An Iron-Antagonized Fungistatic Agent That Is Not Required for Iron Assimilation from a Fluorescent Rhizosphere Pseudomonad

PAUL R. GILL, JR.,†* AND GARETH J. WARREN
Advanced Genetic Sciences, Inc., Oakland, California 94608

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Fluorescent rhizosphere Pseudomonas sp. strain NZ130 promotes plant growth, and may do so in part because of its production of a growth inhibitory factor that is active against phytopathogenic fungi. Analysis of the inhibitory factor that is active against the phytopathogen Pythium ultimum showed that its activity is antagonized at iron concentrations above 10 μM. The iron-antagonized inhibitor was separated from the fluorescent siderophore of this pseudomonad by gel filtration. Mutants that lacked either the iron-antagonized inhibitor or the fluorescent siderophore were isolated. Results of complementation analysis of these mutants by use of a cosmid library indicated that distinct DNA sequences are required for the production of each factor. Analysis of isogenic mutant strains showed that the genetic requirements for the production of the iron-antagonized inhibitor and the fluorescent siderophore are different, and that only the fluorescent siderophore is required for iron assimilation. Fusions of these same sequences to a β-galactosidase gene were used to show that the regions required for the production of both the fluorescent siderophore and the iron-antagonized inhibitor were iron-regulated.

Fluorescent pseudomonads are prominent in the aerobic microflora of the rhizosphere of many plants. These bacteria have been reported to have effects on plants that range from beneficial (plant growth promoting) to deleterious (33, 34). Several mechanisms have been proposed for the growth-promoting effect (12, 30, 33). These bacteria might supply limiting nutrients to the plant or produce growth hormones. A third possibility, that plant growth is increased because colonization by microorganisms that are deleterious to the plants is suppressed, has been strongly supported (17, 31, 34, 39). Microbial antagonism of this sort may be mediated by antibiotic agents which would inhibit plant-deleterious microorganisms.

Antibiotics that are active against fungi have been identified from various plant growth-promoting pseudomonads (12, 14, 15). Results of some experiments done in the field have indicated that production of the antibiotic correlates with the ability to control the fungal pathogen. Fluorescent pseudomonads can synthesize a variety of compounds that might attenuate fungal growth (12, 14, 15, 20, 31).

Microbial siderophores are high-affinity, FeIII-specific chelating agents that enable the producing organism to sequester iron via a specific uptake system (26). Siderophores confer virulence on some animal-pathogenic bacteria by defeating a host defense based on iron deprivation (5, 38). It has been proposed that siderophores, which are produced by plant growth-promoting rhizobacteria, similarly inhibit deleterious microorganisms by iron deprivation (19). This type of inhibitory activity is neutralized by moderately high levels of available iron.

In this study, a collection of Pseudomonas isolates obtained from rhizospheres was screened by using a petri dish assay designed to identify strains with the most active iron-antagonized fungal inhibition. Three isolates that exhibited a potent fungistatic activity that was antagonized by iron were identified. Fluorescent siderophores appeared to be produced by all the isolates in our collection, and the structural features of siderophores produced by such organisms are a variably modified quinoline moiety and a short peptide chain with a variable sequence of amino acids (19, 23). The quinoline moiety and the modified side chains of two of the amino acids were capable of FeIII chelation. Recently, the relevance of siderophores to plant growth and disease (27) and to biological control (19) has been reviewed.

We selected a representative isolate, Pseudomonas sp. strain NZ130, of the three isolates that exhibited the antifungal activity discussed above, to determine whether its fluorescent siderophore was responsible for the activity in our assay. We found that this antifungal activity was distinct from the presence of the fluorescent siderophore of this isolate. Moreover, although the inhibitory activity was antagonized by iron and its expression was regulated by iron, it appeared to play no essential role in iron assimilation.

MATERIALS AND METHODS

Microbial strains and plasmids. Pythium ultimum SB1 and the fluorescent rhizosphere pseudomonad isolates were obtained from the collection of Trevor Suslow (our laboratory), Escherichia coli HB101 (leu proA2 ara-14 xyl-5 galK2 mlt-1 lacY1 thi supEA4 recA13 rpsL20 hsdS20) (2) was used as the host for the cosmid library and subclones. The cosmid vector was pVK102 (18). Conjugal transfer of the cosmid library was done with the helper plasmid pRK2013 (7, 9). pRK2013 was maintained in E. coli HB101. pPR42 is a derivative of pED350 (8) in which the 445-base-pair (bp) HaeII polylinker-containing fragment of pUC18 (29) was blunt-end ligated into the EcoRV site of pED350. pRK767 is a derivative of pRK404 (6) but that lacks the EcoRI site outside of the polylinker (kindly provided by Jonathan Jones, our laboratory), in which the pUC9 polylinker (37) has been replaced with the pUC18 polylinker (29).

Luria broth (LB) and King broth (KB) media were prepared as described previously (16, 24). Antibiotics were included in the media, when appropriate, at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 10 μg/ml.

* Corresponding author.
† Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.
Ultrafiltration and column chromatography. Diaflo ultrafiltration membranes YM10 (M<sub>n</sub> = 10,000 exclusion) and YM2 (M<sub>n</sub> = 1,000 exclusion) (both from Amicon Corp., Lexington, Mass.) were used according to the instructions provided by the manufacturer. Prior to the ultrafiltration of spent media, bacteria were removed by centrifugation and then by filtration with a filter (pore diameter, 0.45 μm; Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.).

Bio-Gel P2 and P6 columns (Bio-Rad Laboratories, Richmond, Calif.) were used in the gel filtration analyses. Columns (30 by 0.7 cm; Econo-column; Bio-Rad) were packed in distilled water to a bed height of 25 cm. Samples of 0.1 to 0.5 ml were admitted to the columns, and 1-ml fractions were collected to monitor the elution of the fluorescent siderophore and the antifungal activity.

Preparation and characterization of the fluorescent siderophore from Pseudomonas sp. strain NZ130. The fluorescent siderophore of Pseudomonas sp. strain NZ130 was prepared by the method described previously that was used for the isolation of pseudobactin (35). Spectral analysis of partially purified spent media or of the purified material was done with a spectrophotometer (model Lambda 5; The Perkin-Elmer Corp., Norwalk, Conn.; or model 25; Beckman Instruments, Inc., Fullerton, Calif.).

Analysis of the antifungal activity. The occurrence of fungal antibiotic activity in Pseudomonas strains was initially analyzed by using a petri dish assay. For a quantitative measurement of the fungal antibiotic activity produced by strain NZ130, bacteria were first grown to the early stationary phase in KB medium and then removed from the medium by centrifugation and filtration with a filter (pore diameter, 0.45 μm; Millipore Corp., Bedford, Mass.). Diluted medium (2 ml) was added to 2 ml of molten LB agar and then poured into a petri dish (diameter, 35 mm). After the agar gelled, a second water agar plug of approximately 0.1 ml in volume containing Pythium ultimum SB1 was placed at the edge of the LB agar dish, and hyphal growth was monitored over the subsequent 48 h.

The fungal hyphae were small and fragile, and growth of the fungus was evident throughout the entire dish but became less dense with the addition of the ferric iron-antagonized inhibitor (FAI). When the fungus was completely inhibited by FAI, no hyphal growth was evident. To estimate the degree of fungal inhibition, the density of hyphal growth was estimated by visually comparing the percentage of transmitted light through a given petri dish containing agar. Calibrated light filters were constructed of differentially exposed film (XAR-5; Eastman Kodak Co., Rochester, N.Y.). Exposed X-ray film was held over a given inhibited culture under dark-field light conditions in a dark room to estimate the percentage of remaining transmitted light. The percentage of exposure of the X-ray film was calibrated by using an empty agar dish (100% inhibition) and therefore required a filter designated as 100% exposed to block the transmitted light. A dish in which fungal growth was uninhibited (0% inhibition) thus required a filter designated as 0% exposed, to block the remaining transmitted light. Therefore, a dilution of FAI that resulted in 20% inhibition required the X-ray film exposed 80% to block the remaining transmitted light. On a given petri dish the amount of fungal inhibition, i.e., increase in transmitted light, was estimated by using 1 of 11 exposed films ranging from 0 to 100% inhibition.

Mutagenesis of Pseudomonas sp. strain NZ130. Pseudomonas sp. strain NZ130 was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Sigma Chemical Co., St. Louis, Mo.), as described by Carlton and Brown (4). Bacteria were incubated with MNNG for 15 min at room temperature. This treatment resulted in a survival frequency of 0.2%. A test of the growth of 500 CFU on M9 medium (24)-0.2% sucrose indicated that approximately 3.5% of the CFU had additional growth requirements.

Cosmid library construction and DNA analysis. Chromosomal DNA was prepared from pseudomonads by the method described by Marmur (22), with minor modifications. To construct the cosmid library of strain NZ130, the chromosomal DNA was partially digested with HindIII and then with XhoI. Partially digested DNA of ≥18 kb was isolated by sedimentation with a 5 to 40% sucrose gradient (21). The cosmid vector pVK102 was digested with HindIII and XhoI, and was similarly purified by using a sucrose gradient. Ligation and phage lambda packaging were done as described previously (21). The products of the packaging reaction were used to transduce E. coli HB101. Tetracycline-resistant transductants were isolated, and 1,600 of them were patched onto and maintained in grids on LB agar containing tetracycline.

Restriction endonuclease analysis of cosmid DNA prepared from 20 randomly selected library clones (1, 13) showed that each clone had a different insert. Restriction enzymes, DNA polymerase I, and DNA ligase were used as suggested by the suppliers (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.). Southern transfer and DNA-DNA hybridization analyses were done as described previously (21).

Conjugation experiments and complementation analyses. The conjugal transfer of pVK102 cosmid clones required the helper plasmid pRK2013 to provide some of the transfer functions. For the transfer of a given plasmid or cosmid clone (see below) from E. coli to a Pseudomonas strain, a triple mating (7) was performed as follows. Two E. coli strains, one containing a plasmid or cosmid clone and one containing the helper plasmid, were grown with antibiotic selection to an A<sub>550</sub> of 0.4 to 0.6 (4 × 10<sup>8</sup> CFU/ml). The bacteria were centrifuged and suspended in fresh LB medium, and then 3 × 10<sup>8</sup> CFU of each strain was mixed with 1.5 × 10<sup>8</sup> CFU of the recipient Pseudomonas strain, which was also washed and suspended in LB medium. The mixture of bacteria was spread onto an LB plate and incubated for 16 h at 28°C. The bacteria in the resulting lawns were suspended, washed, diluted, and then plated onto medium to select the transconjugants. In these matings, Pseudomonas strains were selected by virtue of their natural ampicillin resistance or their ability to grow on M9 medium (24)-0.2% sucrose.

Cosmid clones of the library were transferred to individual MNNG-induced mutants for complementation analysis. Transconjugants were then selected by replica plating. To aid in the selection of transconjugants of these matings, it was helpful to transfer the pRK2013 helper plasmid into the cosmid library before the cosmid library was transferred to a given mutant in a second mating. The highest observed transfer efficiency into strain NZ130 from E. coli was 10<sup>−5</sup> per donor.

Construction of site-directed mutants. The chromosomal restriction fragment containing the target sequence to be analyzed was first subcloned into pPR42. This vector was transferred to strain NZ130 by conjugation, but it was unable to be replicated. The pPR42 subclone was first digested with Sau3A in the presence of a predetermined amount of ethidium bromide (35 to 50 μg/ml), such that the plasmid was cut
present,

was

approximately one time. This treatment resulted in the formation of linear molecules that were separated from the undigested supercoiled and open circular forms of the plasmid by electrophoresis.

The population of linear molecules obtained by the Sau3A treatment described above was purified from the undigested plasmid by using low-melting-temperature agarose in TAE buffer (21). After the melting of the agarose and extraction with phenol, followed by washes in ether, the DNA was precipitated with ethanol. The fragments obtained were then ligated to the 1.43-kbp BamHI fragment of pUC71K (37). This fragment contains the kanamycin resistance gene of Tn903 (11). Insertions were isolated by selection on transformation by virtue of the kanamycin resistance gene. Plasmid DNA was prepared (13), and insertions into the target sequence were identified and mapped with the novel HindIII and XhoI sites introduced by the Tn903 kanamycin resistance gene. Plasmids containing these well-defined insertions were transferred to strain NZ130 by conjugation. Kanamycin-resistant transconjugants were screened for their production of FAI and the fluorescent siderophore (FLU), and restriction endonuclease mapping analysis of the genomic target sequences was done. Only target sequences were altered in the Pseudomonas transconjugants since kanamycin resistance gene-containing derivatives of the vector pPR42 yielded no kanamycin-resistant Pseudomonas transconjugants in parallel matings.

Analysis of the regulation of gene expression for the production of FLU or FAI activity was done by using β-galactosidase gene fusions of these sequences induced by Tn2-HoHo-1 (32). Construction of the Tn2-HoHo-1 mutants was done essentially as described previously (32), except that the target plasmid was transferred to the pTn2-HoHo-1-containing strain by conjugation.

RESULTS

Nature of the antibiotic activity. A petri dish assay was used to screen for the ability of Pseudomonas strains to inhibit Pythium ultimum SB1. From a collection of 38 fluorescent rhizosphere Pseudomonas isolates, three isolates were identified that exhibited high levels of antifungal activity. These three isolates showed no activity when the medium was made to 100 μM FeCl₃ prior to inoculation. This suggests that the mechanism of antibiosis involves iron-assimilation system of the bacterium, the fungus, or both. The three isolates that exhibited antifungal activity, although they were from distinct geographical locations, appeared to be closely related. The restriction endonuclease digestion patterns of the chromosomal DNA of these isolates, when several restriction endonuclease enzymes were used, were indistinguishable after agarose gel electrophoresis. We selected a representative of this group of isolates, designated Pseudomonas sp. strain NZ130, for further study.

When strain NZ130 was grown to the stationary phase in liquid KB medium, antibiotic activity was obtained from the fluid supranautant fluid derived from the spent medium. The supernatant filtrate was diluted into molten LB agar to measure the level of antibiotic activity that was present, and the relative density of the hyphal mat in a given bioassay was determined after 2 days, a time that was sufficient for maximal fungal growth (Fig. 1). Complete inhibition of fungal growth was evident with the addition of 0.4 ml of the filtrate to the 4 ml of bioassay medium. No antibiotic activity was detectable if the supernatant was made to 100 μM in FeCl₃ before a given plate was poured. These results suggest that the inhibitory activity is antagonized by iron and that growth of the fungus in the vicinity of the bacterium is not required for the induction of the activity. These findings were supported by results of other experiments. In these experiments a petri dish assay was used in which a bacterial inoculum was first grown and then killed with chloroform before the fungus was inoculated onto the same agar medium. The zone of fungal growth inhibition was absent in these petri dish assays in which the agar medium was made to 200 μM FeCl₃ before the fungus was inoculated (data not shown). Furthermore, the zone of fungal growth inhibition was the same regardless of whether the bacteria was killed before inoculation of the fungus.

A fungal inoculum that had been previously incubated for 3 days on agar medium containing the antibiotic was transferred to fresh agar medium containing no antibiotic. The fungus grew normally, and the resulting fungal culture was observed to be as sensitive to FAI as was the starting culture. This indicates a fungistatic rather than a fungicidal mode of inhibition.

Purification of the fluorescent sideroaphore and the antibiotic. The FLU produced by strain NZ130 was purified by the procedure developed for pseudobactin, the fluorescent sideroaphore of Pseudomonas sp. strain B10 (35). We did not detect a compound which might correspond to pseudobactin A (36) from strain NZ130. The separation properties and the visible absorption spectrum of the ferric complex of FLU, however, were very similar to those of ferric pseudobactin (Fig. 2) (35). As described previously for the pseudobactin series of siderophores (19), the visible absorption spectrum of deferrated FLU varied with pH, whereas that of the ferric complex was pH invariant (Fig. 2). Only the free ligand was fluorescent (data not shown). A shoulder at A₄₈₀ to A₂₇₀ provided evidence for a ferric complex; the shoulder was indicative of a charge-transfer transition between the metal and the ligand (35).

FLU could be separated from the FAI in either the ultrafiltrate or the spent medium by using either a Bio-Gel P6 (Bio-Rad; data not shown) or a Bio-Gel P2 (Bio-Rad) column (Fig. 3). The visible absorption spectra of the fractions containing FLU were indistinguishable from that of purified FLU. Column fractions containing FAI activity were fungistatic, their antibiosis was antagonized by iron, and they had no detectable visible absorption or fluorescence.

Identification of DNA sequences required for either FLU or FAI production. Mutagenesis with MNNG was used to
obtain mutants of strain NZ130 that were defective in the production of either FLU or FAI. A total of 3,000 colonies derived from MNNG-treated bacteria were screened on KB agar to detect fluorescent siderophore production (25). A total of 27 mutants were identified that had visibly reduced levels of fluorescence on KB agar, and 5 of these were selected for further study. The five Flu' mutant strains were grown to the early stationary phase in KB broth, and the spent medium of each was filter sterilized and then analyzed for FLU levels and FAI activity (Table 1). Among these mutants the level of FLU production varied from <5 to 20% of the wild-type level, yet they all retained full FAI activity. To identify mutants that were deficient in FLU production, 1,600 colonies from MNNG-treated bacteria were analyzed for fungal inhibition by using a petri dish assay, and six strains were analyzed on a given petri dish. A total of 17 mutants with reduced levels of FAI were identified; all of these were Flu'. Three mutants with undetectable levels of FAI activity were chosen for further study.

Results of the mutational analyses described above suggest that the biosyntheses of FLU and FAI require different chromosomal regions. We constructed a cosmid library of the genome of strain NZ130 to identify and characterize these regions. The library was introduced into representative Fai' Flu' or Fai' Flu' mutants by conjugation, and the transconjugants obtained from each mating were then scored for complementation. The transconjugants from the Fai' Flu' mutants were screened on KB agar for the production of FLU, and the transconjugants from Fai' Flu' mutants were screened for FAI by using the petri dish assay. Fai' Flu' mutants PRP41 and PRP45 were complemented by cosmids pPR60, whereas cosmids pPR61 complemented only PRP41. These results indicate the occurrence of at least two closely linked genes for FLU biosynthesis. Fai' Flu' mutants PRP43 and PRP47 were complemented by cosmids pPR67, whereas PRP40 was complemented by pPR62. No cosmid was identified that could complement both Fai' and Flu' mutants. Restriction endonuclease maps of the cosmid clones that complemented various mutants were determined (Fig. 4). These restriction endonuclease maps of these cosmid clones showed no similarities, supporting the idea that different chromosomal regions, i.e., different genes, are required for the biosynthesis of FLU and FAI.

Site-directed mutagenesis of sequences required for FAI or FLU production. The DNA sequences that are responsible for complementation of a Fai' or a Flu' MNNG-induced mutant were identified by subcloning restriction endonuclease fragments from the appropriate cosmid clone into pRK767 and subsequent complementation analysis. After identification, the complementing regions were recloned into pPR42 and then mutated by the insertion of a DNA segment that conferred kanamycin resistance, as described above. These well-defined mutations were then introduced into the chromosome of strain NZ130 by marker exchange. Subclones pPR70 and pPR63 (pRK767) which complemented the MNNG-induced mutant strains PRP40 (Fai' Flu') and PRP41 (Fai' Flu'), respectively, were used to

![FIG. 2. Visible absorption spectra of the fluorescent siderophore of Pseudomonas sp. strain NZ130. The supernatant of KB medium in which strain NZ130 was grown was extracted with chloroform-phenol (1:1; vol/vol). After extraction the aqueous phase was adjusted to the pH indicator prior to analysis. (A) Symbols: —, pH 3.6 and 50 μM FeCl₃; --, pH 3.7 and no iron addition. (B) Symbols: —, pH 6.4 and 50 μM FeCl₃; --, pH 6.5 and no iron addition.](http://jb.asm.org/)

![FIG. 3. Gel filtration of extracellular material produced by strain NZ130. Symbols: ●, Elution profile of a Bio-Gel P2 column showing the fractionation of FLU; ×, antifungal activity of FAI. The elution of FLU was monitored by determination of the fluorescence and the visible absorption (A₄₀₀). The amount of FAI present across the elution profile was analyzed by pooling fractions in groups of three and then by adding this material to 1 ml of molten 2% LB agar; this formed a solid medium which was subsequently inoculated with the fungus for measurement of FAI, as described in the text. The FAI activity of each pool of three fractions analyzed was plotted at the position of the middle fraction. Vₒ and V₁ indicate the void and included volumes, respectively, in the column elution profile.](http://jb.asm.org/)
construct the pPR42 derivatives pPR75 and pPR65, respectively (Fig. 4). pPR75 and pPR65 were used as substrates for mutagenesis.

The types of mutations obtained by the method used were (i) simple insertions of the 1.43-kbp fragment of Tn903 containing the kanamycin resistance gene (11, 37) and (ii) insertions of this 1.43-kbp fragment into locations from which some DNA was deleted, i.e., substitutions. These mutations conferred kanamycin resistance in strain NZ130 and could be used as selectable markers.

Two types of kanamycin-resistant transconjugants were obtained on conjugal transfer of each kanamycin-resistant derivative of a pPR42 subclone to strain NZ130. The first type resulted from the cointegration of the plasmid into the chromosome by homologous recombination of sequences on one side of the insertion mutation. The second type of transconjugant resulted in gene conversion of the target sequences such that the mutation was exchanged into the chromosome.

When insertions inactivated sequences that were essential for FLU or for FAI biosynthesis, the cointegrates and homogenates had the predicted FAI<sup>+</sup> Flu<sup>−</sup> or FAI<sup>−</sup> Flu<sup>−</sup> phenotypes. Transconjugants obtained from kanamycin-resistant insertion derivatives of pPR65 fell into two phenotypic classes. One class was Flu<sup>+</sup>, and the other was partially or completely Flu<sup>−</sup>. The chromosomal structure of these transconjugants was analyzed by DNA-DNA hybridization analyses. The presumed homogenate and cointegrate strains derived from a given kanamycin-resistant pPR65-derived mutation were compared in the same DNA-DNA hybridization analysis. Homogenates were shown to contain only the mutant allele, whereas cointegrates contained a copy of pPR42, as well as both the mutagenized and the wild-type target region. A DNA-DNA hybridization analysis of the chromosomal 7-kbp EcoRI fragment, which was cloned in pPR65, of strain NZ130 was compared with that region in the homogenates, and the cointegrates derived from two pPR65 insertion mutations are shown in Fig. 5.
Two insertion mutations of pPR65 (pPR81, an 8.2-kbp EcoRI fragment, and pPR82, a 7.6-kbp EcoRI fragment), were used to construct pPR62 and pPR63 (pPR81) and PRP59 and PRP60 (pPR82). PRP62 and PRP59 were homogenates since they contained only the mutagenized target region, whereas PRP63 and PRP60 were cointegrates since they contained a copy of pPR42 (4.4-kbp EcoRI fragment), as well as both the mutagenized and the wild-type EcoRI fragments of pPR65. Extensive DNA-DNA hybridization analyses of the four pPR65-derived homogenates, strains PRP59, PRP62, PRP61, and PRP57, indicated that no alterations occurred in chromosomal regions adjacent to the insertions (Fig. 4). Phenotypically, all of the cointegrates were Fai" Flu" and, three of the homogenates were Fai" Flu", indicating that this region is essential for FLU but not for FAI biosynthesis.

A similar analysis was done for the region that is required for the complementation of PRP40 (Fai" Flu"). The pPR75-derived kanamycin-resistant insertion and substitution mutants were tentatively identified as either cointegrates or homogenates based on their Fai phenotype. Results of DNA-DNA hybridization analyses confirmed the identity of the cointegrates, all of which were Fai" Flu", and the homogenates, four of which were Fai" Flu". These were PRP81, PRP79, PRP78, and PRP74 (Fig. 4). Taken together, these results indicate that there are distinct genetic requirements for the biosynthesis of FAI and FLU. The two pPR75-derived homogenates that retained FAI activity (PRP71 and PRP76) and, to a lesser extent, PRP81 appeared to overproduce FLU even when moderately high levels of iron were included in either LB or KB agar medium. PRP71 and PRP76 did not appear to overproduce FLU when they were grown in liquid LB or KB culture, and were indistinguishable from the parent strain NZ130 when grown in liquid LB or KB culture. Furthermore, PRP71 and PRP76 did not overproduce FLU when very high levels of iron were included in the liquid culture medium (P. R. Gill and J. B. Neilands, unpublished data).

To determine the importance of FLU and FAI in iron assimilation by strain NZ130, the Fai" Flu" and Fai" Flu" homogenates, which were otherwise isogenic with strain NZ130, were grown on medium that contained various levels of the iron chelators ethylenediaminediohydroxyphenylacetic acid) or α,α'-bipyridine. Flu" strains were unable to grow as single colonies on media containing either of these chelators at 50 μM. Conversely, Fai" strains grew as well as the wild type, as did PRP71 and PRP76 (data not shown). These results indicate that FLU is essential for iron assimilation and that it probably functions as a siderophore. FAI, however, appears to play no role in iron assimilation. Strains PRP71 and PRP76 were not defective in iron assimilation.

**Expression of chromosomal regions required for FAI or FLU production.** We mutagenized pPR63 and pPR64 with transposon Tn3-HoHo-1, which, on insertion, fused a β-galactosidase gene to a given transcriptional unit at the insertion site (32). To identify the Tn3-HoHo-1 gene fusions of pPR63 and pPR64 that exhibited iron-dependent repression of β-galactosidase, 180 derivatives of pPR64 and 70 derivatives of pPR63 were mobilized into strain PRP40 (Fai" Flu" Lac" Cm"). The PRP40 transconjugants obtained from each derivative were tested for β-galactosidase on LB agar medium containing 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside and on the same medium containing 100 μM FeCl3. A total of 15 pPR64 and 11 pPR63 derivatives exhibited constitutive β-galactosidase activity with or without added iron. Plasmid DNA was prepared from six of each of these derivatives, and in each case the insertion site was mapped to the vector segment of the plasmid.

NZ130 and PRP40 strains containing any of the seven pPR64 or one pPR63 insertion derivatives expressed high levels of β-galactosidase activity when no iron was added to the medium, but they expressed no β-galactosidase activity when 100 μM FeCl3 was added to the medium. All eight of these insertions mapped to the respective pseudomonad DNA (Fig. 4). The direction of low-iron-induced transcription for the seven insertions of pPR64 was left to right (Fig. 4). Only the plasmid that carried the insertion nearest the left HindIII site was unable to complement the Fai" phenotype of PRP40. Additionally, results of complementation analyses with either MNNG-derived mutants or the well-defined mutants described above indicates that these insertions map to regions that are characterized as essential for the production of FAI or FLU (data not shown). These results indicate that the production of FLU and FAI is regulated by the level of available iron in the medium.

**DISCUSSION**

We have shown that an iron-antagonized fungicidal activity, FAI, produced by a rhizosphere pseudomonad is distinct from its fluorescent siderophore. FAI plays no apparent role in iron assimilation, although its expression and activity are modulated by iron. Previously, in a number of biological control studies, fluorescent siderophores from rhizosphere pseudomonads were implicated in iron-antagonized antibiotic activity (17, 19, 31). Furthermore, in a recent study (3) it has been shown that the highly purified fluorescent siderophores that are produced by a number of rhizosphere pseudomonads mediate distinct patterns of antibiotic activity.

As judged by results of petri dish assays, FAI-like activity is not ubiquitous among rhizosphere pseudomonads. Moreover, results of genomic restriction endonuclease mapping analyses of DNA from a number of rhizosphere isolates, by the use of a FAI-specific DNA-DNA hybridization probe, indicated that only the three isolates with potent antifungal activity, NZ130, E6-22, and GV-30, have such sequences (data not shown). These three isolates were indistinguishable by analysis described in this report.

The mechanism of the antagonism of FAI activity by iron in our bioassay is not understood. We cannot distinguish whether iron acts directly on the antibiotic factor or whether
the fungus is sensitive to FAI only when it is grown at low iron levels. Since FAI causes fungistasis rather than lethality, antibiotic mechanisms such as those of albomycin, bleomycin, or ferrimycin (28) appear unlikely. The reversible nature of inhibition by FAI suggests that FAI may deprive the fungus of some essential nutrient.

Strain NZ130 and its Fai" and Flu" derivatives have been tested for antifungal activity by using representatives of different groups of fungi. In all cases, the Fai" phenotype is required for fungal inhibition (T. Suslow, P. R. Gill, and G. J. Warren, unpublished data). Because FAI can inhibit a wide range of fungi, species-specific modes of action can be ruled out. To determine the mechanism of FAI activity, it will first be necessary to purify the factor and then determine its structure.

In this study we used a new method for the construction of well-defined mutants that are stable, nonrevertable, and easy to characterize physically. We intend to use the well-defined Fai" and Flu" derivatives in glasshouse experiments to determine the contribution of FAI and FLU to the plant growth-promoting properties of Pseudomonas sp. strain NZ130. We have transferred pPR62, as well as FAI-like activity, to another rhizosphere pseudomonad that is an efficient root colonizer, i.e., Pseudomonas sp. strain MK 280. Glasshouse experiments will be done to determine the plant growth-promoting properties of the pPR62 transconjugant of strain MK280.

This study was initiated so that we can understand the mechanism of iron transport regulation in fluorescent pseudomonads. Similar studies have been done to analyze the regulation of iron transport in E. coli (10). The results suggest that the production of FLU is regulated by the level of iron in the culture medium.

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LITERATURE CITED


