*	*P. fluorescens*

3.2.2.4 Effect of iron on siderophore production by *Pseudomonas fluorescens* strains CH 16, R 111 and KC 21.

Addition of Fe^{3+} to the growth medium to 2.5 μM concentration completely suppressed the production of siderophores and fluorescent pigments (Table 3.2.4). Neilands (1973b) reported that Fe^{3+} is the key ion controlling the production of siderophores by microbes, as μM levels can directly suppress siderophore production. Emery (1977) reported that 1 μM Fe^{3+} allowed rapid cellular growth of *U. sphaerogena*, but siderophore production was severely inhibited. In a growth chamber study, Kloepper et al. (1980a) showed that addition of Fe^{3+} to a soil to 50 μM suppressed the production of pseudobactin (a siderophore produced by *P. fluorescens*). Further, *in vitro* studies demonstrated that the addition of Fe^{3+} also reversed the antibiosis against *Erwinia carotovora* by pseudobactin. Iron also suppressed siderophore production by *B. edulis*, and the amount of HS released into the culture media was inversely proportional to the amount of iron added (Szaniszlo et al., 1981).

Table 3.2.4 Effect of iron on siderophore production by *Pseudomonas fluorescens* strains CH 16, R 111 and KC 21.

Isolate	Iron level (μM)	Siderophore level[a] (μg DFOM equivalent)	Fluorescent pigment[b]
CH 16	0.0	23.1±2.4[c]	Yes
	2.5	ND[d]	No
	5.0	ND	No
R 111	0.0	21.0±1.9	Yes
	2.5	ND	No
	5.0	ND	No
KC 21	0.0	19.6±2.1	Yes
	2.5	ND	No
	5.0	ND	No

[a] detected by the CAS method
[b] on exposure of colonies on King's B medium to UV light
[c] n = 2
[d] not detectable

3.3 Qualitative characterization of siderophores produced by *Pseudomonas fluorescens*

3.3.1 Materials and Methods

3.3.1.1 Purification of siderophores from crude bacterial supernatant

The supernatants of *P. fluorescens* strains CH 16, R 111, and KC 21 were obtained by growing the bacteria in MSAB-2 medium as described in section 3.2.1.3. Ferric chloride ($FeCl_3 \cdot 6H_2O$) at the rate of 2 g L^{-1} was added to culture supernatant, and the solution was saturated with $(NH_4)_2SO_4$. The preparations were left overnight at 4°C, the precipitates removed by centrifugation, and the supernatants extracted with benzyl alcohol according to the methods of Neilands (1952) and Frederick et al. (1981). Instead of repeated extraction, the first benzyl alcohol addition was left with the supernatant for one week and (during this period) siderophores were extracted from the supernatant into benzyl alcohol. The benzyl alcohol layer was separated and mixed with 5 volumes of diethyl ether and 2 volumes of deionized water. The aqueous layer containing the siderophores was separated and stored at 4°C for further analysis.

The water soluble siderophores were further purified by gel filtration. The aqueous extract was chromatographed on a column (2.5 x 30 cm) of polyacrylamide Bio-Gel P2 (Bio-Rad Laboratories Ltd., Mississauga, ON). The flow rate was maintained at 1.0 ml min^{-1} and effluent was collected in 2 ml fractions. Each fraction was tested for siderophore activity using the CAS method (3.2.1.3)

3.3.1.2 Visible spectroscopy

The purified siderophores (3.3.1.1) of *P. fluorescens* strains CH 16, R 111, and KC 21 were placed into cuvettes and scanned over the visible wave lengths of 380 to 700

nm on a Shimadzu UV-265 Spectrophotometer. The reference cell contained an extract (as described in section 3.3.1.1) of uninoculated MSAB-2 medium .

3.3.1.3 Infrared spectroscopy

The aqueous extracts of the purified siderophores were placed into glass vials and frozen at -40°C. The frozen samples were freeze-dried on a Labconco # 4.5 freeze-drier (Labconco corporation, Kansas City, Missouri) for 48 h. One mg of the freeze-dried siderophore sample was mixed with 200 mg of KBr (BDH Spectrosol). The mixture was transferred into a press and subjected to a pressure of 103 x 10^3 KPa for 2 min. The pellet thus obtained was scanned between wave numbers of 180 to 4000 cm^{-1} on a Perkin Elmer Infrared Spectrophotometer # 983 (Perkin Elmer Ltd., Richmond, B. C., Canada). A potassium bromide pellet was used as a standard.

3.3.1.4 Heat stability of siderophores

The purified bacterial siderophores (3.3.1.1) were filter sterilized, divided into two portions, and one portion was subjected to heating (autoclaved at 121°C for 15 min). Both the portions were then tested for siderophore activity using the CAS method (3.2.1.3).

3.3.2 Results and Discussion

3.3.2.1 Elution pattern of bacterial siderophores

The elution pattern of siderophores through Bio-Gel P2 is shown in Figure 3.3.1. It is apparent that siderophores were eluted immediately after the void volume (16-18 ml). Siderophores appeared to be very close in molecular weight since they eluted at similar peaks. Neilands and co-workers (Garibaldi and Neilands, 1955; Atkin and Neilands, 1968) also used benzyl alcohol as an extractant to extract siderophores from the cell free

supernatant of the rust fungus, *U. sphaerogena*. This method was also used to isolate siderophores from the cell free supernatant of the bacterium, *Azotobacter chrococcum* (Fekete et al., 1989). An alternate procedure involving extraction with a mixture of chloroform: phenol (1:1) may also be used (Meyer and Abdallah, 1978; Kloepper et al.,1980a; Jurkevitch et al., 1989). In the present study, the chemical extractant and Bio-Gel P2 filtration adequately purified siderophores from the cell free culture supernatant.

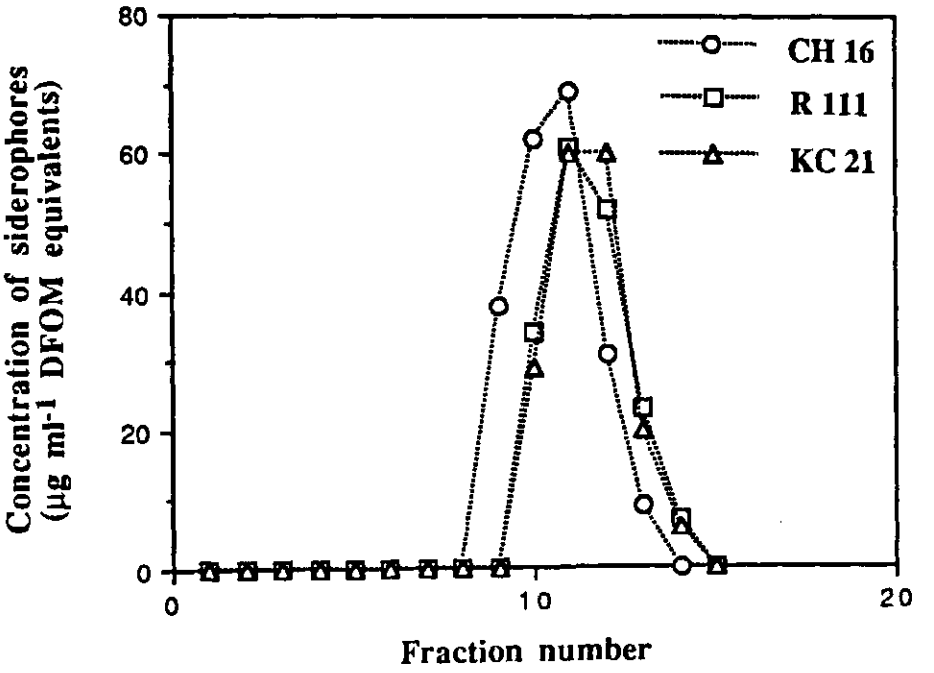


Figure 3.3.1 Elution pattern of siderophores of *Pseudomonas fluorescens* strains CH 16, R 111 and KC 21 through Bio-Gel P2.

3.3.2.2 Visible spectra

Figures 3.3.2 a, b and c show the visible absorbance spectra of siderophores of *P. florescens* strains CH 16, R 111 and KC 21, respectively. The spectra are more or less the same, having absorbance between 375 to 425 nm with a peak close to 400 nm. Neilands (1952) and Philson and Llinas (1982) also reported absorption maximum of ferrichrome from *U. sphaerogena* and siderophores from *P. fluorescens* at 424 and 410 nm, respectively. This test did not provide any information to differentiate these siderophores. However, the closeness of the absorption maxima with the values reported in the literature for siderophores released by *Pseudomonas* species under iron-stress conditions suggest that these siderophores were similar in chemical composition.

3.3.2.3 Infrared spectra

The infrared (IR) spectra of siderophores of the strain CH 16 (Figure 3.3.3a) indicated the presence of methyl groups as evident by the absorption peaks at 2980 and 2880 cm^{-1}. The absence of absorption peaks between 3028 to 3082 cm^{-1} confirmed that the compound was not aromatic in nature. The absorption band at 1660 cm^{-1} indicated the presence of carboxylic and amide groups, suggesting the presence of some kind of amino acid in the structure. Neilands (1952) and Garibaldi and Neilands (1955) also showed the presence of amino acids in ferrichrome and ferrichrome A by the appearance of a peak at 1650 cm^{-1}. The IR spectra did not allow the positive identification of the hydroxamate structural feature of siderophores; however, a sharp peak at 520 cm^{-1} indicated the presence of keto (C = O) groups and this peak could have overlapped with the hydroxamate group. The IR spectra of R 111 (Figure 3.3.3b) and KC 21 (Figure 3.3.3c) siderophores were similar to that of CH 16.

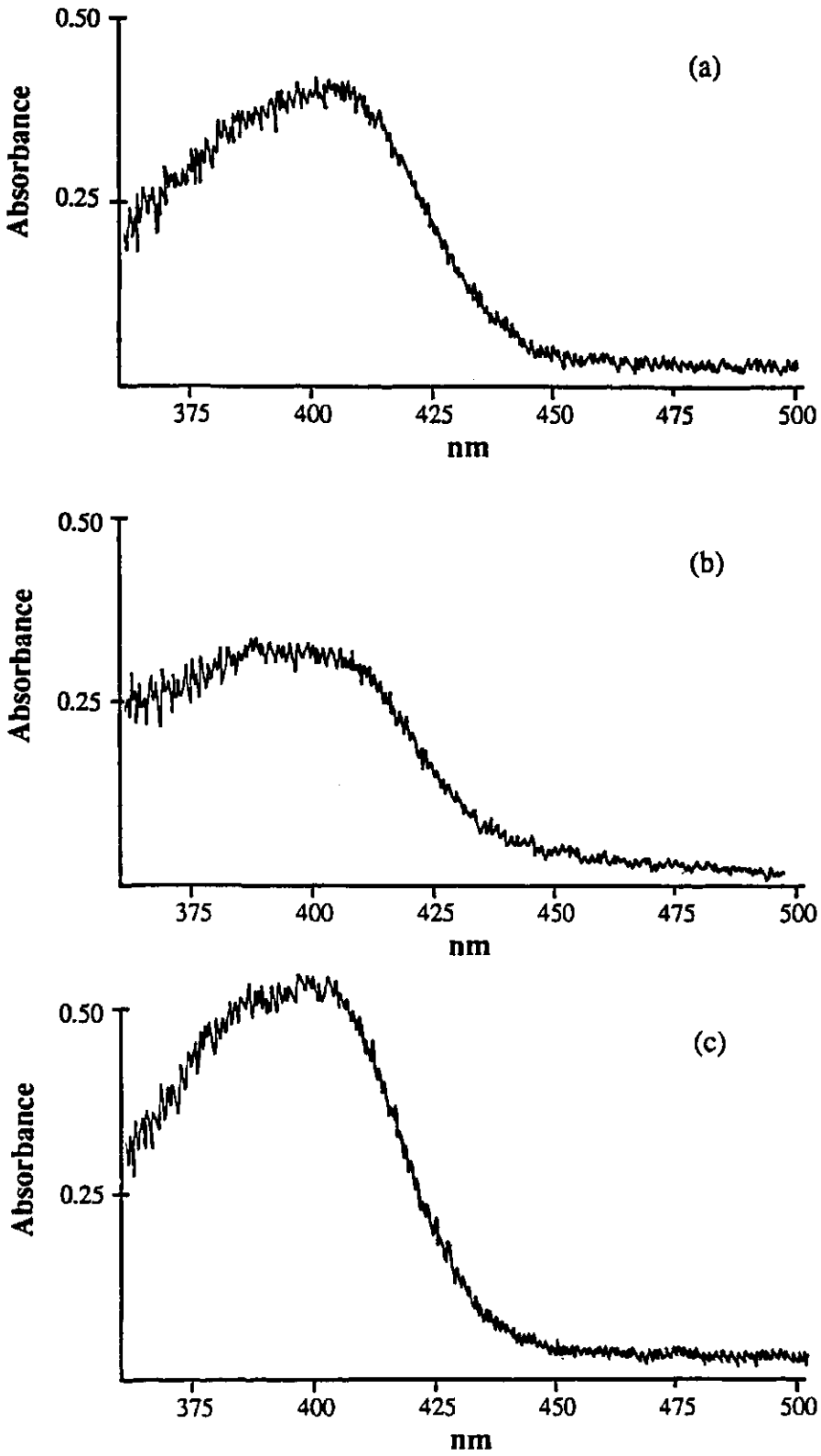


Figure 3.3.2 Visible spectrum of siderophores produced by *Pseudomonas fluorescens* strain CH 16 (a), R 111 (b), and KC 21 (c).

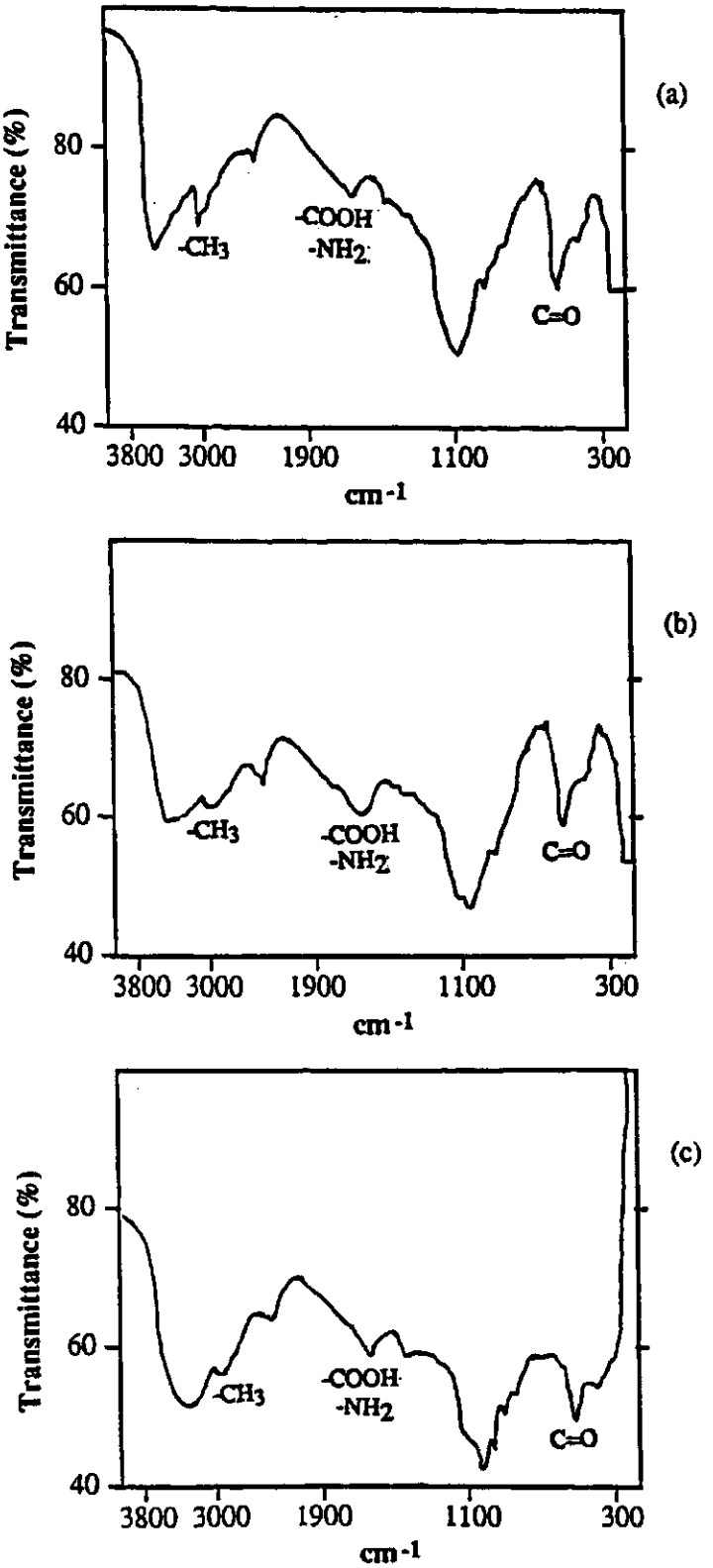


Figure 3.3.3 Infrared spectrum of siderophores produced by *Pseudomonas fluorescens* strain CH 16 (a), R 111 (b), and KC 21 (c).

3.3.2.4 Heat stability of siderophores

The effect of heating (autoclaving) on the activity of siderophores produced by *P. fluorescens* strains CH 16, R 111 and KC 21 is presented in Table 3.3.1. The activity of these siderophores decreased by 11% to 18% as a result of heating. There was no major difference among the three strains for the decrease in activity of siderophores. The reason for the decreased in activity is not known. However, a solution of DFOM was also autoclaved and exhibited no loss in activity (data not reported). The observations might be artifacts of the experimental protocol or, alternatively, the result of contaminants in the "purified siderophores".

Table 3.3.1 Effect of heating on the activity of siderophores of *P. fluorescens* strains CH 16, R 111 and KC 21.

	Siderophore activity ($\mu g\ ml^{-1}$)[a]		
Isolate #	Before heating	After heating	% Decrease
CH 16	25.1	22.3	11.3
R 111	23.4	20.0	14.3
KC 21	22.0	18.1	17.7

[a] DFOM equivalents

3.4 Mechanisms involved in increasing the availability of Cu, Fe, Mn and Zn by siderophores

I propose three ways by which siderophores could increase availability of Cu, Fe, Mn, and Zn in soils. First, due to the reducing conditions in the rhizosphere, Fe^{3+} iron should be reduced to Fe^{2+}, and then siderophores, which are weak chelators of Fe^{2+}, would be available to complex and enhance the availability of Cu and Zn [stability constant of siderophores and the metal ions is in order of $Fe^{2+} > Zn^{2+} > Cu^{2+}$ (Hider, 1984)]. Second, among micronutrient cations, Fe^{3+} is preferred by humic polymers for complexing reactions (Stevenson and Fitch, 1986). Thus the production of siderophores in the rhizosphere would result in complexing of Fe^{3+}, thereby leaving humic polymers available to complex and enhance the availability of Cu, Mn, and/or Zn. Third, it has been reported that iron-hydroxides are important in controlling the availability of Cu, Fe, Mn, and Zn in soil solutions (Lindsay, 1979; Mattigod et al., 1981). Complexing of Fe^{3+} by siderophores will result in the dissolution of iron-hydroxides, thereby releasing co-precipitated and specifically adsorbed Cu, Mn, and Zn in soil solutions.

3.4.1 Materials and Methods

3.4.1.1 Complexing of Cu, Fe, Mn and Zn by siderophores of *P. fluorescens*

The cation exchange resin method is widely used to study complexing reactions between ligands and cations (Randhawa and Broadbent, 1965; Schnitzer and Skinner, 1966; Stevenson and Ardakani, 1972). This method is based on the competition for metal ions between ligands, siderophores in this case, and cation exchange sites on the resin.

To carry out the reaction, a graded amount (0 to 750 µg) of the purified siderophores was added to a series of 50 ml volumetric flasks. Each flask also contained

5 ml of 1M KCl solution, and the required volume of the aqueous metal solution to provide 300 μg each of Cu, Fe, Mn and Zn (the latter solutions were prepared from the following salts: $ZnCl_2$, $CuCl_2 \cdot 4H_2O$, $MnCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$). The pH was then adjusted to 5.0 by the addition of a few drops of 0.1 N KOH or 0.1 N HCl. Finally the volume of each of the flask was made to 50 ml with deionized water. One g of the Dowex-50 resin (K^+ form, 20 to 50 mesh) was weighed into 125 ml Erlenmeyer flasks. The solutions, containing the metal salt, KCl, and siderophores, were transferred to these flasks and shaken at room temperature for 1 hour. The exchange resin was then removed by filtration. The filtrate containing Zn-, Cu-, Mn-, and/or Fe-siderophores complexes were analyzed by an atomic absorption spectrophotometer (Pye Unicam SP 2900, Pye Unicam Ltd., Cambridge, England).

3.4.1.2 Solubilization of iron-hydroxides by siderophores, thereby releasing co-precipitated and specifically adsorbed Cu, Mn, and Zn

Iron-hydroxide precipitates were prepared by titrating a 500 ml solution of 0.16 M $Fe(NO_3)_2 \cdot 9H_2O$ (containing 10 mg each of Cu, Mn, and Zn) with 13% NH_4OH (w/v) to pH 6.5. After aging for 24 h, the suspension was centrifuged (6000 x g for 15 min) and brownish-black precipitates were washed twice with deionized water. The precipitates were air-dried and stored at room temperature.

Five hundred mg of iron-hydroxide precipitates were added to a series of 120 ml beakers and shaken with 20 ml of distilled water. Each beaker then received 0.0, 2.5, 5.0, or 10.0 ml of the purified siderophore solution (3.3.1.1), the pH adjusted to 5.0, and the total volume was made to 50 ml. The mixture was shaken at room temperature for 24 h and then filtered through a 0.45 μm HAWP membrane filter. The filtrate was analyzed for

Cu, Fe, Mn and Zn using atomic absorption spectrophotometry (Pye Unicam SP 2900, Pye Unicam Ltd., Cambridge, England).

3.4.2 Results and Discussion

3.4.2.1 Complexing of Cu, Fe, Mn and Zn by siderophores of *P. fluorescens*

The ability of siderophores produced by *P. fluorescens* strains CH 16, R 111 and KC 21 to keep Cu, Fe, Mn, and Zn in the solution phase is presented in Figures 3.4.1, 3.4.2, and 3.4.3, respectively. In the absence of siderophores, 97 to 98% of the metal ions added was adsorbed onto the cation exchange resin indicating that the resin had a high affinity for the metal ions. On the addition of 250 µg of siderophore (DFOM equivalent) of the strain CH 16 into the system, Fe and Cu started to appear in the solution phase (Figure 3.4.1), indicating the complexing of Fe and Cu by siderophore. The siderophores seems to form more stable complexes with Fe and thereby maintained 60% of the added iron in the solution phase as compared to 8.5% of the added Cu. Iron and Cu continued to increase in the solution phase with the addition of more siderophore (Figure 3.4.1), and finally 98% of the added Fe and 32% of the added Cu was retained in the solution as siderophore complexes. The siderophore of the strain R 111 (Figure 3.4.2) was also effective in complexing and keeping Fe in solution. However, Cu, which appeared in the solution following the addition of 250 µg of siderophore (DFOM equivalent) of the strain CH 16, was not detected until 500 µg of siderophore of strain R 111 was added to the system (Figure 3.4.2). This shows that siderophore of strain R 111 did not complex Cu until no free Fe ion was present in the system. The pattern of metal complexing by

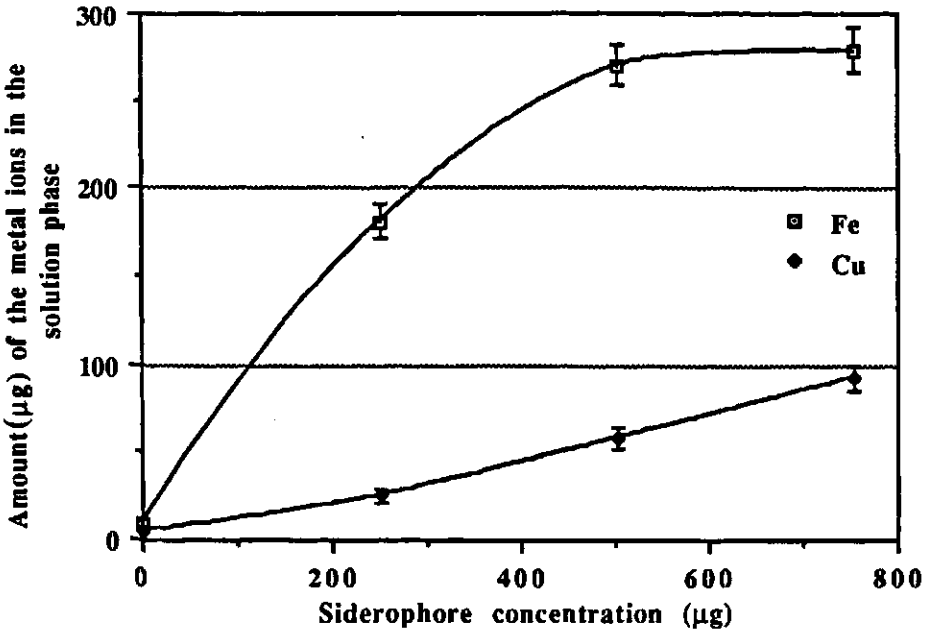


Figure 3.4.1 Ability of *Pseudomonas fluorescens* CH 16 siderophores to keep metal ions in the solution phase.

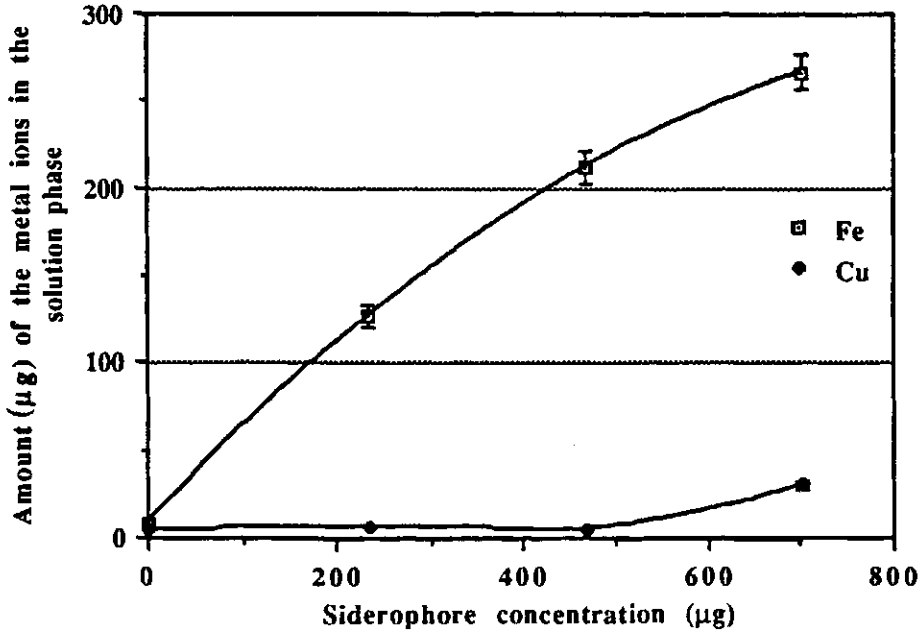


Figure 3.4.2 Ability of *Pseudomonas fluorescens* R 111 siderophores to keep metal ions in the solution phase.

siderophore of strain KC 21 (Figure 3.4.3) was the same as by R 111; however, no complexed Cu was detected in the solution phase. No siderophore from any strain was effective in keeping Mn in solution phase. Comparing siderophores of strains CH 16 and R 111 in terms of their ability to complex and keep Cu in solution (Figures 3.4.1 and 3.4.2), it can seen that complexing of Cu by strain CH 16 siderophore started with the addition of small amounts (250 µg DFOM equivalents) of siderophore and increased with the addition of more siderophore. The complexing seems to have taken place irrespective of Fe complexing. On the other hand, siderophores of strain R 111 were able to complex and keep Cu in the solution phase only when all of the Fe was complexed.

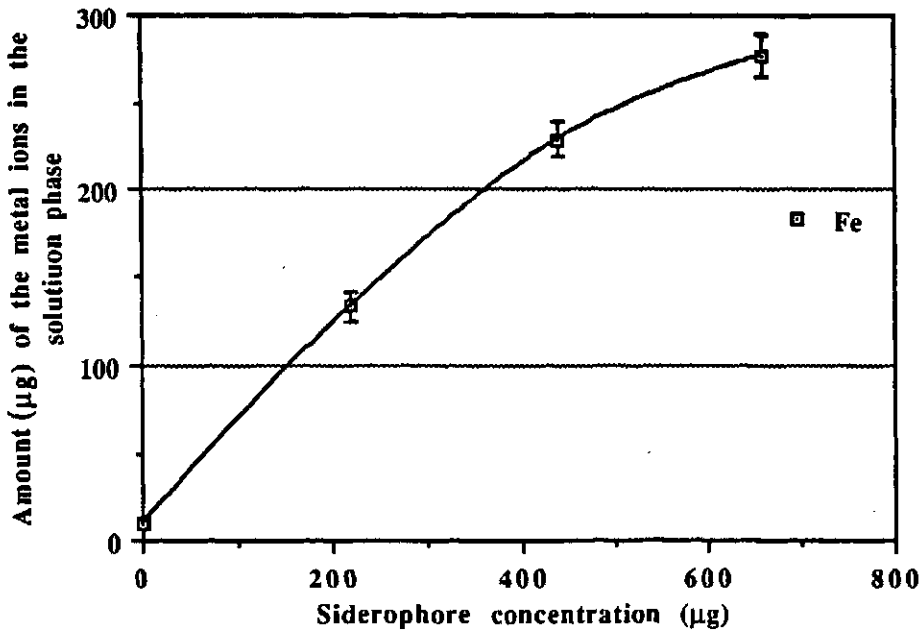


Figure 3.4.3 Ability of *Pseudomonas fluorescens* KC 21 siderophores to keep metal ions in the solution phase.

3.4.2.2 Solubilization of Iron-hydroxides

Solubilization of iron-hydroxides by siderophores of the three rhizobacteria is presented in Table 3.4.1. No metals were detected in the solution phase without the addition of siderophores. Iron appeared in the solution on the addition of 55 to 62.5 μg of siderophores (DFOM equivalent), but the other metal ions were not detected. The continued addition of siderophores solubilized more Fe into the solution, and along with Fe the other metal ions, Cu, Mn and Zn, also appeared in the solution. The percentage of metal ions released from the iron-hydroxide ranged from 3.7 to 10.9% (0.19 to 0.56 μg ml^{-1}). These amounts could be of importance in the rhizosphere for micronutrient nutrition of crops. The siderophore of strain CH 16 appeared to solubilize more Fe and Cu. It appeared that strain CH 16 siderophore not only released Cu by solubilization of iron-hydroxide, but also by direct complexing reactions. Manganese was the metal ion least released in these reactions, indicating that these siderophore do not effectively increase the availability of Mn.

Powell et al. (1982) and Treeby et al. (1989) also found that DFOM to be effective in complexing Fe and to increase its availability. Using computer modelling, Cline et al. (1982, 1983) studied the role of siderophores in complexing Fe in soil solutions and nutrient solutions. They found that siderophores were very effective in complexing 100% of Fe and the complex was stable from pH 4 to 10.

I made an attempt to investigate the mechanisms by which *P. fluorescens* siderophores could increase availability of Fe, and other micronutrient cations, Cu, Mn, and Zn. Two hypothesis, (1) direct complexing of Fe and (2) indirect involvement in releasing Cu, Mn, and Zn by solubilizing iron-hydroxides (a solid phase controlling the availability of Cu, Mn and Zn) were proposed and then tested experimentally. I propose here a scenario by which siderophores could increase the availability of micronutrient

cations to plants. Siderophores are produced by microbes in response to iron stress. Soil solutions never have enough iron to support normal growth of microbes, therefore some amount of siderophores may be expected in the rhizosphere. However, due to the strong adsorption of these organic molecules onto soil particles and the presence of a limited number (13.8% in the present study) of siderophore-producing bacteria in the rhizosphere, the potential significance of siderophores is limited. The inoculation of crop seeds with efficient siderophore-producing *Pseudomonas* to colonize plant roots and constantly produce siderophores in the rhizosphere might overcome this limitation. Siderophores released in the rhizosphere would then directly complex Fe and Cu. This complexing would not only increases the diffusion of Fe and Cu towards roots, but also save these metal ions from unfavorable reactions (precipitation and specific adsorption), and increase their availability. Another way of increasing the availability of Cu, Mn, and Zn is by the solubilization of iron-hydroxides by siderophores. Complexing of Fe by siderophores will result in a shift of the equilibrium towards the soluble side, thereby releasing specifically adsorbed and co-precipitated Cu, Mn, and Zn. From the present study, it can be concluded that siderophores could increase the availability not only of Fe, but also of Cu and Zn. Verification of the mechanisms may prove inoculation of crop seeds with siderophore-producing bacteria as an alternate method to ameliorate micronutrient deficiency.

Table 3.4.1 Solubilization of iron-hydroxides and release of Cu, Fe, Mn, and Zn by siderophores of *Pseudomonas fluorescens* strains CH 16, R 111 and KC 21.

Strain	Siderophores (µg)[a]	Fe	Cu	Zn	Mn
		µg ml^{-1}			
Control	0	ND[b]	ND	ND	ND
CH 16	62.5	0.98	ND	ND	ND
	125.5	1.74	0.31 (6.0)[c]	0.19 (3.8)	ND
	251.0	3.58	0.56 (10.9)	0.34 (6.9)	0.28 (5.7)
R 111	58.5	0.74	ND	ND	ND
	117.0	1.30	0.19 (3.7)	0.22 (4.5)	ND
	234.0	2.42	0.35 (6.8)	0.38 (7.9)	0.24 (4.8)
KC 21	55.0	0.81	ND	ND	ND
	110.0	1.42	0.21 (4.1)	0.23 (4.6)	ND
	220.0	2.61	0.39 (7.6)	0.43 (8.7)	0.24 (4.8)

[a] DFOM equivalents
[b] not detectable
[c] % of total in precipitates

4. SUMMARY AND CONCLUSION

The objectives of this study were *(i)* to determine the occurrence of siderophores in some Saskatchewan soils, *(ii)* to isolate and screen bacteria capable of producing siderophores, *(iii)* to isolate and qualitatively characterize siderophores, and *(iv)* to study the mechanisms by which siderophores could increase availability of Cu, Fe, Mn, and/or Zn. Twenty four soil samples were collected from the five soil zones of Saskatchewan. Siderophores from the soil samples were extracted with deionized water (soil water ratio 1:1), and were quantified by a bioassay using *Arthrobacter flavescens* JG-9, a mutant incapable of producing its own hydroxamate type siderophores (HS). The concentration of HS in soils ranged from 2.1 to 27.3 nM deferrioxamine-methane sulfonate (DFOM) equivalent. These HS levels are within the range of HS values reported in the literature. There was no simple correlation between HS levels and soil pH, % organic carbon, % clay, or DTPA extractable micronutrient cations (Cu, Fe, Mn, or Zn). The widespread occurrence of microbial siderophores in Saskatchewan soils led to an investigation into siderophore-producing bacteria and a study on their potential to enhance availability of micronutrient cations in soils

In a growth chamber study, wheat was grown in the three soils (Medstead, Smeaton, and Choiceland) which showed highest HS levels. After 6 weeks of growth, a total of 210 bacteria were isolated from the rhizosphere of wheat roots. The bacteria were screened for putative siderophore producers using two methods, the JG-9 bioassay and Chrome Azurol-S (CAS). Both methods were efficient in separating out siderophore-producers from non-producers. Twenty-nine siderophore-producing bacteria and 9 PGPR (isolated from rhizosphere of winter wheat [DeFreitas and Germida, 1990]) were further screened to get efficient siderophore-producers strains. They were grown in rhizosphere medium (RSM), siderophore assay broth (SAB), and modified SAB (MSAB-2). The

siderophores produced in the culture media were quantified using the CAS method. The three bacterial strains, CH 16, R 111, and KC 21, were found to be efficient siderophore producers, and identified as *Pseudomonas fluorescens* using the API 20E strips.

Siderophores produced by the three *Pseudomonas* were qualitatively characterized. The activity of siderophores decreased by 11.3 to 17.7% as a result of autoclaving at 121°C for 15 min, thereby indicating that some component(s) of siderophores are heat labile. The visible spectrum of the siderophores showed their absorbance between 375 to 425 nm with a peak close to 400 nm. The siderophores were also scanned in the infrared (IR) region. An absorption band at 1660 cm^{-1} indicated the presence of carboxylic and amide groups, confirming the presence of amino acid(s) in the structure. The IR spectrum did not allow the positive identification of the hydroxamate structural group of siderophores. However, a sharp peak at 520 cm^{-1} indicated the presence of a keto group which could have masked the hydroxamate group.

Mechanisms by which siderophores could enhance availability of micronutrient cations were studied. Siderophores produced by the strain CH 16, R 111 and KC 21 in culture media were purified by chemical extraction using benzyl alcohol and then chromatographed on a column of polyacrylamide Bio-Gel P2. The siderophores produced by the three bacteria eluded as one peak after the void volume, indicating that they had a similar molecular weight.

The first mechanism studied was the direct participation of the siderophores in complexing micronutrient cations. The cation exchange resin (Dowex-50; K^+ form) method was used to study this mechanism. The micronutrient cations, Cu, Fe, Mn, and Zn were allowed to interact with the resin and in the presence of siderophores at pH 5.0. Siderophores produced by the three pseudomonads complexed Fe and thus limited adsorption of Fe onto the resin. Addition of 700 µg DFOM equivalent of the purified

siderophore of either strain complexed 95 to 98 % of the added Fe and kept it in solution phase. However, Cu was only complexed by siderophores of the strains CH 16 and R 111. None of the strains complexed and maintained Mn and Zn in the solution phase.

The second mechanism proposed and studied was the solubilization of iron-hydroxides by the siderophores, thereby releasing co-precipitated and specifically adsorbed Cu, Mn, and/or Zn. Iron-hydroxide precipitates were prepared containing 10 mg each of the Cu, Mn, and Zn. Five hundred milligrams of the precipitate were shaken with the purified siderophores and the amount of the cations released into the solution measured. As a result of solubilization of iron-hydroxide, 3.7 to 10.9% of the Cu, 4.8 to 5.7% of the Mn, and 3.8 to 8.7% of the Zn, and was released into the solution. The release of Cu, Mn and Zn from iron-hydroxide by siderophores indicate that siderophores could be of importance in increasing availability of micronutrient cations in the rhizosphere.

My research showed that many Saskatchewan soils contain siderophore levels high enough to increase availability of Cu, Fe, Mn, and Zn. However, due to the presence of limited number of siderophore producing bacteria (13.8% in the present study) in the rhizosphere, the chances of plant root colonization by siderophore-producers are uncertain. Therefore, the use of siderophore-producing bacteria as seed inoculants give better chances to colonize roots, and facilitate micronutrient nutrition of crops. For example, as seeds germinate, the roots start to form and root exudates would stimulate growth of siderophore-producing bacteria. Siderophores produced by bacteria would complex Fe^{3+} in the rhizosphere and thus not only save Fe^{3+} from unfavorable chemical reactions (e.g. precipitations), but also increases diffusion toward the root surface. Siderophores would also help Fe^{3+} to enter the root and thus facilitate its absorption. Due to the complexing of

Fe^{3+} with siderophores, the equilibrium of the iron-hydroxides would shift to more soluble side, thereby releasing Cu, Mn, and Zn into soil solution.

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6. APPENDIX

6.1 Composition of media used in the study

6.1.1 Siderophore assay broth (SAB)

K_2HPO_4	2.0 g
$(NH_4)_2HPO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Yeast extract (Difco)	1.0 g
Casamino Acids (Difco)	1.0 g
Sucrose	10.0 g
Deionized water	1000.0 ml
pH	7.6

To make agar plates, 15.0 g Bacto - Agar was added to the above medium.

6.1.2 Modified siderophore assay broth (MSAB-1)

KCl	2.0 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Yeast extract (Difco)	1.0 g
Casamino Acids (Difco)	1.0 g
Sucrose	10.0 g
Deionized water	1000.0 ml
pH	7.6

To make agar plates, 15.0 g Bacto - Agar was added to the above medium.

6.1.3 Trypticase soy agar (TSA)

Bacto - Tryptone (Difco)	17.0 g
Bacto - Soytone (Difco)	3.0 g
Bacto - Dextrose (Difco)	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Deionized water	1000.0 ml
pH	7.3
Bacto - Agar	15.0 g

6.1.4 King's B (KB)

Bacto - Peptone	20.0 g
Glycerol	15.0 g
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Bacto - Agar	15.0 g

pH	7.2
Deionized water	1000.0 ml

6.1.5 Chrome Azurol-S (CAS)

1 Chrome Azurol-S dye solution

a.

Chrome Azurol-S	12.2 mg
Deionized water	10.0 ml

b.

HCl (concentrated)	83.0 µl
Deionized water	100.0 ml
$FeCl_3 \cdot 6H_2O$	0.27 g

c.

HDTMA[1]	21.9 mg
Deionized water (warm)	25.0 ml

Mix 7.5 ml of (a) with 1.5 ml of (b), then add slowly to (c) while stirring, and then transfer the mixture to a 100.0 ml volumetric flask

d.

MES buffer	9.76 g
Deionized water	50.0 ml
pH (with 50 % KOH)	5.6

Rinse (d) into the volumetric flask and make to volume of 100 ml with H_2O

2 Chrome Azurol-S agar

a.

Chrome Azurol S	60.5 mg
Deionized water	50.0 ml

b.

HCl (concentrated)	83.0 µl
Deionized water	100.0 ml
$FeCl_3 \cdot 6H_2O$	0.27 g

c.

HDTMA	72.9 mg
Deionized water (warm)	40.0 ml

Mix (a) with 10 ml of (b), then add slowly to (c) while stirring
Autoclave, cool to 50°C.

d. Modified M9 salts

KH_2PO_4	0.3 g
NaCl	0.5 g
NH_4Cl	1.0 g
Deionized water	750.0 ml

e. 50 % (w/w) KOH

Add 30.24 g PIPES[2] to (d) and adjust to pH 6.8 with (e)
PIPES will not dissolve until the KOH increases the pH to near 6
Add 15 g agar
Make to volume of 800 ml with H_2O
Autoclave, cool to 50°C.
Add 30 ml of filter sterilized casamino acids (10 % w/w)

f. Carbon source and growth factor

Glucose	2.0 g
Mannitol	2.0 g
$MgSO_4$ (1 M)	2.0 ml
$CaCl_2$ (1 M)	2.0 ml
NFB micronutrients	2.0 ml

Make to volume of 70 ml
Autoclave, cool to 50°C.

Add solution (f) to mixture of solutions (d) and (e) while stirring
Add mixture of solutions (a), (b), and (c) by pouring along glass wall while stirring with enough agitation to achieve mixing without generating foam

6.1.6 Rhizosphere medium (RSM)

$Ca(NO_3)_2 \cdot 4H_2O$	0.75 g
$MgSO_4 \cdot 7H_2O$	0.246 g
ACES[3]	18.22 g
NaOH	2.0 g
Deionized water	853.0 ml

After autoclaving, the following sterile stock solutions were added:

KH_2PO_4 (1 *M* pH 7)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (7×10^{-4} *M*)	1.0 ml
$MnSO_4 \cdot 4H_2O$ (9×10^{-4} *M*)	1.0 ml
Thiamine.HCl (20 mg L^{-1})	1.0 ml
Biotin (1 mg L^{-1})	1.0 ml
Casamino Acids (10 %)	100.0 ml
Sucrose (30 %)	33.3 ml

The phosphate, casamino acids, and sucrose were auotclaved separately while the metal mixture and vitamins were filter-sterilized.

[1] Hexadecyltrimethylammonium bromide
[2] 1,4-piperazinediethanesulfonic acid
[3] 2-[(2-Amino-2-oxoethyl)-amino]ethanesulfonic acid