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Iron Regulation of Ferric Iron Uptake in a Fluorescent Pseudomonad: Cloning of a Regulatory Gene

Daniel J. O'Sullivan and Fergal O'Gara

Microbiology Department and the National Food Biotechnology Centre, University College, Cork, Ireland.
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Two iron-regulated *lac* gene fusion plasmids (pSP1 and pMSR1) were constructed that showed *lacZ* expression in *Pseudomonas* spp. strain M114 only during growth under low iron conditions. No expression of the reporter *lacZ* genes was detected in *Escherichia coli*. Plasmid pMSR1 was exploited to screen a Tn*5*-induced *Pseudomonas* Fe^{3+} uptake regulatory mutant (strain M114FR1). *lacZ* expression from pMSR1 and pSP1 in strain M114FR1 was not subject to regulation by iron. This strain could produce siderophore when iron was present in the growth medium in contrast to the wild-type strain. The outer membrane of the wild-type strain M114 contains four iron-regulated polypeptides detected following sodium dodecyl sulfate polyacrylamide gel electrophoresis. However in mutant strain M114FR1, three of these proteins were synthesized in the presence of Fe^{3+} whereas the fourth (an 88-kDa protein) was partially deregulated. This strain showed inhibition of both bacterial and fungal indicator organisms under conditions in which the iron concentration was sufficiently high to render the wild-type strain ineffective. Complementation of strain M114FR1 with a pLAFR1-based gene bank derived from parent strain M114 resulted in the isolation of a cosmid clone pMS639 that restored normal regulation by Fe^{3+}. This plasmid was shown to contain a 28-kilobase DNA insert containing the gene(s) responsible for the regulation of the Fe^{3+} uptake system of strain M114FR1 by iron.

Additional keywords: antibiosis, fluorescent *Pseudomonas*, outer membrane protein analysis, Tn*5*-mediated mutagenesis.

The secretion of fluorescent siderophores (iron-chelating agents) by *Pseudomonas* spp. at the plant root has been proposed to be a major factor in the control of pathogenic organisms that may be present in this nutrient rich environment (Kloepper *et al.* 1980; O'Gara *et al.* 1986; Vandenberg *et al.* 1983). It is suggested that these high affinity ferric iron chelating molecules can scavenge effectively for low concentrations of iron present at the plant root and out compete deleterious organisms for this vital micronutrient (Kloepper *et al.* 1980; Leong 1986; Scher and Baker 1982). It has also been shown that there is a strong repression imposed by iron on both the biosynthesis of the siderophore moiety (Cody and Gross 1987; Marugg *et al.* 1985; Marugg *et al.* 1988) and uptake of the Fe^{3+}-siderophore complex (de Weger *et al.* 1988; Magazin *et al.* 1986) in fluorescent *Pseudomonas* spp. Because of this efficient control system, the bacterium switches off production of siderophore when it has acquired sufficient iron. This feature may reduce the effectiveness of exploiting these bacteria for the control of plant root disease. It is therefore desirable that the factors responsible for the regulation of the Fe^{3+} uptake system are fully determined. This would facilitate the construction of strains that could produce siderophore(s) under a wider range of physiological conditions.

The mechanism by which iron regulates siderophore production in fluorescent pseudomonads is as yet unknown. However, regulation is probably mediated at the transcriptional level (Marugg *et al.* 1988). Iron regulation at the transcriptional level has also been shown for exotoxin A production in *P. aeruginosa* (Lory 1986). A positive regulator (Reg A) is specifically regulated by iron in this system (Frank and Iglewski 1988). The regulation of the iron uptake systems in enteric bacteria has been studied extensively. This regulatory mechanism was first determined for *Salmonella typhimurium* by isolating a mutant that could produce the enterochelin siderophore and the outer membrane receptor proteins for the uptake of the Fe^{3+}-enterochelin complex constitutively (Ernst *et al.* 1978). Hantke (1981) isolated an *Escherichia coli fur* (ferric uptake regulation) mutant and cloned the cognate repressor gene (Hantke 1984). Using the purified gene product, a model was proposed suggesting that a repressor protein interacts with a corepressor (for example, Fe^{2+}) and binds to the promoter regions of iron-regulated genes, preventing transcription (DeLorenzo *et al.* 1987). Sequence analysis of a number of different iron-regulated promoters in *E. coli* established that Fur binds a specific domain, overlapping the "−35" region of the promoter, and a consensus sequence was proposed (Griggs and Konisky 1989).

A similar central repressor control system may regulate the iron uptake system of fluorescent *Pseudomonas* spp. However, this has yet to be shown. It is also possible that more than one control system may be present. Alternatively, the possibility of a positive regulatory system, involving a transacting transcriptional activator switching on the genes of the iron uptake system, cannot be overlooked. Such an activator system has recently been postulated for siderophore production from *Vibrio anguillarum* (Tolmasky *et al.* 1988), as well as for exotoxin A production in *P. aeruginosa* (Frank and Iglewski 1988).

To investigate the nature of Fe^{3+}-mediated regulation in fluorescent *Pseudomonas* spp., we have constructed a

mutant of the plant-beneficial strain M114 in which the production of siderophore biosynthetic and uptake proteins is not regulated by iron, and we have also cloned the gene(s) that complement this siderophore regulatory mutation.

MATERIALS AND METHODS

Bacteria, fungi, and plasmids. Bacterial strains and plasmids are listed in Table 1. The plant pathogenic fungus *Rhizopus stolonifer* was obtained from the Commonwealth Mycological Institute, Surrey, U.K.

Media and growth conditions. *E. coli* strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.* 1982) at 37° C. *Pseudomonas* strains were cultured on LB, King's B (KB; King *et al.* 1954), and minimal sucrose-asparagine (SA) media (Scher and Baker 1982). Antibiotics, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), and ethylenediamine-di(*o*-hydroxyphenyl) acetic acid (EDDA) were added as indicated. Chrome azurol S (CAS) indicator medium was used as described by Schwyn and Neilands (1987). *R. stolonifer* was maintained on potato-dextrose agar (PDA).

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
Pseudomonas spp.		
M114	Wild type, Ap^r, Flu^+	O'Gara *et al.* 1986
M114FR1	Ap^r, Km^r, Flu^+, siderophore regulatory mutant	This study
M114FR2	Ap^r, Km^r, Flu^+, putative ferric siderophore uptake mutant	This study
Escherichia coli		
HB101	*recA hsdR hsdM strA pro leu thi*	Boyer and Roulland-Dussoix 1969
SM10	C600, *thi thr leu recA* Muc^+ chromosomally inserted RP4-2-7C::Mu	Simon *et al.* 1982
MC1061 (Nal^r)	*ara leu lacX74 galU galK hsdR hsdM strA*, Nal^r	O'Sullivan and O'Gara 1988
KMBL1164	*del (lac-proAB) thi* F^-	H. Spaink, University of Leiden, Holland
Plasmids		
pHoHo1	Ap^r, Tn*3-lacZYA, tnpA*	Stachel *et al.* 1985
pSShe	Cm^r, Tra^-, $TnpA^+$, p15A replicon	Stachel *et al.* 1985
pRK2013	Km^r, Mob^+, Tra^+, ColE1	Figurski and Helinski 1979
pSUP1011	Cm^r, Km^r, Mob^+, Tra^-, p15A replicon, Tc::Tn*5*	Simon *et al.* 1983
pMS118	Tc^r, Mob^+, Tra^-, IncP, 21.7-kb DNA insert from strain M114 containing siderophore biosynthetic genes	This study
pMSR1	pMS118 with Tn*3*-HoHo1 insertion, iron-regulated *lacZ* expression	This study
pMS639	Tc^r, Mob^+, Tra^-, IncP, 28-kb DNA insert from strain M114 containing siderophore regulatory gene(s)	This study
pSP1	Cm^r, Sm^r, Tra^-, IncQ, iron-regulated *lacZ* expression	This study
pMP190	Cm^r, Sm^r, Tra^-, IncQ, promoterless *lacZ* gene	Spaink *et al.* 1987
pMK19	Tc^r, Tra^-, IncP, iron-regulated *lacZ* expression	Marugg 1988
pMAB25	Tc^r, Tra^-, IncP, iron-regulated *lacZ* expression	Marugg, 1988

Insertion of Tn*3*-HoHo1 onto pMS118. Plasmid pMS118 was transformed into *E. coli* HB101 (pHoHo1; pSShe). Transformants were selected on LB medium containing tetracycline (Tc), 10 μg/ml. They were then checked for the ampicillin resistance (Ap^r) of the Tn*3*-HoHo1 element and chloramphenicol resistance (Cm^r) of pSShe, which contains the transposon gene necessary for transposition of the element onto pMS118. The Tc^r, Ap^r, Cm^r transformants were subsequently conjugated into *E. coli* MC1061 (nalidixic acid-resistant [Nal^r]) in a triparental mating using *E. coli* HB101 (pRK2013) to provide helper functions. Exconjugants were selected on LB medium containing: Tc, 10 μg/ml; Ap, 25 μg/ml; and Nal, 50 μg/ml. These constructs were subsequently mated into *Pseudomonas* spp. strain M114, again using pRK2013 for helper functions. Exconjugants were selected on minimal SA medium containing Tc, 65 μg/ml.

Selection of Fe^{3+}-regulated *lac* gene fusions. Fusion plasmids were conjugated into strain M114 and were screened on SA medium containing X-gal (40 μg/ml) and on the same medium incorporating 50 μM $FeCl_3$. Colonies were selected that had a blue color on low iron medium and a white color in the presence of iron. This indicated iron-regulated *lac* gene expression.

Measurement of promoter activity from fusion plasmids. Strains containing the fusion plasmid were grown to late-logarithmic phase in SA medium with and without 50 μM $FeCl_3$. For some experiments, assays were conducted on cultures scraped off agar plates of the same medium. In assays involving *E. coli*, a mixture of casamino acids (0.2%) was added to the medium to supplement its auxotrophic requirements. Strain M114 secreted fluorescent siderophore during growth on this medium, indicating its low iron content. All glassware was washed in 6 N HCl and thoroughly rinsed with distilled, deionized H_2O. β-Galactosidase assays were performed in duplicate as described by Miller (1972). Units are expressed as a function of the absorbance of the culture at $OD_{600\ nm}$.

Tn*5* mutagenesis. Tn*5*-mediated mutagenesis of *Pseudomonas* was conducted essentially using the method of Simon *et al.* (1983). Briefly, both *E. coli* SM10 1011 and the *Pseudomonas* strain were grown in LB medium to logarithmic phase. Ten milliliters of each culture was concentrated by centrifugation and resuspended in 100 μl of LB broth. Both cultures were then mixed and deposited on a dry LB plate. The plate was incubated at 28° C for 16–20 hr after which the culture was scraped off and resuspended in 1 ml of TY broth (Maniatis *et al.* 1982) incorporating 15% glycerol. Selection for Tn*5*-containing *Pseudomonas* strains was made by plating out aliquots of the mating mixture on SA medium containing 20 μg/ml of kanamycin (Km). The mating mixtures were then stored at −20° C.

Complementation experiments. A pLAFR1-based gene bank of the wild-type strain M114 was conjugated into Tn*5*-induced mutants, using *E. coli* HB101 (pRK2013) to

provide helper functions. Exconjugants were selected on SA medium containing Tc (65 μg/ml) and were subsequently checked for loss of the mutant phenotype.

Absorption spectra of culture supernatants. Strains were grown in SA medium with and without 50 μM $FeCl_3$. Km (20 μg/ml) was also incorporated when assaying Tn*5* mutant strains. Cultures were incubated at 28° C with vigorous shaking until early stationary phase. Cells were removed by centrifugation, and an absorption spectrum of the remaining supernatant was measured at 250–550 nm with a Beckman model 25 recording spectrophotometer.

Analysis of outer membrane proteins. The outer membrane proteins of *Pseudomonas* strains were isolated following the method of Chart and Trust (1983). The proteins were separated by sodium dodecyl sulfate (SDS)-PAGE (Laemmli 1970) and visualized by staining with Coomassie blue (Weber and Osborn 1975). Photographs of the gels were used to obtain densitometer tracings of the outer membrane protein profiles with a Bio-Rad (Richmond, CA) video densitometer model 620.

Antibiosis assays. For all assays, the *Pseudomonas* strain was spot-inoculated on an agar plate (LB or KB) using 10 μl of a freshly grown culture. Plates were then incubated at 28° C for 24 hr before applying the test organism. For inhibition tests with *E. coli*, a logarithmic-phase culture (HB101) grown in LB medium was sprayed over the *Pseudomonas* culture with an atomizer. The plates were further incubated at 28° C for another 24 hr before screening for inhibition zones. In the fungal inhibition assays, a confluent plate of sporulating *R. stolonifer* grown on PDA was inverted and gently tapped over the *Pseudomonas* strain. This had the effect of evenly inoculating the plate with a dense layer of fungal spores. Plates were then incubated at 28° C for approximately 72 hr before scoring.

DNA manipulations. Plasmid DNA isolations were performed using the method of Birnboim and Doly (1979). Restriction enzyme reactions were performed according to the recommendations of the manufacturer (Boehringer Mannheim, London). All other recombinant DNA techniques were performed as outlined by Maniatis *et al.* (1982).

RESULTS

Construction of Fe^{3+}-regulated *lac* gene fusion plasmids. The Fur repressor protein regulates transcription of a group of genes that are scattered throughout the *E. coli* chromosome and which are involved in iron uptake (DeLorenzo *et al.* 1988). It also controls the expression of other iron-regulated genes such as colicin I receptor (*cir*) (Griggs and Konisky 1989) and Shiga-like toxin (*slt*A B) genes (Calderwood and Mekalanos 1987). To investigate if a Fur-like regulatory mechanism exists in *Pseudomonas*, different iron-regulated *lac* gene fusion plasmids were constructed. Two strategies were used for this purpose.

A promoter bank was obtained by cloning total DNA from strain M114 digested to completion by *Bgl*II into the promoter probe plasmid pMP190. Following screening on low and high iron media containing X-gal, one clone (pSP1) was detected that showed iron-regulated *lacZ* expression in strain M114. This was verified by measuring the β-galactosidase levels in broth assays (Table 2).

An additional approach exploited a plasmid (pMS118) containing siderophore biosynthetic genes; it was isolated by complementing five independently isolated siderophore biosynthetic (Sid^-) mutants. These Tn*5*-induced mutants were characterized by checking the following traits: 1) loss of the fluorescent phenotype, 2) reduced ability to grow on low iron media, 3) inability to form an orange halo on CAS medium, and 4) inability to inhibit bacterial or fungal test cultures. Tn*3*-HoHo1, which contains the promoterless *lacZ* reporter gene, was inserted into this plasmid and a fusion plasmid (pMSR1) that showed iron-regulated *lacZ* expression in strain M114 was selected (Table 2). The insertion site of the transposon was mapped by restriction endonuclease analysis and was localized to a 3.0-kilobase (kb) *Sal*I-*Eco*RI fragment (Fig. 1). The iron-regulated promoter of pSP1 was localized to a 2.1-kb *Bgl*II

Table 2. Expression of β-galactosidase from Fe^{3+}-regulated *lac* gene fusion plasmids

Bacterial strain	β-Galactosidase (units)[a]	
	Without Fe^{3+}	With Fe^{3+}
Pseudomonas spp.		
Strain M114	<10	<10
Strain M114		
pMSR1	120	10
pSP1	420	20
pMP190	<10	<10
Escherichia coli KMBL 1164		
pMSR1	<10	<10
pSP1	<10	<10

[a]Units are defined by Miller (1972). See text.

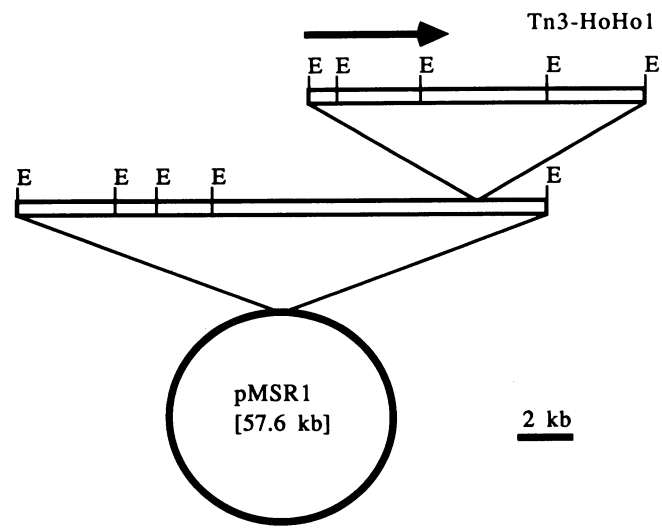

Fig. 1. Physical map of the iron-regulated *lac* gene fusion plasmid pMSR1. The construction of the plasmid is described in the text. The plasmid consists of a 21.7-kilobase (kb) DNA insert from strain M114 cloned into the broad host range vector pLAFR1. The Tn*3*-HoHo1 transposon is inserted within the large *Eco*RI fragment (14.6 kb) of this *Pseudomonas* DNA insert containing siderophore biosynthetic genes. The insertion site of the transposon on this plasmid was mapped by restriction endonuclease digestion with *Sal*I and *Eco*RI. Only the *Eco*RI restriction sites have been included in the diagram and are designated by E.

fragment. This *Bgl*II fragment was not present on pMS118 as shown by restriction endonuclease and DNA hybridization analysis. Consequently, both these iron-regulated promoters were obtained from different loci on the genome of strain M114. While neither of these fusion plasmids were expressed in *E. coli*, both had *in vivo* iron-regulated expression in strain M114 (Table 2).

Isolation of siderophore regulatory mutants. Iron-regulated promoter-*lacZ* fusion plasmids derived from *E. coli* are not regulated by iron in Fur$^-$ mutants. A strategy based on this feature was used to generate Fur$^-$ mutants in *E. coli* (Hantke 1981). Fusion plasmid pMSR1 was exploited in a similar strategy as a convenient selection tool to obtain a mutant of strain M114 that could express the *lacZ* gene of this construct independent of iron in the growth medium. For this purpose, strain M114 was randomly mutated with Tn*5*, and the fusion plasmid pMSR1 was subsequently mated into the Tn*5*-generated mutant clones. Tc^r (pMSR1) and Km^r (Tn*5*) exconjugants were selected on high iron SA medium containing X-gal. Since *lacZ* expression from pMSR1 is switched off in the presence of iron, the majority of colonies were white on X-gal. However, two blue colonies were obtained and were designated strain M114FR1 and strain M114FR2. Expression of β-galactosidase from pMSR1 was no longer regulated by iron in these strains (Table 3). Plasmid pMSR1 was then cured from both strains by subculturing in iron-rich SA medium without Tc. Tc-sensitive, Km^r colonies were readily obtained in this manner and, as expected, had a Lac negative phenotype.

To investigate the possibility that the derepression of the *lac* gene fusion may have been due to a mutation in the transport of the iron-siderophore complex thus causing iron starvation within the cell, the ability of the mutant to grow under low iron conditions was examined. The available iron content of the SA medium was progressively reduced by adding increasing amounts of the chemical ferric iron chelator, EDDA. Mutant strain M114FR1 was capable of growing on SA medium with EDDA concentrations in the range of 0–0.2 mM, which is comparable to the parent strain M114. Strain M114FR2, however, failed to grow at any concentration of EDDA, and adequate growth was only obtained when $FeCl_3$ (> 100 μM) was added to the medium.

Table 3. β-Galactosidase expression from fusion plasmids in mutant strains

Strain[a]	β-Galactosidase (units)	
	Without Fe^{3+}	With Fe^{3+}
M114FR1		
pMSR1	340	380
pSP1	750	700
pMK19	950	1,200
pMAB25	840	600
M114FR2		
pMSR1	ND[b]	280
M114		
pMK19	600	30
pMAB25	470	80

[a]Strain M114FR2 could not grow under low iron conditions (see text).
[b]ND = not determined.

These results indicated that mutant strain M114FR2 was defective in the uptake of iron into the cell. There was, however, no impairment in the major iron-regulated outer membrane proteins as shown by SDS-PAGE, indicating that another component of the ferric-siderophore uptake system may be defective in this mutant. Because no evidence was obtained for a defective iron transport system in mutant strain M114FR1, it was chosen for further study.

The effect of the mutation on siderophore production in strain M114FR1 was then examined in broth assays in the presence and absence of iron. The presence of siderophore can be readily observed by the appearance of a yellow-green pigment under low iron conditions or of a red-brown pigment in the medium when iron is present. This was measured spectrophotometrically and the results are presented in Figure 2. The absorption spectrum of the supernatant from the wild-type strain M114 showed a maximum at 405 nm, which is essentially the same as that found for other pseudobactin siderophores (Marugg *et al.* 1985; Teintze *et al.* 1981). Supernatant from mutant strain M114FR1 also absorbed at 405 nm when grown under low iron conditions. However unlike the wild-type strain, an absorption maximum at 400 nm with a shoulder at

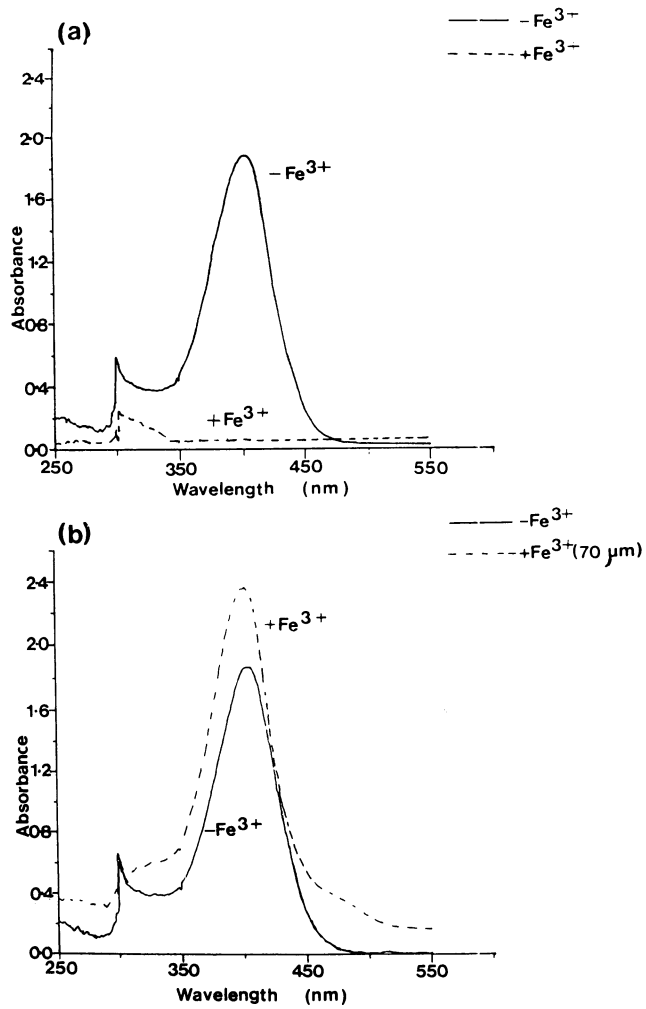

Fig. 2. Absorption spectra of the supernatant from strains M114 (**a**) and M114FR1 (**b**) grown under low (——) and high (- - - -) iron conditions.

450 nm was observed when strain M114FR1 was grown under high iron conditions (Fig. 2), which is consistent with the absorption peak obtained for a ferric-siderophore complex of the pseudobactin type (Marugg *et al.* 1985).

These results show that the presence of iron in the medium does not inhibit the production of siderophore in the mutant strain M114FR1 and indicates that the inserted Tn*5* element may have inactivated a key ferric uptake regulatory gene.

Characterization of siderophore regulatory mutant. To verify that a *fur*-like gene is mutated in strain M114FR1, the expression of other iron-regulated promoters was examined. The iron-regulated fusion plasmids pSP1, pMK19, and pMAB25 were introduced into strain M114FR1. Both of the latter plasmids contain different promoters from the siderophore biosynthetic region of *Pseudomonas* spp. strain WCS358 (Marugg 1988). Unlike the wild-type strain, *lacZ* expression from these constructs in strain M114FR1 is not subject to regulation by iron (Table 3). This showed that numerous Fe^{3+}-regulated transcriptional units can be derepressed in this strain, indicating that a regulatory gene which controls transcription of iron-regulated promoters is mutated.

It was found in *E. coli* that siderophore biosynthesis and uptake were coordinately derepressed in a Fur^- mutant, indicating a common regulatory factor (Hantke 1981). In *Pseudomonas spp.*, iron also imposes a negative regulatory effect on both siderophore biosynthesis and uptake (de Weger *et al.* 1988; O'Sullivan and O'Gara, unpublished results). To determine if this is also mediated by a common regulatory factor as in *E. coli*, the effect of the regulatory Tn*5* mutation in strain M114FR1 on the production of

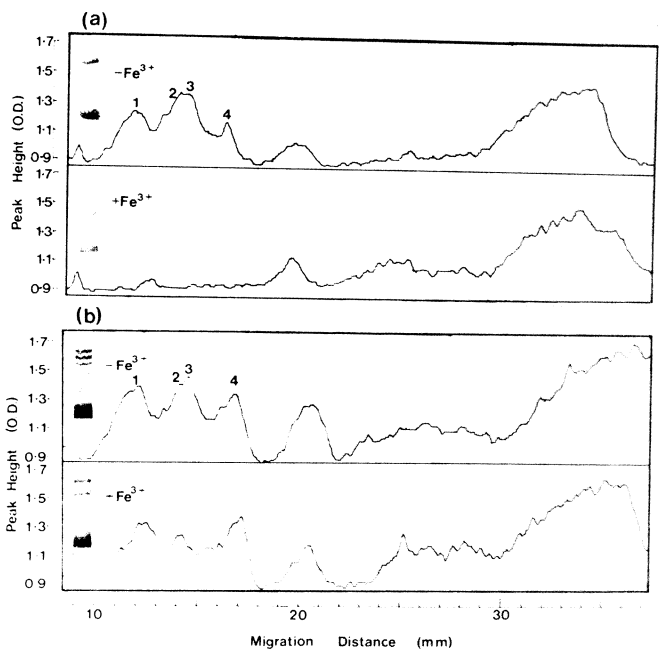

Fig. 3. Densitometer tracings of sodium dodecyl sulfate-PAGE gels of outer membrane proteins of strains M114 (**a**) and M114FR1 (**b**). The section of the gel from which the densitometer tracing was obtained is included as a photographic strip with each tracing. The numbered peaks (1–4) indicate the iron-regulated proteins of 92, 89, 88, and 81 kDa, respectively.

the outer membrane receptor proteins was examined. There are four outer membrane polypeptides produced by strain M114 that are not present when the strain is grown under high iron conditions (Fig. 3a). The size of the polypeptides are 81, 88, 89, and 92 kDa. In mutant strain M114FR1, the production of three of these polypeptides (81, 89, and 92 kDa) is fully derepressed in the presence of iron and the fourth, the 88-kDa polypeptide, is partially derepressed (Fig. 3b). These results indicate that the regulation by iron of the iron uptake receptor proteins is coordinately derepressed in mutant strain M114FR1.

Cloning of the gene for iron-mediated regulation. A complementation strategy, based on the universal siderophore detection CAS medium (Schwyn and Neilands 1987), was exploited to clone the gene(s) encoding iron-mediated regulation of the fluorescent siderophore system of *Pseudomonas* spp. This medium contains a blue Fe^{3+}-dye complex (chrome azurol S-Fe^{3+}-hexadecyltrimethylammoniumbromide), which turns orange when the ferric iron is removed. On this medium, strain M114 produces a small defined orange halo, whereas the siderophore regulatory mutant strain M114FR1 produces a much larger halo.

The complementation strategy involved conjugating a gene bank of the wild-type strain M114 into the mutant strain M114FR1 and then selecting for colonies with a small defined orange halo on the CAS medium. In this manner the complementing cosmid clone pMS639 was isolated. Characterization of the complemented strain established that siderophore was not produced on SA medium containing 50 μM $FeCl_3$. Furthermore, SDS-PAGE analysis of the outer membrane proteins of this strain grown under low and high iron (50 μM $FeCl_3$) conditions indicated the presence of four iron-regulated proteins. These characteristics were essentially the same as the wild-type strain M114, indicating that pMS639 complemented the deregulated phenotype of strain M114FR1. Restriction endonuclease analysis of this plasmid showed that it contained a 28-kb insert of *Pseudomonas* DNA. Because this insert fully complements the mutation of strain M114FR1, it could be concluded that it contains the gene(s) required for regulation by iron of the siderophore system in *Pseudomonas* spp. strain M114.

Fungal and bacterial inhibition by regulatory mutant. *Pseudomonas* spp. strain M114 was originally selected from soil because of its ability to inhibit plant bacterial and fungal pathogens (O'Gara *et al.* 1986). Inhibition was only apparent under low iron conditions and was attributed to the production of a fluorescent siderophore compound. Under high iron conditions, the siderophore moiety is not produced and the ability to inhibit pathogens is drastically reduced. In a normal plate antibiosis assay using KB medium, 10 μM of added ferric iron is sufficient to prevent siderophore production in the wild-type strain M114. However, it is conceivable that under these conditions significant pathogen inhibition could still be attained if siderophore could be produced. The inhibitory properties of mutant strain M114FR1 toward other organisms were therefore examined on iron-containing media. On LB medium strain M114 showed no apparent inhibition of

an *E. coli* HB101 test culture, whereas strain M114FR1 showed strong inhibition of the organism (Fig. 4a). Similarly, inhibition of the *Rhizopus* fungus by the wild-type strain M114 on a high iron KB medium was very poor (Fig. 4b). The small zone of inhibition that was observed is attributed to the production of an inducible antibioticlike substance by strain M114 under these conditions (data not shown). However, strain M114FR1 still showed a significantly increased zone of inhibition against *R. stolonifer* on this medium even though the added ferric iron concentration was 50 μM (Fig. 4b).

DISCUSSION

Pseudomonas spp. strain M114FR1 is a mutant strain that can produce siderophore when iron is present in the growth medium. This is in contrast to the wild-type strain. Coordinate derepression of the outer membrane receptor proteins for the Fe^{3+}-siderophore complex is also observed in this mutant strain. However, evidence indicates that there are a number of transcriptional initiation sites required for the expression of siderophore biosynthetic and uptake genes in the *Pseudomonas* spp. This was established from a mutational analysis of the genes concerned (Magazin *et al.* 1986; O'Sullivan and O'Gara, unpublished results). The derepression observed in mutant strain M114FR1, therefore, strongly suggests that a repressor protein negatively regulates the promoters of these genes involved in the iron uptake process. This model was substantiated by observing the derepression of four independently isolated iron-regulated promoter-*lacZ* fusion plasmids in strain M114FR1 (Table 3). These results indicate that strain M114FR1 is defective in a central regulatory gene which is responsible for the negative regulation by iron of a number of different transcriptional units in *Pseudomonas*. Negative regulation of the iron uptake processes in enteric bacteria has already been confirmed (Calderwood and Mekalanos 1988; DeLorenzo *et al.* 1987).

The two iron-regulated promoter-*lacZ* fusion plasmids (pMSR1 and pSP1) did not show *lacZ* expression in *E. coli* (Table 2). This suggests that this type of promoter is not recognized by the *E. coli* transcriptional apparatus. This is not unusual since it has already been shown from sequence analysis of *Pseudomonas* promoter regions that many do not contain an *E. coli* consensus sequence (Grant and Vasil 1986; Inouye *et al.* 1984; O'Sullivan and O'Gara, unpublished results). Furthermore, there may be activator proteins present in *Pseudomonas* to enable proper promoter functioning, and these may be absent from *E. coli*. *lacZ* expression from four different *lac* gene fusion plasmids was approximately twofold to threefold greater in strain M114FR1 than in the wild-type strain M114 under low iron conditions (Tables 2 and 3). This suggests that the residual iron present in the medium results in reduced promoter expression in strain M114 through its normal iron regulation system, whereas this restriction is absent from strain M114FR1.

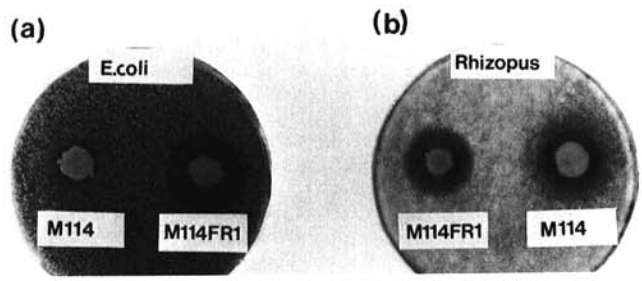

Fig. 4. Inhibition of *Escherichia coli* HB101 (**a**) and *Rhizopus stolonifer* (**b**) by *Pseudomonas* sp. strain M114 and mutant strain M114FR1 on high iron media. *E. coli* inhibitions were conducted on Luria-Bertani agar plates and *Rhizopus* inhibitions on King's B agar incorporating 50 μM $FeCl_3$.

Plasmid pMSR1 may also be useful in the isolation of siderophore uptake mutants. Evidence for this comes from the isolation of a putative ferric-siderophore uptake mutant strain M114FR2, which was shown to express the *lac* gene fusion of pMSR1 in the presence of iron (Table 3). The derepression of the fusion plasmid in this strain may be caused by iron starvation within the cell due to the inability to take up iron. Alternatively, the defect may be in the release of iron from the Fe^{3+}-siderophore complex after it has entered the cell. Mutations of this latter type have been observed in *E. coli* (Langman *et al.* 1972), and the gene affected was designated *fes*. Understanding the nature of the mutation in strain M114FR2 will require further work.

Unlike the other three iron-regulated outer membrane proteins, the 88-kDa protein is apparently present in reduced quantities in strain M114FR1 during growth under high iron conditions (Fig 3b). This may indicate the presence of an additional control in the production of this protein. A similar finding was observed for an *E. coli fur* mutant in which an outer membrane receptor protein (the product of the *fepA* gene) was not fully derepressed and for which an additional control mechanism has been suggested (Hantke 1981). It could be postulated that an additional control at the level of uptake may serve as a protective mechanism so that the uptake of iron may be stringently controlled to prevent iron toxicity within the cell.

Plasmid pMS639 contains the gene(s) for a putative repressor protein of strain M114, which enables the *Pseudomonas* siderophore system to be regulated by iron. This model implies that a repressor protein binds specific domains in the promoter/operator region of the genes encoding siderophore-mediated iron uptake. The corresponding Fur binding site in *E. coli* has been identified for a number of different iron-regulated genes and was found to be conserved in all these genes (Griggs and Konisky 1989). Sequence analysis of the promoter/operator region of fusion plasmid pSP1 did not show homology to the *E. coli* Fur binding consensus sequence (O'Sullivan and O'Gara, unpublished results). Consequently, determination of the repressor binding site in a number of pseudomonad iron-regulated genes is a prerequisite in determining a consensus sequence for this Fur-like repressor protein.

The mutation in strain M114FR1 enabled it to produce a biologically active siderophore in iron-rich media. This was shown by its ability to inhibit bacterial and fungal test organisms under iron conditions that rendered the wild-

type strain M114 ineffective (Fig. 4). It is not yet possible to evaluate if the deregulated strain is a more effective biocontrol agent of plant root disease than is the wild-type, because this Tn*5*-induced mutation reverts readily in the absence of Km selection. Exploitation of the cloned gene to obtain regulatory mutants based on deletions will be necessary to evaluate how these mutants function at the plant root level. Some fluorescent pseudomonads also produce antibioticlike substances that aid in the inhibition of bacteria and fungi. The role of these compounds in the control of plant pathogens is becoming increasingly evident (Gutterson *et al.* 1986; Howell and Stipanovic 1980; Thomashow and Weller 1988). Strain M114 apparently also produces an antifungal compound that enables some fungal inhibition under high iron conditions (Fig. 4b).

The ability to manipulate the regulation imposed by iron on the siderophore system has been shown to increase the capacity of strain M114 to inhibit both bacteria and fungi (Fig. 4). The strategy to exploit pMSR1 may be extended to obtain siderophore regulatory mutants in other strains. This technology may be useful in evaluating whether fluorescent *Pseudomonas* spp. can be improved for biological control purposes.

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