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Outer Membrane Protein Mediating Iron Uptake via Pyoverdin$_{pss}$, the Fluorescent Siderophore Produced by *Pseudomonas syringae* pv. *syringae*†

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In an iron-limited environment *Pseudomonas syringae* pv. *syringae* B301D produces a yellow-green fluorescent siderophore called pyoverdin$_{pss}$ which functions in high-affinity iron transport. Two-dimensional electrophoretic comparisons of the outer membrane proteins of strain B301D identified nine proteins which were expressed at low (50 nM) but not at high (10 μM) iron concentrations. Except for the minor protein 8e, the iron-regulated proteins exhibited high molecular weights ranging from approximately 74,000 to 80,000. A mutant of strain B301D incapable of iron uptake (Iu$^-$) from ferric pyoverdin$_{pss}$ lacked the 74,000-molecular-weight protein 4a, which was the major iron-regulated outer membrane protein. In contrast, a nonfluorescent mutant (Flu$^-$) unable to synthesize pyoverdin$_{pss}$ showed no quantitative or qualitative difference in its outer membrane profile from that of the wild-type strain. In plant pathogenicity tests the Iu$^-$ and Flu$^-$ strains caused typical brown necrotic and sunken lesions in immature sweet cherry fruit which were indistinguishable from those of the wild-type strain. Thus, excretion of pyoverdin$_{pss}$ and subsequent Fe(III) uptake do not have a determinative role in the pathogenicity or virulence of *P. syringae* pv. *syringae*.

Iron is an abundant element, but it exists predominantly as insoluble complexes of Fe(III) in aerobic environments. Because iron is a nutrient required by all living cells, many microorganisms acquire iron by synthesizing siderophores, which are low-molecular-weight Fe(III) chelators that deliver iron to the cell via high-affinity transport systems (33–36). Virulence in several bacteria pathogenic to animals has been correlated with the production and utilization of siderophores during invasion (9, 48). The siderophores supply the iron essential for growth and pathogenesis by sequestering Fe(III) from body fluids, in which iron is mainly complexed with high-affinity proteins such as transferrin and lactoferrin (1). Infection continues only if the pathogen efficiently extracts and assimilates iron from these relatively small and guarded sources.

Despite evidence that some siderophores modulate bacterial virulence in mammalian infections, the role of siderophores in plant pathogenesis is largely unexplored (36). Siderophore production nevertheless has been demonstrated for a number of plant pathogenic bacteria including *Agrobacterium tumefaciens* and *Erwinia carotovora*, which produce the catechol siderophores agrobactin and 2,3-dihydroxybenzoic acid, respectively (28, 37). Plant pathogenic and saprophytic fluorescent pseudomonads produce pyoverdin siderophores which usually contain a hydroxamate group (12, 13, 26, 28, 36, 44). The pyoverdins are the yellow-green, water-soluble fluorescent pigments produced in media when iron is limited. Leong and Neilands (27) concluded that agrobactin production was not required by *A. tumefaciens* for infection and crown gall formation because agrobactin could not be isolated from infected tissues and agrobactin auxotrophs were not impaired in their ability to initiate galls. In contrast, Expert and Toussaint (14) observed in *Erwinia chrysanthemi* that the loss of one to three iron-regulated outer membrane proteins, which probably function as receptors for ferric siderophores, was correlated with a loss of pathogenicity. It appears, therefore, that there is considerable variation in the contribution of siderophores to plant-bacteria interactions.

Of the plant-pathogenic fluorescent pseudomonads, *Pseudomonas syringae* pv. *syringae* is the most widely distributed in temperate climates, causing diseases on most major crop plants (17–19). For example, *P. syringae* pv. *syringae* is a serious pathogen of stone fruit trees such as sweet cherry (*Prunus avium* L.) (19). The bacterium enters through wounds and natural openings, such as stomates, to cause infections of parenchymatous tissues; symptoms appear as necrotic spots on fruit and leaves or as stem cankers. Ample supplies of iron appear to be necessary for pathogenesis because iron tightly regulates the synthesis of the necrosis-inducing phytotoxin, syringomycin (16, 17). For example, at least 2 μM iron is required for maximum yield of syringomycin from strain B301D of *P. syringae* pv. *syringae*. The invading bacterium would consequently need to acquire sufficient quantities of iron from plant constituents, such as citrate or iron-binding proteins, to initiate and sustain disease development.

The siderophore produced by *P. syringae* pv. *syringae* may have a crucial role in iron acquisition and uptake during plant pathogenesis. We have previously demonstrated (7) that strain B301D of *P. syringae* pv. *syringae* excretes large quantities of the siderophore pyoverdin$_{pss}$ in iron-limited environments and that iron from [^{55}Fe]pyoverdin$_{pss}$ is rapidly accumulated in cells of strain B301D, reversing iron starvation. The Fe(III) binding constant for pyoverdin$_{pss}$ at pH 7.0 is 10^{25}, indicating a relatively high affinity for iron. Pyoverdin$_{pss}$ functionally resembles the pyoverdins produced by *Pseudomonas fluorescens* (12, 26, 31, 39, 44), *Pseudomonas putida* (49), and *Pseudomonas aeruginosa* (12, 50). In addition, the spectral characteristics (maximum A_{410} for ferric pyoverdin$_{pss}$) and composition are similar. All

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the pyoverdins characterized thus far consist of a small peptide linked to a fluorescent chromophore, believed to be a dihydroxyquinoline moiety, which has an iron-binding function. Pyoverdin$_{pss}$, however, is unique among previously characterized pyoverdins in amino acid composition, consisting of β-hydroxyaspartic acid, threonine, serine, and lysine in a 2:2:2:1 ratio, respectively; the α-hydroxy acid group from each β-hydroxyaspartic acid residue apparently serves as an iron-binding ligand (7).

Outer membrane proteins have been shown to be necessary for iron uptake in a variety of gram-negative bacteria including *Pseudomonas* species (30, 35, 41, 42). Iron starvation induces synthesis not only of siderophores but also of high quantities of several outer membrane proteins with molecular weights typically between 70,000 and 90,000 (13, 30, 32, 49). However, there is little concrete evidence that specific outer membrane proteins from *P. syringae* function as receptors for ferric siderophores despite evidence that such transport systems exist for pyoverdin, pyochelin, and citrate in *P. aeruginosa* (8, 10, 42). Magazin et al. (30) genetically demonstrated that an 85,000-molecular-weight protein functioned as the receptor for the pyoverdin siderophore, called pseudobactin, from the fluorescent *Pseudomonas* strain B10. An uptake-deficient mutant of strain B10 which lacked the receptor protein was obtained by Tn*5* mutagenesis. The structural gene encoding the 85,000-molecular-weight protein was identified from a pLAFR1 gene bank; the cosmid (pJLM300) fully complemented for the ability to utilize ferric pseudobactin as measured by reversal of iron starvation. Although high-molecular-weight proteins are usually implicated in iron uptake, a study by Sokol and Woods (42) revealed that the receptor for [^{59}Fe]pyochelin in *P. aeruginosa* was an outer membrane protein of approximately 14,000 molecular weight. It is possible that conserved or related iron receptor proteins are found in the outer membrane of *P. syringae* pv. *syringae* since its protein profile resembles that of other fluorescent pseudomonads (23).

This study examined whether a pyoverdin produced by *P. syringae* pv. *syringae* has a role in plant pathogenesis. Pyoverdin$_{pss}$ synthesis and uptake mutants were compared with parental strain B301D for ability to acquire iron via ferric pyoverdin$_{pss}$ and cause disease in susceptible sweet cherry fruit. The iron-regulated outer membrane proteins from strain B301D were characterized, and the probable ferric pyoverdin$_{pss}$ receptor protein was identified.

MATERIALS AND METHODS

Bacterial strains and media. *P. syringae* pv. *syringae* B301D is a highly virulent strain originally isolated in England from a diseased pear (*Pyrus communis* L.) flower (19). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used to obtain a nonfluorescent mutant (Flu$^-$) of B301D unable to synthesize pyoverdin$_{pss}$ (strain W4N62) and an iron uptake (Iu$^-$) mutant which could not grow when iron was supplied as ferric pyoverdin$_{pss}$ (strain W4N2225). All strains were routinely cultured and then stored (4°C) on nutrient broth-yeast extract (NBY) agar (45) slants; lyophilized strains were prepared for long-term storage.

A tryptone-nutrient broth (5 g of tryptone [Difco Laboratories, Detroit, Mich.], 2.5 g of yeast extract [Difco], 1 g of glucose, 8.5 g of NaCl per liter) was used for growth of strain B301D during mutagenesis. For outer membrane protein analysis, cells were grown in N minimal (NM) liquid medium (45); the glucose and salts contained in NM medium were deferrated with 8-hydroxyquinoline (47). After autoclaving, deferrated NM medium (pH 7.0) was supplemented (1 ml/liter) with a filter-sterilized salts solution (200 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$ per liter). Iron-free glassware prepared by the procedure of Waring and Werkman (47) was used in all experiments.

Production and purification of pyoverdin$_{pss}$. Pyoverdin$_{pss}$ from strain B301D was produced, isolated, and purified by standard procedures (31, 39) as modified by Cody and Gross (7). The homogeneity of the pyoverdin$_{pss}$ preparations was checked by thin-layer chromatography and by amino acid analysis.

Mutagenesis and selection of Iu$^-$ and Flu$^-$ mutants. Strain B301D was grown at 25°C in tryptone-nutrient broth (10 ml per culture, 250-ml Erlenmeyer flasks) to the mid-log phase (approximately 10^8 CFU/ml) on a rotary shaker (250 rpm). Nitrosoguanidine was dissolved in distilled water and added to cultures in 25-μg/ml increments to give final concentrations ranging from 0 to 200 μg/ml. Cultures were further incubated without shaking for 15 min, and then cells were pelleted by centrifugation (20 min, 11,700 × *g*).

To isolate Iu$^-$ mutants, we suspended pellets in 10 ml of NBY broth containing 2.5 mg of deferrated (40) ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA; Sigma Chemical Co., St. Louis, Mo.) per ml to induce iron deprivation. After incubation at 25°C for 3 h, purified ferric pyoverdin$_{pss}$ (7) was added (0.1 mg/ml) to reverse iron starvation, and penicillin G (10,000 U/ml; Sigma) and D-cycloserine (100 μg/ml; Sigma) were added to kill growing cells. Cultures were further incubated for 6 h on a rotary shaker (250 rpm) until the A_{640} no longer decreased. Cells were centrifuged (20 min, 11,700 × *g*) and washed twice by resuspending them in 10 ml of sterile NBY broth and recentrifuging. Pelleted cells were resuspended in NBY broth (10 ml) and grown at 25°C with shaking (250 rpm) for 24 h. Appropriate dilutions were plated onto NM agar amended with $FeCl_3$ (10 μM). Colonies were screened on NM agar containing 200 μg of EDDA per ml for inability to utilize ferric pyoverdin$_{pss}$, observed as no growth after 48 h owing to iron starvation. Replicate colonies were maintained on NM agar containing 10 μM $FeCl_3$.

To isolate Flu$^-$ mutants after mutagenesis, pellets were resuspended in NBY broth and incubated at 25°C for 2 h on a rotary shaker (250 rpm). Appropriate dilutions were plated onto NM agar, and plates were incubated at 25°C for 2 days before colonies were screened for lack of fluorescence under a 366-nm light source.

Growth studies. Iu$^-$ and Flu$^-$ mutants were compared with wild-type strain B301D on both NM agar and NM agar containing EDDA (200 μg/ml) for growth in the presence of $FeCl_3$ or ferric pyoverdin$_{pss}$ and production of pyoverdin$_{pss}$. An EDDA concentration of 200 μg/ml was chosen because it fully induced iron starvation of strain B301D and yet allowed growth when overlaid with ferric pyoverdin$_{pss}$. Strains were adjusted to a cell density of 3 × 10^8 CFU/ml in NM liquid medium and then spotted (5 μl) onto the agar medium. After absorption of the liquid from the cell suspension into the agar medium, cells on duplicate plates were overlaid with 5 μl of either $FeCl_3$ (10 μM) or ferric pyoverdin$_{pss}$ (0.1 μg/ml). Plates were incubated at 25°C for 3 days before evaluations of growth and fluorescent pigment production; fluorescent colonies were viewed under a 366-nm light source.

Growth rates of Iu$^-$ strain W4N2225 and Flu$^-$ strain W4N62 were compared with that of strain B301D in NM liquid medium which had been previously deferrated (47).

Just before inoculation the NM medium was amended with one of the following forms of iron: $FeCl_3$ at 10 μM or 50 nM or ferric pyoverdin$_{pss}$ at 100 μg/ml. Strains were grown to the late log phase in NM medium and then transferred to iron-amended NM medium to give a final cell density ranging from 5×10^6 to 8×10^6 CFU/ml. Triplicate cultures were prepared for each strain treatment. Cultures (250 ml/1,000-ml Erlenmeyer flask) were incubated at 25°C for 24 h on a rotary shaker (250 rpm), and growth was measured by A_{420}.

Outer membrane isolation. Outer membranes from cells disrupted in a French pressure cell were isolated and purified by the sodium dodecyl sulfate (SDS) solubilization procedure developed for *P. syringae* by Hurlbert and Gross (23). Cells of *P. syringae* from late-log-phase NM liquid cultures were subcultured (approximately 5×10^6 CFU/ml) to Erlenmeyer flasks (1,000 ml) containing 250 ml of medium and grown at 25°C with rotary shaking (250 rpm) until the mid-log phase (2×10^8 to 5×10^8 CFU/ml). Cells were pelleted by centrifugation at 4°C (20 min, 10,000 × *g*), and outer membranes were immediately isolated; preparations were stored at −80°C before protein analysis. When cells were grown in the presence of high iron, deferrated NM liquid medium was amended with $FeCl_3$ to give a final concentration of 10 μM. Cells grown under iron-limiting conditions were cultured in deferrated NM liquid medium supplemented with $FeCl_3$ (50 nM). The addition of $FeCl_3$ was necessary because deferrated NM medium was too low in iron to support adequate growth of *P. syringae*. To ensure uniform results, outer membranes were isolated under a given set of cultural conditions at least three times and analyzed as described below.

PAGE and IEF. The molecular weights of outer membrane proteins were determined after separation by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE). The slab gels (0.75 mm thick) were prepared and run by the procedure of Hui and Hurlbert (22) except that they contained 11% acrylamide. Membrane samples containing approximately 10 μg of protein were dissolved in an equal volume of sample buffer (0.125 M Tris hydrochloride [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue) and heated at 100°C for 5 min immediately before use. Bovine serum albumin (67,000), egg ovalbumin (43,000), trypsinogen (24,000), and lysozyme (14,300) were used as molecular weight standards. Gels were stained (22) for protein with Coomassie brilliant blue R250. Proteins modifiable by heat or 2-mercaptoethanol were detected as previously reported (22, 23, 25).

Two-dimensional separation of outer membrane proteins by charge and molecular weight was done by the method of Ames and Nikaido (2), except for the following modifications. Outer membrane samples (100 μg of protein) were solubilized (final concentrations, 0.05 M Tris hydrochloride [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 8% Triton X-100 [Sigma], 8 M urea) and subjected to isoelectric focusing (IEF) (0.1 W per tube for 9 h) in tube gels (2.5 by 110 mm) prepared by the method of Lane and Hurlbert (25). The Ampholines (2% final concentration [wt/vol]; LKB Instruments, Inc., Rockville, Md.) were prepared by mixing a 2:2:1 ratio of the pH ranges of 4 to 6, 6 to 8, and 3.5 to 10, respectively, and the gel was polymerized with ammonium persulfate (100 μg/ml). In the second dimension, proteins were separated (25) by SDS-PAGE with a slab gel containing 11% acrylamide (1.5 mm thick) and then stained for protein (22).

Virulence tests. The virulence of Iu$^-$ and Flu$^-$ strains was assessed as described by Gross et al. (19). Immature sweet cherry fruits (cv. Bing) were surface sterilized (20 min in 0.25% sodium hypochlorite containing 0.01% [vol/vol] mild detergent, thoroughly rinsed in water, and injected (five fruits per strain; three injection points per fruit) with approximately 50 μl of a bacterial suspension (10^4 CFU/ml) in sterile deionized water. Cells used for inoculation were grown in deferrated NM liquid medium (supplemented with 50 nM of $FeCl_3$) and then washed three times in sterile deionized water. Companion experiments consisted of injections of fruit with bacteria suspended in sterile deionized water containing EDDA (300 μg/ml). Fruits were evaluated for internal and external disease symptoms after 3 days of incubation in a moist environment at 25°C by counting the number of discolored inoculation points and sectioning fruits to determine the extent of internal discoloration. Each strain was evaluated three separate times and compared with inoculations with the wild-type strain, B301D. Controls consisted of fruit injected with sterile water (or water containing EDDA [300 μg/ml]). Isolations were made from three inoculated fruits onto NBY agar after 3 days of incubation, and representative colonies of the mutants were checked for reversion to either Iu$^+$ or Flu$^+$.

TABLE 1. Effect of ferric chloride and ferric pyoverdin$_{pss}$ on EDDA-induced iron starvation of *P. syringae* pv. *syringae* B301D and its synthesis (Flu$^-$) and iron uptake (Iu$^-$) mutants

Strain	Growth under the following culture conditions[a]			
	NM	NM + EDDA[b]	NM + EDDA[b] + Fe(III)[c]	NM + EDDA[b] + pyoverdin$_{pss}$[d]
B301D	+ (fl)[e]	−	+	+ (fl)
W4N62 (Flu$^-$)	+	−	+	+
W4N2225 (Iu$^-$)	+ (fl)	−	+	−

[a] NM agar medium was spotted with 5 μl of inoculum (3×10^8 CFU/ml) and incubated at 25°C for 3 days. +, Growth; −, no growth.
[b] EDDA added at 200 μg/ml.
[c] Bacterial colony treated with 5 μl of $FeCl_3$ (10 μM).
[d] Bacterial colony treated with 5 μl of ferric pyoverdin$_{pss}$ (0.1 μg/ml).
[e] Fluorescent pigment production (fl) viewed under a 366-nm light source.

RESULTS

Mutant isolation. Iu$^-$ mutants were isolated at a frequency of 5×10^{-3}, and Flu$^-$ mutants were isolated at a frequency of 3.5×10^{-2}. A nitrosoguanidine concentration of 100 μg/ml yielded the highest frequencies of each type of mutant. A range of mutants with limited ability to synthesize pyoverdin$_{pss}$ and mutants suppressed in the ability to utilize ferric pyoverdin$_{pss}$ were detected but not analyzed because they were not fully attenuated. For example, nonfluorescent mutants observed on NM agar grew and were fluorescent on NM agar amended with EDDA (200 μg/ml).

Effects of iron on growth. *P. syringae* pv. *syringae* B301D and the Flu$^-$ and Iu$^-$ derivatives grew well on NM agar medium, but on NM agar containing 200 μg of EDDA per ml growth of all three strains was completely repressed (Table 1). Growth of strain B301D and the Flu$^-$ strain W4N62 was fully restored by supplying iron in the form of either $FeCl_3$ or ferric pyoverdin$_{pss}$. Flu$^-$ strain W4N62 differed from B301D only in its inability to produce the fluorescent siderophore. For Iu$^-$ strain W4N2225, the availability and form of iron greatly affected growth. Although iron starvation was fully reversed when iron was supplied as $FeCl_3$, treatment with ferric pyoverdin$_{pss}$ had no effect. Application of a high concentration of $FeCl_3$ relative to that of ferric pyoverdin$_{pss}$

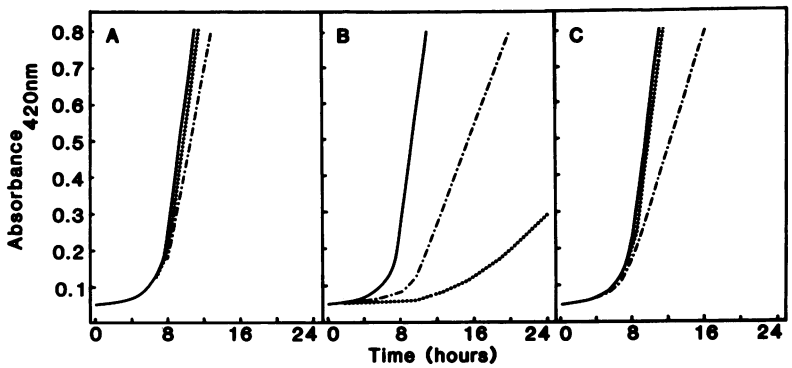

FIG. 1. Growth of *P. syringae* pv. *syringae* B301D (A) and Iu$^-$ (strain W4N2225) (B) and Flu$^-$ (strain W4N62) (C) mutants in NM liquid medium (previously deferrated) under conditions of high iron (10 μM $FeCl_3$) (solid line), low iron (50 nM $FeCl_3$) (broken line), and in the presence of ferric pyoverdin$_{pss}$ (100 μg/ml) (dotted line). Cultures were incubated at 25°C for 24 h on a rotary shaker (250 rpm), and growth was measured by A_{420}. Results depict average absorbancy readings for triplicate cultures.

completely inhibited production of fluorescent siderophore by both strain B301D and Iu$^-$ strain W4N2225.

The quantity and form of iron dramatically affected the growth rates for strain B301D, Iu$^-$ strain W4N2225, and Flu$^-$ strain W4N62 (Fig. 1A, B, and C, respectively) in deferrated NM liquid medium. When $FeCl_3$ was supplied at a high level (10 μM), all three strains grew identically, yielding peak populations between 8 and 10 h after exposure to this quantity of iron. However, when $FeCl_3$ was supplied at a low level (50 nM), peak populations were attained after 10 h for strain B301D and after 18 and 15 h, respectively, for the Iu$^-$ and Flu$^-$ strains. When ferric pyoverdin$_{pss}$ was supplied as the sole source of iron, growth rates for strain B301D and Flu$^-$ strain W4N62 were identical and equivalent to that observed when $FeCl_3$ was supplied at a high concentration. In contrast, Iu$^-$ strain W4N2225 showed no discernible growth by 10 h after exposure to ferric pyoverdin$_{pss}$. Only weak growth was observed by 24 h, which was attributed to the small amount of residual iron contained in the original inoculum.

Iron-regulated outer membrane proteins. Two-dimensional separation of the outer membrane proteins of strain B301D grown under conditions of low iron showed approximately 25 proteins with apparent pI values ranging between 4.8 and 6.2 (Fig. 2A). Protein band 8 was separated by two-dimensional analysis into several proteins labeled 8a to 8f. These may be distinct proteins, or they may have arisen owing to charge heterogeneity of one or more proteins. Protein 9 (40,000 to 41,000 molecular weight) was quantitatively the predominant protein; a study (23) of the outer

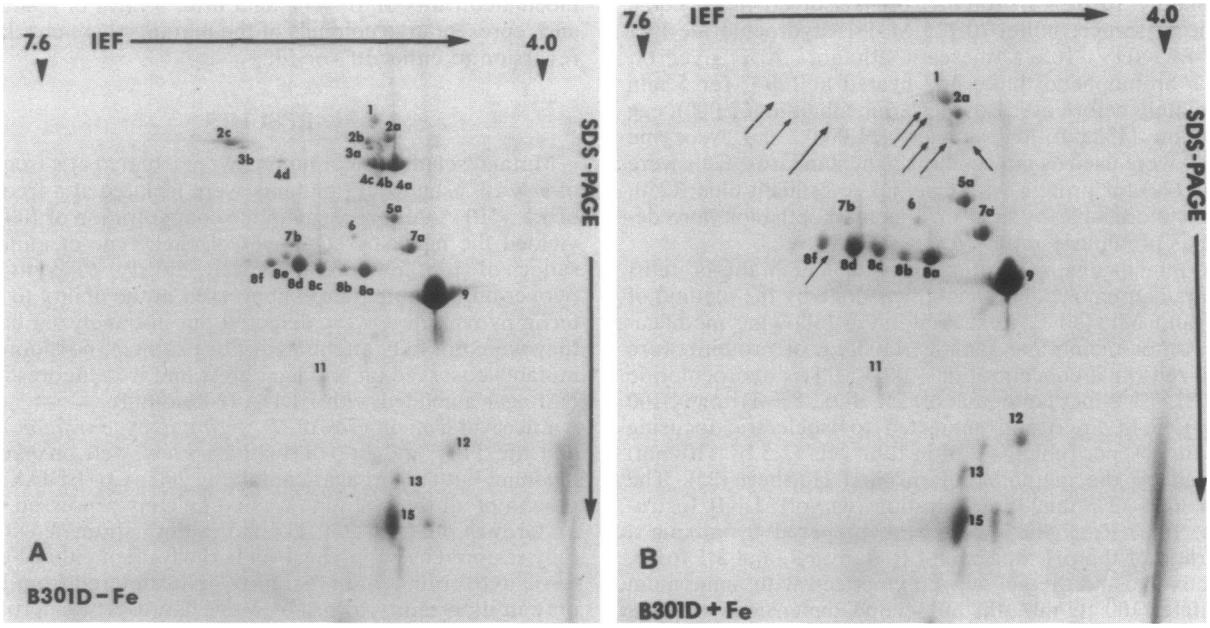

FIG. 2. Two-dimensional IEF and SDS-PAGE analysis of outer membrane proteins from *P. syringae* pv. *syringae* B301D grown under conditions of low iron (50 nM $FeCl_3$) (A) and high iron (10 μM $FeCl_3$) (B). Proteins are numbered by the method of Hurlbert and Gross (23). Samples were solubilized at 100°C for 5 min, and 100 μg of protein was applied to each gel. Direction of development in each dimension is shown at the top and side of both gels. The pH range of the IEF gels was linear and is indicated at the top of each gel; the large arrows indicate the top (left) and bottom (right) of the IEF gels. The small arrows (B) denote approximate positions for iron-regulated proteins.

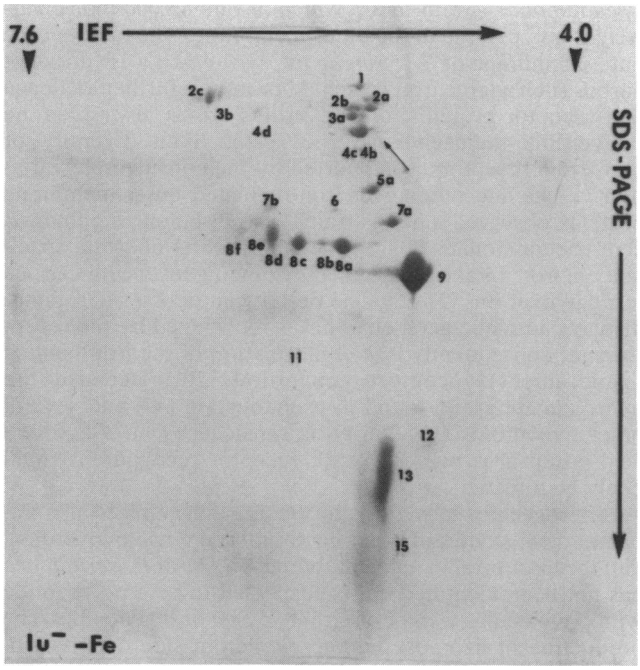

FIG. 3. Two-dimensional IEF and SDS-PAGE analysis of outer membrane proteins from an Iu⁻ mutant (strain W4N2225) of *P. syringae* pv. *syringae* grown under conditions of low iron (50 nM $FeCl_3$). Proteins are numbered as in Fig. 2. The sample was solubilized at 100°C for 5 min, and 100 μg of protein was applied to the gel. Direction of development in each dimension is shown at the top and side of the gel. The pH range of the IEF gel was linear and is indicated at the top; the large arrows indicate the top (left) and bottom (right) of the IEF gel. The small arrow denotes the approximate position of outer membrane protein 4a which appears to function as the receptor for ferric $pyoverdin_{pss}$.

membrane of *P. syringae* pv. *syringae* HS191 has suggested that it is the porin protein. It was also noted that there were numerous proteins of over 70,000 molecular weight.

When strain B301D was grown under high-iron conditions, nine proteins (2b, 2c, 3a, 3b, 4a, 4b, 4c, 4d, and 8e) were not expressed in the outer membrane (Fig. 2B). The apparent molecular weights, determined for the iron-regulated proteins by one-dimensional SDS-PAGE, were 80,000 (2b and 2c), 79,000 (3a and 3b), 74,000 (4a, 4b, 4c, and 4d), and 47,000 (8e). Of these proteins, only 8e was modifiable by heat and 2-mercaptoethanol. Significant quantitative variations were not observed for the other proteins regardless of the iron status of the medium, nor did an abundant supply of iron result in the appearance of unique proteins. After analysis of three or more separately isolated cell wall samples from strain B301D, we surmised that one or more of the iron-regulated proteins might function as a receptor for ferric $pyoverdin_{pss}$ because they were expressed only under conditions of low iron.

The outer membrane of Iu⁻ strain W4N2225 grown under conditions of low iron lacked only protein 4a (Fig. 3), which was the major iron-regulated protein observed for the wild-type strain (Fig. 2A). Protein 4a had an apparent molecular weight of 74,000 and an approximate isoelectric point of 5.5. All the other iron-regulated proteins consistently occurred in the Iu⁻ strain (Fig. 3) at quantities comparable to those of strain B301D (Fig. 2A), although separation of proteins 4b and 4c was sometimes unclear. The outer membrane protein profile of Flu⁻ strain W4N62 was visually identical to that of strain B301D under all growth conditions.

Virulence of Iu⁻ and Flu⁻ mutants as compared with strain B301D. Inoculation of sweet cherry fruit with strain B301D typically caused brown necrotic and sunken lesions that spread from the areas of injection. Visible symptoms occurred after 24 h, and infection enveloped the fruit after 48 to 72 h at 25°C. Injections of sterile water caused no internal or external discoloration of tissue during this time period. Fruit inoculated with cells of either the Iu⁻ (W4N2225) or Flu⁻ (W4N62) strain developed necrotic lesions equivalent to those observed for strain B301D within 48 h. Three days after inoculation, infections had spread extensively throughout the fruit; symptoms were no different from those observed for fruit inoculated with strain B301D. Injections of EDDA (300 μg/ml) into fruit proved to be phytotoxic, causing a brown necrosis which was essentially indistinguishable from that caused by *P. syringae*, obscuring evaluations of the effects of EDDA on *P. syringae* in vivo. Isolations from fruit infected with either the Iu⁻ or Flu⁻ mutant yielded colonies that had not reverted to wild type when tested for either $pyoverdin_{pss}$ uptake or synthesis.

DISCUSSION

Iron uptake, as mediated by production of $pyoverdin_{pss}$, apparently does not play a determinative role in the pathogenicity or virulence of *P. syringae* pv. *syringae*. Regardless of whether the bacterium was unable to synthesize $pyoverdin_{pss}$ or take up iron from its chelated form, the severity of disease was the same as that elicited by wild-type strain B301D at all stages of development. Expression of another high-affinity iron uptake system does not appear to be responsible for growth of either the $pyoverdin_{pss}$ Flu⁻ or Iu⁻ strain in plants. First, catechol and hydroxamate siderophores were not chemically detected in culture supernatants of strain B301D grown in an iron-deficient medium (7). Second, growth was completely repressed for both the Flu⁻ and Iu⁻ strains in the presence of 200 μg of EDDA per ml (Table 1). In contrast, the growth rates of both mutants were about the same as strain B301D when iron was solely supplied as a $FeCl_3$ at a very low concentration (50 nM) (Fig. 1). Finally, growth of the Iu⁻ strain essentially ceased when ferric $pyoverdin_{pss}$ (100 μg/ml) was the sole source of iron, while growth of the Flu⁻ strain was equivalent to that of wild-type strain B301D. Consequently, iron acquisition by the Flu⁻ and Iu⁻ mutants during the infection process probably results from some low-affinity form of uptake.

Ferric citrate may supply the bulk of iron to *P. syringae* pv. *syringae* during pathogenesis. Citrate, which has an iron affinity decidedly lower than that of the typical siderophore (34), is the primary means of Fe(III) transport in the xylem of several crop plants (6). Furthermore, *P. syringae* pv. *syringae* B301D can utilize citrate for growth and probably can acquire iron from ferric citrate via an energy-dependent process, as has been shown for *P. aeruginosa* (8). A problem with this scenario is that *P. syringae* pv. *syringae* is an intercellular pathogen of parenchymatous tissues rather than the xylem where ferric citrate is concentrated. The wounding of cherry fruit during inoculation nevertheless may provide sufficient access to ferric citrate during the early stages of infection for growth of the Flu⁻ and Iu⁻ strains. Ferric citrate is believed to be a primary source of iron for *A. tumefaciens* during plant pathogenesis, negating any substantial role for production of agrobactin in virulence (27). Regardless of the source and form of iron available in cherry

fruit, it can be concluded that substantial quantities of iron are readily accessible to *P. syringae* pv. *syringae* for expression of full virulence. However, different tissues, such as those found in leaves, may not be as abundant in iron, requiring the elaboration of pyoverdin$_{pss}$ to scavenge Fe(III) and sustain the infection process.

Iron regulates the expression of nine proteins in the outer membrane of *P. syringae* pv. *syringae* B301D, accounting for about one-third of all the outer membrane proteins expressed in an iron-deficient environment. The iron-regulated proteins were large and ranged from 74,000 to 80,000 molecular weight; one exception was the minor protein 8e which has a molecular weight of about 47,000. Two-dimensional IEF–SDS-PAGE analysis was essential for separation of unique iron-regulated proteins which were of the same size, as observed for proteins 2b and 2c (80,000 molecular weight), 3a and 3b (79,000 molecular weight), and 4a, 4b, 4c, and 4d (74,000 molecular weight). Among previously characterized pseudomonads, the outer membrane of the *P. fluorescens*-related strain B10 has been shown to contain five large (80,000 to 88,000 molecular weight) iron-regulated proteins (26, 30). Recently, de Weger et al. (13) showed the presence of one to four large iron-regulated proteins (68,000 to 100,000 molecular weight, depending on the strain) in various root-colonizing fluorescent pseudomonads. Meyer et al. (32), in contrast, reported that the outer membrane of *P. aeruginosa* PAO1 contained just two major iron-regulated proteins with molecular weights of 75,000 and 80,000, while one strain each of *P. fluorescens* and *P. putida* possessed only one such major protein with a molecular weight of 80,000. Sokol and Woods (42) verified the presence of the 75,000- and 80,000-molecular-weight proteins in the outer membrane of iron-starved cells of *P. aeruginosa* PAO1, but they also noted that a low-molecular-weight protein of 14,000 molecular weight quantitatively predominated. Because the outer membrane proteins in these studies (13, 30, 32, 41, 42, 49) were characterized solely by one-dimensional SDS-PAGE, enumeration of the high-molecular-weight, iron-regulated proteins is likely to be incomplete for these pseudomonads. In addition, we did not detect the expression of iron-induced proteins in the outer membrane of *P. syringae* pv. *syringae* B301D as was observed (32) for individual strains of *P. fluorescens* and *P. chlororaphis*.

The presence of numerous iron-regulated proteins in the outer membrane of *P. syringae* pv. *syringae* when environmental iron is limited suggests a functional role for them in siderophore-mediated Fe(III) uptake. Evidence that protein 4a, with a molecular weight of about 74,000, serves as the receptor for ferric pyoverdin$_{pss}$ is based on its sole nonexpression in the outer membrane of an Iu$^-$ mutant of strain B301D which cannot obtain iron from ferric pyoverdin$_{pss}$. Furthermore, protein 4a was expressed in quantities higher than that of any other iron-regulated outer membrane protein and was second only to protein 9, which is believed to be the porin protein (23). Because pyoverdin$_{pss}$ is synthesized in large quantities by iron-starved cells of strain B301D and no other siderophore can be readily detected in culture supernatants (7), it follows that protein 4a functions as the receptor for the ferric siderophore. Absolute proof that this protein functions as a receptor for ferric pyoverdin$_{pss}$ requires the isolation of a siderophore-protein complex or cloning of the relevant gene and elucidation of its role in iron uptake.

Magazin et al. (30) demonstrated that 2.4 kilobases of DNA encode the ferric pseudobactin receptor protein in *Pseudomonas* strain B10, which is an 85,000-molecular-weight polypeptide in the outer membrane. The fact that the outer membrane of *P. syringae* pv. *syringae* B301D does not harbor such a large iron-regulated protein is further evidence that receptor proteins for pyoverdins are as diverse as the pyoverdins themselves (12, 21, 26). Such diversity of pyoverdin receptors is supported by the considerable variation in size and number of iron-regulated outer membrane proteins observed among strains of root-colonizing fluorescent pseudomonads (13, 30, 49) and reports of strain specificity in iron uptake determined by binding tests with various ferric pyoverdins (21,26). The Fe(III) center of a siderophore displays a unique geometry that is recognized by the receptor and, consequently, the conformation of the iron-binding ligand affects receptor recognition (20). Because the hydroxamate group found in pseudobactin (44) and several other pyoverdins (12, 26, 49) is replaced by an α-hydroxy acid group in pyoverdin$_{pss}$ (7), receptor recognition would likely be affected.

Heterogeneity in pyoverdin receptor proteins is also apparent from studies of other bacteria. The ferric pyoverdin$_{pa}$ (50) receptor protein in the outer membrane of *P. aeruginosa* has not been identified but is suspected to be a polypeptide with a molecular weight of either 75,000 or 80,000 (32). The production of azotobactin, the pyoverdin siderophore produced by *Azotobacter vinelandii*, was coordinate with the expression of a 77,000-molecular-weight protein in the outer membrane of *A. vinelandii* (38). It remains to be determined whether ferric pseudobactin, ferric pyoverdin$_{pa}$, or ferric azotobactin can complex with the 74,000-molecular-weight receptor protein which appears to facilitate ferric pyoverdin$_{pss}$ uptake in *P. syringae* pv. *syringae*, use one of the other iron-regulated proteins found in the outer membrane of strain B301D, or require the presence of a unique polypeptide which is not expressed in *P. syringae* pv. *syringae*.

It is likely that one or more of the iron-regulated proteins in the outer membrane of strain B301D function as receptors for siderophores other than pyoverdins. Pyochelin is a catechol siderophore for which production was first described in *P. aeruginosa* (11). A protein of 14,000 molecular weight was shown to be the receptor for ferripyochelin in several *Pseudomonas* species including strains of *P. putida* and *P. stutzeri* which did not synthesize pyochelin (41). However, the outer membrane of *P. syringae* pv. *syringae* did not yield any trace of such a small iron-regulated protein in our analysis. Citrate is another iron carrier in *P. aeruginosa* which is believed to require a protein in the outer membrane as a receptor for its ferric complex (8). Despite the characterization of the 80,500-molecular-weight protein receptor for ferric citrate in *Escherichia coli* (46), no concerted effort has been made to identify the ferric citrate receptor protein in any pseudomonad.

A prior study (23) of the outer membrane proteins of *P. syringae* pv. *syringae* HS191, originally isolated from a grass host, indicated that the indigenous plasmid, pCG131, completely repressed the expression of protein 4a, which is known from this study to be the iron-regulated receptor for pyoverdin$_{pss}$. This implies that the plasmid somehow affects the iron status of the bacterial cell. Since the presence of the plasmid was associated with slightly higher virulence (15), one can speculate that the plasmid has some function in high-affinity iron uptake which in turn affects virulence. A plasmid (i.e., ColV) has been shown to be responsible for aerobactin synthesis and transport in *E. coli* (4, 43). Perhaps plasmid pCG131 similarly encodes for synthesis of some type of siderophore. Another possibility is that the 68,000-

molecular-weight protein, which appears to be encoded by pCG131, functions as a siderophore receptor. Strain B301D does not harbor plasmid pCG131; consequently, the effect of the plasmid on siderophore synthesis and iron uptake remains to be determined.

Although we were unable to demonstrate that pyoverdin$_{pss}$ contributed to the virulence of *P. syringae* pv. *syringae*, it appears incongruous that the siderophore does not have a significant function somewhere in the life cycle of the bacterium. In an iron-deficient environment the bacterium produces copious quantities of pyoverdin$_{pss}$, and the outer membrane receptor for ferric pyoverdin$_{pss}$ is expressed at levels higher than those of all other proteins except the porin. *P. syringae* pv. *syringae* exists in the plant environment as a resident epiphyte. For example, on sweet cherry the bacterium can maintain a resident population throughout the year, including dormancy (18). Only when trees are stressed and environmental conditions are suitable for infection will disease develop (19). Perhaps pyoverdin$_{pss}$ has an important role in the epiphytic survival of the bacterium by scavenging iron from plant surfaces. Pyoverdin$_{pss}$ production may also be advantageous by ensuring the dominance of *P. syringae* pv. *syringae* when in competition for iron with other components of the microflora of a plant. Indeed, *P. syringae* is generally conceded to be one of the most aggressive plant colonists (18, 29). Pyoverdin siderophores, moreover, have been shown to change profoundly the microbial composition of rhizospheres (24, 26, 44, 51). As an example, *Pseudomonas* strain B10 is a rhizosphere inhabitant which produces the pyoverdin siderophore pseudobactin, purported to improve plant vigor by suppressing deleterious microorganisms in the rhizosphere through competition for iron (5, 24, 26, 44). Pyoverdin$_{pss}$ resembles pseudobactin in several physical and chemical properties including the binding constant for Fe(III) and, therefore, may have an analogous role in nature.

The source of iron and the means by which it is acquired by *P. syringae* pv. *syringae* during plant pathogenesis remains an intriguing question. High levels of iron are undoubtedly required for the infection process because iron tightly regulates the synthesis of the phytotoxin syringomycin, which is an integral part of disease development (16, 17). It has been estimated that 0.4 ng of Fe(III) is needed to produce 1 unit of the phytotoxin. The elicitation of visible phytotoxic symptoms in leaf tissue would accordingly require between 4 and 40 ng of Fe(III) to stimulate the synthesis of a sufficient yield of syringomycin. Ferric pyoverdin$_{pss}$ and its cognate receptor protein may be expressed only when iron becomes more difficult to obtain by some form of low-affinity iron uptake, which may be mediated, for example, by ferric citrate. Evidence for sequential derepression of siderophores has been observed for *A. vinelandii*, in which azotochelin production is derepressed before that of the pyoverdin azotobactin, which is the superior chelator (38). Additionally, pyoverdin$_{pa}$ was determined by Ankenbauer et al. (3) to be more important than pyochelin for growth of *P. aeruginosa* in human serum. Clearly *P. syringae* pv. *syringae* has evolved a system for iron acquisition which is intricately regulated by the environment. This presents new challenges in understanding the fundamental basis of plant pathogenesis.

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